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Separation and determination of four ganoderic acids from dried fermentation mycelia powder of *Ganoderma lucidum* by capillary zone electrophoresis

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ABSTRACT

Ganoderic acids (GAs) were bioactive secondary metabolites produced by a traditional mushroom *Ganoderma lucidum*. We describe a simple and efficient method for the separation and quantitative determination of four GAs, namely Ganoderic acid T (GA-T), Ganoderic acid Mk (GA-Mk), Ganoderic acid Me (GA-Me) and Ganoderic acid S (GA-S) from dried triterpene-enriched extracts of *G. lucidum* mycelia powder by capillary zone electrophoresis (CZE). Under the optimum conditions, the four GAs reached the baseline separation in 9 min with Glycyrrhetinic acid (GTA) as internal standard. The four GAs and internal standard (GTA) were detected at a wavelength 245 nm. All calibration curves showed good linearity ($r^2 > 0.9958$) within test ranges. Limit of detection (LOD) and limit of quantification (LOQ) were less than 0.6 and 1.8 μg/mL, respectively. The relative standard deviation (R.S.D.) values of precision and recoveries were less than 5% and recoveries ranged from 91.4% to 103.6%. This was the first report on simultaneous determination of the four GAs and the results provided a firm basis for the trace analysis of GAs in dried fermentation mycelia powder of *G. lucidum* with high accuracy.

1. Introduction

Ganoderma lucidum Karst ("Lingzhi" in Chinese, "Youngzhi" in Korean and "Reishi" in Japanese) as a kind of very popular and valuable traditional Chinese medicine has gained more and more attention. It was used as a medicinal herb to treat a variety of diseases [1] including hypertension, hypercholesterolemia, hepatitis, gastritis, diabetes, bronchitis, and cardiovascular problems. Highly oxygenated lanostane-type triterpenoids, such as GAs and lucidenic acids, were identified as primary bioactive ingredients in *G. lucidum* [2]. Among them GAs had a broad spectrum of pharmacological activities, such as significant cytotoxicity of hepatoma cells, hepato-protection, inhibition of histamine release, stimulation of platelet aggregation and anti-HIV activity [3,4].

Several modern analytical methods to the qualitative analysis of GAs in *G. lucidum* were reported, including thin-layer chromatography [5–7] and high-performance liquid chromatography (HPLC) [8–14]. For example, a method combining HPLC and pressurized liquid extraction was developed by Zhao et al. for simultaneous quantification of nine components, including eight triterpenes (ganoderic acid A, ganoderic acid Y, ganoderic acid DM, ganoderol A, ganoderol B, ganoderal A, methyl ganoderate D and ganoderate G) and a sterol (ergosterol), in Ganoderma and applied the method to quantify the nine components in two species of Ganoderma, i.e. *G. lucidum* and *G. sinense*, used as Lingzhi in China [10]. Wang et al. reported a HPLC method for the quantitative determination of six major triterpenoids (namely ganoderic acids C_2, B, AM_1, K, H and D) in *G. lucidum* and its related species and the method was applied to quantitative determination of constituents of triterpenoids in 36 different samples of *G. lucidum* and its related species [11]. Later, the same author also reported a HPLC method for the determination and pharmacokinetic studies of four triterpenoids in rat plasma after oral administration of *G. lucidum* extract and evaluating the clinical applications of this medicinal fungus [12]. Zhong and co-workers [15] reported the effective use of reversed phase liquid chromatography with ultraviolet and mass spectrometry detections for the separation of GA-T and GA-Me from *G. lucidum*. However, it was rarely reported that these separative methods mentioned above were used for simultaneous separation of up to this four kinds of GAs (GA-T, GA-Mk, GA-Me and GA-S,

