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Preparative isolation and dual column high-performance liquid chromatography of ginkgolic acids from *Ginkgo biloba*

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Abstract

A chromatographic procedure for the preparative isolation of six different 6-alkylsalicylic acids (syn. ginkgolic acids) with as alkyl substituents C13:0, C15:0, C15:1, C17:1, C17:2 and, tentatively C17:3 from *Ginkgo biloba* leaves was developed. The procedure consisted of a combination of normal-phase, reversed-phase and argentation chromatography. The compounds were characterised by means of UV, ^{1}H-NMR and ^{13}C-NMR spectroscopy, and mass spectrometry after silylation. A 15 cm C_{18} RP-HPLC column connected in series with a 20 cm silver(I) loaded cation exchanger HPLC column in combination with the solvent methanol–water (93:7) acidified with 0.1% formic acid was capable of separating the ginkgolic acids C13:0, C15:1, C17:2, C15:0 and C17:1 within 21 min on an analytical scale. The separation is based on a combination of reversed-phase mechanisms and double bond complexation. Detection took place by UV at 311 nm. The separation is a good starting point for the development of a quantitative procedure for the five major ginkgolic acids in Ginkgo leaves and standardised extracts.

Keywords: *Ginkgo biloba*; Preparative chromatography; Ginkgolic acids; Alkylphenols; Anacardic acid; 6-Alkylsalicylic acid

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. Unfortunately the leaves also contain considerable quantities of — under certain conditions — allergenic compounds (ginkgolic acids, cardanols and cardols) which are removed by most manufacturers during processing [3–10]. Most suppliers limit the concentration of alkylphenols to 5 or 10 ppm. However it is often unclear how these limits are exactly defined and how these compounds, which are not commercially available, are determined. So far no fast, simple, validated method has been published for extracts or leaves.

The approach for leaves used by Irie et al. has not been validated and the HPLC separation takes 82 min [11]. The extract remaining after a procedure published in a draft monograph of the European Pharmacopeia on standardised Ginkgo extracts shows still many other, much larger peaks and the

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baseline returns only to zero after 30 min. Peak identification is not totally clear and quantitation is against a mixture of C13, C15 of C17 alkylsalicylic acids [12]. Two recent papers showed that ginkgolic acids can be well analysed by LC–ESI–MS in the negative mode [13,14]. However LC–MS is a rather heavy method for routine quality control of medicinal plants and their extracts. The only quantitative study on cardols using a lengthy sample clean-up and a colorimetric assay reported total concentrations of 0.0027–0.0087% in leaves depending on the specimen [15]. One other method uses supercritical extraction with carbon dioxide modified with 8% methanol with on-line sample clean-up over silica gel [16]. An SFE apparatus is required for this elegant approach. Thus, there is a clear scope for new rugged procedures using readily available equipment and capable of separating all ginkgolic acids.

A prerequisite for the development of any phytochemical procedure is the availability of pure, well characterised reference compounds and a good and robust separation of the major compounds involved within a reasonable time. In this paper we report on a preparative isolation procedure for ginkgolic acids and the development of an HPLC system capable of separating the five major ginkgolic acids.

2. Experimental

2.1. Plant material

Leaves were collected from a Ginkgo tree (accession nr. C558) in the botanical garden of Wageningen University during the Summer of 1999. The leaves were immediately dried at 45°C under forced ventilation in a drying oven. After 24 h they were stored in plastic bags in the dark at room temperature until use.

2.2. Solvents

All solvents were either HPLC grade and used as such or technical grade and redistilled prior to use. Ultrapure water was from a combined Seradest LFM 20 Serapur Pro 90 C apparatus (Seral, Beun de Ronde, Abcoude, Netherlands). Prior to use HPLC solvents were filtrated over a 0.45 μm membrane filter (Type RC, Schleicher & Schuell). Formic acid and acetic acid were from Acros (Geel, Belgium).

