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Preparative isolation of terpene trilactones from *Ginkgo biloba* leaves

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Abstract

This study investigated and compared some techniques for the preparative isolation of terpene trilactones, including ginkgolides (GA and GB, etc.) and bilobalide (BB), from *Ginkgo biloba* leaves. The crude *Ginkgo biloba* L. extracts (GBE) were prepared using an extractor with solvent refluxing operated under an optimal extraction condition. The extraction yield was 20–23% and the purity of terpene trilactones was about 1.0–1.4 wt%. Before the isolation operations, the extracts were dissolved in de-ionized water. The isolation procedures included the method of liquid–liquid extraction and the method of column chromatography. For the method of liquid–liquid extraction using ethyl acetate as the organic solvent operated under the optimal extraction conditions, the purity, concentration ratio, and yield of terpene trilactones were 13.5–18.0%, 15–16, and >99%. For the method of column chromatography, XAD-7HP, XAD-4, and C-18 adsorbents with different polarities were used as the packing materials. Only for the XAD-7HP column, a part of more polar impurities was efficiently separated with the majority of terpene trilactones by a proper step-gradient elution, which resulted in an efficient isolation: the purity, concentration ratio, and yield of terpene trilactones were ∼20, ∼15, and ∼80%. In comparison, the XAD-7HP column achieved the highest purity, but at the expense of the yield of terpene trilactones; on the contrary, the liquid–liquid extraction method, achieving the highest yield but with a slightly lower purity, was proved to be superior to the method of column chromatography in the current isolation stage.

Keywords: *Ginkgo biloba* leaves; Preparative isolation; Liquid–liquid extraction; Terpene trilactones; Bilobalide; Ginkgolides

1. Introduction

The terpene trilactones of *Ginkgo biloba* leaves, containing mainly ginkgolides (ginkgolides A, B, C, and J, abbreviated as GA, GB, GC, and GJ) and bilobalide (abbreviated as BB), are selective platelet-activating factor (PAF) antagonists and possess the neuro-protective effect. Especially, GB has the most strong potent among these terpene trilactones and is now used as the PAF antagonist for the prevention and treatment of thrombus, illness of blood vessel of heart and brain, arrhythmia, asthma, bronchitis, senile dementia and allergic reaction [1,2]. Fig. 1 shows the chemical structures of terpene trilactones, which are cage-like molecules full of oxygen [3].

Because of the potential usefulness of pure GB or a mixture of ginkgolides as novel drugs as well as for quality control purpose, there is a high demand for a high purity of these compounds. Current procedures for the isolation of terpene trilactones have been discussed in the analysis of terpene trilactones in extracts from *Ginkgo biloba* L. (GBE), where the liquid–liquid extraction method [3–6] and the adsorption separation method [1,3,6–8] have been used. Only a few literatures discussed the isolation of terpene trilactones in preparative scale. Van Beek and Lelyveld [9] preparatively isolated and separated the pure compounds of terpene trilactones using medium-pressure liquid chromatography on silica impregnated with 6.5% NaOAc with a gradient elution. Yu et al. [10] preparatively isolated the mixture of terpene trilactones using the extraction with organic solvent and adsorption with resins, where the final product containing over 70% terpene trilactones with a yield of 68%. In spite of the above studies, there still exists a need for processes which are efficient, relatively inexpensive, and easy to scale-up into industrialized usage.

This study is aimed to develop an efficient and economic technique for the preparative isolation of ginkgolides and bilobalide with high purity, high yield and high production rate. The entire isolation procedure for ginkgolides and

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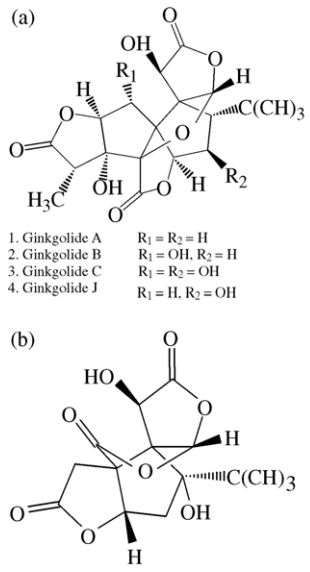

Fig. 1. Structures of terpene trilactones. (a) Ginkgolides and (b) bilobalide.

bilobalide from *Ginkgo biloba* leaves is represented in the flow diagram depicted in Fig. 2. The isolation procedures investigated in this study included the method of liquid–liquid extraction and the method of column chromatography. In the method of liquid–liquid extraction, ethyl acetate was chosen as the organic solvent and the effects of volume ratio, temperature, duration time, and times of extraction on the extraction performance were investigated. In the method of column chromatography, three different packing materials, i.e. XAD-7HP, XAD-4, and C-18 adsorbents, with different polarities were used. The column operation included the adsorption, washing, elution and regeneration steps in sequential. The effects of packing material and corresponding column operating conditions on the column performance were investigated. Finally, the isolation performances, in terms of purity, concentration ratio, and yield of terpene trilactones, between these two methods were compared.

2. Experimental

2.1. Materials

Dry green *Ginkgo biloba* leaves were obtained from Zhejiang province, Mainland China. Terpene trilactones standards including Ginkgolide A and B (GA and GB, 90% purity) and bilobalide (BB, 95% purity) were purchased from Sigma (St. Louis, MO, USA). Purified water was obtained using a Milli-Q purifier (Millipore, USA). Methanol, acetonitrile, ethyl acetate, and tetra-hydrofuran (THF) were bought from TEDIA (Fairfield, OH, USA), and ethanol was from TCI (Tokyo, Japan). All the organic solvents used were of high-performance liquid chromatography (HPLC) grade and were ultrasonically degassed before use.

