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Design of Countercurrent Separation of *Ginkgo biloba* Terpene Lactones by Nuclear Magnetic Resonance

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Abstract

Terpene lactones such as bilobalide, ginkgolides A, B, C, and J are major bioactive compounds of *Ginkgo biloba* L. Purification of these compounds is tedious due to their similar chemical properties. For the purpose of developing an effective and efficient method for both analytical and preparative separation of terpene lactones in *G. biloba*, an innovative orthogonality-enhanced high-speed countercurrent chromatography (HSCCC) method was established. Taking advantage of quantitative ¹H NMR (qHNMR) methodology, partition coefficients (*K*) of individual terpene lactones were calculated directly from crude *G. biloba* leaf extract, using their H-12 signals as distinguishing feature. The partitioning experiment assisted the design of a two dimensional (2D) HSCCC procedure using a pair of orthogonal HSCCC solvent systems (SSs), ChMWat +4 and HEMSoWat +3/0.05%. It was surprising that the resolution of ginkgolides A and B was improved by 25% in the HEMWat +3 SS modified with 0.5% DMSO. Consequently, all five terpene lactones could be well separated with qHNMR purity > 95% from *G. biloba* leaf extract. The separation was further evaluated by offline qHNMR analysis of HSCCC fractions associated with Gaussian curve fitting. The results showed less than 2% error in HSCCC retention predicted from the partitioning experiment. This compelling consistency demonstrates that qHNMR-derived *K* determination (“*K*-by-NMR”) can be used to predict CCC fractionation and target purification of analytes from complex mixtures. Furthermore, Gaussian curve fitting enabled an accurate prediction of less than 2% impurity in the CCC fraction, which demonstrates its potential as a powerful tool to study the presence of minor constituents, especially when they are beyond the detection limit of conventional spectroscopic detectors.

Keywords

Ginkgo biloba; terpene lactones; HSCCC; quantitative ¹H NMR; *K*-by-NMR; Gaussian curve fitting

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1. Introduction

Ginkgo biloba L. is known as a living fossil in the plant kingdom and has been valued for its pharmaceutical properties for centuries. *G. biloba* leaf extract is one of the most popular dietary supplements in the United States and worldwide [1]. It has shown beneficial pharmacological effects against neurodegenerative diseases such as Alzheimer's, cardiovascular diseases, oxidative stress, cancer, tinnitus, geriatric, and psychiatric disorders [2,3]. Pharmacognostic studies have established flavonoids and diterpene lactones as the main bioactive compounds. The five major terpene lactones found in *G. biloba* leaf are bilobalide (BB), ginkgolides A (GA), B (GB), C (GC), and J (GJ; Fig. 1).

Previously, purification of ginkgo terpene lactones has been accomplished mainly by absorption chromatography. However, these compounds are poorly resolved on normal silica gel. BB has the least retention, followed by GA/GB as a pair and GC/GJ also as a pair. The poor separation of GA and GB is caused by the strong hydrogen bonding between the 1- and 10-hydroxyl groups in GB. As a result, GB has polarity almost equal to that of GA, despite the presence of an extra hydroxyl group. The same phenomenon occurs with the GC/GJ pair. A complete separation of BB, GA, GB, GC, and GJ has previously been obtained by impregnating the silica gel absorbent with sodium acetate prior to fractionation and using neat methyl acetate as eluent [4]. Apparently, the sodium acetate interrupts the intramolecular hydrogen bonding in GB and GC, and results in a much better resolution. Another approach takes advantage of enhanced susceptibility of GB and GC to benzylation when separating them from their non-reactive GA and GJ counterparts, respectively [5]. Reversed phase HPLC also allowed separation of the five terpene lactones by using optimized eluents [6,7]. However, all these methods have limitations. Absorption chromatography has a tendency of permanently retaining analytes on the stationary phase, resulting in sample loss and empirical quantitative relationships. In addition, modification of silica or sample treatment with a derivation agent is tedious and time-consuming and/or alters the natural products diversity. Finally, although HPLC has high resolving power, it is not very economical or environmentally friendly when conducting preparative chromatography.

In order to provide a mechanistically different approach, and also to overcome these limitations, the present study focuses on the design of a high-speed countercurrent chromatography (HSCCC) procedure for separation of terpene lactones in *G. biloba* L. This approach takes advantage of ^1H NMR spectroscopy for various aspects of design and assessment of the CCC separation method. By using the ^1H -NMR signal of the H-12 protons as a distinguishing characteristic (Fig. 2), it becomes feasible to perform simultaneous qualitative and quantitative analysis of these compounds, even in mixtures, and without a preceding separation [8-10]. In extension of previous uses of quantitative ^1H NMR (qHNMR), a qHNMR methodology was established capable of measuring the partition coefficients (K) of terpene lactones in crude ginkgo preparations. This qHNMR method of determining K values by NMR (K -by-NMR) was used for the selection of most suitable HSCCC solvent systems (SSs). It is noteworthy that, traditionally, the polarity of a SS has been given more consideration in countercurrent separation (CS) than the selectivity of the SS. However, the selectivity significantly affects the separation characteristics, especially for compounds with similar chemical properties such as the ginkgolides. Therefore, an orthogonal approach was explored in this study by using SSs with different selectivity to optimize the CS of terpene lactones from *G. biloba*. To evaluate the separation performance predicted by the partitioning experiments, HSCCC fractions of interest were analyzed by offline qHNMR, followed by Gaussian curve fitting which fully matched the elution profiles of the target compounds.

