1H NMR-based metabolomics combined with HPLC-PDA-MS-SPE-NMR for investigation of standardized Ginkgo biloba preparations

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ORIGINAL ARTICLE

^{1}H NMR-based metabolomics combined with HPLC-PDA-MS-SPE-NMR for investigation of standardized *Ginkgo biloba* preparations

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Abstract Commercial preparations of *Ginkgo biloba* are very complex mixtures prepared from raw leaf extracts by a series of extraction and prepurification steps. The pharmacological activity is attributed to a number of flavonoid glycosides and unique terpene trilactones (TTLs), with largely uncharacterized pharmacological profiles on targets involved in neurological disorders. It is therefore important to complement existing targeted analytical methods for analysis of *Ginkgo biloba* preparations with alternative technology platforms for their comprehensive and global characterization. In this work, ^{1}H NMR-based metabolomics and hyphenation of high-performance liquid chromatography, photo-diode array detection, mass spectrometry, solid-phase extraction, and nuclear magnetic resonance spectroscopy (HPLC-PDA-MS-SPE-NMR) were used for investigation of 16 commercially available preparations of *Ginkgo biloba*. The standardized extracts originated from Denmark, Italy, Sweden, and United Kingdom, and the results show that ^{1}H NMR spectra allow simultaneous assessment of the content as well as identity of flavonoid glycosides and TTLs based on a very simple sample-preparation procedure consisting of extraction, evaporation and reconstitution in acetone-d_6. Unexpected or unwanted extract constituents were also easily identified in the ^{1}H NMR spectra, which contrasts traditional methods that depend on UV absorption or MS ionizability and usually require availability of reference standards. Automated integration of ^{1}H NMR spectral segments (buckets or bins of 0.02 ppm width) provides relative distribution plots of TTLs based on their H-12 resonances. The present study shows that ^{1}H NMR-based metabolomics is an attractive method for non-selective and comprehensive analysis of *Ginkgo* extracts.

Keywords *Ginkgo biloba* · ^{1}H NMR-based metabolomics · HPLC-PDA-MS-SPE-NMR · Multivariate data analysis · Principal component analysis

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1 Introduction

Extracts of *Ginkgo biloba* leaves are among the best-selling herbal preparations worldwide (Warrier and Corzine 2000). Originally, only the seeds were used in traditional Chinese medicine (Bensky et al. 2004), but in the 1960s it was discovered that leaf extracts improved central and peripheral blood circulation (DeFeudis 1991; Drieu and Jaggy 2000). This led to the development of standardized *Ginkgo biloba* leaf extracts (named EGb 761) containing 6% of TTLs (3.1% of ginkgolides and 2.9% of bilobalide) and 24% of flavonol glycosides (Drieu and Jaggy 2000). Since then, the interest in crude as well as standardized *Ginkgo biloba* extracts has increased dramatically, and a series of excellent reviews of the chemistry and biology

(Strømgaard and Nakanishi 2004; Singh et al. 2008), pharmacology (Maclennan et al. 2002; Ahlemeyer and Krieglstein 2003; Schulz 2003), analytical methods (van Beek 2002; van Beek and Montoro 2009), and chromatographic and spectroscopic properties (van Beek 2005) have been published.

The primary constituents of standardized leaf extracts are flavonol glycosides represented by structures **1–8** identified in this work, ginkgolides A (GA, **9**), B (GB, **10**), C (GC, **11**), and J (GJ, **12**), and bilobalide (BB, **13**) (Fig. 1). Other major classes of compounds (content >5%) found in the standardized extract are proanthocyanidins, carboxylic acids, and non-flavonoid glycosides, whereas biflavones and alkylphenols (ginkgolic acids, ginkgols and bilobols) are removed during the manufacturing process (van Beek and Montoro 2009). The standardized extracts are amongst other used for symptomatic treatment of dementia, Alzheimer's disease, peripheral occlusive arterial disease, and tinnitus (Mahady 2001), and both the TTLs and flavonoid glycosides are thought to contribute to the neuroprotective effect. Thus, in 1985 it was discovered that ginkgolides are antagonists of the platelet-activating factor receptor, which is involved in slowing the progression of neurodegenerative diseases (Singh and Saraf 2001). Recently it was found that GB is an antagonist of the glycine receptors and BB is an antagonist of the γ-aminobutyric acid receptors (Ivic et al. 2003). Flavonoid glycosides are antioxidants that can potentially prevent neurodegenerative diseases caused by oxidative stress (Ramassamy 2006), and quercetin has been shown to enhance serotonin uptake in synaptosomes from mouse cortex (Ramassamy et al. 1992). Several animal studies and clinical trials support the efficacy of the standardized extract, but the exact mechanism and the constituents responsible for the effect remain largely unknown due to contradictory results. One reason for this could be that the majority of investigations are based on extracts that are standardized using procedures, which do not assure the same batch-to-batch or brand-to-brand distribution of individual TTLs and flavonoid glycosides. In addition, other constituents than TTLs and flavonoid glycosides may contribute to pharmacological activity without being included in the standardization.

To ameliorate the above problems, there is a need for a non-selective analytical technique that allows assessment of the global composition of the extract. Such a method should be complementary to the existing targeted methods based on HPLC coupled with evaporative light-scattering detector for analysis of TTLs (Ganzera et al. 2001), with UV–VIS or PDA for analysis of flavonoid glycosides (Hasler et al. 1992b), and with various types of MS for separate or simultaneous detection of TTLs and flavonoids (Li et al. 2002; Sun et al. 2005; Ding et al. 2006).