Amberlite XAD-7HP and XAD-4 polymeric adsorbents were from Rohm and Haas company (Philadelphia, PA, USA), each with the particle size of 250–840 μm (20–60 mesh). Octadecyl bonded silica gel (C-18) adsorbent was from J.T. Baker (Pillipsburg, NJ, USA), with the particle size of 40 μm. Based on the "like attracts like" principle [11], XAD-7HP adsorbent, possessing intermediate polarity due to its both aliphatic and acrylic structure [12], is effective for adsorbing non-polar compounds from polar solvents and polar compounds from non-polar solvents; XAD-4 and C-18 adsorbents, possessing non-polarity due to their aromatic

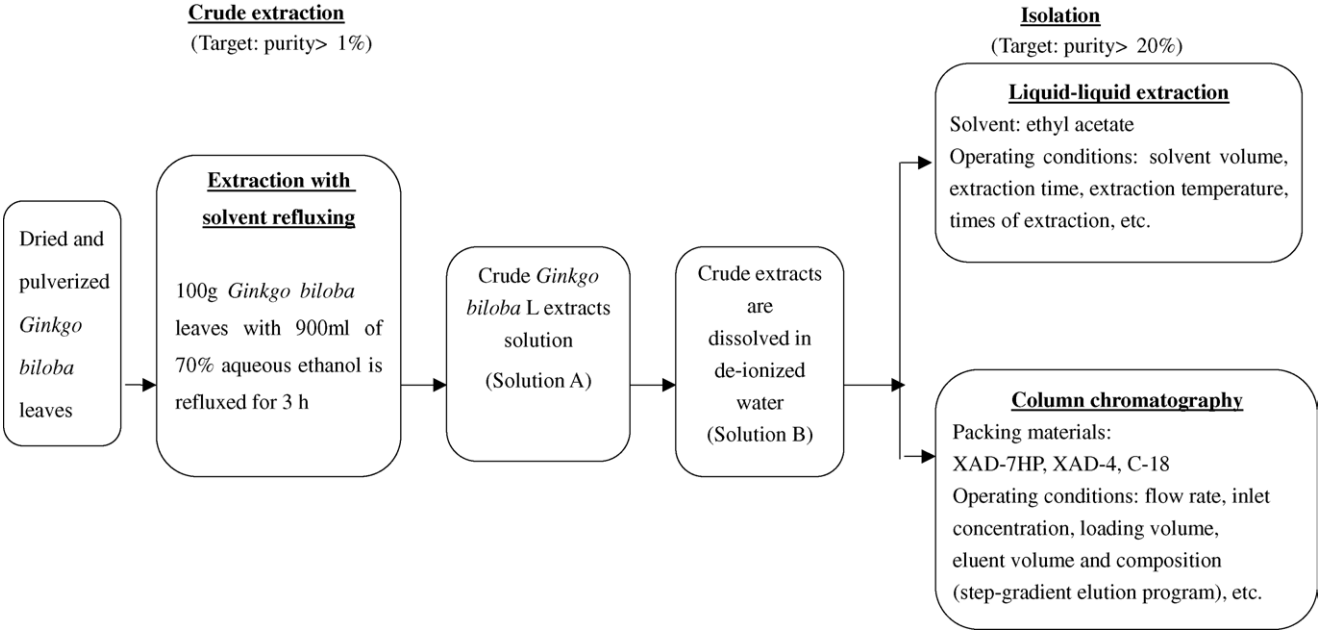

Fig. 2. Flow diagram of the entire isolation procedure for terpene trilactones from *Ginkgo biloba* leaves.

structure [13] and long hydrocarbon chain structure [14], respectively, are effective for adsorbing non-polar compounds from polar solvents.

2.2. Preparation of the crude Ginkgo biloba L. extracts

Prior to use, dry *Ginkgo biloba* leaves were further dried in an oven at 60 °C for 5 h and then pulverized. A batch of 100 g dried and pulverized *Ginkgo biloba* leaves and 900 mL of 70% aqueous ethanol were placed in a 1000 mL conical flask, and the solution was refluxed on a hot-plate for 3 h. The concentrated extracts of the first extract solution (Solution A) were then dissolved in a certain amount of de-ionized water to form the second extract solution (Solution B). Some water-insoluble lipophilic components were precipitated and separated. Solution B will be used as the aqueous phase solution for the liquid–liquid extraction and the feed solution for the adsorption separation using column chromatography.

The amount and mass fraction of terpene trilactones in the concentrates of Solutions A and B were analyzed by HPLC in the next section. The loss and concentration ratio of terpene trilactones of the extracts from Solution A (70% ethanol aqueous solution) to Solution B (de-ionized water) were calculated.

2.3. Quantitative analysis of the active ingredients

2.3.1. High-performance liquid chromatography system

The high-performance liquid chromatography system included a Jasco Model PU-980 solvent metering pump, a Showa Denko Model Shodex RI-71 RI detector (Tokyo, Japan), a Rheodyne Model 71256-way syringe loading valve fitted with a 20 μL sample loop (Cotati, CA, USA), and a Sunway Model 940-CO column oven (Taipei, Taiwan). The HPLC column was Hypersil-100 C_{18}, 250 × 4.6 mm i.d., 5 μm (Hypersil, UK). All analyses were carried out at 30 °C.

2.3.2. Quantitative analysis of terpene trilactones

In this study, because there are no commercial GC and GJ standards available for calibration, only the contents of GA, GB, and BB were used as the quantitative indices of terpene trilactones. To analyze the terpene trilactones in the crude *Ginkgo biloba* L. extracts, the extracted solution (dissolved in methanol) was directly analyzed by the reversed-phase HPLC. The isocratic mobile phase was H_2O–acetonitrile–THF (23:4:2, v/v/v). The flow rate was 1.0 mL/min and the peaks were monitored with the RI detector. Fig. 3 shows the corresponding chromatogram of one particular example where a successful separation of BB, GA, and GB was obtained. The result is similar to that obtained with the RP- C_{18} column by van Beek et al. [1]. This isocratic mobile phase was also proved workable and accordingly adopted in the rest of this work.

BB, GA, and GB were then identified and quantified by direct comparison with reference standards. Multiplying BB, GA, and GB concentrations by the total extracted solution volume, the contents of BB, GA, and GB and the total terpene trilactone content (an under-estimated value calculated by only the sum of the contents of BB, GA, and GB) in the crude extract solution were determined. It is noted that averaging the results of several batches of the crude *Ginkgo biloba* L. extracts, the percentage of the content of BB ranged from 45 to 50% and the percentage of the content of GA and GB ranged from 50 to 55% in the calculated total terpene trilactone content.