2.3. Preparative isolation of ginkgolic acids

The isolation procedure is depicted in Fig. 2. The filter paper was from Schut (Renkum, Netherlands). The 40–64 μm silica gel 60 was from Fluka (Buchs, Switzerland). Fractions were collected with a Gilson 202 fraction collector (Meyvis, Bergen op Zoom, Netherlands). The presence of phenolics was checked by spotting a fraction on a silica gel TLC plate and spraying with 1% Fast Blue B (Janssen, Beerse, Belgium) solution in water after removal of any residual HOAc. Fractions of the second separation over silica gel were screened for the presence of ginkgolic acids by RP-TLC on $F254_S$ C_{18} plates (Merck, Darmstadt, Germany) in combination with the solvent MeCN–H_2O–HCO_2H (90:10:1). Detection occurred by UV 254 nm and spraying with Fast Blue B. C17:0 ginkgolic acid was available as a reference compound. Both the RP and argentation MPLC separations were carried out on axial compression columns (Jobin Yvon, Longjumeau, France) of 2 and 4 cm I.D. respectively. The 40 μm Bakerbond C_{18} material was from Mallinckrodt Baker (Deventer, Netherlands). The coating of the 40 μm silica based sulfonic acid cation exchanger with Ag(I) ions was carried out as described earlier [17].

2.4. Instrumentation

Analytical separations were performed with a Gilson 302 HPLC pump in combination with a Gilson 802C pulse damper (Meyvis, Bergen op Zoom, Netherlands) and a Marathon XT autosampler (Separations, H.I. Ambacht, Netherlands) equipped with a 5 μl loop. The separation took place on Alltech (Alltech Assoc., Deerfield, USA) C_{18} EC HPLC column (15 cm×4.6 mm, 5 μm particle size) connected in series with a Macherey and Nagel (Düren, Germany) Nucleosil 100-5SA cation-exchange column (20 cm×4 mm, 5 μm particles) coated with Ag(I) ions. The coating procedure has been described earlier [17]. The solvent consisted of MeOH–H_2O–HCO_2H (929:70:1%v/v) and was prepared by accurately weighing the amounts required. The flow was 1.0 ml/min and the column oven

temperature 35°C. Detection took place with an Applied Biosystems (Foster City, USA) UV detector at 311 nm. Peaks were recorded with a Shimadzu (Tokyo, Japan) C-R3A integrator.

Gas chromatographic analyses took place on a HP 6890 GC equipped with a HP 5973 Mass Selective Detector (Hewlett-Packard, Germany). The injection temperature was 250°C, the oven programme was 80°C (5 min hold) and then at 10°C/min to 300°C (5 min hold). The split ratio was 50:1, pressure 9.3 p.s.i., He carrier gas, MS source 230°C, column 30 m HP 5ms 0.25 mm I.D., 0.25 μm d_f.

Silylation took place by heating a few mg of pure alkylphenol with 0.10 ml BSTFA containing 1% TMCS (art. nr. 15239, Fluka, Buchs, Switzerland) and 0.2 ml DMF (Fluka, Buchs, Switzerland) at 60°C during 30 min. The mixture (1 μl) was injected directly into the GC.

NMR spectra were recorded in $CDCl_3$ on a Bruker AC-200 spectrometer (Bruker, Rheinstetten, Germany).

3. Results and discussion

3.1. Preparative isolation

Until recently only the occurrence of ginkgolic acids (synonyms: 2-hydroxy-6-alkylbenzoic acids, 6-alkylsalicylic acids or anacardic acids) and ginkgols (synonyms: 3-alkylphenols, or cardanols) had been reported in Ginkgo leaves [18]. Of these two groups, the former group occurs in approximately ten times higher concentrations and is from a point of view of allergenic potential more important [10]. According to Nguyen Tu et al. however, the concentration of the cardanols is higher than that of the ginkgolic acids [19]. The structural formulae of the ginkgolic acids are given in Fig. 1. Recently also 5-alkylresorcinols (synonyms: cardols or bilobols), which were known to occur in Ginkgo fruits, have been detected in Ginkgo leaves, albeit in 200 times lower concentrations than the ginkgolic acids [15]. For the reasons above, this paper is restricted to the analysis of the ginkgolic acids. For setting up an HPLC system capable of separating the most important ginkgolic acids access to pure and well characterised reference compounds is necessary.