2. Experimental

2.1. Materials and reagents

The standardized *G. biloba* leaf extract (Lot number G96/63/A9) was acquired from Naturex (South Hackensack, NJ, USA). All chemical solvents were analytical grade and purchased from Pharmco-AAPER (Brookfield, CT, USA). NMR solvents were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Nomenclature of partition coefficients (*K*)

At present, no IUPAC recommendation exists for the nomenclature of partition or distribution behavior of analytes in countercurrent or partition chromatography, and the literature is inconsistent with regard to nomenclature [11,12]. In keeping with previous reports, the present study uses the simple variable, *K*, to designate the partition coefficient (or distribution coefficient) of individual phytoconstituents in either static or dynamic systems. This choice is made with the awareness that, in theory, a distinction has to be made for compounds that exists in multiple individual forms, such as ionizable compounds. However, under the conditions chosen herein, the ginkgo compounds like many natural products do not show such behavior, and in particular are insensitive to pH effects. While efforts to harmonize the nomenclature and establish IUPAC recommendations are underway, it should be pointed out that the variable, *K*, used here and called *partition coefficient* is equivalent to the variable, *K_C*, recently proposed and designated to be called *distribution coefficient*.

2.3. Preparation of terpene lactones-enriched sample

G. biloba leaf extract (600 mg) was added to the biphasic SS of hexane-ethyl acetate-methanol-water (1:9:1:9, v/v) with 5 mL each of upper and lower phase. The solution was shaken vigorously and centrifuged to break up any emulsion and speed up phase separation. The upper phase was then separated from the solution. Another 5 mL of fresh upper phase was added to the remaining lower phase. The abovementioned partitioning procedure was repeated twice. The upper-phase solutions were combined and evaporated in a SpeedVac (Thermo-Fisher Scientific, Waltham, MA, USA) to dryness and yielded ca. 200 mg of terpene lactone-enriched sample (TLES).

2.4. High-speed countercurrent chromatography (HSCCC)

HSCCC was performed on two instruments: A CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research, Baltimore, MD, USA), equipped with a 120 mL column consisting of three serially connected coils of 1.6 mm i.d. PTFE tubing; a TBE-20A high-speed countercurrent chromatograph (Tauto Biotech, Shanghai, China), equipped with a 20 mL coil of 0.8 mm i.d. PTFE tubing. Both HSCCC systems were equipped with a six-port valve with a loop (3 mL and 0.5 mL, respectively) for sample injection and a PTR HPLC pump (Pharma-Tech Research, Baltimore, MD, USA) for solvent delivery. Fraction collection was accomplished with a Foxy Jr. HPLC fraction collector (Teledyne Isco, Lincoln, NE, USA).

2.5. Selection of HSCCC SSs

Several SSs in the families of chloroform-methanol-water (ChMWat) and hexane-ethyl acetate-methanol-water (HEMWat) [13] were tested in preliminary partitioning experiments for the suitability of HSCCC separation of terpene lactones from *G. biloba* leaf extract. Shake-flask tests were used to determine the partition coefficients (*K*) of the terpene lactones as follows: An enriched sample (TLES, 30 mg) was dissolved in 5 mL each of upper and lower phase of the biphasic SS. The solution was shaken vigorously and

centrifuged to break up any emulsion and speed up phase separation. The two phases were separated and dried down in a SpeedVac. Then, the upper and lower-phase samples were each dissolved in 500 μL of methanol- d_4 , and the ^1H -NMR spectrum was acquired under identical experimental conditions (see 2.7). In normal-phase countercurrent chromatography, the partition coefficient is the ratio of concentrations of an analyte in aqueous and organic phases $K = C_{\text{aqueous}} / C_{\text{organic}}$ (1). Therefore, the K value of each terpene lactone was calculated by determining the ratio of the absolute integrals of individual H-12 signals in the aqueous and the organic phases.

