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ORIGINAL PAPER

Supercritical fluid extraction and identification of isoquinoline alkaloids from leaves of *Nelumbo nucifera* Gaertn

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Abstract Isoquinoline alkaloids from leaves of *Nelumbo nucifera* (*N. nucifera*) were extracted using supercritical CO_2. The effects of the parameters such as the dynamic extraction time, temperature, pressure, various modifiers, and flow rate of the modifier on the yield of nuciferine and the ratio of total isoquinoline alkaloids to the total extract were investigated. Nuciferine content of the extract was determined by high-performance liquid chromatography (HPLC). The results indicated that the yield of nuciferine increased with increases in the dynamic extraction time, pressure, temperature, and flow rate of the modifier. The highest nuciferine yield of 325.54 μg/g was obtained when the extraction was carried out for 2 h at 70 °C under 30 MPa, with 10% (v/v) diethylamine and 1% (v/v) water in methanol as the modifier which kept a flow rate of 1.2 mL/min. The ratio of total isoquinoline alkaloids to the extract was 49.85% at the highest nuciferine yield. Five kinds of isoquinoline alkaloids extracted from *N. nucifera* leaves were identified by high-performance liquid chromatography combined with ion trap/time-of-flight mass spectrometry (LC/MS-ITTOF). They were Dehydronuciferine, N-nornuciferine, O-nornuciferine, Nuciferine, and Roemerine in the order of retention time.

Keywords Supercritical fluid extraction (SFE) · Isoquinoline alkaloids · *Nelumbo nucifera* (*N. nucifera*) · High-performance liquid chromatography (HPLC) · High-performance liquid chromatography-ion trap/time-of-flight mass spectrometry (LC/MS-ITTOF)

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Introduction

Nelumbo nucifera Gaertn, namely Lotus, is a perennial aquatic plant grown and consumed throughout Asia [1, 2]. All parts of *N. nucifera* have been used in oriental medicine for various medicinal purposes. Its leaves are known for diuretic and astringent properties and are used to treat fever, sweating, and strangury, and also used as a styptic [3]. Moreover, the extract of *N. nucifera* leaves has been found to inhibit digestion, slower absorption of lipids and carbohydrates, accelerate lipid metabolism, up-regulate energy expenditure [2], reduce the development of atherosclerosis in cholesterolfed rabbits [4], also show hepatoprotective and antioxidant activity [5], and inhibit human peripheral blood mononuclear cells proliferation in vitro [6]. Recent studies show that *N. nucifera* is a rich source of pharmaceutically important isoquinoline alkaloids. Alkaloids were one of the main active substances existing in the leaves and more than 15 kinds of alkaloids were found [7, 8]. Five isoquinoline alkaloids, such as (+) -1 (R) - Coclaurine, (−) -1 (S) - Norcoclaurine, Nuciferine (Fig. 1), Nornuciferine (Fig. 1), and Roemerine (Fig. 1), were isolated from the leaves of *N. nucifera* and demonstrated potent anti-HIV activity [9]. N-Nornuciferine and roemerine have exhibited inhibitory activity to CD_{45} protein tyrosine phosphatase [10]. Consequently, it is very important to develop effective methods for isolating and purifying these alkaloids from the leaves of *N. nucifera*.

Traditionally, extraction of alkaloids from *N. nucifera* leaves usually used organic solvents, subsequently, the alkaloids were separated and analysed by thin-layer

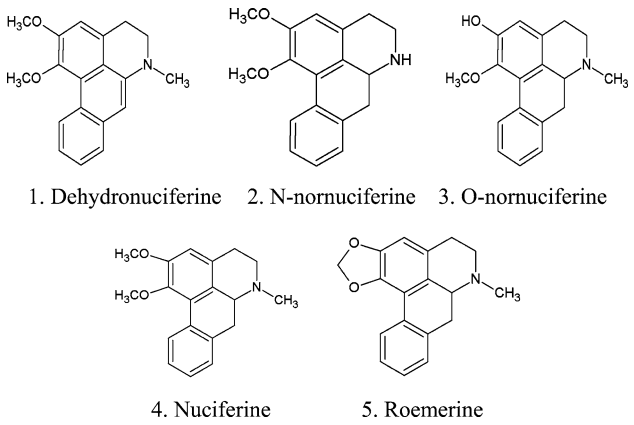

Fig. 1 Structures of Dehydronuciferine, N-nornuciferine, O-nornuciferine, Nuciferine, and Roemerine