High-field ^{1}H NMR spectroscopy can, in a single spectrum acquired within a few minutes, provide a non-selective metabolic fingerprint of all hydrogen-containing organic constituents in an extract present above the detection threshold. Due to this, ^{1}H NMR-based metabolomics (Nicholson et al. 2007) has proven valuable for data-driven analysis of complex mixtures like herbal preparations and medicinal plants, including *Hypericum perforatum* (Rasmussen et al. 2006; Lawaetz et al. 2009), *Tanacetum parthenium* (Bailey et al. 2002), *Ephedra* species (Kim et al. 2005), *Strychnos* species (Frédérich et al. 2004), *Matricaria recutita* (Wang et al. 2004), and *Cannabis sativa* (Choi et al. 2004). In the current work, ^{1}H NMR-based metabolomics was used for investigation of the global composition of 16 commercially available *Ginkgo biloba* preparations, and HPLC-PDA-MS-SPE-NMR (Staerk et al. 2006) was used for unambiguous identification of eight major flavonoid glycosides.
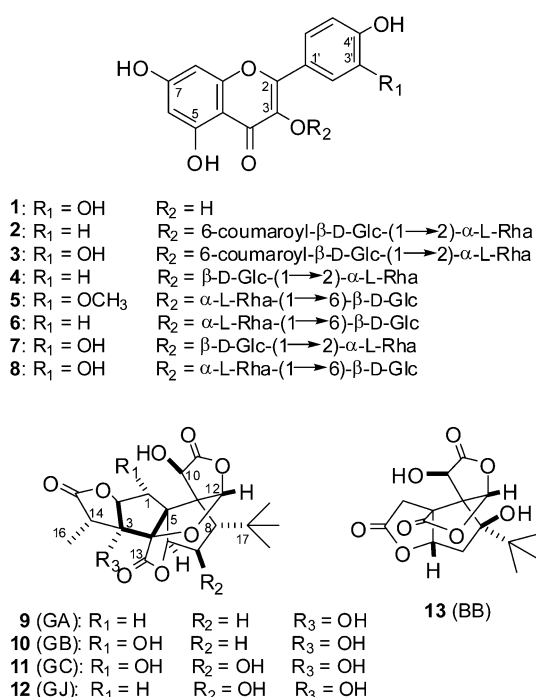

1: R₁ = OH   R₂ = H
2: R₁ = H   R₂ = 6-coumaroyl-β-D-Glc-(1→2)-α-L-Rha
3: R₁ = OH   R₂ = 6-coumaroyl-β-D-Glc-(1→2)-α-L-Rha
4: R₁ = H   R₂ = β-D-Glc-(1→2)-α-L-Rha
5: R₁ = OCH₃   R₂ = α-L-Rha-(1→6)-β-D-Glc
6: R₁ = H   R₂ = α-L-Rha-(1→6)-β-D-Glc
7: R₁ = OH   R₂ = β-D-Glc-(1→2)-α-L-Rha
8: R₁ = OH   R₂ = α-L-Rha-(1→6)-β-D-Glc

9 (GA): R₁ = H   R₂ = H   R₃ = OH
10 (GB): R₁ = OH   R₂ = H   R₃ = OH
11 (GC): R₁ = OH   R₂ = OH   R₃ = OH
12 (GJ): R₁ = H   R₂ = OH   R₃ = OH

13 (BB)

Fig. 1 Structure of flavonoid glycosides **1–8** and terpene trilactones **9–13** identified in commercially available *Ginkgo biloba* preparations

2 Materials and methods

2.1 General experimental procedures

^{1}H NMR spectra of extracts of *G. biloba* preparations were recorded at 25°C using a Bruker Avance spectrometer (^{1}H resonance frequency 600.13 MHz) equipped with a

5 mm ^{1}H{^{13}C} probe. HPLC-PDA-MS-SPE-NMR experiments were performed on a system consisting of an Agilent 1100 LC system, a Knauer K100 Wellchrom pump, a Prospekt 2 solid-phase extraction device, an EsquireLC ion-trap mass spectrometer, and the above-mentioned Bruker Avance spectrometer equipped with a 30 µl ^{1}H{^{13}C} flow-probe, as described in details elsewhere (Schmidt et al. 2008; Sprogøe et al. 2008). NMR experiments were controlled with Bruker XWINNMR version 3.1 software, whereas all other operations were controlled with Bruker HyStar version 2.3 or 3.2 software. HPLC-PDA experiments were performed using a Shimadzu HPLC system (SCL-10A system controller, SIL-10AD autoinjector, LC-10AT pump, CTO-10AC column oven, FRC-10A fraction collector, and SPD-M10A PDA detector, all controlled with Shimadzu Class-VP version 6.10 software). Semipreparative HPLC separations were performed on an Agilent 1200 system consisting of an autosampler, a quaternary pump, a thermostated column compartment, a photo-diode array detector, and a fraction collector. Operations were controlled by ChemStation revision B.03.02 software. Reference samples of GA, GB, GC, GJ, and BB were in-house material from earlier studies (Jaracz et al. 2004; Jensen et al. 2007). Reference samples of glycerol and 3,4-dihydroxybenzoic acid as well as HPLC-grade solvents for all HPLC separations were from commercial suppliers. Water was purified by deionization and 0.22 µm membrane filtration (Millipore).

2.2 Sample preparation

Sixteen commercial *Ginkgo* preparations were purchased from health food- and pharmacy stores in Denmark (1–7), Italy (8–9), United Kingdom (10–14) and Sweden (15–16) during January–February 2007. Preparations 2, 8, 9, 11, 13 were capsules and the remaining were formulated as tablets. Preparations 5, 6, and 7 belong to the same brand but were sold under different names. The same applies to preparations 11 and 12 and to 13 and 14. Tablets were powdered whereas the content of capsules was used as is. The amount of material used for extraction was chosen according to information obtained from the package leaflet, as summarized in Table 1. The material was extracted three times by sonication with 100 ml of 70% MeOH for 30 min, filtered, and the combined extracts were evaporated to dryness at temperatures below 40°C. The extract was redissolved in HPLC-grade methanol and evaporated to dryness before removal of residual solvent by freeze-drying. The extractions were made in triplicates (labeled a, b and c) for each preparation.