2.6. HSCCC fractionation

The HSCCC fractionation included two steps. The first step fractionation was carried out on the 120 mL HSCCC in normal-phase mode. Initially, the stationary phase (upper phase of ChMWat or lower phase of HEMWat SS) was pumped into the coil at a flow rate of 5 mL/min. After the coil was completely filled with stationary phase, the mobile phase was pumped into the coil at a flow rate of 1 mL/min in tail-to-head (ChMWat) or head-to-tail (HEMWat) mode, with the centrifuge spinning at 800 rpm. The stationary phase displaced by the mobile phase was collected in a graduated cylinder. The injection sample was prepared by dissolving 100 mg of the enriched sample in 1.5 mL each of upper and lower phase of the SS. Injection of the sample from the 3 mL sample loop occurred after the hydrodynamic equilibrium was established in the column, i.e., only mobile phase eluted from the column. The volume of stationary phase that was extruded during the equilibration process was measured and the stationary phase retention factor (S_f) calculated. Fraction collection was started at the time of injection. In total, 60 fractions were collected in 3 min intervals. These fractions were combined according to their composition, evaporated to dryness in the SpeedVac, and accurately weighed.

The second-step HSCCC fractionation was performed on either 120 mL or 20 mL HSCCC in normal-phase mode. Operation of the 120 mL HSCCC was the same as in the first-step, except that fractions were collected in 4 min intervals. Fractionation on the 20 mL HSCCC was performed at a flow rate of 0.50 mL/min, and the fractions were collected in 2 min intervals. The number of fractions collected was related to the retention volume of the compound to be isolated, which was determined by the partition coefficients of the target compounds and the stationary phase retention of the HSCCC system: $V_R = V_m + K \cdot V_s$ (2), where V_R , V_m , and V_s denote volume of retention, mobile phase, and stationary phase, respectively. In preparation for further ^1H -NMR analysis, all fractions were evaporated to dryness in the SpeedVac and stored in a desiccator *in vacuo* to eliminate moisture.

2.7. ^1H -NMR analysis

The ^1H -NMR spectra were recorded on a Bruker DPX-400 NMR spectrometer (Karlsruhe, Germany) in 5 mm NMR tubes (Norell, Landisville, NJ, USA) with the sample dissolved in 500 μL methanol- d_4 accurately dosed with an analytical syringe (Valco Instruments, Baton Rouge, LA, USA). The temperature was maintained at 300 Kelvin. Identical acquisition and processing parameters were used for all samples: Pulse program zg30, 65536 (64k) complex data points, spectral width 20.69 ppm, acquisition time 4.0 s, and receiver gain 1000. Depending on the concentration for each pair of upper and lower-phase samples, 256 or 512 scans were accumulated to achieve an adequate signal-to-noise ratio (S/N) for both qualitative and quantitative analysis. Raw data were processed with MestReNova v6.2.1-7569 (Mestrelab Research, Santiago de Compostela, Spain) software. Line resolution was improved by Lorentzian-Gaussian (LG) window functions (LB -1.0, GB 0.1) and three times of zero-filling, prior to Fourier transformation of the FID data.

3. Results and discussion

3.1. Measurement of *K* values of Ginkgo terpene lactones

The partition coefficient (*K*) is not only an important theoretical parameter in CS, but also of much practical value. It determines when a particular compound elutes from the CS column, i.e., the retention volume. In addition, *K* allows prediction of whether any two particular compounds can be separated, i.e., their resolution (R_s) from one another. The measurement of *K* values of target compounds is an important aid in the selection of appropriate CS SSs. The simplest method is to partition the pure analyte in the candidate biphasic SS and calculate the concentration ratio of the compound distributed into the two phases. However, for natural products and other target compounds which are unavailable commercially or otherwise in pure form, this requires purification from crude mixtures or synthesis. Both often preclude the practicality of the CS experiment from the start. While HPLC or GC is frequently used to work with mixtures, these methods are often associated with detection problems. Due to the absence of carbon-carbon unsaturation, terpene lactones show only a weak maximum UV absorption around 220 nm [14]. Therefore, UV is unsuitable for detection and quantitation purposes, leaving refractive index (RI), evaporative light scattering (ELSD), NMR or MS as likely potential techniques. RI and ELSD lack selectivity for distinction of the individual terpene lactones. Moreover, both of RI and ELSD, as well as MS require calibration with authentic standards for quantitative analysis. However, qHNMR is not limited by these factors due to the almost ubiquitous occurrence of protons in organic compounds. In addition, the absolute integral of the ^1H -NMR signal of an analyte has a linear relationship with analyte concentration in the sample [15]. Taking these advantages from this property of NMR, this study used a combination of classical shake-flask experiments and qHNMR analysis to simultaneously determine multiple *K* values of target analytes in mixtures (Fig. 3). The signals of H-12 of the five terpene lactones are well-separated singlets with δ 6.311 (BB), 6.029 (GA), 6.081 (GB), 6.097 (GC) and 6.044 (GJ) ppm and therefore, they were used for determination and quantitation purposes. Because the upper and lower-phase samples were analyzed under identical experimental conditions, the *K* values of each terpene lactone could be calculated as the ratio of absolute integral of corresponding individual H-12 signals in the two phases.

