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Extraction of Pharmaceutical Components from *Ginkgo biloba* Leaves Using Supercritical Carbon Dioxide

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Ginkgo biloba extract (GBE) has many remarkable pharmacological and clinical effects, and it is the most frequently used product as a phytomedicine in many countries. The combination of primary extraction with 70% ethanol followed by extraction using supercritical carbon dioxide provides an efficient and economical means for obtaining flavonoids and terpenoids from *Ginkgo biloba* leaves. The supercritical fluid extraction (SFE) is affected by pressure, temperature, and the concentration of modifier in the extractant. At the most favorable experimental conditions of 300 MPa, 60 °C, and carbon dioxide containing 5% ethanol as modifier, the yield of GBE powder is 2.1% (based on the air-dry weight of *Ginkgo biloba* leaves) compared to a yield of only 1.8% by conventional solvent extraction. The contents of flavonoids and terpenoids in SFE products are 35.9% and 7.3%, respectively, which are significantly higher than the general standards of 24% and 6%, respectively.

KEYWORDS: Supercritical fluid extraction; carbon dioxide; *Ginkgo biloba* leaves; GBE

INTRODUCTION

Ancient *Ginkgo biloba* survived from the era when dinosaurs became extinct over 200 million years ago. Its amazing vitality has attracted an increasing exploration into its potential application in health foods and supplements. It is proved that *Ginkgo biloba* leaves contain a large number of active compounds, the most important of which are flavonol glycosides (flavonoids) and terpenoids. These effective pharmaceutical substances, in particular the terpenoids, regulate blood flow to the brain, thus helping to counteract memory loss, depression, and lack of alertness which may develop in old age. The major therapeutic uses of *Ginkgo biloba* extract (GBE) are in many areas such as vascular insufficiency and hearing disorders (*1*−*4*).

As is well-known, conventional solvent extraction is rather tedious, and it inevitably introduces hazardous residual of organic solvent into the products. To get high-quality GBE, supercritical carbon dioxide (SC CO_2) is regarded as an ideal substitute. SC CO_2 has unique physicochemical properties such as high diffusivity, lower viscosity, and lower surface tension, which make it an ideal extractant for the extraction of target components from the hosting matrixes. Once extracted and fractionated into its different components, the products are completely free of residual solvent. Nowadays, supercritical fluid extraction (SFE) technology has been widely used for the analysis of natural products and pharmaceuticals (*5*−*7*), and this technology is receiving increased attention for the extraction and determination of effective components present in *Ginkgo biloba* (*8*−*11*). However, even though SFE extraction of *Ginkgo biloba* has many advantages, research and development in this field is still not sufficient. Actually, so far, there are very few references describing the process in detail, possibly due to the lack of technical confidence. In this paper SC CO_2 was tentatively applied to extract pharmaceutical components from *Ginkgo biloba* leaves and raw GBE, and the variables such as pressure, temperature, and modifier were optimized in order to achieve satisfactory results.

MATERIALS AND METHODS

General Experimental Procedures. Green *Ginkgo biloba* leaves were collected in autumn in Nankou near Beijing. The leaves were rinsed with distilled water and air-dried in the laboratory. The dried leaves were pulverized to pass No. 5 mesh screen (5-mm openings) using a FZ 103 plant pulverizer (Qijiawu Plant of Scientific Instruments, Heibei, China).

In this study, extraction with ethanol was considered as the primary treatment prior to the SFE extraction. Pulverized *Ginkgo biloba* leaves (500 g) were extracted with 70% ethanol (1 L × 2 times) for 2 h under reflux. More extraction steps are often needed in order to completely extract the effective components from *Ginkgo biloba* leaves. After filtration, the aqueous solution was distilled to remove extractant, where the raw product denoted as GBE_{Raw} contains approximately 4% of total flavonoids (abbreviated as TFL) and less than 1% of total terpenoids (TTE). The yield of GBE_{Raw} was approximately 16% based on the air-dry weight of *Ginkgo biloba* leaves. In the conventional solvent extraction, further refinement of extractives with organic solvents such as chloroform and acetone was followed by a series of purifications with columns filled with polycaprolactam, alumina oxide, and resins such as XAD and GDX. These steps can lead to generally satisfactory GBE product (GBE_{SE}).