chromatography [6], column chromatography, HPLC [1], and high-performance liquid chromatography-photodiode array detection-electrospray mass spectrometry [11]. Recent years, SFE has been widely used in many fields, especially in natural products [12]. Many reports have revealed that SFE was an almost pollution-free method and had many advantages such as having faster and more efficient extraction and giving the extracts with no residual organic solvents [12–15]. And many researchers have reported that SFE was capable of extraction alkaloids such as indole alkaloids [13], purine alkaloids [14] and cocaine [16], thus SFE can be used to extract isoquinoline alkaloids from *N. nucifera* leaves. In this method, carbon dioxide (CO_2) is one of the most commonly used supercritical fluids for the extraction of alkaloids due to its low critical point, low toxicity, chemical inertness, non-flammability, non-corrosiveness, odourlessness, and inexpensiveness [16–19]. Additionally, it allows supercritical operations at relatively low pressure and at near-room temperature and the possibility of obtaining products free of solvent residuals [20, 21]. However, since supercritical CO_2 with low polarity was not efficient for the extraction of most alkaloids, some polar solvents, such as methanol and water, were useful as modifiers to raise polarity of CO_2 [17, 22], thus enhance significantly the extraction efficiency and, consequently, reduce the extraction time [23, 24].

Many references [25–27] have shown that liquid chromatography/multi-stage mass spectrometry (LC/MS^n) offers both high sensitivity and selectivity for the unambiguous identification and quantification of multiple analytes in complex samples such as herbal materials; therefore, LC/MS^n has been proven to be a powerful analytical method for rapid determination of ingredients in botanic extracts [11, 28]. By producing the MS^n ions associated with these basic structural features as a substructural template based on the parent substance, the substance's structure may be rapidly characterized by comparing their product ions with those of the parent substance, even without standards [29, 30]. A high-performance liquid chromatography-photodiode array detection-electrospray mass spectrometry method has been developed for the analysis of four aporphine alkaloids from the leave of *N. nucifera* [11].

In the present study, we determined the best conditions in supercritical fluid extraction for highest nuciferine yield from *N. nucifera* leaves. Furthermore, a rapid and sensitive method for the identification of isoquinoline alkaloids of *N. nucifera* leaves attained by SFE was first developed based on LC/MS-ITTOF.

Experimental

Plant material

The leaves of *N. nucifera* (cultivar: wuzhierhao) were harvested from Honghu District (Hubei, China) in late August, 2008, and were sun-dried to about 8% moisture. Moisture content was determined by oven method of AOAC (1984) [31]. The dry leaves were crushed to pass through a 40 mesh/inch sieve. Particle size is one of major factors impacting on the extraction efficiency. As a result, in this experiment, leaves of 40–60 mesh (350–245 μm) were chosen for improving the extraction efficiency and simultaneously protecting the equipment from damage [32]. Part of the leaf powder was wetted with ammonia water (10%) and then dried at 60 °C [22]. The basified sample was generated by this treatment.

Chemicals and reagents

Carbon dioxide (99.9% purity) was obtained from the Daxing Gas Co., Beijing, China. HPLC-grade acetonitrile was purchased from Merck Co. (Germany). HPLC-grade methanol was obtained from Fisher Co. (USA). Water was purified using a Milli-Q system (Millipore, MA, USA). Diethylamine (99%), triethylamine (99%), ammonia water (28%), and NaOH were analytical grade reagents purchased from Shanghai Chemical Factory (Shanghai, China). Nuciferine standard was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, and diluted to the desired concentration prior to use.