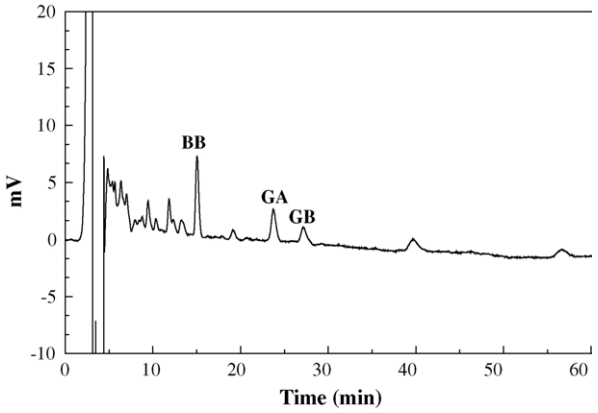

Fig. 3. Typical HPLC analysis results of the crude *Ginkgo biloba* L. extracts. Column: RP-C_{18} (250 mm × 4.6 mm i.d., 5 μm); injection volume: 20 μL; injection concentration: 85.29 mg/mL; flow rate: 1 mL/min; monitor: RI detector; isocratic mobile phase: H_2O–acetonitrile–THF (23:4:2, v/v/v); oven temperature: 30 °C.

2.4. Isolation of terpene trilactones using liquid–liquid extraction

2.4.1. Liquid–liquid extraction system

The aqueous extract (Solution B) was extracted two times with ethyl acetate. The volume ratio of aqueous phase to organic phase each time was fixed to be 1:2. The extraction was done by placing the mixture in a 1000 mL conical flask and shaking it in a thermostat water bath set at 30 °C for 2 h. Ethyl acetate was chosen because it is an excellent solvent for all compounds of interest, has a relatively low cost, exhibits low toxicity, and has good biodegradability [9]. Upon extraction, the two phases were separated and collected by a 1000 mL separatory funnel. The combined ethyl acetate layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness under reduced pressure. The aqueous layer was dried overnight in a vacuum oven. The residues from both layers were dissolved in methanol and kept for HPLC analysis.

2.4.2. Evaluation of the extraction performance

The residues of the ethyl acetate layer and the aqueous layer were analyzed by HPLC. The extraction performance indices of the ethyl acetate layer, including purity, concentration ratio, and yield of terpene trilactones, are defined as

follows:

$$\text{Purity} = \text{mass fraction of terpene trilactones in the ethyl acetate layer} \quad (1a)$$

$$\text{Concentration ratio} = \frac{\text{purity of terpene trilactones in the ethyl acetate layer}}{\text{purity of terpene trilactones in Solution B}} \quad (1b)$$

$$\text{Yield} = \frac{\text{mass of terpene trilactones in the ethyl acetate layer}}{\text{mass of terpene trilactones in Solution B}} \quad (1c)$$

Meanwhile, the mass of the residual and the mass of terpene trilactones in the aqueous layer were also calculated to examine the mass balances of the residue and terpene trilactones.

2.5. Isolation of terpene trilactones using column chromatography

2.5.1. Medium pressure liquid chromatograph (MPLC) system

The medium pressure liquid chromatograph system included a Lab Alliance Model Series II medium pressure pump (Lemont, PA, USA), a ISCO Model 2360 gradient programmer, and a ISCO Model Retriever® 500 fraction collector (Lincoln, NE, USA). Each of the three packing materials, i.e. XAD-7HP, XAD-4, and C-18, was dry packed in a glass column. The column was located in a water bath, where the temperature was controlled at 30 °C.

2.5.2. Adsorption separation process

It is noted that the triangular relationship among the adsorbate, the adsorbent, and the eluent is formed based on the "like attracts like" principle [11], i.e. whether the adsorbate will stay in the adsorbent or in the eluent depends on the relative strength of attraction between them. Applying the principle to the current system, as intermediate polar and non-polar adsorbents, all the three packing materials can adsorb intermediate polar terpene trilactones [15] from aqueous solutions; on the contrary, using an eluent at a higher percentage of ethanol is favorable for the desorption of terpene trilactones.

A whole adsorption separation process thus included four steps in sequential: conditioning, adsorption, washing, and elution steps in sequential. First, the column was conditioned with de-ionized water. Then, the feed solution (Solution B) was loaded onto the column. At the end of adsorption, the column was washed with a certain volume of de-ionized water in order to remove the impurities remaining in the pore of the adsorbents and the packing spacing. Finally, the packed column was eluted with a certain volume of the ethanol aqueous solution varying step-gradiently from 20 to 100%.

2.5.3. Evaluation of the column performance

The effluent for each step in the adsorption separation process was collected for compositional analysis. Each effluent was concentrated and each concentrate was weighed. Based on the compositions of terpene trilactones in all effluents, the following column performance was evaluated. First, the dynamic adsorption curve (breakthrough curve) of terpene trilactones was established to evaluate the suitable loading volume for the adsorption step. Then, the dynamic elution curve of terpene trilactones was established to find the proper step-gradient elution program for achieving an efficient isolation in the elution step. The column performance indices, including purity, concentration ratio, and yield of terpene trilactones, are defined as follows:

$$\text{Purity} = \text{mass fraction of terpene trilactones in the concentrated elution effluent fraction} \quad (2a)$$

$$\text{Concentration ratio} = \frac{\text{purity of terpene trilactones in the concentrated elution effluent fraction}}{\text{purity of terpene trilactones in Solution B}} \quad (2b)$$

$$\text{Yield} = \frac{\text{mass of terpene trilactones in the concentrated elution effluent fraction}}{\text{sum of the masses of terpene trilactones in Solution B}} \quad (2c)$$

Finally, the column performances among the three different packing materials were compared.

3. Results and discussion

3.1. Preparation of the crude Ginkgo biloba L. extracts

The crude extraction results of three batches of Solution A were as follows: the masses of the concentrated extracts were 20.58, 21.86, and 22.06 g and the masses of terpene trilactones were 283.79, 236.84, and 270.58 mg, respectively. The results showed that the yield of the crude extracts (mass of the concentrated extracts/mass of dry leaves used) ranged from 20 to 23% (w/w) and the mass fraction (or purity) of terpene trilactones in the concentrated extracts ranged from 1.0 to 1.4% (w/w). These results are close to the values reported in the literature [2].