Table 1 Information about the commercial preparations, their declared content, and the obtained amounts of extract

Prep.	Origin	Formulation	Extract[a] (mg/item)	Flavonoids[a] (mg/item)	TTLs[a] (mg/item)	Indications[a] (items/day)	Flavonoids[b] (mg/day)	TTLs[b] (mg/day)	No. items used[c]	Extract obtained[d] (mg/item)
1	Denmark	Tablet	100	24	6	1–2	24–48	6–12	6	120.9 ± 15.4
2	Denmark	Capsule	100	24	6	1–2	24–48	6–12	6	170.7 ± 1.9
3	Denmark	Tablet	100	24	6	1	24	6	6	106.5 ± 5.6
4	Denmark	Tablet	40	9.6	2.4	3–4	28.8–38.4	7.2–9.6	15	156.1 ± 7.3
5	Denmark	Tablet	60	14.4	3.6	2	28.8	7.2	10	65.0 ± 5.2
6	Denmark	Tablet	60	14.4	3.6	2	28.8	7.2	10	74.4 ± 5.2
7	Denmark	Tablet	60	14.4	3.6	2	28.8	7.2	10	87.0 ± 22.2
8	Italy	Capsule	277	6	2	4	24	8	20	156.4 ± 5.0
9	Italy	Capsule	300	18	n.a.	2	36	n.a.	8	176.7 ± 35.8
10	U.K.	Tablet	50	12.5	n.a.	3	37.5	n.a.	12	163.3 ± 18.3
11	U.K.	Capsule	120	28.8	7.2	1	28.8	7.2	5	128.4 ± 11.5
12	U.K.	Tablet	120	28.8	7.2	1	28.8	7.2	5	117.1 ± 22.7
13	U.K.	Capsule	120	28.8	7.2	1	28.8	7.2	5	128.3 ± 6.5
14	U.K.	Tablet	30	7.2	1.8	3–4	21.6–28.8	5.4–7.2	20	34.0 ± 7.1
15	Sweden	Tablet	62.5	15	3.8	2	30	7.6	10	63.5 ± 1.5
16	Sweden	Tablet	60	14.4	3.6	2	28.8	7.2	10	62.0 ± 5.2

[a] Declared content according to package leaflet

[b] Daily amount according to indications

[c] Chosen to obtain a total of 144 mg of flavonoids and 36 mg of TTLs

[d] Mean ± SD, $n = 3$

2.3 NMR experiments

Samples for NMR analysis were prepared by dissolving 50 mg of the extract in 1 ml of acetone-d_6 containing 0.02% of TMS. After sonication for 20 min, the samples were centrifuged at 13000 rpm for 1 min to remove insoluble material and 700 μl of liquid was transferred to 5-mm NMR tubes. All ^{1}H NMR spectra for multivariate data analysis were acquired consecutively within a 48 h time-interval with samples prepared immediately before data acquisition. Repeated control experiments after 48 h showed no additional variation. For each sample, 256 transients were recorded as 64 k data points with a spectral width of 20 ppm using 30° pulses, 2.73 s acquisition time, and an inter-pulse delay of 2.84 s. The cumulative FID was Fourier transformed to 128 k data points using line broadening of 0.1 Hz. All spectra were manually phase corrected, baseline corrected, and calibrated to TMS (δ 0.0) in XWIN-NMR version 3.1.

2.4 HPLC-PDA-MS-SPE-NMR analysis

Separations of *G. biloba* extracts were performed at 40°C on a 150 × 4.6 mm i.d. Phenomenex $C_{18}(2)$ Luna column (3 μm, 100 Å) with a flow rate of 0.8 ml/min using a mixture of water–acetonitrile 95:5 + 0.1% formic acid (eluent A) and acetonitrile–water 95:5 + 0.1% formic acid (eluent B). Eight repeated separations were performed using the following gradient profile: 0 min, 0% B; 10 min, 10% B; 70 min, 40% B; 75 min, 100% B; 77 min, 100% B; 80 min, 0% B; 90 min, 0% B. Injection volumes were 20 μl of a 10 mg/ml solution (1:1 mixture of eluents A and B), and analytes were trapped on GP phase SPE cartridges [general-purpose poly(divinylbenzene)-based resin from Spark Holland, 10 × 2 mm i.d.; post-column dilution with water, 2 ml/min] using threshold-based absorbance at 254 nm for selection of analytes. The adsorbed material was eluted to the NMR flow probe with acetone-d_6. ^{1}H NMR spectra were acquired using the NOESYPRESAT pulse sequence with dual presaturation of solvent resonances (H_2O and CD_2HCOCD_3) during mixing time (100 ms) and relaxation delay (2.4 s).

2.5 HPLC-PDA analysis

HPLC-PDA data were obtained for each of the 16 preparations using the above-mentioned gradient elution profile. Injection volumes of 20 μl of a 10 mg/ml solution (1:1 mixture of eluents A and B) were used. UV spectra were acquired from 190 to 620 nm with a resolution of 1 nm/point at a sampling rate of 1.56 s^{-1} and exported as a 3D ASCII file into MATLAB, version 7.6.0, software (MathWorks, Natick, MA). UV-traces at 254 nm from 1 to 58.6 min were aligned using a correlation optimized warping algorithm (Schmidt et al. 2008). Optimum segment length and slack size were found for each sample (Skov et al. 2006). The dataset was imported into SIMCA-P version 12 software (Umetrics, Umeå, Sweden) and PCA was performed on mean-centered data without any normalization.

2.6 NMR data analysis

The ^{1}H NMR spectra were reduced to 425 or 850 sequentially integrated spectral regions ("buckets") of 0.02 or 0.01 ppm width, respectively, from δ 0.5 to 9 using AMIX version 3.6.8 software (Bruker BioSpin, Germany). The NMR data reduction file from AMIX was imported into a Microsoft Excel spreadsheet for addition of labels. Principal component analysis was performed with the SIMCA-P version 12 software (Umetrics, Umeå, Sweden) after mean-centering of data and, in some models, exclusion of δ 1.28–1.40, δ 2.04–2.12, and δ 3.28–3.36 regions. The significance of differences between the samples was assessed by ANOVA test (95% probability level) of the data matrix using Multi-Experiment Viewer version 4.0.

2.7 Semipreparative HPLC isolation

Extract (700 mg) of preparation 8 was dissolved in 40 ml of water and extracted three times with 10 ml of HPLC-grade petroleum ether (60–80°C). Evaporation of the pooled petroleum ether fractions yielded 19.5 mg, which was redissolved in 500 μl of eluent B. Five separations (50 μl each) were performed at 40°C on a 150 × 10.0 mm i.d. Phenomenex $C_{18}(2)$ Luna column (3 μm, 100 Å) with a flow rate of 4.0 ml/min of eluent B. This yielded 1.7 mg of Δ^8-15:1-anacardic acid.