Traditional solvent extraction [5, 33, 34]

Each 15 g sample of basified or non-basified leaf powder was placed into a 500 mL round-bottomed flask containing 300 mL of solvent mixture (either 90% aqueous ethanol or pH 2.5 HCl aqueous solution), and the flask was fitted with

a reflux condenser. The extraction was performed at 84 °C for 2 h in a thermostated water bath (HH-2S, Tianjin, China). The extract was filtered, then the filtrate collected was adjusted to pH 10 with 1% NaOH and evaporated in a RE111 Rotavapor (Switzerland) to remove the solvent at 50 °C. After that, the residue was dissolved using methanol in an ultrasonic bath (KQ-50B ultrasonic cleaner, Kunshan Ultrasonic Instrument Co., Ltd, Jiangsu, China). Subsequently, the methanol solution was transferred to a 10 mL volumetric flask and made-up exactly to the mark with methanol. Before HPLC analysis, the solution was filtered through a 0.45 μm membrane and was further diluted if necessary.

Procedure for supercritical fluid extraction

SFE was performed on a supercritical fluid extractor HA221-50-06 equipped with two separators and two extraction vessels (1 and 5 L) (Nantong Huaan Above-critical Extraction Co., LTD). The equipment can work well under the pressure up to 30 MPa and temperatures up to 100 °C. The flow rate of CO_2 can go up to 416.7 mL/min during the process of static and dynamic extraction. A metering valve is used to control the flow rate of the modifiers. The extract is collected at room temperature and atmospheric pressure.

In the study, leaf powder (150 g) was placed in an extraction vessel (1 L). Before the start of extraction, the extraction vessel was preheated to aim temperature (50, 60, or 70 °C) for 20 min. The extraction conditions were as follows: extraction time: static extraction (1 h) and then dynamic extraction (0.5, 1.0, 1.5, or 2 h); temperature (50, 60, or 70 °C); pressure (10, 20, or 30 MPa); modifiers (methanol, 10% (v/v) diethylamine in methanol, 1% (v/v) water in methanol, 10% (v/v) diethylamine, or 1% (v/v) water in methanol); flow rate of CO_2 (416.7 mL/min); flow rate of modifiers during dynamic extraction (0.4, 0.8, or 1.2 mL/min). The extract was collected in a brown reagent glass flask (500 mL) containing 50 mL of methanol, and then evaporated at 50 °C to remove the solvent and the modifier [35]. The residue was dissolved with methanol in an ultrasonic bath. Subsequently, the methanol solution was transferred to a 25 mL volumetric flask and made-up exactly to the mark with methanol. The solution was filtered through 0.45 μm membrane and further diluted suitably before HPLC analysis and LC/MS-ITTOF analysis.

Reverse-phase HPLC analysis

The reversed-phase HPLC analysis of isoquinoline alkaloids was performed on a Varian liquid chromatograph with a photodiode array detector. The column was a Diamonsil C_{18} column (5 μm, 200 × 4.6 mm i.d., Dikma, Beijing, China). The mobile phase was acetonitrile/water/triethylamine (56/44/0.2, v/v/v) at a flow rate of 1 mL/min. The absorbance of the eluate was monitored at 270 nm. Twenty μL of standard or the extract was uploaded in this study.

A series of standards of nuciferine in the range of 6.4–32 μg/mL were prepared in methanol. A linear response with a correlation coefficient of 0.999 ($n = 6$) was obtained for the standard.

LC/MS-ITTOF analysis of the extract of *N. nucifera* leaves

For the identification of alkaloids in the extract acquired from SFE with 10% (v/v) diethylamine and 1% (v/v) water in methanol as the modifier, an ITTOF mass spectrometer system (Kyoto, Japan) coupled with a high-performance liquid chromatography (HPLC) system (Shimadzu) was used. The LC system was equipped with a solvent delivery pump (LC-20AD), an autosampler (SIL-20AC), a DGU-20A3 degasser, a photodiode array detector (SPD-M20A), a communication base module (CBM-20A), and a column oven (CTO-20AC).

The separation was performed on a VP-ODS column (5 μm; 150 × 2.0 mm i.d.) using an isocratic mobile phase (acetonitrile/water/triethylamine (56/44/0.2, v/v/v)). The injection volume was 20 μL, the flow rate was 0.2 mL/min. Detection wavelength was over the range of 200–400 nm and the peaks were simultaneously determined at 270 nm. The sample chamber in the autosampler was maintained at 4 °C, while the column was set at 40 °C. The entire analysis sequence took 30 min.