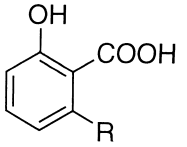

Fig. 1. Structures of the five most important ginkgolic acids occurring in Ginkgo leaves. Double bonds have the *Z*-configuration. Ginkgolic acids. R=$C_{13}H_{27}$ (C13:0), R=$C_{15}H_{31}$ (C15:0), R=$C_{15}H_{29}$ (C15:1), R=$C_{17}H_{33}$ (C17:1), R=$C_{17}H_{31}$ (C17:2).

Preparative isolations from ginkgolic acids (syn. anacardic acids) from Ginkgo fruits or leaves, or cashew nuts or oil (*Anacardium occidentale*) have been described among others in [11,14,20–29]. As starting material Ginkgo leaves were selected as these were both readily available and Ginkgo leaves or extracts thereof also constitute the material to be analysed later on. It was further decided to roughly follow the procedure described in [11]. The entire isolation and purification procedure is schematically presented in Fig. 2. The main purpose of the two initial crude isocratic separations was to increase the ginkgolic acid concentration three-fold by removing chlorophyll and other classes of co-extracted impurities. As all ginkgolic acids share the same polar groups, they eluted together in this normal-phase system. In a second step an MPLC reversed-phase separation was carried out on 40 μm C_{18} material. Four relatively pure 'ginkgolic acid' containing fractions were obtained (Fig. 2). These fractions were exceedingly difficult to purify any further by either normal or reversed-phase mechanisms. However as each fraction contained only ginkgolic acids differing in the number of double bonds, a separation by argentation chromatography should be simple [17,30]. And indeed an MPLC separation on a 40 μm Ag(I) impregnated silica based strongly acidic cation exchanger of each fraction yielded the pure ginkgolic acids C13:0, C15:0, C15:1, C17:1, C17:2 and tentatively C17:3 (Fig. 2). Advantages of this procedure are (1) the selectivity and low price of the initial extraction solvent (petrol ether) yielding only 3% crude extract, (2) the simplicity and low price of the two initial normal-phase steps (silica gel, gravity column chromatography, isocratic conditions), (3) the efficiency of the third reversed-phase MPLC separation yielding four fractions containing only ginkgolic acids differing in the number of double

