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Quantification of a Botanical Negative Marker without an Identical Standard: Ginkgotoxin in *Ginkgo biloba*

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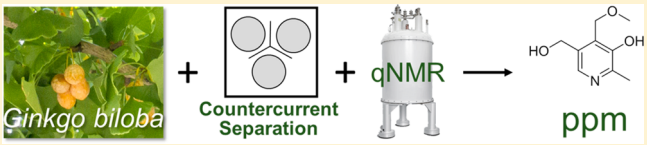
Supporting Information

ABSTRACT: A new strategy for the analysis of natural products uses a combination of quantitative ^{1}H NMR (qHNMR) and adsorbent-free countercurrent separation (CS) methodology to establish a quantification method for ginkgotoxin (4′-*O*-methylpyridoxine) in *Ginkgo biloba* preparations. The target analyte was concentrated in a one-step CS process using the ChMWat +2 solvent system ($CHCl_3$−MeOH−H_2O, 10:5:5) and subsequently assayed by qHNMR. While commercial *G. biloba* seeds contained 59 μg of ginkgotoxin per seed, the compound was below the limit of detection (9 ppm) in a typical leaf extract. Due to the enrichment potential and loss-free operation of CS, the combination of CS and qHNMR is a generally suitable approach for threshold assays aimed at quantifying target compounds such as botanical negative markers at the low ppm level. As the proof of principle is demonstrated for relatively small CS capacities (20 mL, 1:40 loading) and modest NMR sensitivity (n = 16, 400 MHz, 5 mm RT probe), the approach can be adapted to quantification at the ppb level. The procedure enables the quantification of a botanical negative marker in the absence of identical reference material, which otherwise is a prerequisite for LC-based assays.



Ginkgo biloba L., also known as "*Yin Xing*" in China and "*Ginnan*" in Japan, has documented use in China and Japan for the treatment of cough, bronchial asthma, irritable bladder, and alcohol abuse for over one thousand years.[1] Its seeds are used as food or dietary supplements to improve memory, reduce neuronal disorders, and increase blood flow.[1,2] Its leaf extract is known to have antioxidant activity and anti-inflammatory effects and to stimulate neurotransmission.[3]

Ginkgotoxin (4′-*O*-methylpyridoxine, **1**; Scheme 1) is considered the major toxic compound in ginkgo seeds, and it may be present in the leaves as well (see below). It behaves as an antagonist of vitamin B_6 (pyridoxine, **2**), acting as a pseudosubstrate of pyridoxal kinase.[4,5] Clinical reports indicate that overconsumption of *G. biloba* seeds has induced seizures or even caused death.[6] The National Institutes of Health has warned that roasted seeds could also be dangerous.[7]

The WHO has estimated that 80% of people worldwide rely on herbal medicines for the treatment of health problems. Sales of *G. biloba*-related products placed this botanical among the top-10 selling herbal products in the U.S. in 2007.[8] Virtually all of the dietary supplement products sold in the U.S. and Europe are extracts of the leaves. Although the presence of **1** in seeds is well established, the presence in leaves is controversial. Using RP-HPLC/UV, Wada et al.[9] were unable to detect the presence of **1** and, thus, concluded that there was no toxic potential in leaf extracts. Later, Arenz et al.[10] found that the concentration in leaves varied over time and at its peak exceeded even that in seeds.

Wada and Haga[11] have summarized adverse reactions to ginkgo seeds reported from 1930 to 1996 with more than 100 cases, most of which involved the occurrence of convulsions. Children under 6 years of age accounted for 74% of these cases, and mortality was up to 27%. Considering more recently reported cases,[6,12−14] it is clear that the high-incidence population was that of infants and of the aged. However, the dose/concentration threshold for **1**-induced seizures in patients is still unclear.[15] The presence and effect of **1** and its relationship to other bioactive markers of *G. biloba* have also been addressed in recent publications.[1,16]

Although **1** was first reported from *G. biloba* in 1985,[5] virtually no effective and convenient standard detection methods have been established to date. Considering botanical safety in general, and the challenge of identifying and quantifying a low-level negative marker (i.e., an undesirable compound in a botanical extract, such as a toxin), there is a need to establish methods for the accurate analysis of **1**. Herein, we report a method based on a combination of countercurrent

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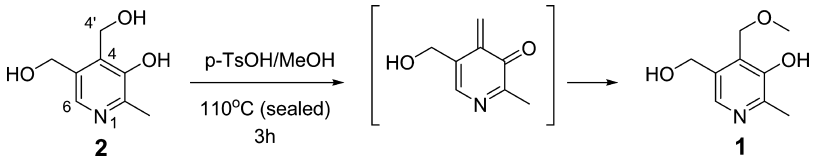
Scheme 1. Route for the Microwave-Assisted Synthesis of Ginkgotoxin (1) from Pyridoxine (2)