Mass spectral data for isoquinoline alkaloids were obtained by using an ITTOF mass spectrometer which was equipped with an electrospray ionization (ESI) source conducted in the negative ion mode. Liquid nitrogen was used as a nebulizing gas at a flow rate of 1.5 L/min. The interface and detector voltages were set at −3.5 and 1.56 kV, respectively. The curved desolvation line (CDL) and heat block temperatures both were 200 °C. Mass spectrometry was performed in the full-scan mode (MS^1) and automatic multiple-stage fragmentation-scan modes (MS^2) over an m/z scan range of 100–400. The MS^2 spectra were generated by collision-induced dissociation (CID) of the selected precursor ions with argon as the collision gas. The ion accumulation time and relative collision energy were set at 50 ms and 50%, respectively. Data acquisition and processing were carried out using the LCMS solution version 3.41 software supplied with the instrument [36].

Results and discussion

Effect of temperature and pressure on the yield of nuciferine

Effects of temperature and pressure on the yield of nuciferine were studied because many literatures have reported that temperature and pressure influenced the extraction efficiency dramatically [12, 32]. Figure 2 illustrates the effect of extraction temperature (50, 60, or 70 °C) on the yield of nuciferine which was obtained from basified leaves of *N. nucifera* by supercritical CO_2 with methanol (0.8 mL/min) under 30 MPa. The yield of nuciferine increased dramatically as temperature increased. The increasing trend of the nuciferine yield slowed from 60 to 70 °C after a significant rise over the range of 50–60 °C. Taking the nuciferine yield of 144.72 μg/g obtained at 70 °C for 2 h as the control, the nuciferine yield was 72% at 50 °C and 93% at 60 °C. According to these results, it is obvious that with the increasing dynamic extraction time, the yield of nuciferine rose regularly. It was possible to extract over 80% of the nuciferine (all yields being based on the yield obtained after 2 h) within 1 h, and approximately 94% of the nuciferine within 1.5 h.

Figure 3 reveals the effect of extraction pressure (10, 20, or 30 MPa) on the yield of nuciferine which was obtained from basified leaves of *N. nucifera* by supercritical CO_2 with methanol (0.8 mL/min) at 70 °C. The yield of nuciferine was strongly enhanced with the increase in pressure. Compared with the nuciferine yield of 144.72 μg/g under 30 MPa for 2 h, the nuciferine yield obtained under 10 and 20 MPa was 52% and 81% of that, respectively. Until reaching a dynamic extraction time of 1.5 h, the yield of nuciferine increased notably along with the extending dynamic extraction time.

Considering the economic influence, 70 °C was chosen as the extraction temperature and 2 h as the dynamic extraction time. In view of the energy consumption of the equipment, 30 MPa extraction pressure was employed.

Obviously, these results were in accord with former researchers' results.

Effect of different modifiers and traditional solvent on the extraction yield

Supercritical CO_2 fluid with 4 kinds of modifiers, including methanol, 10% (v/v) diethylamine in methanol, 1% (v/v) water in methanol, 10% (v/v) diethylamine, and 1% (v/v) water in methanol, were used to extract isoquinoline alkaloids from leaves of *N. nucifera* in order to evaluate the feasibility of SFE and the effect of selected factors on the nuciferine yield.

No extract was obtained when pure supercritical CO_2 was used to extract isoquinoline alkaloids from shattered leaves of *N. nucifera*. This was attributed to the fact that most of the alkaloids in the vacuoles of plant cells are often stored in the form of salts which cannot be extracted by pure CO_2 with a weak polarity [17]. As a consequence, polar modifiers should be considered. Usually, the addition of a small amount of liquid modifier can significantly enhance the extraction yields of polar alkaloids such as hyoscyamine [17], sinomenine [22], and berberine [37], consequently, reducing the extraction time. The report by Choi et al. [17] revealed that the addition of methanol to CO_2 drastically improved the extraction yield of hyoscyamine and scopolamine.