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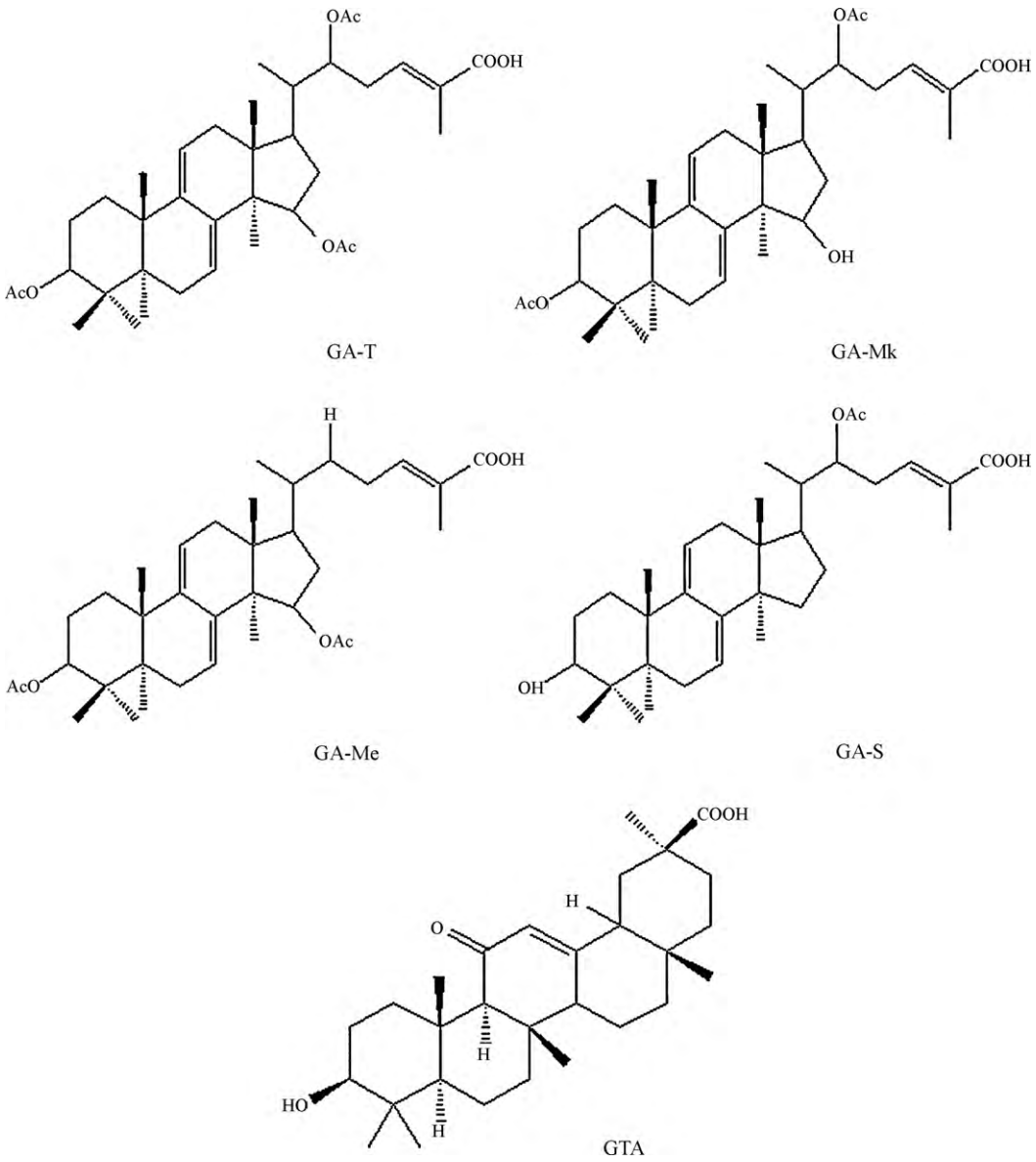

Fig. 1. Chemical structures of GA-T, GA-Mk, GA-Me, GA-S and GTA.