Typically, multi-step CS is performed by repeatedly fractionating the overlapping peaks using SSs from the same family only differing in the ratio of solvent composition. While the polarity match between the SS and the target compounds is important in CS, the different selectivity of the SS for the target compounds also matters. Therefore, it is usually worthwhile to pre-fractionate the sample in one SS, and then re-fractionate the overlapping peaks in an “orthogonal” SS, i.e., a system that is qualitatively different but has similar overall polarity. Chloroform and ethyl acetate, which are commonly used as solvents in chromatography, exhibit different relative dipole moments, and acid/base properties. Accordingly, they have been classified into two groups of organic solvents with distinct selectivity [16]. Thus, for an optimized separation, two families of CS SSs derived from these two solvents, ChMWat and HEMWat, were evaluated as candidates for the separation of the terpene lactones. In our previous studies, the ChMWat SS family was standardized to eight SSs with different composition ratios. The SSs were coded by a series of integer values from -3 to +4 which indicate the increasing polarity. Similarly, the HEMWat family consists of sixteen standardized SSs with a polarity range from -7 to +8 [13]. The *K* values of the five terpene lactones in three of these CS SSs were calculated as expressed in normal-phase HSCCC, as shown in Table 1. Based on the *K* values, BB, GA and GB were predicted to be separated in ChMWat +4 (10:7:3, v/v), but the GC/GJ pair was poorly resolved. In HEMWat +3 (4:6:4:6, v/v), however, the GC/GJ pair could be well separated, while the GA/GB pair was unresolved. Apparently, neither ChMWat +4 nor HEMWat +3 alone could be able to separate all five terpene lactones in a single HSCCC step.

3.2. Alteration of the hydrogen bonding characteristics of Ginkgo terpene lactones

Dimethyl sulfoxide (DMSO) is a practically universal solvent for small organic compounds, capable of dissolving compounds of a wide range of polarities. Two previous reports described the use of DMSO as a sample injection solvent to increase sample load or solubility in CS [17, 18]. However, the effect of DMSO on CS SSs has not yet been systematically studied. In the present study, a novel SS was created by adding 0.50% of DMSO (v/v) to HEMWat +3 (HEMSoWat +3/0.5%). Compared with the distribution properties in HEMWat +3, all five terpene lactones favored more the aqueous phase of HEMSoWat +3/0.5%. The *K* value increased 26% for GA, but only 2% for GB, which resulted in a much improved resolution of these two ginkgolides. The *K* values of GC and GJ both increased ca. 20% and their resolution was similar to HEMWat +3. This selective change of solubility behavior in aqueous and organic phase was apparently a consequence of the effect of DMSO upon the intermolecular and/or intramolecular hydrogen bonding of the terpene lactones in solvents. Another interesting observation was that the elution order of the GA/GB pair in HEMSoWat +3/0.5% was GA > GB. In ChMWat +4, however, their *K* values are reversed such that GA < GB, which is consistent with their polarities based on their number of hydroxyl groups. These observations further emphasize the different selectivity of HEMWat and ChMWat SSs and the value of selectivity changes that can be achieved by solvent modification in CS.

3.3. Design of orthogonality-enhanced HSCCC procedure

Several reports have described the application of multidimensional CS for the isolation of pure compounds from plant extracts using same SSs at various volume ratios [19-21]. However, strong chromatographic overlap was occasionally observed even in the 2nd CS dimension. As a result, sample cutting was needed to recover small amounts of pure compounds from the overlapping peaks. Based on *K* values measured by qHNMR-guided shake-flask experiments, it was initially determined that all five terpene lactones could be separated in a single run of HSCCC fractionation by using HEMSoWat +3/0.5%. In practice, however, the enriched sample (TLES) contained other compounds such as flavonoids and benzoic acid derivatives which exhibit chromatographic overlap with terpene lactones. Trial fractionation of the enriched sample was performed on a 20 mL HSCCC by using ChMWat +4 and HEMSoWat +3/0.5%, respectively. ¹H-NMR analysis of the ChMWat +4 fractions indicated that kaempferol, isorhamnetin and quercetin overlapped with BB, GA/GB and GC/GJ, respectively. In HEMSoWat +3/0.5%, however, kaempferol and isorhamnetin eluted at the front, while quercetin overlapped with GA/GB. Based on these results, the most practical procedure for purifying the five terpene lactones from the enriched sample was a two-step HSCCC fractionation procedure, using ChMWat +4 and HEMSoWat +3/0.5% as a pair of orthogonal SSs (Fig. 4). Using this orthogonality-enhanced HSCCC, the three originally interfering flavonoids, which are also considered bioactive compounds in *G. biloba*, can be purified as well.