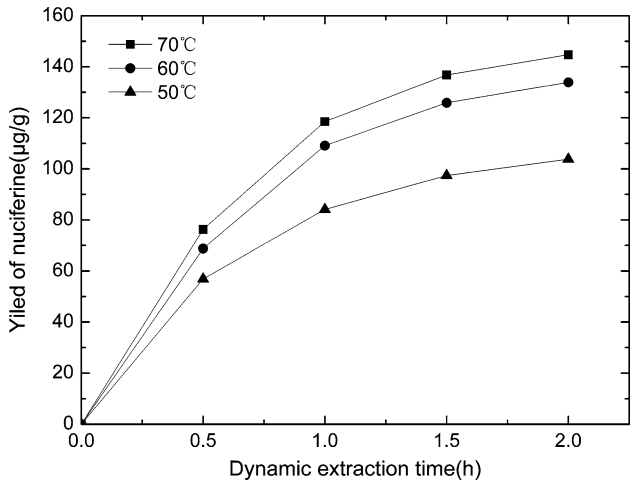

Fig. 2 Yield of nuciferine obtained from basified leaves of *N. nucifera* by supercritical CO_2 with methanol (0.8 mL/min) over a range of temperature under 30 MPa

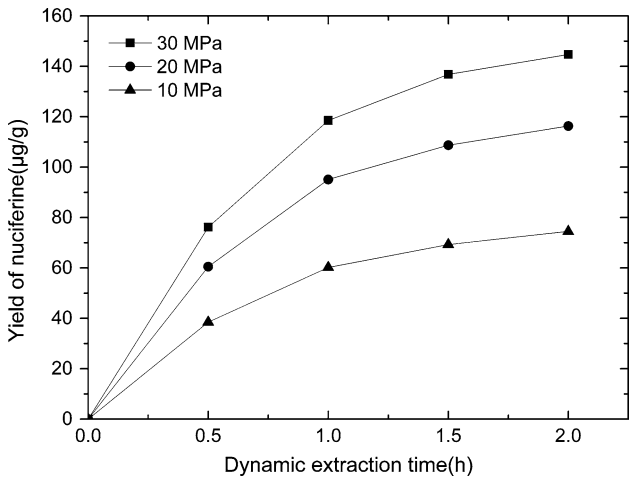

Fig. 3 Yield of nuciferine obtained from basified leaves of *N. nucifera* by supercritical CO_2 with methanol (0.8 mL/min) over a range of pressure at 70 °C

Methanol-modified supercritical CO_2 and solvent mixtures (90% aqueous ethanol or pH 2.5 HCl aqueous solution) were used to extract isoquinoline alkaloids from basified and non-basified leaves of *N. nucifera*. The results are displayed in Table 1. Conspicuously, the yield of nuciferine gained from basified leaves using SFE with methanol was twice as much as that obtained from non-basified leaves. Simultaneously, the ratio of total isoquinoline alkaloids to the extract of the former went up to by nearly two times that of the later. The above results suggested that alkaloids existed in the form of combinations with organic acids, and these results agree with the reports by Choi et al. and Liu et al. Therefore, basified leaves were applied in further steps of this study. The yield of nuciferine acquired by methanol-modified supercritical CO_2 was only 60% of the yield obtained by 90% aqueous ethanol. Nevertheless, the ratio of total isoquinoline alkaloids to the extract of the former was increased by about two times, compared with that of the latter. Apparently, it was inefficient to extract isoquinoline alkaloids using methanol-modified supercritical CO_2.

To improve the yield of nuciferine, diethylamine and water were taken into account for the addition of diethylamine to methanol dramatically enhancing the yield of scopolamine and hyoscyamine [17] and a low content of water in supercritical CO_2 benefiting the extraction process [38, 39]. Supercritical CO_2 with 3 kinds of modifiers including 10% (v/v) diethylamine in methanol, 1% (v/v) water in methanol, 10% (v/v) diethylamine, and 1% (v/v) water in methanol were investigated to evaluate the feasibility of SFE. The results are shown in Table 1. The addition of diethylamine (10%, v/v) to methanol enhanced the yield of nuciferine largely in comparison with pure methanol. The yield of nuciferine was got a little rise when water (1%, v/v) was added into methanol, which may agree to K. Jackson's study that only 0.5% (v/v) of water can be completely miscible with CO_2, thus water did not appear to improve the polarity of CO_2 as much as did methanol [40]. However, adding diethylamine (10%, v/v) and water (1%, v/v) to methanol together led to a sharp rise of the nuciferine yield. The higher nuciferine yield, 292.35 μg/g, won by 10% (v/v) diethylamine and 1% (v/v) water in methanol as the modifiers were far more than that obtained by other methods, so it can be deduced that water can increase the polarity of CO_2 and diethylamine can make alkaloids more easily extractable from basified leaves. Furthermore, the ratio of total isoquinoline alkaloids to the extract reached 49.31%. The results were in accordance with Choi's study that diethylamine (10%, v/v) can be treated as a basifying agent for dissociating isoquinoline alkaloids and water (1%, v/v) as an agent for increasing the polarity of CO_2 in SFE of alkaloids [17].