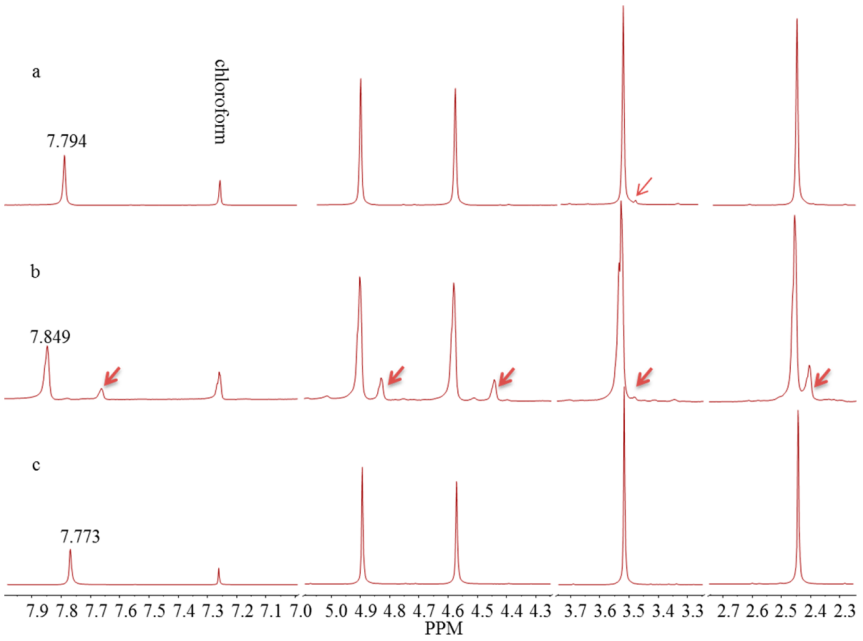


Figure 1. Purity evaluation by qHNMR (under identical quantitative experimental conditions; 400 MHz, in $CDCl_3$) of synthetic **1** after different purification: TLC (a) yielded 96% pure material, while NP-silica gel column chromatography resulted in a much lower purity of 81%. Purification by HSCCC gave the highest purity (98%). Only the resonance of H-6 exhibited a slight δ shift (0.08 ppm), which was attributed to pH changes due to the various degrees and patterns of impurities.

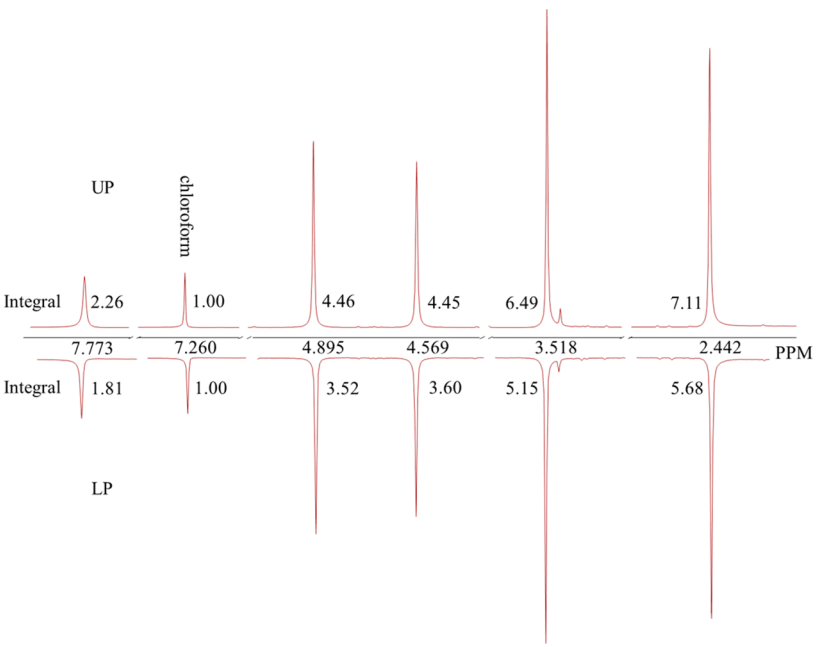


Figure 2. Example of the determination of the CS K value of synthetic **1** by qHNMR, directly from the upper and lower phases of a shake flask experiment with the ChMWat +2 solvent system (dried and redissolved in $CDCl_3$). The K values were calculated by (eq 2), as follows: $A_{\text{upper phase}}$ = (2.26/1 + 4.46/2 + 4.45/2 + 6.49/3 + 7.11/3)/5 = 2.25; $A_{\text{lower phase}}$ = (1.81/1 + 3.52/2 + 3.60/2 + 5.15/3 + 5.68/3)/5 = 1.79; K value = 2.25/1.79 = 1.26.