see Fig. 1) owing to they had many similar properties, such as the same electric charge, high hydrophobicity and extreme similarity in their chemical structures. Recently, capillary electrophoresis (CE) has become a well-adapted analytical technique possessed both high resolving power and efficiencies with fast analysis times [16,17]. These features have attracted many researchers to the technique [18–20] especially the contributions about separating complex metabolites [21] and several analogous compounds [22]. A cyclodextrin-modified CE method was developed by Guo et al. [23] for the separation of bioactive pentacyclic triterpene acids from the fruits of *Rubus chingii*. Emara et al. [24] advanced a CE method for the separation and quantitation of naturally occurring oleanene triterpenoidal saponins. Qi et al. [25] reported a novel and simple nonaqueous capillary electrophoresis (NACE) method for the complete separation of three bioactive triterpenes ursolic acid, oleanolic acid and 2α,3β,24-trihydroxy-urs-12-en-28-oic acid and the method was successfully utilized for separation and determination the three analytes in six Chinese herbs extraction.

The purpose of this work was to separate four bioactive ganoderic acids of GA-T, GA-Mk, GA-Me and GA-S that were structurally very close to each other, using the powerful CZE separation method, and thereby to determine their quantities within the dried fermentation mycelia powder. For the sake of exact quantitative analysis, GTA (Fig. 1) was used as internal standard after a series of screening, considering that GTA did not exist in the matrix of *G. lucidum* and could be completely separated from these four GAs.

2. Experimental

2.1. Apparatus

The experiment of relevant separation analysis was carried out with an ACS 2000 HPCE apparatus which purchased from Beijing Cailu Scientific Inc. (Beijing, China), equipped with a power supply (up to voltage 30 kV), a HW-2000 chromatography workstation from Qianpu Software Co. Ltd. (Nanjing, China) and a UV–vis detector (double light beams, λ = 190–740 nm, set at 245 nm). Two fused-silica capillaries which were purchased from Factory of Yongnian Optical Fiber (Hebei, China) were used and their dimensions were 55 cm (46 cm to the detector) × 75 μm I.D. and 69 cm (60 cm

to the detector) × 75 μm I.D. Water from an SG Ultra Clear system (Wasseraufbereitung und Regenerierstation Gmbh, Germany) was used to produce ultra-pure water with specific conductivity down to 0.055 μS cm^{-1}. All the pH measurements were performed with the Metter-Toledo (Zurich, Switzerland). A centrifuge from Factory of Shanghai Anting Scientific Instrument (Shanghai, China), a HS-1200 ultrasonic apparatus from Shanghai Scientific Institute of Biotechnology (Shanghai, China) and a vacuum air pump from Shanghai Deying Vacuum & Lighting Equipment Co. Ltd. (Shanghai, China) were used for extraction.

2.2. Chemicals

The standard GAs (GA-T, GA-Mk, GA-Me and GA-S, analytical reagent, AR) and dried fermentation mycelia powder containing the four GAs were supplied by the Key Laboratory of Microbial Metabolism of Shanghai Jiao Tong University (Shanghai, China). GTA (AR) was purchased from the Aladdin Reagent Inc. (Shanghai, China). Boric acid (guarantee reagent, GR, 99.8%), disodium tetraborate decahydrate (GR), hydrochloric acid (AR), sodium hydroxide (GR), disodium hydrogen phosphate dodecahydrate (AR) and sodium dihydrogen phosphate (AR) were purchased from Shanghai Chemistry Reagent Company (Shanghai, China). Tris (hydroxymethyl) aminomethane (Tris, AR) was purchased from Shanghai Shisheng Cell Biotechnology Co. Ltd. (Shanghai, China). Acetonitrile (ACN, AR), methanol (AR), ethyl acetate (AR) and ethanol (AR) were purchased from Shanghai Lingfeng Chemical Reagent Co. Ltd. (Shanghai, China).