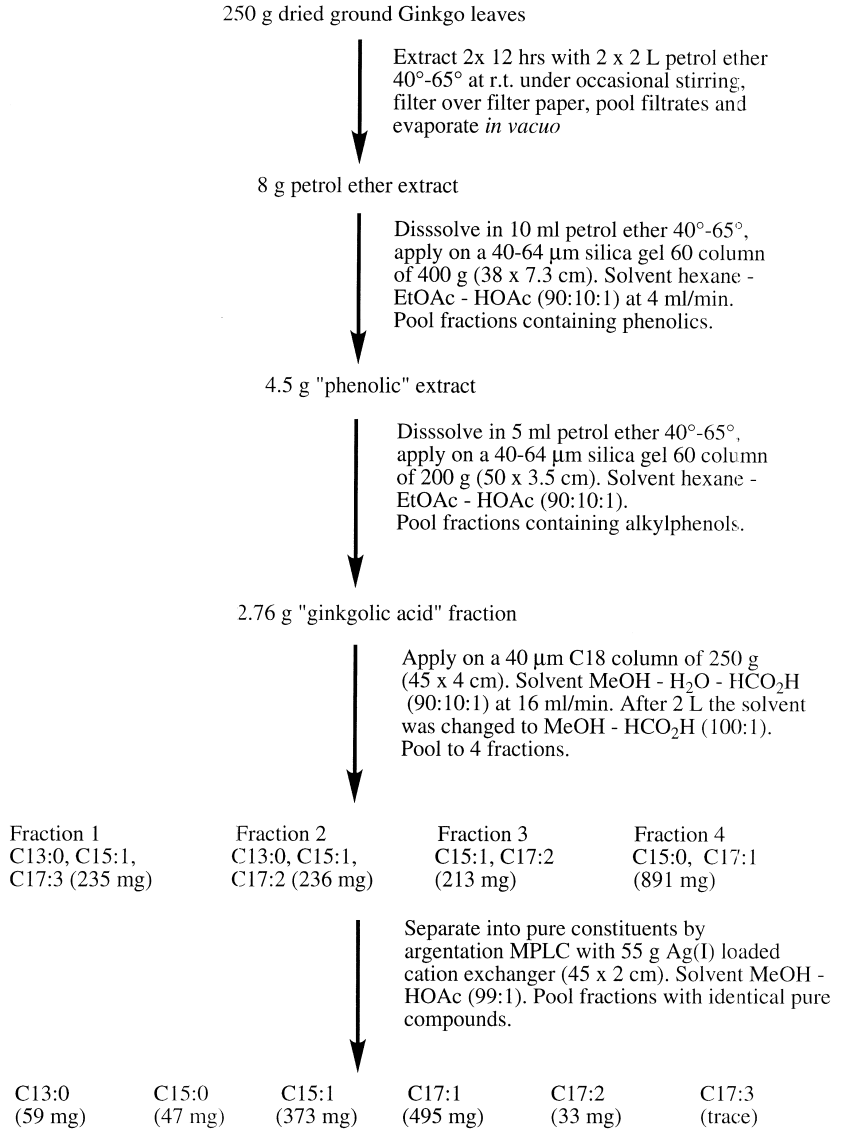

Fig. 2. Schematic presentation of the used isolation and purification procedure for ginkgolic acids from Ginkgo leaves.

bonds, (4) the very high selectivity of the last argentation chromatography step leading to six pure ginkgolic acids and (5) the absence of any chlorinated solvents or preparative HPLC steps in the whole procedure.

3.2. Identification of ginkgolic acids

The five major compounds were identified by UV, ^{1}H-NMR and ^{13}C-NMR spectroscopy and GC–MS after silylation (Table 1). We could confirm the observation that C15:1 ginkgolic acid actually consists of two isomers occurring in the ratio (1:2) differing only in the position of their *Z* double bond (Δ8 and Δ10) [19,20,29,31]. These two compounds should be considered as inseparable by HPLC and can only be separated by capillary GC after derivatisation. The recently reported RP-HPLC sepa-

Table 1
Recorded ^{1}H-NMR, ^{13}C-NMR and mass spectral data of isolated Ginkgo alkylphenols