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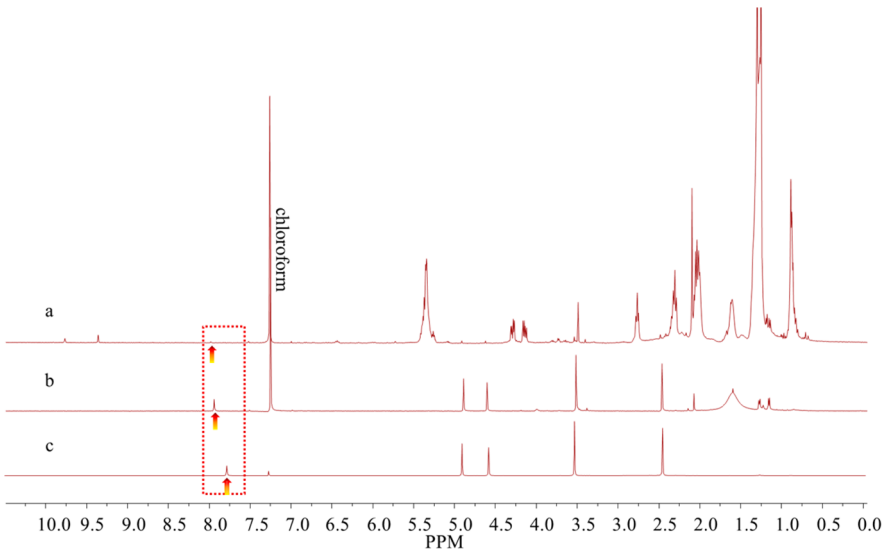

Figure 3. Enrichment and identification of ginkgotoxin (**1**) in *G. biloba* seeds. Quantitative (400 MHz, $CDCl_3$) ^{1}H NMR analysis of (a) the extract of a single seed; (b) the targeted HSCCC fraction corresponding to a *K* value of 1.62 (ChMWat, 10:5:5); (c) synthetic **1**.

separation (CS)[17] and NMR techniques. This approach can be used to detect (limit of detection [LOD]: 9 ppm) and quantify (limit of quantification [LOQ]: 30 ppm) **1** in methanolic gingko extracts. While we found **1** in seeds at 59 ppm, the content in a commercial ginkgo leaf sample was below the LOD.

Since the establishment of the ginkgo lactones and (bi)flavonoids as positive markers (unique botanical markers),[18−21] HPLC has been the mainstay of quality control of ginkgo preparations, and accordingly LC has dominated the analytical methodology applied to ginkgo. The development of alternative, multitargeted analytical methods for the quality control of *G. biloba* preparation has been the subject of previous reports.[22−24] The present study focuses on the low-level detection of the toxic negative marker, **1**, with subsequent quantification performed by an off-line high-speed counter-current chromatography (HSCCC) and quantitative ^{1}H NMR (qHNMR) analysis, respectively. Compared with solid-phase chromatographic methods, the particular advantage of HSCCC as a liquid-only CS method is the avoidance of absorption or degradation losses, as the sample recovery is in practice close to 100%.[25] Moreover, NMR spectroscopy is a nondestructive and near-universal detection method and can simultaneously perform both qualitative and quantitative analyses.[23,26,27] Hence, qHNMR was used initially for the determination of the distribution constant (*K* value, *K*-by-NMR)[23] for the selection of an HSCCC solvent system (Figure 2). Furthermore, the present study uses qHNMR for the quantification of **1** in *G. biloba* extracts. The qHNMR approach utilizes external calibration[28] and does not rely on the availability of high-purity **1** as an identical calibrant,[28,29] as would be the case for LC-based quantification methods. This approach can be used more generally not only to detect and establish threshold assays for botanical negative markers, but also to quantify minor components or unwanted additives in plant extracts, herbal medicines, food, and other complex nature-derived commercial preparations. The lack of a requirement for identical calibrants is advantageous for natural products and other rare chemical entities, such as **1**, which are not readily available commercially.

RESULTS AND DISCUSSION

Synthesis of Authentic Reference Standard. Despite the prospect of establishing an analytical method that does not rely on the authentic compound as a calibrant, the availability of an authentic standard remained indispensable for establishing an analytical method with high specificity for the analyte, in this case, **1**. Because commercial samples of **1** were unavailable, **1** was synthesized in-house. The method of synthesis was a modification of Harris' method[30] using *p*-toluenesulfonic acid for activation of pyridoxine via the intermediate *o*-quinone methide. While this reaction is per se reversible, excess MeOH pushes it to the product. The synthetic route is outlined in Scheme 1, and the yield of **1** was 56%. In order to assess the efficiency of CS and solid-phase-based preparative purification methods, preparative-TLC and silica gel column chromatography (both using 5% MeOH in EtOAc) were compared with HSCCC using ChMWat (10:5:5) using qHNMR, as summarized in Figure 1. The pH characteristics of the samples and/or residual impurities induced a 0.08 ppm downfield shift of only the H-6 (s, 1H), resonating at δ 7.773 in the HSCCC purification product, which showed the highest qHNMR purity (98%, Figures 1 and 3). Although the effect of pH on the pyridine derivative, **1**, was a primary consideration, the chemical shift effect was not observed in the sample neutralized by $NaHCO_3$ (data not shown), indicating that residual traces of, for example, reactants and/or other impurities are able to affect the exact chemical shift of H-6.

The ^{1}H NMR spectrum in Figure 3c can function as a reference standard for identity of the target marker, **1**, and the specificity of the subsequently developed qHNMR method. The synthesized **1** was also used for determination of the best CS solvent system to be used for the HSCCC procedure. Due to the low concentration of **1** in *G. biloba* extracts, synthesized **1** was also employed for piloting the enrichment of **1** in assay samples.