Effect of flow rate of the modifier on the yield of nuciferine

Flow rate of the modifier is also a significant factor impacting the yield of nuciferine [32]. In the above experiments, flow rate of modifier was controlled at 0.8 mL/min. In order to evaluate the effect of flow rate of the modifier (0.4, 0.8 or 1.2 mL/min) on the yield of nuciferine, a series of experiments were carried out with

Table 1 Effect of different modifiers and traditional solvent on the yield of nuciferine (μg/g), and the ratio of total isoquinoline alkaloids to the extract (%) from leaves of *N. nucifera*

Sample	Modifier or solvent	Yield[f]	Ratio[g]
Nonbasified powder	SFE[a], Methanol	71.12 ± 3.53	20.86 ± 0.98
Basified powder	SFE[a], Methanol	144.72 ± 8.54	41.43 ± 1.64
Basified powder	SFE[a], Diethylamine-Methanol[c]	211.77 ± 10.88	48.05 ± 2.42
Basified powder	SFE[a], Water-Methanol[d]	170.91 ± 8.12	41.06 ± 1.88
Basified powder	SFE[a], Diethylamine-Water-Methanol[e]	292.35 ± 9.74	49.31 ± 1.96
Nonbasified powder	Solvent extraction[b], 90% aqueous ethanol	236.47 ± 7.45	11.33 ± 0.91
Nonbasified powder	Solvent extraction[b], pH 2.5 HCl aqueous solution	121.35 ± 6.15	14.70 ± 1.26
Basified powder	Solvent extractiont[b], 90% aqueous ethanol	225.29 ± 10.16	21.20 ± 1.69

[a] supercritical fluid extraction. The temperature and pressure were 70 °C and 30 MPa, respectively. Flow rate of CO_2 and the modifier were 416.7 mL/min and 0.8 mL/min, respectively. Dynamic extraction lasts 2 h

[b] Traditional solvent extraction. The extraction was performed at 84 °C for 2 h

[c] 10% (v/v) diethylamine in methanol

[d] 1% (v/v) water in methanol

[e] 10% (v/v) diethylamine and 1% (v/v) water in methanol

[f] The yield of nuciferine (μg/g) (Mean ± SD)

[g] The ratio of total alkaloids to the extract (%) (Mean ± SD)

10% (v/v) diethylamine and 1% (v/v) water in methanol as the modifier at 70 °C under 30 MPa for 2 h. The results illustrated that varying the flow rates of the modifier from 0.4 to 0.8 mL/min, the yield of nuciferine had increased from 186.12 to 292.35 μg/g, and then the yield of nuciferine increased by 33.56 μg/g when flow rate of the modifier improved from 0.8 to 1.2 mL/min. That is to say, the increasing flow rate of the modifier caused an increasing yield of nuciferine, but the increasing trend became less after a sharp rise in the range of 0.4–0.8 mL/min. The highest nuciferine yield of 325.54 μg/g was obtained when the extraction was carried out for 2 h at 70 °C under 30 MPa, 10% (v/v) diethylamine and 1% (v/v) water in methanol as the modifier, with a flow rate of 1.2 mL/min for supercritical CO_2. The corresponding ratio of total isoquinoline alkaloids to the extract was the highest, reaching up to 49.85%.