3 Results and discussion

Samples for ^{1}H NMR spectral data were prepared in triplicate, by extraction of commercially available preparations of *Ginkgo biloba* with 70% methanol, using sonication for 30 min as previously described (Choi et al. 2003; Li et al. 2004). However, contrary to these studies, all samples were prepared without hydrolysis of flavonoid glycosides (Li et al. 2004) in order to enable simultaneous analysis of TTLs and flavonoid glycosides, and without prepurification based on solvent partitioning (Choi et al. 2003; Li et al. 2004) in order to assure that the samples reflect all the extract constituents. Information from the package leaflet was used to choose the number of tablets or capsules used for extraction, in order to obtain similar amounts of TTLs and flavonoids in each extract. Table 1 summarizes

information about the preparations, their declared content and the obtained amount of extract. There is clearly a large variation in the amount of extract in each tablet or capsule, and with indications obtained from the package leaflet, the recommended intake of TTLs ranges from 5.4 to 12 mg/day whereas the amount of flavonoids ranges from 21.6 to 48 mg/day.

Based on earlier ^{1}H NMR investigations of *Ginkgo biloba* extracts (van Beek et al. 1993; Choi et al. 2003; Li et al. 2004), spectra of all samples were acquired in acetone-d_6 with an inter-pulse delay of 2.84 s, which assured full relaxation of magnetization between excitations. The spectra constitute the dataset for the multivariate data analysis, and an overview of the spectra is shown in Fig. 2. Assignment of individual ^{1}H NMR resonances of GA, GB, GC, GJ, and BB were performed by comparison with spectra of authentic samples as well as with literature data (van Beek 2005). Due to lack of reference compounds, identification of flavonoid glycosides and assignment of their ^{1}H NMR resonances were performed using HPLC-PDA-MS-SPE-NMR. Thus, a gradient-based reversed-phase HPLC method was developed and single HPLC-PDA data of each preparation were obtained. Based on UV-traces at 254 nm shown in

Supplementary Fig. 1, eight major peaks were chosen for the HPLC-PDA-MS-SPE-NMR analysis. Thus, preparations 9 and 15 were chosen for identification of peak 1 and 2–8, respectively, and data from the HPLC-PDA-MS-SPE-NMR analysis are given in Supplementary Table 1. This enabled identification of 3′,4′,5,7-tetrahydroxyflavonol [quercetin] (**1**) (Schmidt et al. 2008), 3-(2-*O*-[6-*O*-(*p*-coumaroyl)-*β*-D-glucopyranosyl]-*α*-L-rhamnopyranosyloxy)-4′, 5,7-trihydroxyflavone (**2**) (Gao et al. 1996), 3-(2-*O*-[6-*O*-(*p*-coumaroyl)-*β*-D-glucopyranosyl]-*α*-L-rhamnopyranosyloxy)-3′,4′, 5,7-tetrahydroxyflavone (**3**) (Tang et al. 2001), 3-(2-*O*-*β*-D-glucopyranosyl-*α*-L-rhamnopyranosyloxy)-4′,5,7-trihydroxy flavone (**4**) (Kazuma et al. 2003), 3′-methoxy-3-(6-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranosyloxy)-4′,5,7-trihydroxyflavone [isorhamnetin 3-*O*-rutinoside] (**5**) (Sang et al. 2002), 3-(6-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranosyloxy)-4′,5,7-trihydroxyflavone [kaempferol 3-*O*-rutinoside] (**6**) (Sang et al. 2002), 3-(2-*O*-*β*-D-glucopyranosyl-*α*-L-rhamnopyranosyloxy)-3′,4′,5,7-tetrahydroxyflavone (**7**) (Hasler et al. 1992a), and 3-(6-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranosyloxy)-3′,4′,5,7-tetrahydroxyflavone [quercetin 3-*O*-rutinoside, rutin] (**8**) (Clarkson et al. 2005). HPLC-PDA-MS-SPE-NMR analysis was performed as described

Fig. 2 ^{1}H NMR spectra used for principal component analysis. Shown are the first replicates of preparation 1–16 [600 MHz, acetone-d_6, 256 transients, δ 0.5–9.0]
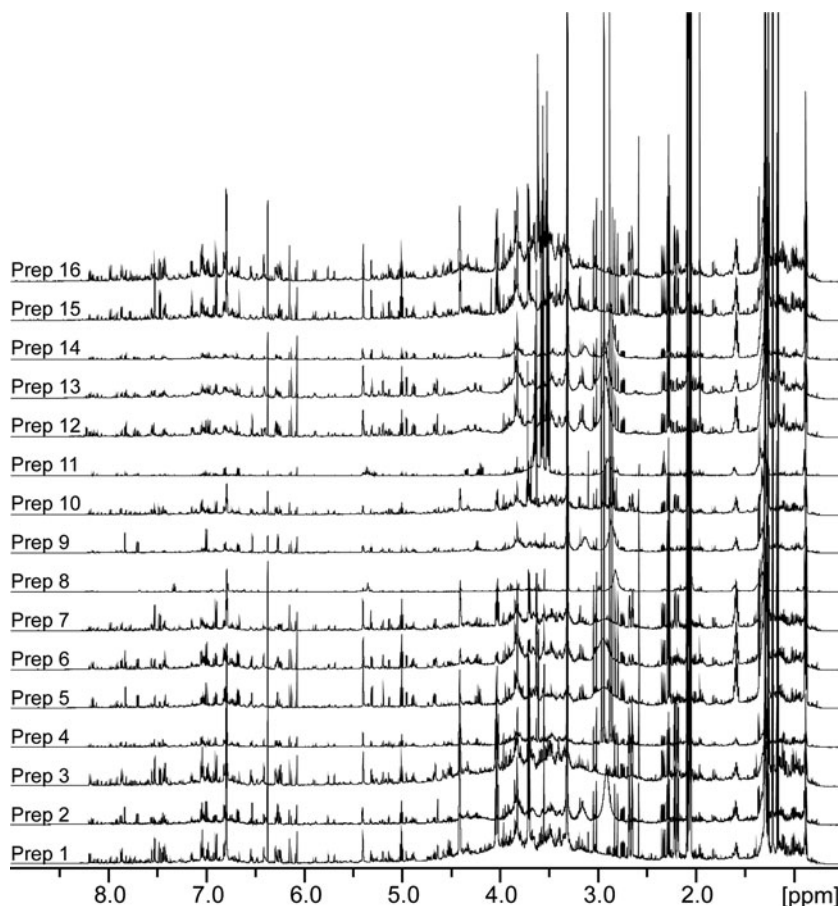
elsewhere (Staerk et al. 2006; Pedersen et al. 2009), except that the content of the dried SPE cartridges was eluted to the NMR probe with acetone-d_6 instead of acetonitrile-d_3. This enabled direct identification of resonances observed in the ^{1}H NMR spectra of the preparations by comparison with the ^{1}H NMR data of the pure compounds.