Targeted Purification by High-Speed Counter-Current Chromatography. The generally useful estimation of solvent systems (GUESS)[31] method represents a rapid and practical method to link CS *K* values and TLC retention characteristics (R_f values). By using GUESS, a ChMWat system was chosen

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Table 1. *K* Values of the Target Compound, Ginkgotoxin (1), in the Shaking Trials Using Two CS Solvent System (SS) Families Based on $CHCl_3$ ($CHCl_3$–MeOH–H_2O, syn. ChMWat) and EtOAc (*n*-Hex–EtOAc–MeOH–H_2O, syn. HEMWat), Which in the GUESS System[31] Correspond to the $CHCl_3$- and EtOAc-Based TLC SSs, SSC and SSE, Respectively

	TLC			HSCCC		
trial no.	solvent system	volume ratio	R_f value	solvent system	volume ratio	*K* value
1	SSE 5	5:5	<0.05	HEMWat 0	5:5:5:5	0.06
2	SSE 7	7:3	<0.05	HEMWat +4	3:7:4:6	0.33
3	SSE 9	9:1	<0.05	HEMWat +7	1:9:1:9	0.29
4	SSC 1	100:0:0	0.31	ChMWat −3	10:0:10	8.03
5	SSC 6	85:15:0.5	0.47	ChMWat +2	10:5:5	1.26

that would deliver **1** to the "sweet spot" of the chromatogram. Based on previous studies,[31] compounds with R_f values between 0.29 and 0.71 (optimal 0.5) will have *K* values between 0.4 and 2.5 (optimal = 1) and be eluted in the sweet spot of an HSCCC run. Table 1 summarizes all partition trial results. Using TLC solvent systems (SSs) based on $CHCl_3$–MeOH–H_2O (SSC, Table 1) as guidance, the R_f value of **1** in the SSC6 is 0.47, which is around an optimal value (0.5). The partition trials were also analyzed using the *K*-by-NMR approach,[23] to give a *K* value of **1** in ChMWat +2 of 1.26, comfortably in the CS sweet spot between 0.4 and 2.5. The systems HEMWat 0, +4, and +7 as well as ChMWat −3 were also tested. A partition trial using ChMWat +2 is shown in Figure 2 as an example of a *K*-by-NMR determination. The *K* value was calculated by eq 2 (Experimental Section).

HSCCC has previously been employed to effect chemical subtraction of a single target compound from a crude plant extract.[32] The method can work in a single step and with high selectivity. Accordingly, the recommended working range for the HSCCC chemical subtraction is $1< K < 5$. Thus, a *K* value of 1.26 indicates that the compound is an applicable target for this procedure. Indeed, this *K* value was used to perform a pilot HSCCC run and determine the congruence between predicted elution volume and the actual fraction(s) containing **1**. Under experimental conditions, **1** was eluted in a fraction with a *K* value centered at 1.47. Considering potential pH effects in crude extract samples (see also the above discussion of ^{1}H NMR data), this matched the predicted elution behavior well. According to this result, as long as the content of **1** is over the limit of detection, enrichment of **1** could be performed in a single HSCCC run. This also complemented the outcome of the trial of three different preparative purification methods for the synthetic material of **1** (Figure 1).

The *K* value of a compound in any two-phase SS is a physical constant, which is specific to that compound and that solvent system.[17] One additional consideration is that particularly natural products extracts typically contain complex chemical matrixes, which together with varying sample concentrations (see Figure 5 in ref 33) and pH effects can affect the actual partition behavior in a CS experiment via, for example, solvent strength modification of solutes and analyte–analyte interaction. However, the *K* value of **1** in an HSCCC enrichment procedure with *G. biloba* extracts should still be around 1.47. This was confirmed by an HSCCC separation of seed extract (Figure 3), in which **1** was indeed enriched in a pooled fraction centered at a *K* value of 1.62. On the basis of these results, the *K* range from 1.4 to 1.7 was chosen as a target for the collection of HSCCC fractions of the leaf extract for subsequent quantification by qHNMR.

Quantitative ^{1}H NMR Analysis. $CDCl_3$ was selected as the solvent for all qHNMR analyses. Using this method, the power of HSCCC as a means of chemical subtraction is clearly demonstrated in Figure 3, which shows the difficulty in quantifying **1** in a seed extract sample (Figure 3a), compared with the ready quantification after a single-step HSCCC enrichment (Figure 3b). Using the qHNMR results, the CS enrichment factor achieved for **1** was 282, which in our experience is a remarkably high value. The molarity-based quantification of **1** was achieved using caffeine as external calibrant (EC) and the residual solvent signal ($CHCl_3$ in $CDCl_3$) for a combined external/internal calibration (ECIC; Table 2; see also ref 28 for details about qHNMR calibration).

Table 2. Integral Values of the qHNMR Quantification of 1 in *G. biloba* Seed Extract Using the Combined Internal/External Calibration with Caffeine and the Residual Solvent Signal

caffeine			**1** in HSCCC fraction 6 of seed extract		
	δ	normalized integral		δ	normalized integral
1	7.260[a]	1.00	1	7.260[a]	1.00
2	7.501[b]	1.04 (1H)	2	7.947[b]	0.10 (1H)
3	3.992[b]	3.22 (3H)	3	4.908[b]	0.20 (2H)
4	3.588[b]	3.23 (3H)	4	4.622[b]	0.20 (2H)
5	3.412[b]	3.31 (3H)	5	3.537[b]	0.35 (3H)
			6	2.490[b]	0.31 (3H)

[a]Caffeine. [b]Residual solvent signal.