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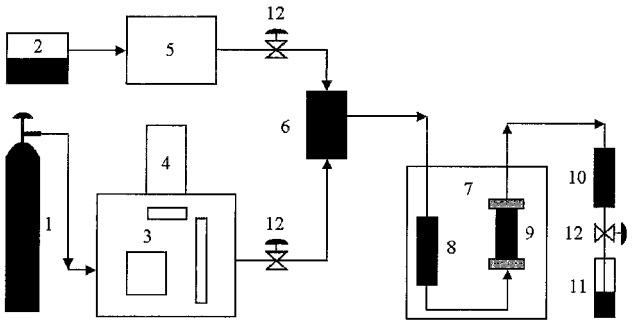

Figure 1. Schematic diagram of extraction of *Ginkgo biloba* leaves using supercritical CO_2: (1) CO_2 tank; (2) reservoir for modifier; (3) SFE pump; (4) cooling jacket; (5) modifier pump; (6) mixer; (7) SFE-931 extractor; (8) preheater; (9) extraction chamber; (10) heater; (11) collection vessel; and (12) valves.

The schematic process of SC CO_2 extraction of GBE is depicted in **Figure 1**. The laboratory-scale SFE-931 processing unit was designed and constructed in-house by the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China. The 316 stainless steel chamber with an inner diameter of 10 mm, a length of 50 mm, and a wall thickness of 4 mm, was designed to withstand high temperatures and high pressures (cf. 40.0 MPa). A known amount of sample (typically 1.0 to 5.0 g) was placed in the extraction chamber, and silica gel was used to eliminate the dead volume. Liquid CO_2 and absolute ethanol were separately introduced to a mixer at a predetermined volumetric ratio by two pumps. Liquid CO_2 into the SFE pump was supplied by means of condensation or by placing the tank in a position reverse to that used for direct liquid CO_2 transfer. Extractant from the mixer was transferred to the extraction chamber. In the first operating mode, enough extractant was introduced to allow for a 5-min static conditioning for sufficient contact between matrixes and extractant. The second operating mode was followed by a steady flow of SC CO_2 under the dynamic extraction condition. Certain temperature and pressure were maintained to allow CO_2 in the supercritical state. Dynamic extraction lasted 30 min to 1.0 h with an approximate flowrate of 1.0 mL/min. Products from the chamber were collected in a vessel containing 10 mL of 70% ethanol solution. The solvent was vaporized, and the GBE was obtained for subsequent qualitative and quantitative analysis by high-performance liquid chromatography (HPLC).

Measurement of Composition of GBEs by HPLC. According to related literature (*12*−*15*) pretreatment of GBE samples was essential in order to obtain accurate results. GBE powder (0.1 g) was weighed and dissolved into a methanol/water solution. With respect to the measurement of the total flavonoids in samples, hydrolysis of GBE was conducted in 2% HCl aqueous solution under 90 °C before HPLC−UV analysis. To determine the total terpenoids, including ginkgolides and bliobalide, GBE aqueous solution was loaded on an acidic alumina column and eluted with methanol. The methanol in the eluate was vaporized under a nitrogen gas stream to a predetermined volume for assaying by HPLC method using a refractive index (RI) detector.

Separation and assaying of components of GBE were conducted on an HPLC (LC-1050, Hewlett-Packard Co., Palo Alto, CA) equipped with an UV detector and a refractive index detector (LC-25 Perkin-Elmer Co., Wellesley, MA). The separation was performed on a C_{18} column (4.0 mm i.d. × 150 mm length, particle size 5.0 μm). The mobile phase was a methanol/water/phosphoric acid (58:41.5:0.5, v/v) mixture under the optimal flow rate for the individual analyte to achieve complete analytical separation with a short analysis time. The concentrations of the analytes were determined with external standards for calibration. The method detection limits are 1 ng level for three flavonoids: quercetin (QU), kaempferol (KA), and isorhamnetin (iso-RH) by HPLC/UV, and 1 μg level for ginkgolides A, B, and C (GA, GB, and GC) and bilobalide (BB) by HPLC−RI (*14*, *15*).