HPLC chromatograms of the extracts obtained

Figure 4 exhibits HPLC chromatograms of the nuciferine standard and the extract obtained from basified leaves by supercritical CO_2 modified with 10% (v/v) diethylamine and 1% (v/v) water in methanol at 70 °C under 30 MPa for 2 h. By comparison with the peak of the nuciferine standard which appeared at a retention time of 12.57 min, the nuciferine peak of the extract can be identified rapidly. Chromatograms of all other extracts were similar to Fig. 4b. Five peaks appeared at 4.47, 5.97, 7.58, 12.57, and 14.71 min, which probably belonged to isoquinoline alkaloids including the nuciferine which appeared at 12.57 min.

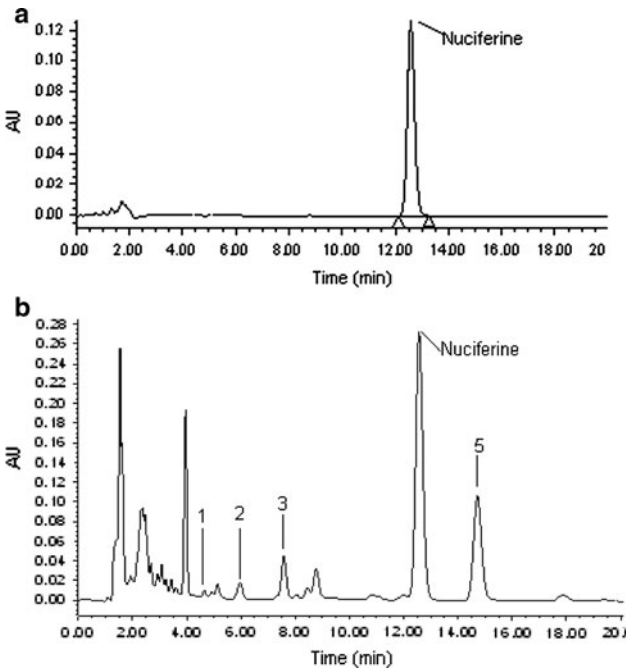

Fig. 4 **a** HPLC chromatogram of Nuciferine standard, **b** HPLC chromatogram of the extract obtained from basified leaves with methanol-modified supercritical CO_2

LC/MS-ITTOF analysis of nuciferine and other isoquinoline alkaloids

The extract of basified leaves acquired by SFE with 10% (v/v) diethylamine and 1% (v/v) water in methanol as the modifier was analysed by LC/MS-ITTOF. Its total ion current chromatogram in negative mode is shown in Fig. 5. Five peaks appeared at retention times of 7.6, 9.3, 12.1, 20.8, and 22.8 min under the conditions described in Section "LC/MS-ITTOF analysis of the extract of *N. nucifera* leaves". This was consistent with the five peaks in the HPLC chromatograms such as illustrated in Fig. 4b.

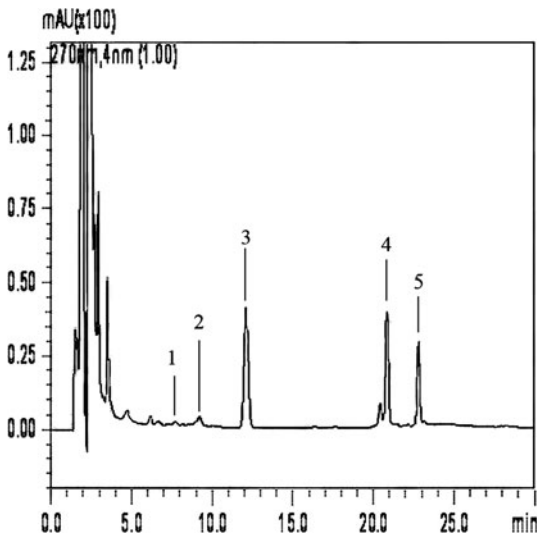

Fig. 5 LC/MS-ITTOF total ion current chromatogram in negative mode of the extract of *N. nucifera* leaves obtained by SFE with 10% (v/v) diethylamine and 1% (v/v) water in methanol as the modifier