The ^{1}H NMR data for principal component analysis were subjected to a data reduction step before analysis. A common data reduction procedure is integration of discrete spectral segments (bucketing) of typically 0.04 ppm (Lindon et al. 2001), in order to minimize effects of chemical shift variations. However, important information might be lost using this procedure, and a recent study showed that for herbal preparations where only very small chemical shift variations are observed, the use of full resolution data is preferable in terms of analyzing the loading plots (Rasmussen et al. 2006). In this study, PCA analysis was initially performed with 0.04, 0.02, and 0.01 ppm bucket widths, which showed the same overall PCA score plots. However, 0.04 ppm bucket widths resulted in grouping of H-12 and *tert*-butyl resonances of GB + GC and GC + GJ, respectively, in the same buckets. Bucket widths of both 0.02 and 0.01 ppm allowed assessment of the relative distribution of TTLs by observing their H-12 and *tert*-butyl resonances in individual buckets. However, the 0.01 ppm bucket yielded more information-rich loading plots, which proved more valuable for identification of flavonoid glycosides. Furthermore, inspection of the calibrated ^{1}H NMR spectra showed small variations of the resonances of the TTLs (*tert*-butyl group: $\Delta v < 3.0$ Hz; H-12: $\Delta v < 2.0$ Hz) and of the flavonoid glycosides ($\Delta v < 1.5$ Hz) (Supplementary Fig. 2), and the data for principal component analysis were therefore integrated with spectral segments of 0.01 ppm, corresponding to a resolution of 6 Hz per data point.

Initial principal component analysis was performed using the entire dataset without any normalization procedures or exclusions to obtain a global overview of the raw extract of all preparations. This resulted in a model with seven principal components accounting for 94.1% of total variance. The first principal component accounted for 29.0% of the variance and the score plot differentiated preparation 11 from all the others (data not shown). This differentiation was due to very intense resonances (δ 3.51, 2H, *dd*, $J = 10.9$ Hz and $J = 5.9$ Hz; δ 3.57, 2H, *dd*, $J = 10.9$ Hz and $J = 5.0$ Hz; δ 3.51, 1H, *br p*, $J \approx 5$ Hz), which were assigned to glycerol by spiking with an authentic sample. Although glycerol is generally considered a non-toxic additive used in pharmaceutical preparations, its identification by the PCA model clearly demonstrates that the ^{1}H NMR-based method identifies constituents that would not have been identified by PCA analysis of, e.g., HPLC-UV data due to lack of

chromophores. A significant portion of the remaining variance in the data was due to the residual solvent signal (δ 2.05, *p*, CD$_3$COCD$_2$H), residual acetone (δ 2.09, *s*), residual methanol (δ 3.31, *s*), and the methylene envelope of fatty acids (δ 1.29, *br s*). All these signals can usually be recognized by manual inspection of the ^{1}H NMR spectra and the use of principal component analysis for their identification might appear trivial in this limited study. However, in the unsupervised analysis of larger datasets this approach can be used as a preliminary screening for automatic removal of outliers or large resonance signals from constituents that are of no importance for characterization of the extracts.

Preparation 11 was subsequently excluded from the dataset, and so were regions corresponding to residual acetone-d_6, acetone, methanol, and the methylene envelope. A new PCA model with four components accounting for 86.3% of the variance was constructed using the rest of the preparations without normalization but with mean-centering. The resulting score plots of PC1 vs. PC2 and PC2 vs. PC3 are shown in Fig. 3a and b, respectively, and the corresponding loading plots in Supplementary Fig. 3. Although a few of the samples displays a large variance between the three replicates, supposedly caused by matrix effects, the inter-group variance is more significant than the intra-group variance. This was shown using an ANOVA test at 95% probability level, and high F-values were confined to spectral regions corresponding to constituents that in the remainder of the work display the highest discriminant power. Other scaling methods like univariate scaling and Pareto scaling were also attempted, but they all resulted in increased intra-group variance without any change in inter-group variance.

In Fig. 3a, the samples separate along PC1 in a group comprising preparations 1, 3, 5, 6, 12, 13, 15, and 16 (right-hand side) and a group comprising preparations 2, 4, 7, 8, 9, 10, and 14 (left-hand side). According to the loadings, the former group separates due to a higher relative concentration of TTLs and a constituent displaying the following ^{1}H NMR resonances: δ 2.20, 1H, *dtt*, $J = 18.0$ Hz, $J = 5.5$ Hz, and $J = 1.7$ Hz; δ 2.67, 1H, *dtt*, $J = 18.0$ Hz, $J = 4.8$ Hz, and $J = 1.9$ Hz; δ 3.71, 1H, *dd*, $J = 7.2$ Hz and $J = 4.2$ Hz; δ 4.03, 1H, *dt*, $J = 7.2$ Hz and $J = 5.2$ Hz; δ 4.41, 1H, *m*; δ 6.80, 1H, *dtd*, $J = 3.6$ Hz, $J = 1.8$ Hz, and $J = 0.5$ Hz. These data are in accordance with those of shikimic acid (Talapatra et al. 1989). Along PC2, preparations with positive score values are differentiated according to loadings corresponding to the *tert*-butyl group of the ginkgolides and bilobalide; i.e., preparations 5, 6, 12, and 13 in the upper part of the plot contain relatively higher amounts of ginkgolides and bilobalide. Preparations with negative score values along PC2 (preparations 1 and 3)
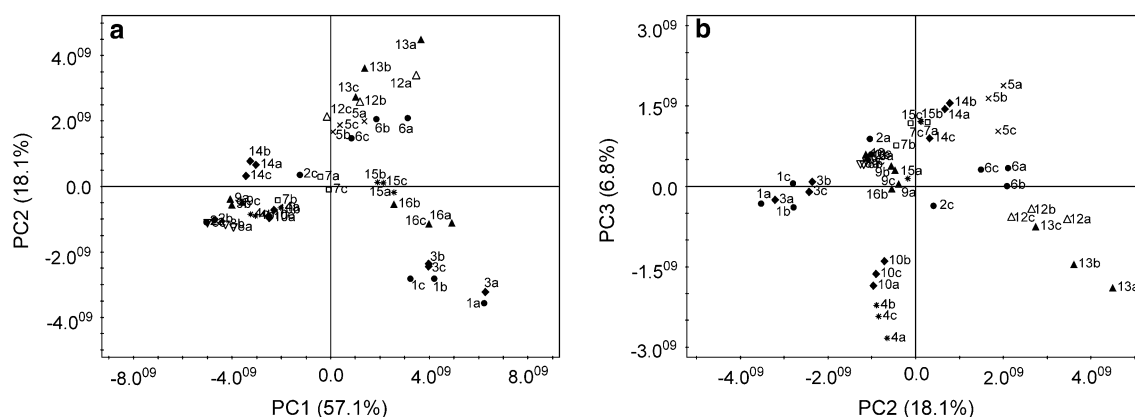
Fig. 3 Score plots of four-component PCA model of the region δ 0.5–9.0 of the ^{1}H NMR spectral data of preparation 1–10 and 12–16. **a** PC1 vs. PC2. **b** PC2 vs. PC3 [preparation 11 excluded and δ 1.28–1.40, δ 2.04–2.12, and δ 3.28–3.36 regions excluded, 0.01 ppm buckets, data mean-centered]