3.4. HSCCC fractionation monitored by offline qHNMR

Efficient detection and rapid characterization of natural products plays an important role in the study of complex biological matrices, such as crude plant extracts. The preliminary identification of target components at the earliest stage of separation is a strategic element in guiding selective isolation procedures [22]. NMR is the most widely used method for structural elucidation of phytochemicals. This explains recent attempts to couple chromatography to NMR. Currently, LC-NMR is emerging as a powerful tool for separation and identification of unknown compounds in mixtures [23]. In contrast with other LC-coupled techniques such as UV, RI, ELSD or MS, NMR is not only capable of performing quantitative analysis, but also it provides a great deal of information for the full structural elucidation of natural products. While HSCCC shows excellent separation performance in

some aspects, coupling NMR to HSCCC has received little attention [24]. Much like HPLC-NMR, the construction of online HSCCC-NMR depends on the development of special interfaces and multiple solvent suppression techniques. Although further work is needed to overcome these difficulties, this study made extensive use of offline NMR to perform post-chromatographic analysis of the HSCCC fractions. A distinct advantage of offline qHNMR analysis is that the elution of identified compounds can be monitored and the separation performance can still be evaluated by constructing the molarity-based chromatograms corresponding to the eluting compounds.

Based on the proposed fractionation scheme (Fig. 4), 100 mg of enriched sample (TLES) was initially fractionated on a 120 mL HSCCC in normal-phase mode by using ChMWat +4. Stationary phase retention was 50% when the hydrodynamic equilibrium was established within the column. According to Eq. (2), the retention volume was 180 mL at K equals 2 in the chromatogram. Therefore, 60 fractions were collected in 3 mL intervals. In the range of fractions 21 to 52 where K values are 0-1.5, four fractions were combined into one fraction, yielding eight combined fractions (CFs). The mass distribution of these eight CFs is shown in Fig. 5. According to offline ^1H NMR analysis, CF-3 ($K = 0.35\text{-}0.55$) contained GA, GB, kaempferol and isorhamnetin; CF-4 ($K = 0.55\text{-}0.75$) contained BB and kaempferol; CF-7 ($K = 1.15\text{-}1.35$) contained GC, GJ and quercetin.

After the 1st step of HSCCC fractionation, CF-3 was subjected to the 2nd step of HSCCC fractionation in normal-phase mode using HEMSoWat +3/0.5%. Due to the smaller sample size (10.1 mg), a 20 mL HSCCC machine was selected to perform the fractionation. Stationary phase retention volume was 80% when the hydrodynamic equilibrium was established within the column. A total of 40 fractions were collected in 1 mL intervals. qHNMR analyses of eleven fractions CF-3-21 to CF-3-31 showed that the first three fractions contained GB and the six fractions CF-3-26 to CF-3-31 contained GA. The elution of GB and GA partly overlapped in three fractions CF-3-23 to CF-3-25. The mass ratio of GB and GA in these three fractions was 7.20 (86% GB), 0.99 (1:1 mixture of GB and GA), and 0.15 (85% GA), respectively.

3.5. Reconstruction of CS chromatogram and K analysis by ^1H NMR

As all fractions were analyzed offline by ^1H -NMR under the same experimental condition, the absolute integral of the ^1H -NMR signal of H-12 had a linear relationship with the concentration of terpene lactones in the sample. By using this absolute integral as a measure of the relative molar mass of each sample, the elution pattern of GA and GB were reconstructed as given in Fig. 6. Theoretically, all HSCCC elution, which does not exhibit sample solubility or overloading problems, follows a Gaussian distribution curve. Thus, the chromatographic peaks of GA and GB were simulated by Gaussian curve fitting following established the mathematical forms [25,26] as follows:

$$y_{\text{GA}} = 4.6533e^{-\left(\frac{n-27.19}{2.8604}\right)^2} \quad (R^2=0.995) \quad (3)$$

$$y_{\text{GB}} = 1.3014e^{-\left(\frac{n-22.62}{1.9700}\right)^2} \quad (R^2=0.997) \quad (4)$$

where n is the fraction number and y is the absolute integral of H-12. According to these simulated chromatographic peaks, the achieved experimental resolution (R_s) of GA and GB was approximated as follows. For retention time-based chromatography like HPLC or GC, $R_s = 2(t_{\text{R}2} - t_{\text{R}1}) / (w_1 + w_2)$. Similarly, for retention volume-based HSCCC, R_s can be calculated using the equation $R_s = 2(V_{\text{R}2} - V_{\text{R}1}) / (w_1 + w_2)$. In Fig. 6-C, it was easily

known that $V_{R/GA} \approx 27.2$ mL, $w_{GA} \approx 6.7$ mL, $V_{R/GB} \approx 22.6$ mL, and $w_{GB} \approx 5.6$ mL. Thus, R_s of GA and GB was ca. 0.74.