All data were obtained based on NMR peak area (integration). From the qHNMR analysis, the method specificity for **1** (structural ID) is clearly confirmed, and the content of **1** contained in the investigated commercial seeds was shown to be 59 μg per seed. This matches well with a previous report,[15] according to which one *G. biloba* seed contains about 80 μg of **1**. The commercial leaf sample used in the present study showed no detectable qHNMR signal in the extract (Figure 4) nor in the combined HSCCC fractions that represented the target *K* value range from 1.4 to 1.7. Meanwhile, both spiked and unspiked samples were extracted using the USP standard method and analyzed by qHNMR. Results from the spiked sample indicate that **1**, if contained in ginkgo leaf extract, does indeed elute in the target HSCCC fractions. However, **1** could not be detected in a ginkgo leaf extract prepared by using the USP standard method, which eliminated the possibility that the extraction method has a significant effect on the present method. Hence, it can be concluded that the content of **1** in the investigated sample of ginkgo leaves was less than 9 ppm, corresponding to the LOD.

Ginkgotoxin Levels in Ginkgo Preparations. A review of the reported cases of convulsion induced by **1** found that the range of serum concentrations in affected patients who typically consumed three daily doses of 1–2 mL of ginkgo preparation

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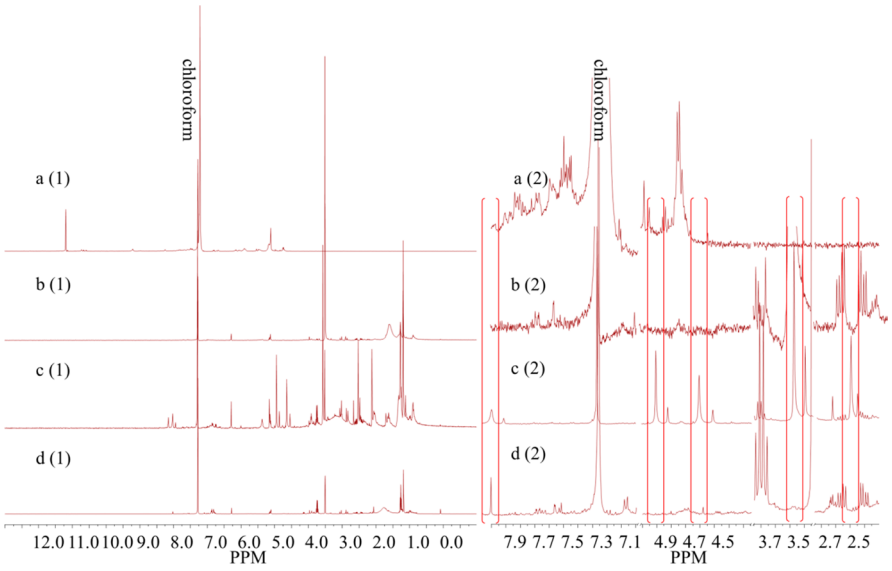

Figure 4. Results of the qHNMR analyses (400 MHz, in $CDCl_3$) of a commercial *G. biloba* leaf sample: (a) crude methanolic extract; (b) enriched HSCCC fraction of (a), corresponding to the target *K* value range of 1.4 to 1.7; (c) *G. biloba* extract prepared using the USP and spiked with 2 mg of **1** and subject to the same HSCCC enrichment as in (b); (d) method for the same extract as in (c), but unspiked and also subjected to the HSCC enrichment as for (b). Shown are the entire spectra (1) and representative expansions (2) for the signals of **1**. The chemical shifts of **1** are bracketed in red.

was between 90 and 484 ng/mL.[2] Accordingly, a reasonable approximation of a safety threshold for **1** that would likely avoid lethality can be calculated as follows:

$$m = cV \quad (1)$$

where m is the mass of intake of **1** by the patient, c is the serum concentration, and V is the blood volume in an adult (averaging around 5 L). Although the threshold level for **1** required to induce seizures is still unclear, a blood level of 90 ng/mL could potentially be reached by the consumption of as few as 8 ginkgo seeds or an equivalent preparation thereof. In a survey of commercial liquid ginkgo preparations sold in Europe, the detected amounts of **1** ranged from 0.01 to 9.77 μg/mL.[10] The survey further showed that the content in seeds and leaves depends on the time of the year of harvest and that widely used ginkgo preparations are not necessarily ginkgotoxin-free products.