The feed solution for the following isolation processes was prepared by collecting the crude extract solution (Solution A), concentrating it, dissolving the concentrates in 700 mL of de-ionized water, centrifuging the solution, and then filtrating it

Table 1
Liquid–liquid extraction[a] results of three batches of Solution B with different volumes

Volume of Solution B (mL)	Solution B			Aqueous phase			Organic phase				
	Mass of concentrate (mg)	Mass of terpene trilactones (mg)	Purity of terpene trilactones (%)	Mass of concentrate (mg)	Mass of terpene trilactones (mg)	Purity of terpene trilactones (%)	Mass of concentrate (mg)	Mass of terpene trilactones (mg)	Purity of terpene trilactones (%)	Yield (%)[b]	Concentration ratio (%)[c]
50	1025	8.65	0.844	731.12	~ 0	~ 0	63.62	8.62	13.55	99.71	16.05
150	3330	37.63	1.13	2938.5	~ 0	~ 0	208.17	37.2	17.87	98.87	15.81
250	6000	61.38	1.02	5450.15	~ 0	~ 0	359.85	60.85	16.91	99.13	15.54

[a] Liquid–liquid extraction conditions: water–oil (1:2, v/v), two times, shaken in a thermostat water batch at 30 °C for 2 h.
[b] Yield of terpene trilactones (%) = (mass of terpene trilactones in the ethyl acetate layer/mass of terpene trilactones in Solution B) × 100%.
[c] Concentration ratio of terpene trilactones = purity of terpene trilactones in the ethyl acetate layer/purity of terpene trilactones in Solution B.

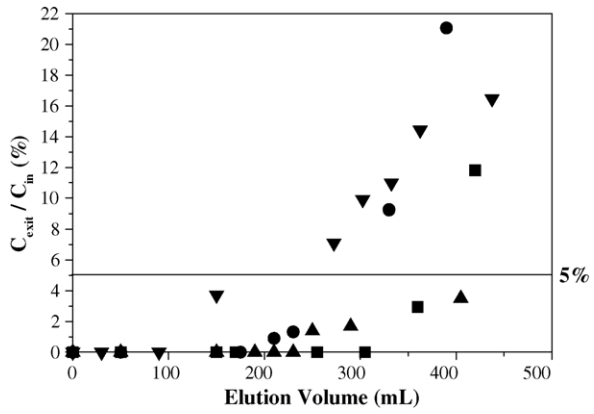

Fig. 4. The dynamic adsorption curves of terpene trilactones for the XAD-7HP column. Column size: 96 mm × 15 mm i.d., 250–840 μm; temperature: 30 °C; (■) flow rate: 1 mL/min, C_{in}: 0.3132 mg/mL; (●) flow rate: 2 mL/min, C_{in}: 0.3132 mg/mL; (▲) flow rate: 2 mL/min, C_{in}: 0.1858 mg/mL; (▼) flow rate: 3 mL/min, C_{in}: 0.1858 mg/mL.

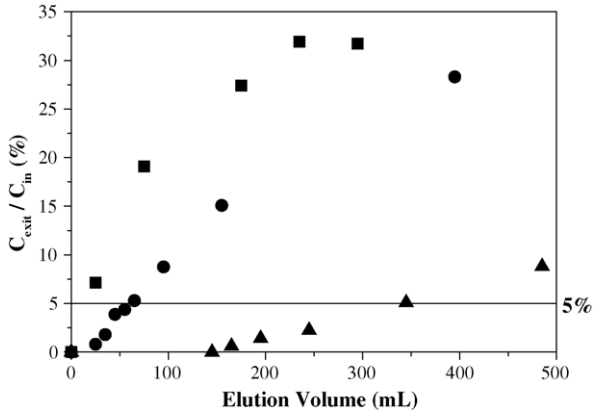

Fig. 5. The dynamic adsorption curves of terpene trilactones for the XAD-4 column. Column size: 98 mm × 15 mm i.d., 250–840 μm; temperature: 30 °C; (■) flow rate: 2 mL/min, C_{in}: 0.226 mg/mL; (●) flow rate: 1 mL/min, C_{in}: 0.241 mg/mL; (▲) flow rate: 1 mL/min, C_{in}: 0.112 mg/mL.

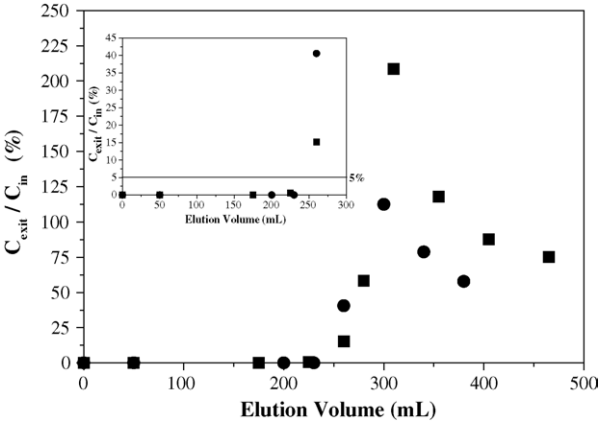

Fig. 6. The dynamic adsorption curves of terpene trilactones for the C-18 column. Column size: 95 mm × 15 mm i.d., 40 μm; temperature: 30 °C; (■) flow rate: 2 mL/min, C_{in}: 0.3969 mg/mL; (●) flow rate: 2 mL/min, C_{in}: 0.5105 mg/mL. Insert: the enlarged figure for the first 260 mL elution.

to get the filtrate (Solution B). Averaging the results of three batches of Solution B obtained from the above three batches of Solution A, the mass of the concentrated extracts ranged from 13 to 17 g, the mass of terpene trilactones ranged from 140 to 180 mg, and the mass fraction (or purity) of terpene trilactones ranged from 1.0 to 1.3% (w/w), which was about the same as that in the concentrates of Solution A. From Solution A (70% ethanol aqueous solution) to Solution B (deionized water), the loss of the concentrated extracts ranged from 30 to 40% and the loss of terpene trilactones of the extracts ranged from 30 to 40%. Finally, the concentration of terpene trilactones of the feed solution (Solution B) ranged from 0.2 to 0.4 mg/mL.