RESULTS AND DISCUSSION

Direct SFE from *Ginkgo biloba* Leaves. This study attempted to directly extract effective contents from *Ginkgo biloba* leaves using SC CO_2. The product appears viscous and dark green, which demonstrates that the contents of flavonoids and terpenoids were both very low, whereas chlorophyll and glucides remained in the products. Efforts including changing the operating temperature and pressure, as well as adding modifier, were applied to improve the process; unfortunately, none were noticably effective. This demonstrates that it is not feasible to obtain high-quality GBE via a single-step direct SFE extraction from *Ginkgo biloba* leaves. On the other hand, the contents of TFL and TTE are only around 1.2 and 0.4 g, respectively, in 100 g of dry *Ginkgo biloba* leaves (*16*). The use of direct SFE extraction from *Ginkgo biloba* leaves would involve a greater CO_2 consumption, thereby it is not an economical means. In this case, a pretreatment step is essential, resulting in significant reductions in the volume and mass of material and the elimination of many unwanted substances, by which the SFE of the effective contents is more cost-effective and practicable.

Table 1. $L_4(2^3)$ Tests and Results for Supercritical CO_2 Extraction of *Ginkgo biloba*

	variables			GBE products	
test no.	*T* (°C)	P (MPa)	ethanol (%)	*W* (g)	color
1	50	10.0	1.0	0.17 ± 0.01	light yellow
2	50	30.0	5.0	0.48 ± 0.02	orange yellow
3	80	10.0	5.0	0.44 ± 0.06	orange yellow
4	80	30.0	1.0	0.38 ± 0.02	yellow
level I (g) [a]	0.33	0.31	0.28		
level II (g)	0.41	0.43	0.46		
ΔW^2 (g^2) [b]	0.0064	0.0144	0.0324		

[a] The values for level I and level II are the averages of the weight of GBE products at lower values and higher values of the variable, respectively. [b] ΔW^2 represents the square of the difference between the values of levels I and II.

Optimization of Experimental Conditions. Before the SC CO_2 extraction of GBE_{Raw}, experimental conditions were optimized first. Three variables, including pressure (*P*), temperature (*T*), and modifier volume, were selected as possible factors which influence the SFE extraction process. The experimental design by orthogonal method [$L_4(2^3)$] focused on the three variables, each chosen at two levels (**Table 1**), and all experiments were performed in triplicate using 3.5 g of GBE_{Raw} powder. In this work, the experimental results collected are weight (*W*) of GBE and the product coloration, because they could vary with the experimental conditions and could reflect the overall product quality. In general, yellow GBE product contains more flavonoids than orange or brown GBE products.

To determine the effect of the variability in the experimental system caused by variables, the two averages of the weight of GBE products for level I and level II are examined according to the results. The squares of the differences between the two levels (ΔW^2) are then computed from the averages. As can be noted, the largest difference is obtained from the results for the variable of modifier, which indicates that the modifier present in SC CO_2 affects extraction of GBE in a most significant way. Thereby, it can be concluded that the most important influential factor is the modifier, followed by pressure and temperature. Moreover, in view of the results summarized in **Table 1**, the optimized conditions are higher content of modifier, higher temperature, and higher operating pressure.

Although increasing pressure and temperature can enhance the extracting efficiency of SC CO_2 and improve the product quality of active compounds from *Ginkgo biloba* leaves, because carbon dioxide is a nonpolar extractant, the polarity change by means of altering temperature and pressure has limited effect

Table 2. Influence of Modifier on the Extraction of GBE with Supercritical CO_2 at 30.0 MPa and 60 °C

ethanol (%)	yield (%)	color	TFL (%)	TTE (%)
0	0.3	light yellow	15.3 ± 0.5	1.2 ± 0.1
1.0	1.6	light yellow	23.4 ± 1.1	4.8 ± 0.3
5.0	2.1	golden yellow	35.9 ± 1.3	7.3 ± 0.6
10.0	2.2	brown	29.4 ± 1.2	5.8 ± 0.6

Table 3. Effects of Pressure and Temperature on the Extraction of GBE with Supercritical CO_2 Containing 5% Ethanol

experimental conditions		GBE products	
P (MPa)	*T* (°C)	yield (%)	color
8.0	60	0.8	light yellow
15.0	60	1.4	light yellow
25.0	60	1.8	yellow
30.0	60	2.1	golden yellow
30.0	40	1.7	light yellow
30.0	80	2.1	orange yellow
30.0	100	2.3	brown

on the extraction of more polar substances, such as flavonoids and terpenoids. To increase the solubility of the moderately polar solutes and improve their isolation selectivity, small amounts of polar cosolvent can be used to modify the polarity and selectivity of SC CO_2. Ethanol is by far the most common solvent used to enhance the supercritical extraction for food or medical processes because of its low toxicity.