The present study employed a 20 mL HSCCC instrument equipped with a 500 μL sample loop, which was used to inject the extract from ∼1 g of crude ginkgo preparation. The qHNMR spectrum used for the quantification of **1** in the ginkgo seed sample exhibited an average signal-to-noise (S/N) value of the target signals of 39:1, and this was achieved with the basic configuration of a 400 MHz NMR instrument equipped with a 5 mm RT probe and acquiring 16 transients (ns = 16). Considering the result of our recent qHNMR validation study,[26] the LOD of the present off-line HSCCC−qHNMR method at 9 ppm corresponds to a qHNMR S/N of 3:1. Accordingly, as the LOQ is 3.3 times the LOD, this basic qHNMR procedure already works quantitatively at 30 ppm and above. This method can readily be adapted to accommodate a much lower level for both LOD and LOQ, and this can be achieved by two straightforward means: (i) increase of HSCCC sample loading; (ii) increase of qHNMR sensitivity. Regarding (i), the use of abundant CS instruments with 200−300 mL total volume will enlarge the sample loading capability by 10- to 15-fold compared to the present setup. This takes advantage of the fully predictable scale-up capability of CS.[34] With respect to qHNMR sensitivity (ii), there are numerous options: possibly the foremost is to increase the number of scans (ns) from the ns = 16 used presently, keeping in mind that the sensitivity increase is proportional to the square root of the ns increase. Other options include the use of higher magnetic fields (e.g., 500 MHz to 1 GHz) and cryoprobe and/or microcryoprobe technology.[35] In summary, for example, the use of ns = 256 on a 600 MHz 5 mm cryoprobe-equipped instrument will result in a 10- to 20-fold or better overall sensitivity increase. Together with the potentially increased CS loading capacity, this will enhance the overall sensitivity by >100-fold to an LOD of 0.3 μg, equivalent to detecting 0.09 ppm (LOD) of **1** in analytical liquid ginkgo preparation. This shows that the method can be adapted even to practically unimportant levels of **1** in commercial liquid ginkgo preparations if needed.

CONCLUSION

To summarize, the present study established an off-line HSCCC−qHNMR detection method for determining the ginkgotoxin (**1**) content in ginkgo preparations. The key aspects of this methods are as follows: (i) using a modified procedure, an authentic standard of **1** was synthesized and characterized; (ii) The GUESS method was applied to rapidly select an efficient CS solvent system, and as a proof of principle for the suitability of the GUESS prediction, the *K* value of **1** was rapidly measured using the *K*-by-NMR approach. The compound was then successfully enriched by HSCCC of an extract of ginkgo seeds, which are known to contain **1**; (iii) quantification of **1** was performed by qHNMR in $CDCl_3$ solution, using the spectroscopic characteristics of synthetic **1** as reference for identity and assay specificity, and by applying combined external/internal calibration with high-purity caffeine as EC and the residual solvent signal as IC.

This methodology could have broader impact in various fields: generally and with regard to (i), it facilitates access to authentic reference material of **1** by synthesis and subsequent CS purification, which could be useful for LC quality control

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purposes. Considering the recent availability of automated CS instrumentation, the enrichment−detection approach (above points ii and iii) could potentially be employed for exploiting threshold levels of **1** in body fluids related to seizures and for the determination of **1** content in commercial products. In more generalized terms, this approach can potentially be transferred to other low-level metabolites and can be used to detect ppm levels of any target compound from a natural or commercial source. Finally, the case of **1** shows the enrichment potential of CS and its power for chemical subtraction[32] and targeted isolation of toxins and other bioactive principles of interest.

EXPERIMENTAL SECTION

Materials and Reagents. Whole *G. biloba* seeds (commercial name: White Nut) were purchased in a local grocery store of Chinatown, Chicago (origin of material: Hongchanglong, China). Powdered *G. biloba* leaves were sourced from Mountain Rose Herbs (lot no.: M10579, Eugene, OR, USA). Both samples were stored at −20 °C until analyzed. Pyridoxine hydrochloride, caffeine (>99%), and $CDCl_3$ (99.8 atom % D) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). All solvents were ACS grade, purchased from Pharmco-AAPER (Crookfield, CT, USA), and were re-distilled before use.

Extraction. The outer shells of the seeds were peeled off, and the meat was dried at 37 °C, then powdered by trituration, and stored at −20 °C. Extraction was performed according to ref 10 briefly as follows: 100 g of the powder was transferred to a 1 L flask, 500 mL of freshly distilled MeOH was added, and the mixture was shaken horizontally at low speed for 1 h. The extract was filtered, and each extraction was performed three times. The combined extract solutions were concentrated on a rotary evaporator at 30 °C under vacuum. The concentrated sample was stored at −20 °C. Leaf extractions were performed either in the way mentioned above or by the USP standard method.

Synthesis of Ginkgotoxin (syn. 4′-*O*-methylpyridoxine, 1). The method of Harris et al.[30] was modified in order to produce **1**. Pyridoxine hydrochloride (100 mg) and *p*-toluenesulfonic acid (4 mg) were combined in MeOH (1 mL) and heated at 110 °C (sealed) for 3 h in a microwave. The CEM Explorer 48/72/96 automated microwave synthesizer was used for microwave-heated and sealed reactions, controlled by an external computer loaded with the Synergy application software (version 1.1). The solution was cooled, the mixture filtered, and the solvent evaporated in vacuo. Aliquots of the crude residue were purified by preparative-TLC (5% MeOH in EtOAc), silica column (5% MeOH in EtOAc), and HSCCC (ChMWat, 10:5:5, v/v/v). The structural characterization of **1** was based on NMR (1D ^{1}H and ^{13}C NMR and 2D ^{1}H,^{13}C-HMBC in DMSO-d_6; see Supporting Information) and HRESIMS. Related physical properties are listed below.