Compound	H/C Nr Spectrum	COOH	1	2	3[a]	4[a]	5[a]	6	Sidechain
C13:0	^{1}H[b]	11.09	–	–	6.84	7.34	6.76	–	2.95 (2H, t, 8 Hz, H1′), 1.58 (2H, m, H2′), 1.24 (m), 0.86 (3H, t, 6.4 Hz, H13′) [11,26]
	^{13}C[b]	176.0	110.5	163.6	115.9	135.4	122.8	147.8	36.5 (C1′), 32.0, 31.9, 29.8, 29.7, 29.5, 29.4, 29.3, 29.2, 22.7 (C12′), 14.1 (C13′) [11,26]
	MS	m/z 464 (M^+), 449 (100%), 359. MS data fit the data given in [31]							
C15:0	^{1}H	11.10	–	–	6.85	7.34	6.75	–	2.95 (2H, t, 8 Hz, H1′), 1.62 (2H, m, H2′), 1.24 (m), 0.87 (3H, t, 6.4 Hz, H15′) [32,33]
	^{13}C	175.8	110.5	163.6	115.8	135.3	122.7	147.8	36.5 (C1′), 32.1, 31.9, 29.8, 29.7, 29.5, 29.4, 22.7 (C14′), 14.1 (C15′) [32,33]
	MS	m/z 492 (M^+), 477 (100%), 387. MS data fit the data given in [31]							
C15:1	^{1}H	11.02	–	–	6.86	7.35	6.76	–	5.33 (2H, m, CH=CH), 2.97 (2H, t, 8 Hz, H1′), 2.00 (4H, m), 1.59 (2H, m, H2′), 1.27 (m), 0.88 (3H, t, 6.4 Hz, H15′) [11,26,32]
	^{13}C	176.4	110.4	163.6	115.9	135.5	122.8	147.9	130.0, 129.9, 36.5 (C1′), 32.0, 31.8, 29.9, 29.8, 29.6, 29.5, 29.4, 29.3, 29.0, 27.2, 26.9, 22.4 (C14′), 14.0 (C15′) [11,26,32]
	MS	m/z 490 (M^+), 475 (100%), 400, 385. MS data fit the data given in [31]							
C17:1	^{1}H	11.05	–	–	6.86	7.35	6.76	–	5.33 (2H, m, CH=CH), 2.96 (2H, t, 8 Hz, H1′), 1.99 (4H, m), 1.59 (2H, m, H2′), 1.26 (m), 0.88 (3H, t, 6.4 Hz, H17′) [11,26]
	^{13}C	176.5	110.3	163.6	115.8	135.4	122.7	147.8	129.8, 129.7, 36.4 (C1′), 31.9, 29.7, 29.6, 29.5, 29.3, 29.0, 27.2, 26.9, 22.3 (C16′), 14.0 (C17′) [11,26]
	MS	m/z 518 (M^+), 503 (100%), 428, 413. MS data fit the data given in [31]							
C17:2	^{1}H	11.10	–	–	6.85	7.35	6.76	–	5.34 (4H, m, CH=CH), 2.96 (2H, t, 8 Hz, H1′), 2.76 (m, 2H, =CH–C$\underline{H}_2$–CH=), 2.04 (4H, m), 1.59 (2H, m, H2′), 1.32 (m), 0.87 (3H, t, 6.4 Hz, H17′) [11]
	^{13}C	176.1	110.3	163.6	115.9	135.4	122.8	147.8	130.2, 130.1, 128.0, 127.9, 36.5 (C1′), 32.0, 31.5, 29.7, 29.6, 29.4, 29.2, 27.2, 25.6 (=CH–C$\underline{H}_2$–CH=) 22.6 (C16′), 14.1 (C17′) [11]
	MS	m/z 516 (M^+), 501 (100%), 426, 411. MS data fit the data given in [31]							
C17:3	UV	λ_{max} 210, 240 and 310 nm. This UV spectrum is typical of a 6-alkylsalicylic acid							
	MS	m/z 514 (M^+, 0.3), 501 (6), 500 (8), 499 (17), 425 (6), 424 (17), 409 (3), 391 (5), 355 (2), 293 (7), 273 (7), 265 (8), 233 (9), 219 (36), 217 (18), 207 (17), 205 (13), 204 (10), 203 (11), 147 (41), 73 (100)							

[a] In all ^{1}H-NMR spectra H3 appeared as a d with J = 8.4 Hz, H4 as a dd with J = 7.6 and 8.4 Hz and H5 as a d with J = 7.6 Hz.
[b] NMR values in ppm relative to TMS.

ration of C15:1–Δ8 and C15:1–Δ10 should be reconfirmed [16]. Perhaps C13:0 was mistaken for C15:1–Δ8. A similar observation was made for C17:1 which even gave three distinct peaks with exactly the same mass spectrum after GC–MS. They occurred in a ratio of approximately (2:1:12). It is assumed that the two minor peaks correspond with isomers with the double bond at a different position.

According to Gellerman and Schlenk the main C17:1 ginkgolic acid has a Δ12 double bond [29]. At least one minor C17:1 isomer was also visible in the GC–MS profile after silylation given in [19]. Ginkgolic acids with an extra hydroxyl substitution in the benzene ring at C4 were not detected in this study. Such compounds have been reported from Ginkgo seeds [34] and were recently possibly identified by LC–ESI–MS in Ginkgo leaf extracts [13]. Strikingly, in another recent and independent LC–ESI–MS study on alkylphenols in Ginkgo leaves, this compound was specifically mentioned to be absent [14]. In our study one trace ginkgolic acid never reported before from any part of *Ginkgo biloba* was tentatively identified as 6-heptadecatrienylsalicylic acid (C17:3 ginkgolic acid). It was easy to separate from all other ginkgolic acids by its strong retention on the Ag(I) loaded cation exchanger. It was identified by UV and GC–MS after silylation. For further proof and the exact location of its three double bonds reisolation in larger amounts followed by NMR will be necessary.