3.2. Isolation of terpene trilactones using liquid–liquid extraction

The liquid–liquid extraction results of three batches of Solution B with different volumes (50, 150, and 250 mL) are shown in Table 1. For each batch, the extraction was done two times and the volume of ethyl acetate was chosen according to 1:2 volume ratio of aqueous phase to organic phase each time.

The extraction performances of the ethyl acetate layers of three batches show that the purity ranged from 13.5 to 18.0%, the concentration ratio ranged from 15 to 16, and the yield ranged from 99 to nearly 100%. It is noted that the mass of the residue from the second time of extraction was less than 2% (data not shown), therefore, only single extraction operated at the above conditions was proved to be efficient. Also, the liquid–liquid extraction was proved to be successfully scaled-up.

It is also noted that the sums of the masses of the residues and the masses of terpene trilactones in both layers were very close to their corresponding amounts in Solution B and the masses of the residues and terpene trilactones were proved to be conserved.

3.3. Isolation of terpene trilactones using column chromatography

The bed lengths of the three glass columns (i.d. = 1.5 cm), packed with approximately 8.59, 5.60, and 11.01 g of XAD-7HP, XAD-4, and C-18 adsorbents, were 9.6, 9.8, and 9.5 cm, respectively.

3.3.1. Dynamic adsorption performance

Solution B was used as the feed solution for the dynamic adsorption experiment. It was carried out on each of the three columns at a certain flow rate and feed concentration (C_{in}). For each column, the effluent fractions of the adsorption step were collected in several intervals, e.g. 20–30 mL for each interval, for compositional analysis. The dynamic adsorption curves of terpene trilactones are shown in Figs. 4–6 for the XAD-7HP, XAD-4, and C-18 columns, respectively. To avoid the loss of terpene trilactones from the adsorption stage, the break point was set at the point as the exit concentration of terpene trilactones approached 5% of the feed concentration. The loading volume of the adsorption process was then selected according to the elution volume at the break point.

It can be seen from each figure that the break point took place earlier as the flow rate and/or the feed concentration increased. The earlier breakthrough at higher flow rate is believed to be caused by the increasing turbulent mixing in the axial dispersion [16]. On the other hand, due to the

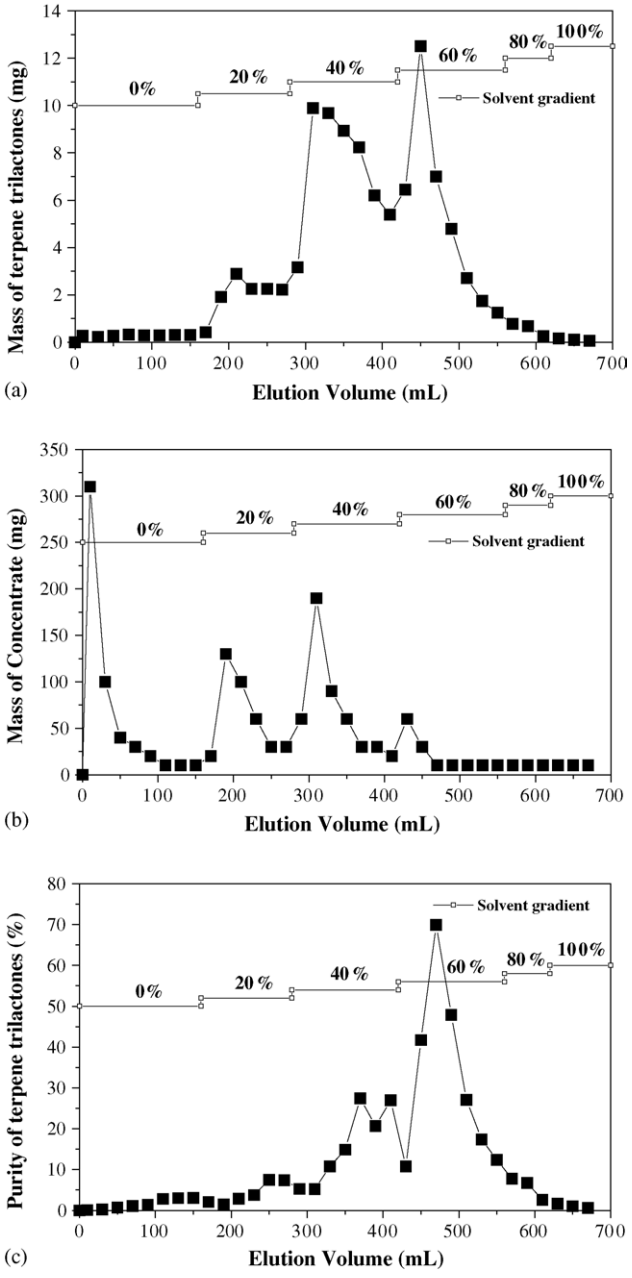

Fig. 7. The dynamic elution curves for the XAD-7HP column: (a) mass of terpene trilactones, (b) mass of concentrate and (c) purity of terpene trilactones. Column size: same as Fig. 4; feed solution: 200 mL Solution B; feed concentration: 0.3716 mg/mL; feed flow rate: 2 mL/min; elution solution: 0, 20, 40, 60, 80, 100% aqueous ethanol; elution flow rate: 2 mL/min; temperature: 30 °C.

loading amount (=loading volume × feed concentration) is about fixed for a certain column operated at a certain flow rate, the increase in the feed concentration leaded to the decrease in the loading volume. Based on the dynamic adsorption behaviors of Figs. 4–6 and in order to have an efficient column operation in terms of the loading volume and the operating time, for the XAD-7HP and C-18 columns, the flow rate of 2 mL/min was selected and the loading volumes were estimated to be 250–400 mL at the feed concentration of 0.20–0.40 mg/mL and 200–300 mL at the feed concentration of 0.35–0.50 mg/mL, respectively; for the XAD-4 column, the flow rate of 1 mL/min was selected and the loading volumes were estimated to be 150–250 mL at the feed concentration of 0.15–0.30 mg/mL. The loading volume has to be carefully adjusted when the flow rate or the feed concentration changes.