In analytical chromatography, the emphasis is on the resolution, while the critical parameters for preparative chromatography are the amount of compound produced per unit time, product purity, recovery and separation cost [27]. Although R_s of GA and GB (0.74) was less than 1.0, which is considered insufficient resolution in analytical chromatography, the recovery of GB was remarkably high at > 75% with purity of 95% when only three fractions CF-3-21, CF-3-22 and CF-3-23 are combined. This exemplifies the high preparative efficiency of CS.

By using the equation $K = (V_R - V_m) / V_s$, the K values of GA and GB were calculated as 1.45 and 1.16, respectively. In the shake-flask experiment (see 3.1), the K values of these two ginkgolides were measured as 1.46 and 1.18. The consistency between two experiments (1.45 vs. 1.46, $\Delta = -0.7\%$; 1.18 vs. 1.16, $\Delta = -1.7\%$) demonstrated that the measurement of K values by NMR (K -by-NMR) can efficiently guide targeted isolation by quickly locating the compounds of interest in a series of CS fractions without any further chemical analysis.

3.6. Prediction of achievable purity by ^1H NMR offline analysis of CS fractions

Due to the limit of detection (LOD) in qHNMR, a compound at the beginning or ending of elution which is present at a very low concentration level may not be detected. As a result, it can be difficult to determine if two compounds exhibit overlap and which fractions should be combined for the best balance of recovery and purity. However, this problem can be solved when considering that the chromatographic peaks are approximated as Gaussian distribution curves. With the help of Gaussian curve fitting, the breadth of chromatographic peaks can be predicted using the values of more concentrated fractions in the peak center. To establish this method, this study used the elution of GA and GB as an example (Fig. 7-A). The relative quantity of GB in fractions before CF-3-21 and after CF-3-24 was predicted to be below the LOD under the chosen conditions of the NMR experiment (mainly: S/N). In order to facilitate a prediction, the chromatographic peak of GB was first reconstructed by Gaussian curve fitting using the relative quantities of the four major fractions CF-3-21 to CF-3-24 as:

$$y_{GB} = 1.3240e^{-\left(\frac{n-22.60}{1.9040}\right)^2} \quad (R^2=0.998) \quad (5)$$

Thus, by using Eq. (5), the relative molar masses of GB in CF-3-20 and CF-3-25 were predicted as 0.2061 and 0.2782, respectively. It was also expected that CF-3-26 contained ca. 1.7% of GB which was not actually detected by NMR using the standard acquisition parameters (NS = 256). To evaluate the accuracy of this predicted value, CF-3-26 was re-analyzed by qHNMR with a much increased NS value of 8192 (8k) for 4-fold improved LOD. As expected, GB was confirmed to be present at 2.0 mol% (Fig. 7-B). Similarly, the chromatographic peak of GA was best fitted to five fractions CF-3-25 to CF-3-29 as a Gaussian equation:

$$y_{GA} = 4.5899e^{-\left(\frac{n-27.14}{2.8844}\right)^2} \quad (R^2=0.994) \quad (6)$$

The calculated relative molar masses of GA in CF-3-30 and CF-3-31 were as 1.0888 and 0.3339, respectively, and well matched the qHNMR values of 1.0826 ($\Delta = 0.57\%$) and 0.3530 ($\Delta = -5.4\%$), respectively.

This example demonstrates that Gaussian curve fitting enables prediction of the quantities of target compounds in each fraction, even if the concentration is below the LOD of the qNMR experiment. In addition, the breadth of each chromatographic peak as well as peak overlap can be assessed. Therefore, it is possible to determine which fractions need to be combined to achieve the best yield with a required level of purity without performing chemical analysis of all fractions.

3.7. Separation of bilobalide and ginkgolides C and J

CF-4 (12.9 mg) was also further fractionated on a 20 mL HSCCC using HEMSoWat +3/0.5%. Fractions were collected in 1 mL intervals. With the stationary phase retention of 80%, BB was present in fractions CF-4-18 to CF-4-22. Based on the retention volume, the K value of BB was calculated as 0.84, which was consistent with the values from a shake-flask experiment ($\Delta = 0.0\%$).