2.3. Standard solution and buffer

Sodium borate buffer was finally chosen as the background electrolyte (BGE) in our experiment, its pH values ranged from pH 8.0 to 10.0 and the concentrations ranged from 10 to 35 mM. The pH of the buffer was adjusted by addition of equivalent concentration boric acid or NaOH, respectively. ACN was used as organic modifier and its concentrations were from 30% to 60% (v/v). Owing to the hydrophobicity of GAs and GTA, the stock standard solutions (1.0 mg/mL) of the four GAs and GTA were prepared by dissolving 1.0 mg sample powder in 1.0 mL methanol and stored at 4 °C prior to use. A series of standard working solutions were further made by diluting the stock standard solutions with running buffer.

0.5, 1.0, 5.0, 10.0, 20.0, 30.0, 50.0, 60.0, 80.0 and 100.0 μL of the four GAs solutions (1.0 mg/mL) and 20.0 μL internal standard solution (1.0 mg/mL) were added into blank sample powder in sequence from low to high concentration and extracted using the procedures described in Section 2.4. The final concentrations of GAs were 0.5, 1, 5, 10, 20, 30, 50, 60, 80 and 100 μg/mL and that of GTA was 20 μg/mL. It was necessary to state that the blank sample was the matrix of *G. lucidum* mycelia powder but free of GAs, it was obtained by collecting the *G. lucidum* mycelia powder which had been extracted the four ganoderic acids, and no GAs peak appeared in CE electrophoretogram (in Fig. 6A).

2.4. Extraction procedures

GA-T, GA-Mk, GA-Me, GA-S and GTA could be well extracted from dried fermentation mycelia powder with ethanol. The extraction was performed as follows: the finely ground dried fermentation mycelia powder (30 mg) and 20 μL internal standard (GTA, 1 mg/mL) were mixed well with 980 μL ethanol, then the mixture was strongly vibrated with ultrasonic for 1 h followed by centrifuged at 8000 rpm for 10 min. For the purpose of maximum extraction efficiency, the extraction was repeated for three times. All the ethanol was combined together and evaporated to dryness. The dried residue was dissolved with 1 mL running buffer for CZE analysis.

2.5. Analytical procedures

Before use, each new capillary was conditioned by flushing successively with 1 M NaOH for 20 min, followed by ultra-pure water for 20 min, 1 M HCl for 20 min, ultra-pure water for 20 min and finally rinsed with running buffer for 30 min. Pressure injection was selected and the pressure was set at 13 mbar with the injection time controlled at 10 s. The running buffer was changed every 5 injections for fear of influence on migration time drift. The temperature of air-cooling was maintained at 25 °C.

3. Results and discussion

3.1. Optimization of conditions

There were some factors that had notable impact on the resolution, sensitivity, migration time, peak shape, and column efficiency of CE. Consequently, an appropriately designed, rapid and sensitive method was needed. To investigate these factors effectively, a shorter capillary with total length of 55 cm was used at first.

3.1.1. Selection of running buffer and buffer pH value

Because GAs were weak acid, alkaline buffer should be a better choice. According to the literature, the normally used BGE for the separation of triterpenoids in CE were sodium borate buffer [23,24], phosphate buffer and Tris–HCl buffer [25]. In our experiment, it was observed that there were several interferential peaks and the peaks of the GAs were overlapped when Tris–HCl buffer was used as BGE, and only two sharp GAs peaks appeared in one broad peak when the phosphate buffer was used. However, if borate buffer was applied as BGE, the separation of GAs became much better in contrast to these with Tris–HCl and phosphate buffers. It was demonstrated in Fig. 2A that the four GAs were partially separated. Furthermore, the electric current of borate buffer was low, hence, borate buffer was chosen as BGE in the following study.

The effect of pH value on the separation was studied over a range of 8.0–10.0, but it had a less substantial effect on resolution of GAs. When the pH value of sodium borate buffer was set at pH 9.0, it was observed that the separation of four GAs had good reproducibility, short migration time and stable base line of detection, in addition, sodium borate buffer at pH 9.0 had a quite sufficient buffer capacity. Hence, pH 9.0 was finally chosen as an optimized parameter.