The data in **Table 2** show that the addition of ethanol in SC CO_2 can substantially improve the extraction efficiency of flavonoids and terpenoids. This demonstrates that a minor fluctuation in the volume composition of modifier has a rather considerable impact on the polarity of extractant, and subsequently on the overall extraction efficiency of GBE. It was also found that the contents of GBEs in terms of flavonoids and terpenoids vary with the volume composition of modifier in SC CO_2. It is noted that the TFL and TTE in GBE for SC CO_2 extraction modified with 10% ethanol are both lower than those in GBE for 5% ethanol modifier. One possible way to explain the decrease of the purity is that more polar substances were extracted together with active compounds because the addition of modifier also increases their solubility in the extractant. On the other hand, as the modifier in the extractant increases to a certain content, a higher temperature is required to reach the increased critical temperature (T_c). Therefore, the content of ethanol should be in a range of 5−10 vol %.

On the basis of the results from the orthogonal experiments, pressure and temperature have effect on the extraction of GBE using SC CO_2. The influences of the two variables were further investigated in order to achieve the best results. **Table 3** shows the experimental conditions and the yield and color of GBE products. As can be seen, while the temperature was set at 60 °C and with a volume of 5% of ethanol as modifier, the yield of GBE increases from 0.8 to 2.1% with an increase of pressure from 8.0 to 30.0 MPa. This is explained by the fact that the density and viscosity of supercritical carbon dioxide change and, therefore, its extracting power increases. It is also worth mentioning that high pressure is bound to result in greater cost for the extraction operating system, as well as stringent operation design and increased energy demand. Likewise, increased temperatures have a favorable effect on the extraction efficiency. However, when the temperature reaches 80 °C, the effective contents possibly begin to decrease according to the color of GBEs. Moreover, elevated temperature could lead to decomposition of thermally liable active ingredients present in the finished products. Therefore, in general, it is recommended that operation be maintained at appropriate pressure and medium temperature, say 30.0 MPa and 60 °C.

Table 4. Comparison of GBEs Produced by Conventional Solvent Extraction and by Supercritical CO_2 Extraction

		flavonoids (%)			terpenoids (%)			
GBE	yield[a] (%)	QU	KA	iso-RH	GA	GB	GC	BB
GBE_{SE}	1.8 ± 0.2	20.6	6.2	5.0	0.5	0.3	0.2	1.7
GBE_{SFE}	2.1 ± 0.1	19.6	8.8	7.5	1.7	1.0	0.4	4.2

[a] Calculated from the weight of final GBE products and the feed of dry *Ginkgo biloba* leaves.

Extraction of Active Components from Raw GBE using Supercritical CO_2. According to the results mentioned in the previous sections, the SFE of GBE_{Raw} took 30 min with SC CO_2 containing 5 vol % of ethanol as modifier, at 30.0 MPa and 60 °C. The product of GBE_{SFE} obtained under these SFE conditions appeared as golden-yellow powder with bitter taste and is completely soluble in ethanol and 50% methanol in water.

Table 4 presents the comparison of GBEs extracted from GBE_{Raw} with conventional organic solvents and SF CO_2. The 2.1% yield of GBE_{SFE} is 15% greater than the 1.8% yield of GBE_{SE} by conventional solvent extraction. The contents of total flavonoids and total terpenoids in GBE_{SFE} are both higher than the generally recognized critical values of 24% and 6%, respectively. The 7.3% of terpenoid contents is 2.7 times higher than the value of 2.7% obtained from GBE_{SE}. In addition, although the whole process of GBE_{SFE} involves ethanol used in the primary step and SFE step, in fact, ethanol is often selected as an extractant or an additive in food and drug processing. Therefore, the presence of a trace amount of ethanol has no or little negative influence on the GBE quality.

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