Table 2 summarizes the formulas, retention time (t_R), UV (λ_{max}), observed masses and predicated masses, double bond equivalents (DBE), and mass errors of the fragment ions of isoquinoline alkaloids component of Fig. 5. Five alkaloids exhibited their maximum UV absorption over the wavelength range: 270–272 nm, which conforms to the characteristic of isoquinoline alkaloids in *N. nucifera* leaves. The fragment ion at m/z 277.1143 in ingredient 1 indicated the fragment $[M–H–CH_3]^-$. The fragment ion at m/z 249.1009 in ingredient 2 implied the fragment $[M–H–OCH_3]^-$ which was in accord with the fragment ion at m/z 251 in N-nornuciferine in Luo's study. The fragment ions at m/z 265.1094 and 250.0858 in ingredient 3 are supposed to infer fragments $[M–H–CH_3]^-$ and $[M–H–CH_3–CH_3]^-$, respectively. From the fragment ions at m/z 279.1212 and 263.1236 in ingredient 4, namely Nuciferine, fragments

Table 2 Formulas, Retention time (t_R), UV (λ_{max}), Observed and Predicated masses, Double bond equivalents (DBE), and Mass error of the fragment ions of alkaloids' components of Fig. 5

Ingredient	Predicated formula $[M–H]^-$	t_R (min)	UV (λ_{max}) (nm)	Predicated mass (Da)	Observed Mass (Da)	DBE	Error (mDa)	Error (ppm)	Proposed compound
1	$C_{19}H_{19}O_2N^-$	7.6	272	292.1343	292.1317	11	−2.6	−8.90	Dehydronuciferine
				277.1108	277.1143	12	3.5	12.63	
2	$C_{18}H_{19}O_2N^-$	9.3	270	280.1343	280.1303	10	−4.0	−14.28	N-nornuciferine
				249.1033	249.1009	11	−2.4	−9.63	
3	$C_{18}H_{19}O_2N^-$	12.1	272	280.1343	280.1431	10	8.8	31.41	O-nornuciferine
				265.1108	265.1094	12	1.4	5.30	
				250.0874	250.0858	11	−1.6	−6.40	
4	$C_{19}H_{21}O_2N^-$	20.8	270	294.1500	294.1560	10	6.0	20.40	Nuciferine
				279.1265	279.1212	11	−5.3	−18.99	
				263.1316	263.1286	11	−3.0	−11.40	
5	$C_{18}H_{17}O_2N^-$	22.8	272	278.1187	278.1229	11	4.2	15.10	Roemerine
				263.0912	263.0925	8	1.3	4.94	

$[M–H–CH_3]^-$ and $[M–H–OCH_3]^-$ can be concluded, respectively. The fragment ion at m/z 263.1286 accorded with the fragment ion at m/z 265 in Nuciferine in Luo's study. The fragment ion at m/z 263.0925 in ingredient 5 indicated the fragment $[M–H–CH_3]^-$. The peak sequence of ingredients 2, 3, 4, and 5 was consistent with that in Luo's study. All five isoquinoline alkaloids could be identified in leaves of *N. nucifera* by comparing their t_R, UV, and MS data with those of the reference standards. The mass error was relatively large, perhaps as a result of lack of instrument calibration. The mass errors of the fragment ions were in the range of −5.3 to 8.8 mDa.

Conclusions

It was notable that the yield of nuciferine from *N. nucifera* leaves was influenced by using various modifiers in SFE, among which the highest yield was obtained by using 10% diethylamine (v/v) and 1% water (v/v) in methanol as the modifier, while the yield obtained by using pure methanol as the modifier was the lowest. Supercritical CO_2 with 10% diethylamine (v/v) and 1% water (v/v) in methanol was more efficient than the traditional solvent method. Diethylamine (10%, v/v) as a basifying agent for liberating the alkaloids and water (1%, v/v) as an agent for increasing the polarity of CO_2 are useful for SFE of alkaloids. Basified leaves are more efficiently extracted for alkaloids by SFE rather than non-basified leaves, in that basifiers such as ammonia water can dissociate alkaloids from their combination with organic acids. These results will prove useful for the SFE of other alkaloids from other types of plant materials. According to LC/MS-ITTOF, five kinds of isoquinoline alkaloids such as Dehydronuciferine, N-nornuciferine, O-nornuciferine, Nuciferine, and Roemerine extracted from *N. nucifera* leaves were found.

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