contain higher amounts of shikimic acid. Thus, preparations 1 and 3 contain high amounts of shikimic acid, whereas preparation 15 and 16 contain relatively high amounts of both shikimic acid and TTLs.

In Fig. 3b, positive scores in PC2 are once again due to high amounts of TTLs whereas negative scores are due to high amounts of shikimic acid. Interesting, however, are the groupings along PC3 in the right-hand side of the score plot. Positive score values are due to relative higher amounts of GC, GJ, and BB, whereas negative score values are due to higher amounts of GA and GB. Thus, of the preparations 5, 6, 12 and 13 containing relatively high amounts of TTLs, as observed by their positive score values in PC2, preparations 5 and 6 contain relatively high amounts of GC, GJ, and BB, whereas preparations 12 and 13 contain relatively high amounts of GA and GB. Finally, the grouping of preparations 4 and 10 is due to loadings corresponding to an AB system (δ 2.86, AB doublet, $J = 15.8$ Hz; δ 2.95, AB doublet, $J = 15.8$ Hz). These data are in accordance with citric acid (Couto Alves et al. 2009), which is a frequently used excipient in pharmaceutical preparations, even though its use may give rise to interactions with active constituents via cyclic anhydride formation (Higuchi et al. 1963; Larsen et al. 2009; Silver and Sundholm 1987).

Because a very large part of variance explained in the first three principal components in the above PCA model was due to shikimic acid and citric acid, variations in the level of other constituents were not easily identifiable with this model. Thus, a second PCA model was constructed, using mean-centered data of the spectral region δ 6.0–9.0, which also enabled re-inclusion of preparation 11. This region contains the singlet ^{1}H resonance signals of H-12 of all TTLs, as well as the aromatic ring signals from both the flavonoid glycosides and the flavonoid aglycones. The discrimination between different flavonoid glycosides will

thus be based on discrete ^{1}H resonance signals of the aromatic rings of the aglycones instead of the signals of the glycosidic moieties. Thus, this model enables simultaneous analysis of TTLs, flavonoid glycosides and flavonoid aglycones, together with all other constituents that display signals in this region. Excluded was only the region δ 6.78–6.81 to avoid contribution from the signal of H-2 of shikimic acid.

Principal component analysis resulted in a model (Fig. 4a, b) with six principal components accounting for 96.2% of the total variance. Principal component 1 accounts for 80.6% of the variance (Fig. 4a), and as observed with the first model, positive scores in PC1 differentiate preparations 1, 3, 5, 6, 12, 13, 15, and 16, which contain relatively higher amounts of rutin, **2**, **3**, GA, GB, GC, BB, and 3,4-dihydroxybenzoic acid than preparations 2, 4, 7, 8, 9, 10, 11, and 14 (negative score values). Principal component 2 accounts for 7.4% of the variance, and positive score values cluster preparation 5, 6, 9, 11, 12, and 13 due to relatively high amounts of GA, GB, rutin, and quercetin, the two latter being discriminated by their H-2′ resonances at δ 7.82 and 7.83, respectively, and their H-6′ resonances at δ 7.74 and 7.71, respectively. Negative score values cluster preparations 1, 3, 4, 7, 8, 10, 15, and 16 due to high levels of **2**, **3**, GC, BB, and a constituent identified as 3,4-dihydroxybenzoic acid [protocatechuic acid] based on comparison of ^{1}H NMR data (δ 7.53, 1H, d, $J = 2.0$ Hz, H-2; δ 7.47, 1H, dd, $J = 8.3$ Hz, $J = 2.0$ Hz, H-6; δ 6.90, 1H, d, $J = 8.3$ Hz, H-5) with those reported in literature (Chiji et al. 1980). Loading plots are shown in Supplementary Fig. 4. Especially interesting is the fact that preparation 9, having one of the lowest score values in PC1 because of low intensity of all signals, is grouped together with the preparations with high amounts of GA, GB, rutin, and quercetin. Close inspection of the ^{1}H spectrum shows that the sample contains very small amounts of TTLs and
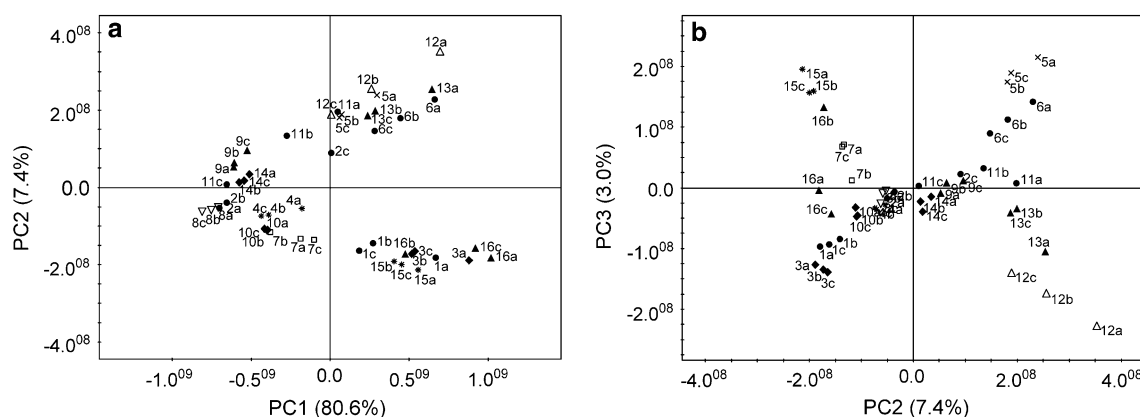
Fig. 4 Score plots of six-component PCA model of the region δ 6.0–9.0 of the ¹H NMR spectral data of all preparations. **a** PC1 vs. PC2. **b** PC2 vs. PC3 [δ 6.78–6.81 region excluded, 0.01 ppm buckets, data mean-centered]