3.1.2. Effect of organic additive

Organic additive as modifiers had been shown to affect both CZE and MEKC separations [26–28], the addition of them reduced the electroosmotic flow (EOF) velocity leading to prolong the migration time. For further investigating the effect of different organic solvents on separation, methanol and ACN which were the most commonly used solvents [29,30] were studied in this section. Under the same conditions, they were equivalently (57%, v/v) added into the BGE, respectively. The results showed that methanol had little effect on resolution, but ACN appeared to markedly improve resolution. This was because (1) ACN was a dipolar aprotic solvent with a high dipole moment and dielectric constant while MeOH was a protic solvent [31]; (2) the ratio of dielectric constant to viscosity of ACN (110) was higher than that of MeOH (60) [32]; (3) MeOH/water mixtures had been found to consist of a ternary mixture [33] while ACN/water mixture formed up to six different compositions on the microscopic scale [34] and so on. The above reasons resulted in ACN/water had larger influence on the zeta potential and EOF than those of MeOH/water. Therefore, the separation efficiency of

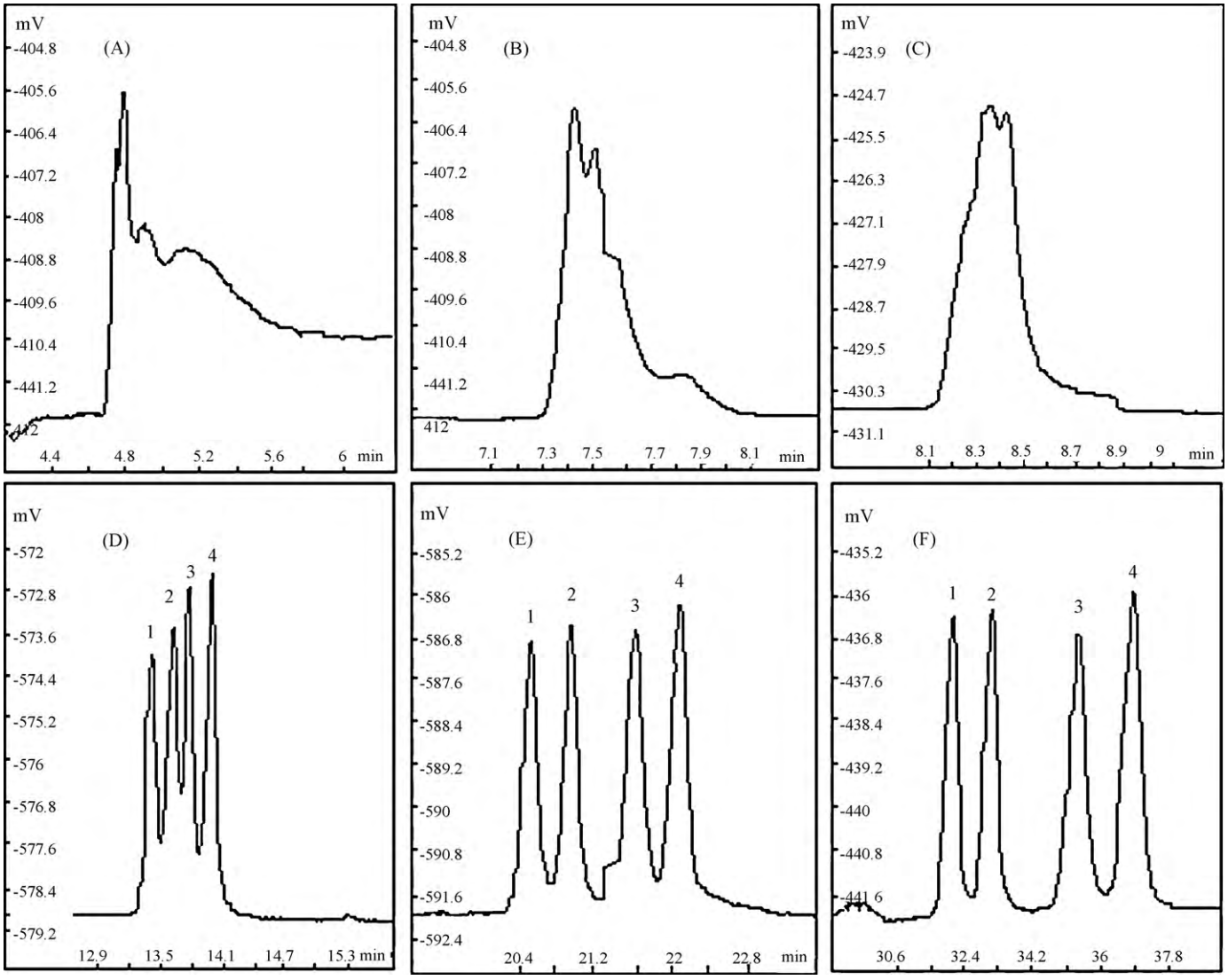

Fig. 2. Effect of acetonitrile concentrations on resolution and migration time of (1) GA-T, (2) GA-Mk, (3) GA-Me, and (4) GA-S. (A) 0% ACN; (B) 30% ACN; (C) 40% ACN; (D) 50% ACN; (E) 55% ACN; (F) 60% ACN. Conditions: 0–60% concentrations (v/v) of ACN, 20 mM pH 9.0 sodium borate buffer, 15 kV applied voltage, the concentration of each sample were 20 μg/mL, 55 cm total length (46 cm to the detector) and 75 μm i.d. capillary, 245 nm detection wavelength, 13 mbr 10 s pressure sample injection, 25 °C air-cooling.