3.3. Analytical separation

A problem with the reversed-phase (RP) separation of ginkgolic acids is the difficult separation of C13:0, C15:1 and C17:2, and of the pair C15:0 and C17:1. The introduction of one double bond more or less equals the effect on the retention of two additional carbon atoms under RP conditions. In Fig. 3 the RP-HPLC separation on a 25 cm 5 μm column is depicted showing coelution of C13:0 and C15:1, a marginal separation of these two compounds from C17:2 and the co-elution C15:0 and C17:1. Changing the organic solvent did not result in any higher selectivity. Increasing the percentage of water increased the resolution at the expense of time but a baseline separation of the more difficult pairs could not be obtained within 30 min. Thus, a different separation mechanism was called for. As normal-phase chromatography was not an option, argentation HPLC was considered. Argentation HPLC can be practised in two different modes, either by addition of Ag(I) ions to the eluent in combination with an RP column [35,36], or by coating an HPLC cation exchanger with Ag(I) ions. As we had good experiences with the latter mode of operation and as it offers a more pure separation mechanism and no special precautions against light are necessary, the cation exchanger was chosen [17]. As expected, this column which solely separates on type and number of double bonds gave an excellent separation of the

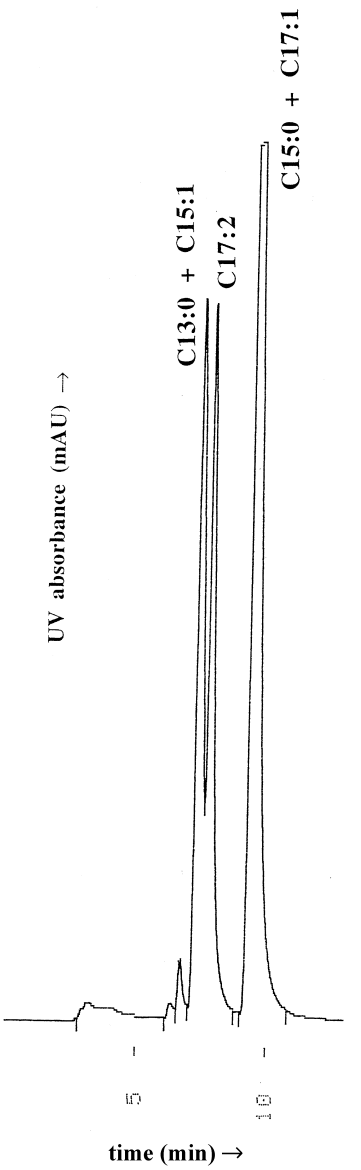

Fig. 3. Chromatogram of a reconstituted mixture of pure C13:0, C15:0, C15:1, C17:1 and C17:2 ginkgolic acids. Column: 250× 4.6 mm 5 μm Alltima C_{18} EC HPLC column. Eluent: MeOH–HCO_2H (999:1) at 1.0 ml/min. Temperature ≈25°C. Detection UV at 311 nm.

pairs C13:0/C15:0 and C15:1/C17:1. C17:2 eluted much later (Fig. 4). As the solvent (MeOH with 0.1% formic acid) was the same in both the RP and argentation separation, the columns could be connected for a combined separation. As expected the retention times were additive within experimental error (Fig. 5). Thus, for the first time a modest separation was obtained of the five major ginkgolic acids with one column separating on van der Waals forces (C13:0 from C15:0 and C15:1 from C17:1) and the other column separating on the number of