It is noted that a higher loading volume was attributed to a stronger interaction between the adsorbate (terpene trilactones) and the packing material. Since terpene trilactones

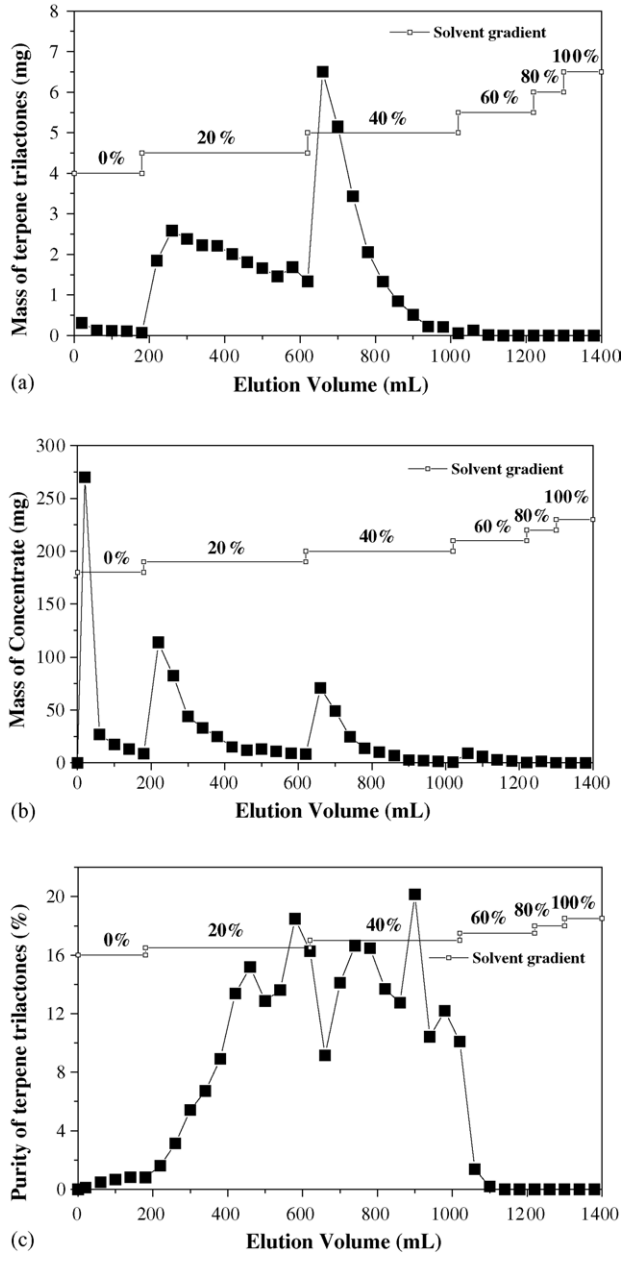

Fig. 8. The dynamic elution curves for the XAD-4 column: (a) mass of terpene trilactones, (b) mass of concentrate and (c) purity of terpene trilactones. Column size: same as Fig. 5; feed solution: 200 mL Solution B; feed concentration: 0.2493 mg/mL; feed flow rate: 1 mL/min; elution solution: 0, 20, 40, 60, 80, 100% aqueous ethanol; elution flow rate: 2 mL/min; temperature: 30 °C.

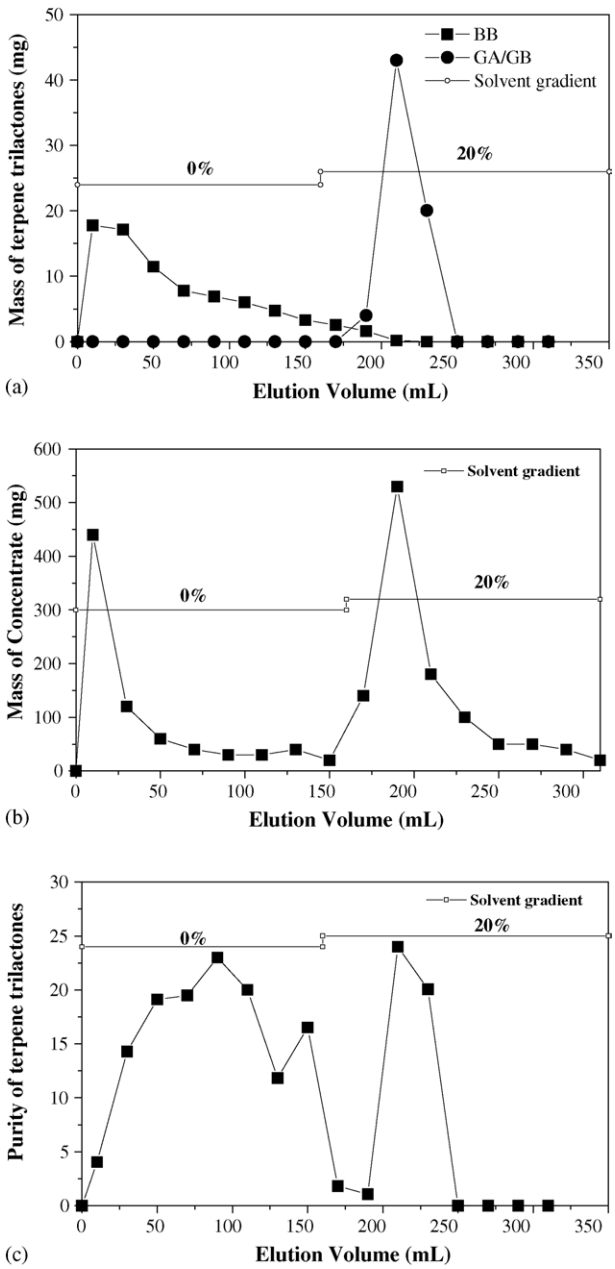

Fig. 9. The dynamic elution curves for the C-18 column: (a) mass of terpene trilactones, (b) mass of concentrate and (c) purity of terpene trilactones. Column size: same as Fig. 6; feed solution: 275 mL Solution B; feed concentration: 0.3969 mg/mL; feed flow rate: 2 mL/min; elution solution: 0, 20% aqueous ethanol; elution flow rate: 2 mL/min; temperature: 30 °C.