ACN/water was superior to that of MeOH/water. Hence, ACN was selected as the organic modifier solvent.

The electropherograms displayed in Fig. 2B–F investigated the effect of different concentrations of ACN (0–60%) with pH 9.0 sodium borate buffer introduced into the buffer system on resolution and migration time. With increasing the percentage of ACN, the resolutions were improved at the expense of an increase of the migration time. The four analytes were separated well when the concentration reached at 55%, but bubbles and crystal of sodium borate buffer occurred frequently at 60% even if the resolutions increased. Thus, the influence of different concentrations of ACN on the resolutions of GAs from 50% to 60% was further studied (see Fig. 3). On account of better resolutions and faster analysis of samples, the addition of 57% ACN to the buffer was chosen as the most suitable condition.

To further clarify the best BGE, the equivalent of phosphate buffer was designed to replace the sodium borate buffer in present buffer system, the data indicated that the resolutions were inferior to that of sodium borate buffer when ACN reached at 57% and the resolutions were worse in spite of increasing or decreasing the amount of ACN, which further indicated that the sodium borate buffer was indeed fit for this work. Further, it is

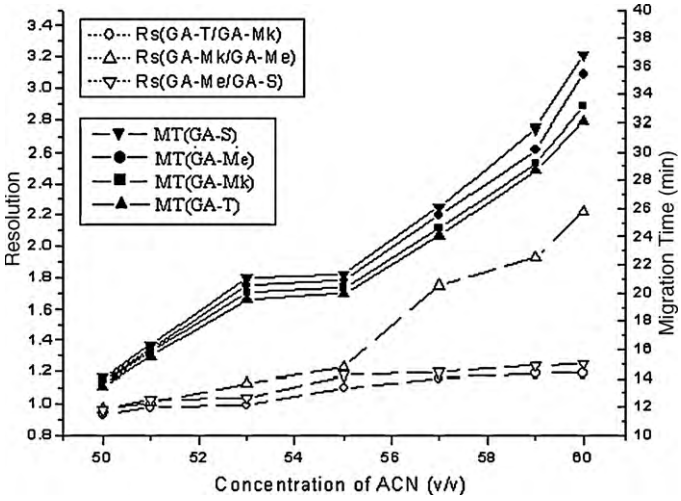

Fig. 3. Effect of acetonitrile concentrations on resolution and migration time of GA-T, GA-Mk, GA-Me, GA-S. Conditions: 50–60% concentrations (v/v) of acetonitrile, other conditions were the same as those in Fig. 2.