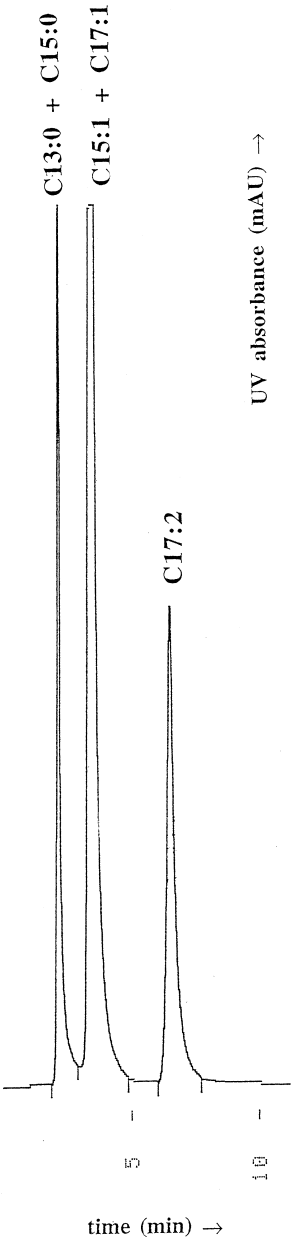

Fig. 4. Chromatogram of a reconstituted mixture of pure C13:0, C15:0, C15:1, C17:1 and C17:2 ginkgolic acids. Column: 200×4 mm 5 μm Nucleosil SA cation-exchange HPLC column loaded with Ag(I) ions. Eluent: MeOH–HCO_2H (999:1) at 1.0 ml/min. Temperature ≈25°C. Detection UV at 311 nm.

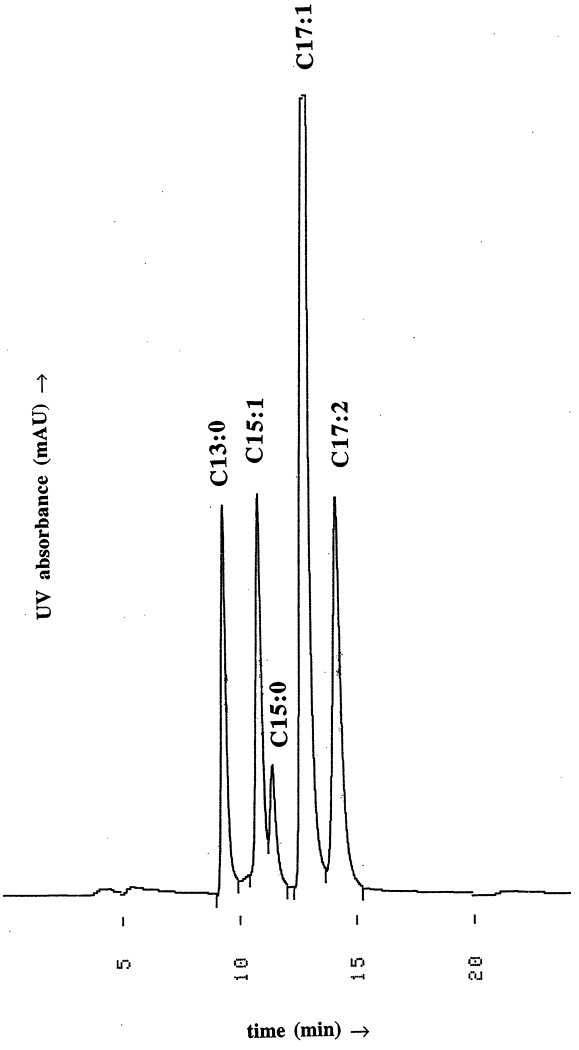

Fig. 5. Chromatogram of a reconstituted mixture of pure C13:0, C15:0, C15:1, C17:1 and C17:2 ginkgolic acids. Column: 250×4.6 mm 5 μm Alltima C_{18} EC HPLC column in series with a 200×4 mm 5 μm Nucleosil SA cation-exchange HPLC column loaded with Ag(I) ions. Eluent: MeOH–HCO_2H (999:1) at 1.0 ml/min. Temperature ≈25°C. Detection UV at 311 nm.