almost no flavonoid glycosides, but a high amount of quercetin (δ 7.83, 1H, *d*, *J* = 2.2 Hz, H-2′; δ 7.70, 1H, *dd*, *J* = 8.5 Hz, *J* = 2.2 Hz, H-6′; δ 7.00, 1H, *d*, *J* = 8.5 Hz, H-5′; δ 6.53, 1H, *d*, *J* = 2.1 Hz, H-8; δ 6.26, 1H, *d*, *J* = 2.1 Hz, H-6) This suggests that preparation 9 is presumably fortified with quercetin. Fortification of *Ginkgo biloba* extracts with commercially available quercetin is a well-known problem that is not disclosed by traditional analytical methods, because these involve hydrolysis of flavonoid glycosides and measurement of the amounts of the aglycones quercetin, isorhamnetin, and kaempferol.

Looking only at preparations with positive scores in PC1 in this model, preparations 5, 6, 12, and 13 are grouped together along PC2 (positive score values) due to high amounts of GA, GB, rutin, and quercetin. However, preparations 5 and 6 are differentiated from preparations 12 and 13 along PC3 (Fig. 4b). The loadings show that the negative score values for PC3 are due to high levels of GA, GB, and rutin, whereas positive score values are due to high level of quercetin. Thus, by using PC3, it is possible to discriminate samples with relatively higher levels of quercetin from samples with relatively higher levels of GA, GB, and rutin. Whether preparation 5 and 6, which otherwise are rich in TTLs and flavonoid glycosides, are fortified with quercetin cannot be conclusively decided, but several studies indicate that the levels of flavonoid aglycones are very limited in standardized *Ginkgo* extracts (Sticher [1993]; Liu et al. [2005]). Thus, the above qualitative model can be used to select preparations for further targeted quantitative analyses. PC3 is also able to differentiate preparations 1, 3, 15 and 16, which cluster in the lower right part of Fig. 4a due to high levels of **2**, **3**, GC, BB, and 3,4-dihydroxybenzoic acid. In the left part of Fig. 4b, preparations 1 and 3 are separated from 15 to 16. Loadings show that this is primarily due to higher amounts of **2** and **3** in preparations 1 and 3, and higher amounts of

3,4-dihydroxybenzoic acid and to a smaller degree GC and BB, in preparations 15 and 16.

A comparison between the relative amounts of TTLs based on integrals of 0.02-ppm buckets representing the H-12 resonances of GA, GB, GC, GJ, and BB is shown in Fig. 5. The total integral area representing all H-12 resonances in each preparation was normalized and expressed as a fraction of 6, which is the standard composition of the extract EGb 761 containing 6% of TTLs (3.1% of ginkgolides and 2.9% of bilobalide) (Drieu and Jaggy [2000]), assumed to be the case for the commercial preparations studied here. The majority of the preparations follow this distribution between the ginkgolides and bilobalide, although preparation 11 and to a smaller degree preparations 9, 12, 13, and 14 differ from this by having relatively high values of GA, and to some degree of GB. It is worth emphasizing that Fig. 5 shows the distribution between the individual components, and not absolute amounts. However, because the exact pharmacological role of the individual constituents remains unknown and because the plot is easily prepared from information already available from the data reduction, this method is suggested to be implemented as a routine procedure for characterization of *Ginkgo biloba* extracts to be used in future pharmacological studies as a supplement to determination of absolute amount of total TTLs. To show that the relative distribution of TTLs obtained by automatic integration of buckets is, as expected, in agreement with the distributions obtained by manual integration of individual peaks as described elsewhere (Choi et al. [2003]; Li et al. [2004]), a correlation plot was prepared (Fig. 5). It demonstrates excellent correlation for GA ($R^2 = 0.9942$), GB ($R^2 = 0.9955$), GC ($R^2 = 0.9918$), and BB ($R^2 = 0.9927$). Only GJ showed a significantly lower correlation ($R^2 = 0.9038$), which is presumably due to the low *S/N*-ratio for this signal.