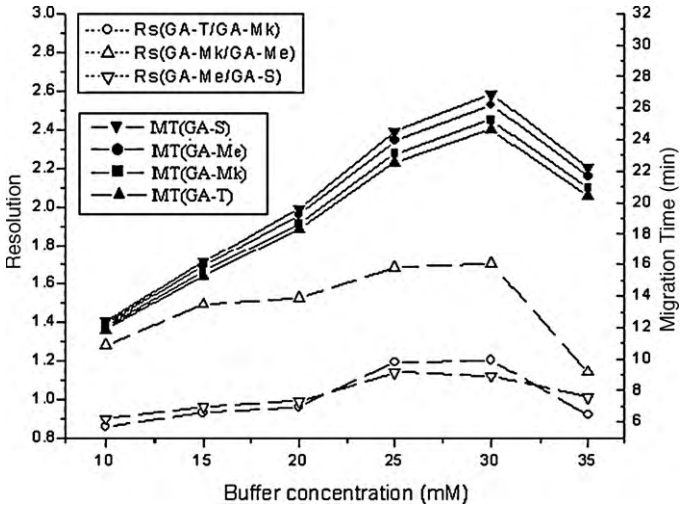

Fig. 4. Effect of buffer concentrations on resolution and migration time of GA-T, GA-Mk, GA-Me, GA-S. Conditions: 10–35 mM concentrations of buffer, 57% (v/v) ACN, other conditions were the same as those in Fig. 2.

easy to form bubble when phosphate buffer and Tris–HCl buffer were used.

3.1.3. Effect of buffer concentration and applied voltage

Buffer concentration and applied voltage affected the resolution, migration time and peak shape. It was displayed that (in Fig. 4) the resolutions increased and the migration time prolonged obviously when the buffer concentrations increased from 10 to 30 mM, however, the buffer often formed bubbles and crystal during the experiment when the buffer was 30 mM. After a comparative study, the 25 mM buffer was selected in the following experiments.

Attempt was made to optimize the separation conditions by using different applied voltages ranging from 15 to 30 kV. The migration time decreased with increasing applied voltage and the resolution was decreased. When the voltage reached at 30 kV, it induced a poor resolution of peaks as well as a deteriorated baseline because of the much higher Joule's heating and electric current at higher voltage. In order to get shorter migration time and better resolution, 27.5 kV was selected as the working voltage.

3.1.4. Effect of extraction solvent

The ganoderic acids were easily dissolved in organic solvent. Hence, ACN, methanol, ethyl acetate and ethanol were tried for the extraction of GAs from sample matrix. The finely ground dried fermentation mycelia powder (30 mg) and 20 μL internal standard (GTA, 1 mg/mL) were mixed well with 980 μL different organic solvent, respectively. The further extraction steps were the same as those described in Section 2.4. In view of the impact on extraction efficiency, instrument noise, toxicity of organic solvents, and ethanol were used as the optimum extractant.

3.1.5. Optimized separation conditions

As the conclusions mentioned above, the optimized separation conditions were obtained as follows: 25 mM pH 9.0 sodium borate buffer with 57% ACN (v/v), 27.5 kV applied voltage, 55 cm total length (46 cm to the detector) and 75 μm i.d. capillary, 245 nm detection wavelength, 10 s 13 mbr pressure injection. Under the optimal experimental conditions, the electrophorogram of the four GAs and GTA were shown in Fig. 5. In figure, the width of peak 5 was wider than that of the other four peaks, this was caused by the following two reasons. The first is that the concentration of peak 5 was higher than that of the others, and the second is that the migration time of peak 5 was longer than that of the others, and the longer the migration time, the serious the diffusion. However, at high concentration, a longer capillary was need.