double bonds (C13:0/C15:0 from C15:1/C17:1 from C17:2). To further optimise the separation three parameters were varied: Length of the RP C_{18} column (effect theoretically predictable), temperature (higher temperatures leading to less retention on both columns) and % water (higher % of water having a neglible effect on the retention on the argention column but giving a strong increase in retention on the C_{18} column). An optimised separation was eventually achieved by combining a 15 cm C_{18} column and the 20 cm cation-exchange column with the eluent methanol–water–formic acid (929:70:1) at 1.0 ml/min at 35°C (Fig. 6). Lowering the temperature did not alter the selectivity, the separation can also be carried out at room temperature at the expense of some time. For instance at 20°C retention times of 16.1, 17.2, 19.3, 24.9 and 26.5 min were measured for C13:0, C15:1, C17:2, C15:0 and C17:1 respectively. The separation was found to be robust and could be accurately reproduced if the coating procedure was adhered too and if the solvent was prepared by weighing the three different constituents. After several hundred of injections over a period of 3 months no shift in retention time or loss of resolution was observed. No displacement of Ag(I) ions by protons derived from the 0.1% formic acid was observed. Detection can be carried out at 210, 245 or 311 nm. Although the sensitivity is optimal at 210 nm, detection at 311 nm is far more selective, thus, reducing the need for an extensive sample clean-up. Even at 311 nm stock solutions of 2 ppm of any ginkgolic acid could be reproducibly integrated offering good prospects for a quantitative phytochemical procedure.

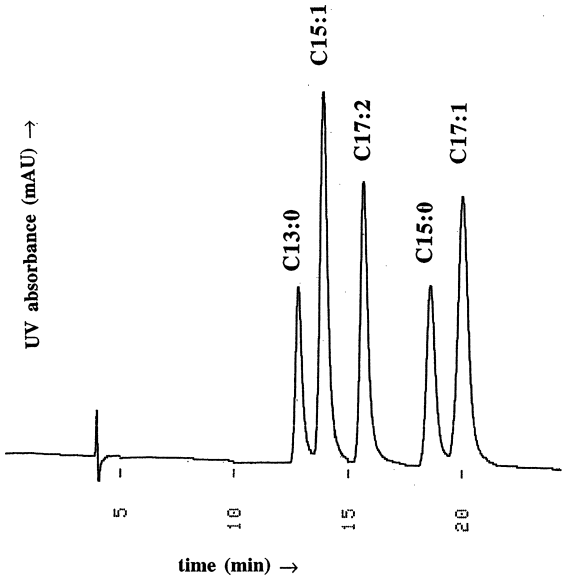

Fig. 6. Chromatogram of a reconstituted mixture of pure C13:0, C15:0, C15:1, C17:1 and C17:2 ginkgolic acids. Column: 150× 4.6 mm 5 μm Alltima C_{18} EC HPLC column in series with a 200×4 mm 5 μm Nucleosil SA cation-exchange HPLC column loaded with Ag(I) ions. Eluent: MeOH–H_2O–HCO_2H (929:70:1) at 1.0 ml/min. Temperature 35°C. Detection UV at 311 nm.

4. Conclusions

The proposed straightforward 4-step preparative separation procedure characterised by a selective extraction, a crude normal-phase group separation, a reversed-phase separation and an argentation separation, led to six different pure ginkgolic acids. One of them, occurring in low concentrations, was tentatively identified as C17:3 ginkgolic acid and had not been reported before. The method foregoes troublesome partitionings, slow and expensive preparative HPLC procedures and does not need any chlorinated solvents. Starting with 250 g dry Ginkgo leaves gives sufficient amounts to construct calibration curves for the 5 major ginkgolic acids.

The developed HPLC system consisting of two entirely different columns (argentation and reversed-phase) connected in series which use however the same eluent, gives an almost baseline separation of the five major ginkgolic acids occurring in Ginkgo leaves. The separation appears robust and combined with the good selectivity and sensitivity of the used UV detector (311 nm, 2 ppm) this offers good prospects for the development of a simple and cheap phytochemical procedure capable of analysing all five ginkgolic acids simultaneously.

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