Table 2
Column performances of the XAD-7HP column

Step of column operation	Operating volume (mL)	Concentrate				Terpene trilactones					
		Mass (mg)		Mass ratio (%)[b]		Mass (mg)		Yield (%)[c]		Purity (%)[d]	
Adsorption[a]	220	4330		75.87		0.08		0.10		~ 0	
Washing (0% ethanol)	160	550		9.64		1.50		1.79		0.27	
Step-gradient elution											
20% ethanol	60	220		3.85		4.14		4.94		1.88	
30% ethanol	60	260		4.56		7.94		9.47		3.05	
40% ethanol[e]	60	200	Subtotal: 340	3.50	Subtotal: 5.95	22.04	Subtotal: 67.52	26.30	Subtotal: 80.56	11.02	Subtotal: 19.86
50% ethanol[e]	90	120		2.10		31.33		37.38		26.11	
60% ethanol[e]	90	20		0.35		14.15		16.88		70.75	
70%+80% + 100% ethanol	90	7		0.12		2.63		3.14		37.57	
Total	–	5707		100		83.81		100		–	

[a] Feed solution (Solution B): loading volume = 200 mL; concentration of concentrate = 28.11 mg/mL; concentration of terpene trilactones = 0.3986 mg/mL; purity of terpene trilactones = 1.42%.
[b] Mass ratio of concentrate (%) = (fractional mass of concentrate/total mass of concentrate) × 100%.
[c] Yield of terpene trilactones (%) = (fractional mass of terpene trilactones/total mass of terpene trilactones) × 100%.
[d] Purity of terpene trilactone (%) = (fractional mass of terpene trilactones/fractional mass of concentrate) × 100%.
[e] The fraction was collected as a part of the concentrated product.

possess intermediate polarity [15], the order of the strength of interaction, based on the "like attracts like" principle [11], is predictably in the order of the similarity of the polarities from both of them, i.e. XAD-7HP column (intermediate polar) >XAD-4 column (non-polar) >C-18 column (most non-polar). However, when operated at the same flow rate and feed concentration, the loading volume was found to be in the order: XAD-7HP column >C-18 column >XAD-4 column. The reason for a higher loading volume for the C-18 column than expected was due to the very steep shape of the dynamic adsorption curve compared to the elongated S-form shape for the XAD-7HP and XAD-4 columns. According to the theory of fixed-bed adsorption [16], the very narrow mass transfer zone, achieved by the very steep breakthrough curve, was formed due to the negligible mass transfer resistance from the very small particle size (40 μm) used for the C-18 column.

3.3.2. Dynamic elution performance

A dynamic elution experiment was then carried out on each of the three columns. Each column was eluted with a certain volume of the ethanol aqueous solution varying step-gradiently from 20 to 100% at a flow rate 2.0 mL/min, following the adsorption step of a suitable loading volume of the feed solution and the washing step of 150–200 mL de-ionized water. The effluent fractions of the elution step were collected in several intervals, e.g. 20–30 mL for each interval, for compositional analysis. Their dynamic elution curves are shown

Table 3
Column performances of the XAD-4 column

Step of column operation	Operating volume (mL)	Concentrate				Terpene trilactones					
		Mass (mg)		Mass ratio (%)[b]		Mass (mg)		Yield (%)[c]		Purity (%)[d]	
Adsorption[a]	200	3631		79.93		7.27		12.4		0.20	
Washing (0% ethanol)	150	316		6.96		0.65		1.11		0.20	
Step-gradient elution											
10% ethanol	50	88		1.94		1.52		2.59		1.73	
20% ethanol	50	100		2.20		3.25		5.55		3.25	
20% ethanol[e]	450	192	Subtotal: 395	4.23	Subtotal: 8.70	26.29	Subtotal: 45.92	44.87	Subtotal: 78.36	13.69	Subtotal: 11.63
40% ethanol[e]	350	203		4.47		19.63		33.49		9.67	
60% ethanol	200	13		0.29		0		0		0	
80% + 100% ethanol	100	0		0		0		0		0	
Total	–	4543		100		58.60		100		–	

[a] Feed solution (Solution B): loading volume = 200 mL; concentration of concentrate = 22.95 mg/mL; concentration of terpene trilactones = 0.2612 mg/mL; purity of terpene trilactones = 1.14%.
[b] Mass ratio of concentrate (%) = (fractional mass of concentrate/total mass of concentrate) × 100%.
[c] Yield of terpene trilactones (%) = (fractional mass of terpene trilactones/total mass of terpene trilactones) × 100%.
[d] Purity of terpene trilactone (%) = (fractional mass of terpene trilactones/fractional mass of concentrate) × 100%.
[e] The fraction was collected as a part of the concentrated product.

Table 4
Column performances of the C-18 column

Step of column operation	Operating volume (mL)	Concentrate		Terpene trilactones				
		Mass (mg)	Mass ratio (%)[b]	Mass (mg)		Yield (%)[c]	Purity (%)[d]	
				BB	GA/GB		BB	GA/GB
Adsorption[a]	275	5600	71.25	1.84	0	1.67	0.03	0
Washing[e] (0% ethanol)	200	780	9.92	53.49	0	48.51	6.86	0
Step-gradient elution								
20% ethanol	35	580	7.47	0.43	2.71	2.85	0.07	0.47
20% ethanol[e]	45	330	4.20	0	51.79	46.97	0	15.69
40–100% ethanol	150	570	7.25	0	0	0	0	0
Total	–	7860	100	110.26		100	–	

[a] Feed solution (Solution B): loading volume = 275 mL; concentration of concentrate = 28.50 mg/mL; concentration of terpene trilactones = 0.3969 mg/mL; purity of terpene trilactones = 1.39%.
[b] Mass ratio of concentrate (%) = (fractional mass of concentrate/total mass of concentrate) × 100%.
[c] Yield of terpene trilactones (%) = (fractional mass of terpene trilactones/total mass of terpene trilactones) × 100%.
[d] Purity of terpene trilactone (%) = (fractional mass of terpene trilactones/fractional mass of concentrate) × 100%.
[e] The fraction was collected as a part of the concentrated product.

in Figs. 7–9 for the XAD-7HP, XAD-4, and C-18 columns, respectively. There are three parts in each figure: the mass of terpene trilactones, the mass of concentrate, and the purity of terpene trilactones versus the elution volume shown in parts (a)–(c), respectively.