Ginkgotoxin (1): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.773 (s, 1H, H-6), 4.895 (s, 2H, H-5), 4.569 (s, 2H, H-4), 3.518 (s, 3H, H-4), 2.442 (s, 3H, H-2); HRESIMS *m*/*z* 184.08954 $[MH]^+$ (calcd for $C_9H_{13}NO_3$, 183.08956).

Selection of Solvent Systems for Countercurrent Separation. Five biphasic solvent systems from the HEMWat and ChMWat SS families[31] (Table 1) were assayed using partition shaking trials. To confirm an optimal *K* value for the HSCCC experiment, the following procedure was used: in a separatory funnel, 5 mg of **1** was added to 10 mL of a mixture containing equal volumes of the upper and lower phases of the two-phase SS, and the mixture was shaken adequately to equilibrate the sample. The two phases, UP and LP, were separated and evaporated.

$$K\text{values} = A_{\text{upper phase}}/A_{\text{lower phase}} \quad (2)$$

where $A_{\text{upper phase}}$ is the average NMR peak area of the target compound in the upper phase and $A_{\text{lower phase}}$ is the average NMR peak area of the target compound in the lower phase. An example is given in Figure 2.

High-Speed Counter-Current Chromatography. CS was performed using a TBE-20A HSCCC (20 mL, Tauto Biotech, China), equipped with a 500 μL sample loop, pump, UV detector, thermostatic circulator, and data processing system (Cherry One, Cherry Instruments, Chicago, IL, USA). The procedure was as follows: 100 mg of extract was dissolved in 500 μL of ChMWat (10:5:5) made up from equal parts of the two phases. The solution was loaded into the injection loop. Then, the column was rotated at 800 rpm and filled with UP as the stationary phase. The LP was pumped as the mobile phase in a head-to-tail direction at a flow rate of 0.5 mL/min, and the column was rotated at 2000 rpm.[36] After the hydrodynamic equilibrium between the two phases was reached, i.e., the eluate had changed from stationary to mobile phase, the sample solution was injected through a six-port valve. Throughout the separation, the *K* values were monitored by the Cherry One system. Outflow was collected by a Foxy Jr. fraction collector (Teledyne Isco, Lincoln, NE, USA) and matched to these *K* values.

General Instrumentation. HRESIMS was performed on a Waters Synapt mass spectrometer (Waters, USA). NMR measurements were performed on a Bruker DPX-400 spectrometer (Karlsruhe, Germany) in 5 mm NMR tubes (Norell, Landisville, NJ, USA), using Bruker standard pulse sequences at 25 °C (298 K).

Quantitative ^{1}H NMR (qHNMR) Analysis. The samples were dissolved in 600 μL of $CDCl_3$ using an analytical syringe (Valco Instruments, Baton Rouge, LA, USA). A total of 16 scans (ns) were acquired, collecting 64 k of time domain data and using a 30 degree excitation pulse as well as a relaxation delay (D1) of 30 s. The spectra were analyzed using MestReNova v6.2.1−7569 (Mestrelab Research, Santiago de Compostela, Spain) software. Line resolution was improved by applying a Gaussian−Lorentzian window functions (GB 1.0, LB −0.3) and zero-filling to 256 k prior to Fourier transformation of the FID. Baseline correction used a fifth-order polynomial function, and phase correction was done manually.

ASSOCIATED CONTENT

Supporting Information

NMR spectra (1D ^{1}H and ^{13}C NMR and 2D ^{1}H,^{13}C-HMBC) for **1** in DMSO-d_6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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DEDICATION

Dedicated to Prof. Dr. Otto Sticher of ETH-Zurich, Zurich, Switzerland, for his pioneering work in pharmacognosy and phytochemistry.

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Quantification of a Botanical Negative Marker without an Identical Standard – Ginkgotoxin in *Ginkgo biloba*‖