Although the sample size of CF-7 (23.9 mg) was within the loading capacity of a 20 mL HSCCC, quercetin was poorly soluble in the SS. Hence, quercetin eluted with tailing towards a greater retention. As a result, some co-elution with GC was observed despite their significantly different K values, as observed in trial fractionation (see 3.3). To increase the solubility of quercetin, 10 mg of CF-7 was fractionated a 120 mL HSCCC machine. Using HEMSoWat +3/0.5%, HSCCC fractionation was performed in normal-phase mode at a flow rate of 1 mL/min. At the time of injection, the system was equilibrated showing a S_f of 50%. However, there was a loss of stationary phase at an average rate of 0.1 mL/min until quercetin eluted from the column. After that, the system was re-equilibrated with a S_f of ca. 40%. According to the findings by Berthod, the stationary phase retention volume is directly related to the phase density difference [28]. Thus, it is possible that a high content of quercetin changed the phase density enough to cause a further loss of stationary phase until a new equilibrium was established. While the separation performance was not significantly influenced by 10% loss of stationary phase, in practice it was still difficult to mathematically monitor the dynamic process of stationary phase loss. qHNMR analyses of fractions CF-7-53 to CF-7-59 and fractions CF-7-64 to CF-7-69 followed by Gaussian curve fitting indicated that GC and GJ eluted following Gaussian distribution with equations as follows:

$$y_{GC}=2.1008e^{-\left(\frac{n-55.67}{2.5586}\right)^2} \left(R^2=0.985\right) \quad (7)$$

$$y_{GJ}=0.9341e^{-\left(\frac{n-66.58}{2.8228}\right)^2} \left(R^2=0.991\right) \quad (8)$$

In Fig. 8-C, it can be observed that GC and GJ were completely separated. Their R_s was calculated as ca. 1.50 (GC: $V_R \approx 222.7$ mL and $w \approx 28.5$ mL; GJ: $V_R \approx 266.3$ mL and $w \approx 29.4$ mL) which was considered a baseline resolution.

4. Conclusions

K -by-NMR did not require any pre-fractionation process or standard compounds but directly determined the K values of the five terpene lactones in the enriched sample. The K values not only reveal the resolution of target compounds in a particular SS, but also predict when the compounds elute from the column. The consistency between partitioning in shake-flask experiments and analyte retention in experimental CS demonstrated that K values can guide targeted purification of compounds of interest in complex mixtures, such as the terpene lactones in crude Ginkgo preparations.

In RP LC, solvent selectivity is often explored as a means of resolving difficult-to-separate analytes. Similarly, the selectivity of SSs is an important aspect of CS separation performance. This study proposes an approach using a pair of orthogonal SSs assembled in a sequential CS method. This study took advantage of the fact that chloroform-based and ethyl acetate-based SSs show very different selectivity on terpene lactones and flavonoids. This approach allows for a more efficient separation of terpene lactones from *G. biloba* than repeatedly using SSs from the same family.

At the onset of this study, it was observed that when trace quantities of DMSO were used to increase sample loading with a HEMWat+3 CS, a better resolution of GA and GB was obtained. Upon further investigation it was found that the addition of 0.5% DMSO increased the resolution of GA and GB, thus allowing for a separation of these two, otherwise almost equipolar lactones. Although the ability of an additional solvent to change partitioning behavior is well known, the effect of such a trace amount is without parallel. Clearly, DMSO has potential as a modifier in CS SSs by acting as a “resolution enhancer” for compounds with similar properties. Future work will focus on a systematic study of DMSO-doped CS SSs for better understanding the role of DMSO in CS of natural products.

While the Ginkgo terpene lactones are readily identified and quantified by their H-12 signals, even in crude extracts, peak overlap is frequently observed in the 1D ¹H (q)NMR analysis of natural products and especially complex mixtures. Thus, spectral dispersion represents an intrinsic limitation, even when applying ultra-high field NMR. However, as we have now also demonstrated for Ginkgo phytoconstituents [30], precise quantitation by NMR can be achieved for overlapping and/or complex signals and in crude preparations. The approach uses a combination of computer-aided ¹H NMR full spin analysis (“fingerprinting”) and 1D qNMR to quantitate several constituents simultaneously. Methods based on 2D NMR represent another alternative and are currently being developed. These advanced tools can be implemented into the CS methodologies present here, such as *K*-by-NMR, and have the potential to further enhance the “resolving” power of CS for the chemical complexity of natural products.

In summary, this study, for the first time, uses the qHNMR technique as an offline detection method for HSCCC fractionation. This combination of methods is generally applicable to CS and enables the assessment of chemical compositions in HSCCC fractions by providing structural information. The tandem of CS and qHNMR provides information well beyond elution curves generated by UV, RI or ELSD detectors. Moreover, combining qHNMR analysis with Gaussian curve fitting enables the quantitative representation of elution profiles of any detectable analytes, regardless of whether it has been identified or not. This provides unique information about the composition of fractions, which can be used to study the presence of minor constituents and analytes with overlapping elution/extrusion behavior, i.e., residual complexity (RC) of the resulting CS fractions (see [29] and references therein). The observed consistency of partition coefficients (*K* values) in concurrent shake-flask, experimental HSCCC/CS, and qNMR assays (*K*-by-NMR) is striking and underscores the future potential of combinations of NMR and CS methodology.