The results of part (a) show that the proper eluent composition for an efficient elution of the majority of terpene trilactones ranged from 40 to 60%, 20 to 40%, and 0 to 20% ethanol for the XAD-7HP, XAD-4, and C-18 columns, respectively. It was reflected on the fact that the stronger the interaction between the adsorbate and the packing material is, the higher percentage ethanol aqueous solution is needed to elute terpene trilactones. The results of parts (b) and (c) show that for the XAD-7HP column, a part of more polar impurities, eluted by 0–40% ethanol, was efficiently separated with the majority of terpene trilactones, eluted by 40–60%, which resulted in an efficient isolation of terpene trilactones (purity ∼20%) from the fractions collected at 40–60% ethanol; on the contrary, for the XAD-4 and C-18 columns, the majority of impurities were nearly eluted together with the majority of terpene trilactones, from which terpene trilactones were not efficiently isolated (purity ∼10%).

It is worth noting that for the XAD-7HP and XAD-4 columns, all the contents of terpene trilactones were eluted about at the same time course; however, for the C-18 column, BB, eluted by 0% ethanol, was efficiently separated with GA and GB, eluted by 20% ethanol.

3.3.3. Comparison of the column performance

The dynamic elution curves of Figs. 7–9 were then referred and their step-gradient elution programs were modified in order to enhance their isolation efficiencies. Using the modified step-gradient elution programs, the column performances, in terms of purity, concentration ratio, and yield of terpene trilactones, among the three different packing materials were compared as presented in Tables 2–4 for the XAD-7HP, XAD-4, and C-18 columns, respectively.

It can be seen from each table that, in the adsorption step, the loss of terpene trilactones was less than 10% when the selected volume of feed solution was loaded. Since nearly 70–80% of the total mass of concentrates was removed without too much loss of terpene trilactones in the adsorption step, the active ingredient contents were first isolated more than 4.0 times (concentration ratio >4.0) after the adsorption step and were further isolated by an efficient separation from the other impurities in the elution step. In the elution step, for all the effluent fractions collected, the sum of concentrates weighed around 5700, 4500, and 7800 mg and the sum of terpene trilactones weighed around 84, 59, and 110 mg for the XAD-7HP, XAD-4, and C-18 columns, respectively. When compared the collected amounts with their loading amounts, the errors in mass balances of the concentrate and terpene trilactones were about <2 and <10%, respectively. The mass conservation was satisfactory and each column was proved to be sufficiently regenerated.

The elution peaks of terpene trilactones, formed as happened in the dynamic elution experiments, were collected as the isolation product for each of the three columns. The purity, concentration ratio, and yield of terpene trilactones were ∼20, ∼15, and ∼80% for the XAD-7HP column and ∼12, ∼10, and ∼80% for the XAD-4 column. For the C-18 column, BB and GA/GB were separated effectively, from which their purity, concentration ratio, and yield of terpene trilactones were ∼7, ∼10, and ∼95% for BB and ∼16, ∼23, and ∼95% for GA/GB. In comparison, the purity and the concentration ratio were in the order: XAD-7HP column >C-18 column >XAD-4 column and the yield was in the order: C-18 column >XAD-7HP column >XAD-4 column.

4. Conclusions

The preparation of the crude *Ginkgo biloba* L. extracts, using an extractor with solvent refluxing operated at 900 mL

of 70% ethanol aqueous solution and 3 h for a batch of 100 g dry leaves gave a satisfactory result. The yield of the crude extracts ranged from 20 to 23% (w/w) and the purity of terpene trilactones in the concentrated extracts ranged from 1.0 to 1.4 wt.%. In order to be effectively processed in the isolation processes, the crude extracts were dissolved in de-ionized water. This procedure led to a about the same purity of terpene trilactones but with a 30–40% loss for the concentrated extracts and a 30–40% loss for terpene trilactones.

For the method of liquid–liquid extraction using ethyl acetate as the organic solvent, only single extraction with a 1:2 volume ratio operated at 30 °C for 2 h was proved to be efficient for the isolation. The purity, concentration ratio, and yield of terpene trilactones were 13.5–18.0, 15–16, and >99%. Also, the process was successfully scaled-up to a preparative scale.

For the method of column chromatography using three different packing materials (XAD-7HP, XAD-4, and C-18 adsorbents) with different polarities, the order of the strength of interaction between terpene trilactones and the packing material was proved to be: XAD-7HP column (intermediate polar) >XAD-4 column (non-polar) >C-18 column (most non-polar). The loading volume of the adsorption process was selected according to the elution volume at the break point of the dynamic adsorption curve. The loading volume was in the order: XAD-7HP column >C-18 column >XAD-4 column. The reason for a higher loading volume for the C-18 column than expected was due to the very steep shape of the dynamic adsorption curve formed by the small size of particles (40 μm) packed in it. An efficient elution of the majority of terpene trilactones ranged from 40 to 60%, 20 to 40%, and 0 to 20% ethanol for the XAD-7HP, XAD-4, and C-18 columns, respectively, reflected the order of the strength of interaction. Only for the XAD-7HP column, a part of more polar impurities was efficiently separated with the majority of terpene trilactones by a proper step-gradient elution, which resulted in an efficient isolation: the purity, concentration ratio, and yield of terpene trilactones were ∼20, ∼15, and ∼80%. It is worth noting that for the XAD-7HP and XAD-4 columns, all the contents of terpene trilactones were eluted about at the same time course; however, for the C-18 column, BB was efficiently separated with GA and GB by the step-gradient elution.

In comparison, the orders of purity and concentration ratio were both found to be XAD-7HP column >liquid–liquid extraction >C-18 column >XAD-4 column and the order of yield was found to be liquid–liquid extraction >C-18 column >XAD-7HP column >XAD-4 column. The XAD-7HP column achieved the highest purity, but at the expense of the yield of terpene trilactones; on the contrary, the liquid–liquid extraction method achieved the highest yield, but the purity of terpene trilactones was slightly lower. Therefore, the method of liquid–liquid extraction was superior to the method of column chromatography in the current isolation stage (∼20%). Finally, a synthesis of the different processes, e.g. using the liquid–liquid extraction method first and followed by the method of column chromatograph, is suggested for a further isolation and separation of terpene trilactones. Work along this direction is currently in progress.

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