Fig. 6 Score plot of PC1 vs. PC2 of 11-component PCA model of the region δ 6.0–9.0 of the 600 MHz ^{1}H NMR spectral data of all preparations [0.01 ppm buckets, δ 6.78–6.81 region excluded, data normalized to total intensity of the region and mean-centered]
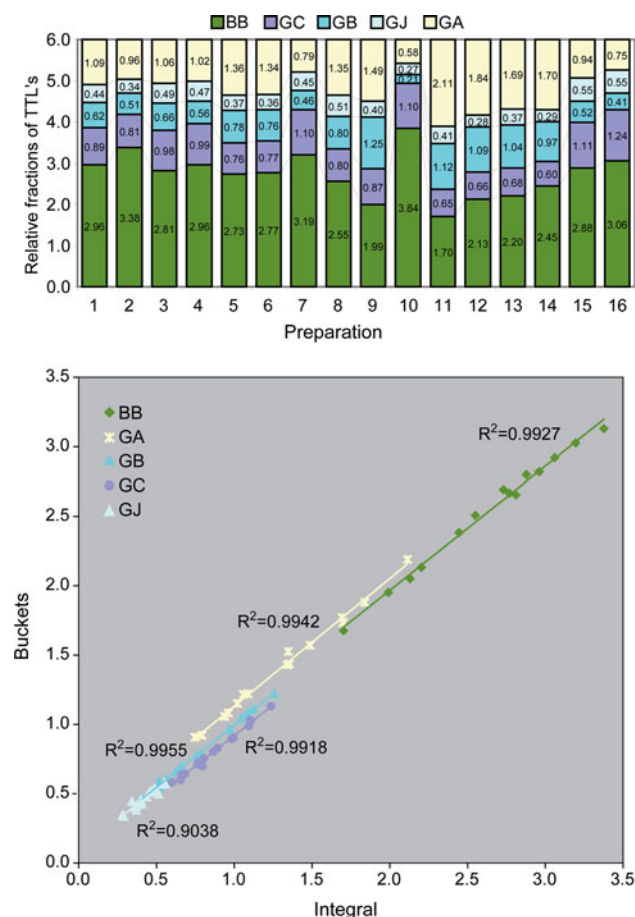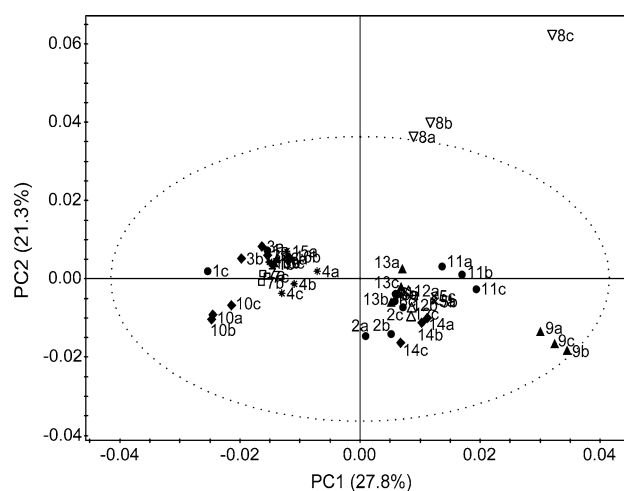
Fig. 5 *Top*: Relative distribution plots of GA, GB, GC, GJ, and BB obtained from buckets, i.e., automatically integrated spectral segments of 0.02 ppm, of the H-12 singlet. The total sum of intensities of the H-12 singlets of GA, GB, GC, GJ, and BB are normalized and expressed as a fraction of 6 (standard composition of EGb761 is 6% TTLs). *Bottom*: Correlation plots comparing automated integration (AMIX) of bucketed data obtained as above with data from manual integration of H-12 resonances

2H, *m*; δ 2.95, 2H, *t*, *J* = 8.0; δ 2.01, 4H, *m*; δ 1.58, 2H, *m*; δ 1.32, 16H *m*; δ 0.89, 3H, *t*, *J* = 7.0 Hz) with values obtained from literature (Itokawa et al. 1987). The identification of Δ^8-15:1-anacardic acid, which is one of the potential allergenic ginkgolic acids, the content of which should be less than 5 ppm in standardized *Ginkgo biloba* extracts, once again demonstrates the value of ^{1}H NMR-based metabolomics as a complementary method to other targeted analytical techniques.

For comparison with the data obtained by ^{1}H NMR, a PCA model based on aligned HPLC-UV traces at 254 nm (Supplementary Fig. 1) was made. The score plot of PC1 vs. PC2 is shown in Supplementary Fig. 6, and displays some of the same characteristics as Fig. 4. Thus, preparations 2, 5, 6, 9, 11, 12, 13, and 14 have higher levels of quercetin than preparations 1, 3, 15, and 16. In addition, preparations 12 and 13 are separated from the other due to a much higher relative amount of rutin. However, the scores are not influenced by the TTLs and other constituents that do not absorb at 254 nm. On the other hand, HPLC enables a detailed analysis of flavonoid distributions (Hasler et al. 1992b), and chromatographic fingerprint-shapes are broadly used for quality assessment of complex extract (Liang et al. 2004; Xie et al. 2006). However, both HPLC-fingerprint analysis and pattern recognition methods based on PCA models of HPLC-DAD data suffer from the limited structural information content of UV spectra, which makes identification of individual constituents difficult without access to authentic samples. These problems may be circumvented by use of the HPLC-PDA-MS-SPE-NMR technique as demonstrated in this work by the identification of flavonoids, even though the analysis was limited to the eight major constituents **1–8** due to sensitivity limitations.

Another PCA model with 11 components accounting for 94.5% of total variance was constructed using the same spectral region δ 6.0–9.0 but normalizing all spectra to the same total intensity in this region and mean-centering. The score plot of PC1 vs. PC2 (Fig. 6) shows once again grouping into two main clusters due to differences in the content of flavonoid glycosides, but importantly, preparations 8 and 9 are clearly differentiated from the others. Results from the loadings (Supplementary Fig. 5) show that preparation 9 is now separated from the others due to the presumed fortification with quercetin, whereas the separation of preparation 8 is due to the presence of Δ^8-15:1-anacardic acid, i.e., (*Z*)-2-hydroxy-6-(pentadec-8-enyl)benzoic acid, which was subsequently isolated by semipreparative HPLC and identified by comparison of its ^{1}H NMR data (δ 7.32, 1H, *t*, *J* = 8.0 Hz; δ 6.84, 1H, *dd*, *J* = 8.0, 1.0 Hz; δ 6.76, 1H, *dd*, *J* = 8.0, 1.0 Hz; δ 5.34,

4 Concluding remarks

In conclusion, this work demonstrates that ^{1}H NMR-based metabolomics combined with HPLC-PDA-MS-SPE-NMR is an attractive technology platform for comprehensive characterization of the global composition of standardized *Ginkgo biloba* preparations. ^{1}H NMR spectroscopy is intrinsically non-selective and information-rich, and PCA analysis of ^{1}H NMR spectra therefore enabled simultaneous assessment of TTLs and flavonoid glycosides. The method also allowed identification of fortification with quercetin, simultaneous generation of relative distribution data for TTLs, which disclosed cases of non-conformity to standard ratio between ginkgolides and bilobalide, and detection of the potentially harmful Δ^8-15:1-anacardic acid. In addition, the analysis demonstrated the presence of constituents that are usually not considered in the standardization protocols (shikimic acid and 3,4-dihydroxy benzoic acid) as well as excipients (glycerol and citric acid). All these features are of likely importance in relation to consumer experience with *Gingko* products as well as for pharmacological or clinical studies using *Ginkgo* extracts. A paradigm shift from targeted analyses towards more non-selective data-driven analytical approaches is needed in future pharmacopoeias encompassing standardized *Ginkgo biloba* extracts, and a full or partial incorporation of the presented methods is therefore of utmost importance.

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