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Highlights

- Novel qHNMR-based approach to countercurrent partition coefficients (K values)
- Enabled targeted countercurrent separation (CS) of *Ginkgo biloba* terpene lactones.
- Established orthogonal solvent systems (SSs) for separation of Ginkgo lactones
- Developed DMSO-doped SSs as a new means of resolution enhancement in CS.
- Evaluated ID and residual complexity of eluents by qHNMR with Gaussian fitting.

Fig. 1.
The major terpene lactones in *Ginkgo biloba* L leaves.

Fig. 2.

The ¹H-NMR spectra of five terpene lactones (400 MHz, methanol-*d*₄). The ¹H-NMR signals of individual H-12 protons are singlets with chemical shifts (δ) 6.311 ppm (BB), 6.029 ppm (GA), 6.081 ppm (GB), 6.097 ppm (GC) and 6.044 ppm (GJ).

Fig. 3. An example of K -by-NMR

The upper and lower-phase samples of HEMWat +3 partition of the enriched sample (TLES) were subjected to ^1H -NMR analysis under the same quantitative experimental conditions. The K values of each terpene lactone were calculated as the ratio of absolute integral of individual H-12 signals in the lower and upper phases.

Fig. 4. The proposed orthogonality-enhanced HSCCC procedure

The scheme visualizes how the five terpene lactones can be well separated and isolated by using orthogonality-enhanced HSCCC in a pair of specially designed SSs. An enriched sample (TLES) is initially separated by ChMWat +4 into three combined fractions, zones ①-③: ①a mixture of isorhamnetin, GA and GB; ②a mixture of kaempferol and BB; and ③a mixture of quercetin, GC and GJ. These three samples are subsequently separated by an orthogonal SS of HEMSoWat +3/0.5%.

Fig. 5. 1st step of HSCCC fractionation of the enriched sample (TLES)

Fig. 5-A shows the mass distribution of the eight combined fractions (CFs) from the 1st dimension of HSCCC fractionation. The ^1H -NMR signals of the H-6 protons of the three major flavonoids in *G. biloba* were doublets with distinct chemical shifts: quercetin 6.192, kaempferol 6.199, and isorhamnetin 6.204 ppm. Together with the characteristic H-12 signals of the terpene lactones, they were used to determine the compositions of the CFs. The preliminary qHNMR analysis revealed that the masses of ginkgolides mainly accumulated in CF-3, -4 and -7 (Fig. 5-B). CF-3 contained ginkgolides GA and GB with a total content of ca. 70%. CF-4 contained BB with a purity of ca. 80%. While CF-7 had the greatest mass, only ca. 15% of it was a mixture of ginkgolides GC and GJ.

Fig. 6. Offline HSCCC-qHNMR analysis of the final purification step of ginkgolides A and B
 Panel A shows the stacked plots of the ^1H -NMR spectra of eleven fractions CF-3-21 to CF-3-31. Panel B shows a contour plot of the range of 5.990-6.130 ppm of these spectra. Using the absolute integral of H-12 as a measure of the relative molar mass of the terpene lactones in each fraction, the individual terpene lactone content of the eleven fractions were shown in Panel C. In addition, the corresponding elution curves were reconstructed by Gaussian fitting. Asterisks (*) represent the omitted residuals solvent signals (3.320 and 4.860 ppm).

Fig. 7. Matching and prediction of compound purity by Gaussian fitting of qHNMR-based elution profiles of GA and GB

A: The elution of GA and GB was each mathematically described as a Gaussian equation. The relative molar mass of these two ginkgolides in the fractions shown in the shaded area was predicted from the equations. **B:** GB was identified with a content of 2.0 mol% in Fr CF-3-26 by qHNMR analysis, employing 8k scans (NS = 8192).

Fig. 8. Offline HSCCC-qHNMR analysis of the final purification step of ginkgolides C and J
 Panel A shows the stacked plots of the ^1H -NMR spectra of thirteen fractions CF-7-53 to CF-7-59 and CF-7-64 to CF-7-69. Panel B shows a contour plot of the range of 6.099-6.130 ppm of these spectra. Using the absolute integral of H-12 as a measure of the relative molar mass of the terpene lactones in each fraction, the individual terpene lactone content of the thirteen fractions were shown in Panel C. The relative molar mass of seven fractions shown in shaded area was predicted from Gaussian curve fitting.

Table 1

Partition coefficients of five *Ginkgo biloba* terpene lactones in three polarity-adjusted SSs.

	ChMWat +7 (10:7:3)	HEMWat +3 (4:6:4:6)	HEMSoWat +3/0.5%
BB	0.68	0.78	0.84
GA	0.35	1.16	1.46
GB	0.53	1.16	1.18
GC	1.24	3.74	4.63
GJ	1.21	4.47	5.34