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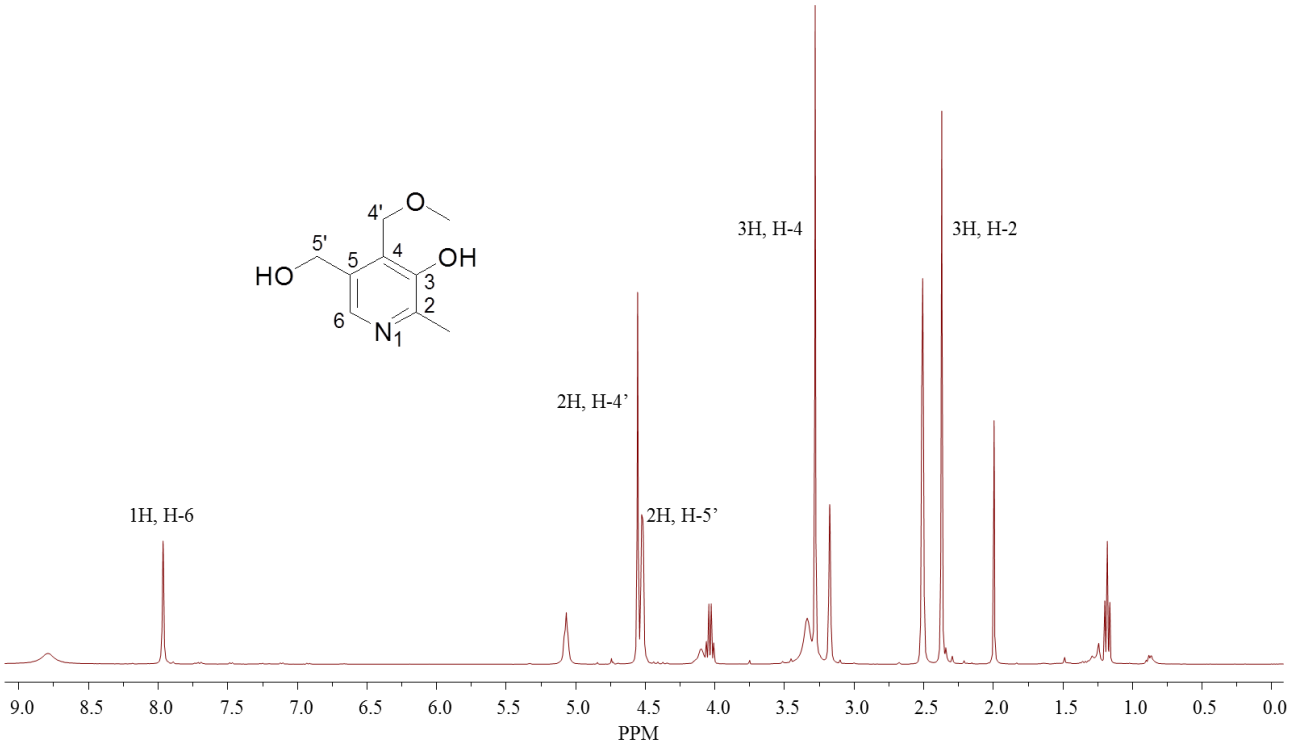
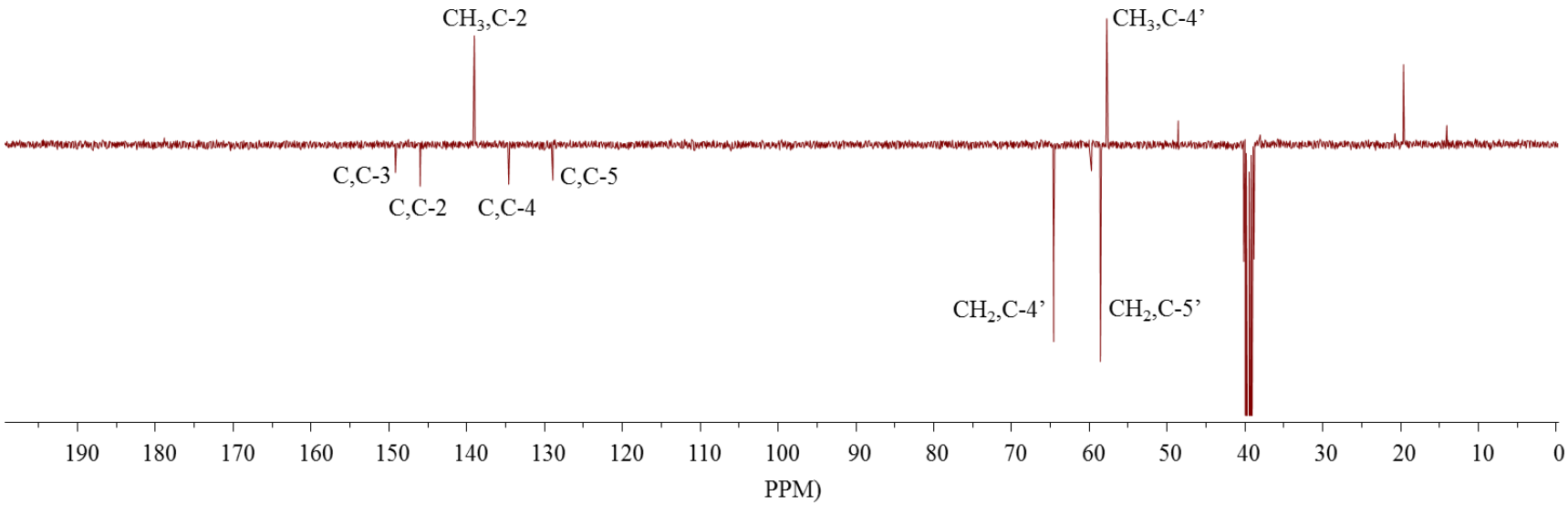
■ SUPPORTING INFORMATION

S1. NMR Spectral Identification of Ginkgotoxin (1) (400/100 MHz, DMSO-*d*$_6$)

1D ^{1}H NMR Spectrum



1D ^{13}C NMR Spectrum



2D $^{1}H,^{13}C$-HMBC Spectrum