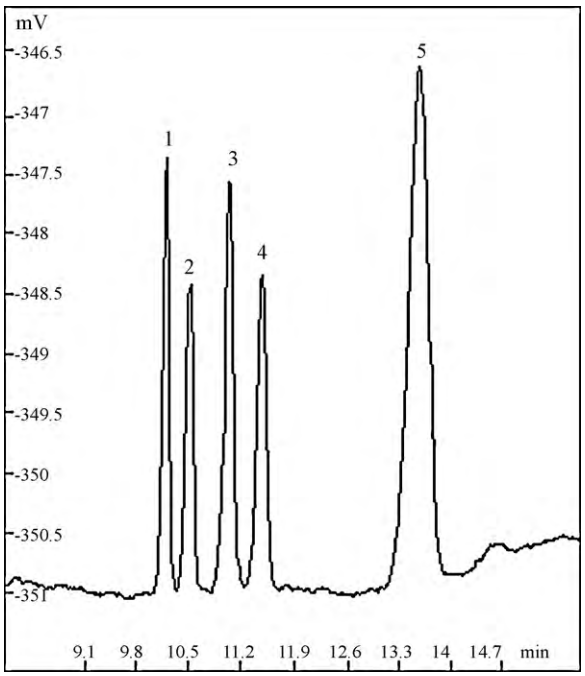

Fig. 5. Samples and internal standard peaks under the optimal results. (1) GA-T; (2) GA-Mk; (3) GA-Me; (4) GA-S; (5) GTA. Conditions: 27.5 kV applied voltage, 57% (v/v) ACN, 25 mM buffer, other conditions were the same as those in Fig. 2.

3.2. Validation

To improve resolution in high concentration, a longer capillary with total length of 69 cm was used in the following experiments.

3.2.1. Specificity

A proper amount of dried mycelia powder containing the four GAs spiked with the internal standard solution of GTA was extracted in accordance with the procedure in Section 2.4. It was identified that (in Fig. 6) 1, 2, 3, 4 and 5 peaks were GA-T, GA-Mk, GA-Me, GA-S and GTA, respectively. The results in Fig. 6 coupled with Fig. 5, demonstrated the good specificity of the method for the determination of GAs.

3.2.2. Linearity, LOD and LOQ

The linearities of GA-T, GA-Mk, GA-Me and GA-S were determined by spiking their standard solutions into blank samples (details see Section 2.3). The final concentrations of four GAs in

Table 1
The linearity, LOD and LOQ of four GAs in the *G. lucidum* mycelia powder samples.

GA	Calibration curves	R^2	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
T	$y = 0.0589x - 0.0926$	0.9958	1.0–100	0.3	1.0
Mk	$y = 0.0521x - 0.0521$	0.9968	2.0–100	0.6	1.8
Me	$y = 0.0456x - 0.0712$	0.9976	2.0–100	0.5	1.6
S	$y = 0.0764x - 0.1026$	0.9990	1.0–100	0.3	1.0

Table 2
The intra-day, inter-day R.S.D. and the recovery of samples at different concentrations ($P = 0.95$, $n = 5$).

Spiking level (μg/mL)	Intra-day R.S.D. (%)				Inter-day R.S.D. (%)				Recovery (%)			
	T	Mk	Me	S	T	Mk	Me	S	T	Mk	Me	S
5	1.5	0.7	2.9	2.0	1.9	2.0	3.3	1.6	100. 1 ± 2.5	98.1 ± 1.9	95.6 ± 2.1	99.3 ± 1.2
30	1.2	0.5	0.3	1.5	1.3	1.5	1.2	1.9	97.6 ± 3.5	95.1 ± 2.1	91.4 ± 1.3	94.8 ± 1.2
80	0.6	0.9	1.2	1.4	1.6	2.9	2.9	2.5	97.7 ± 1.0	97.6 ± 3.3	99.5 ± 1.1	103.6 ± 2.3

P = the confidence level.

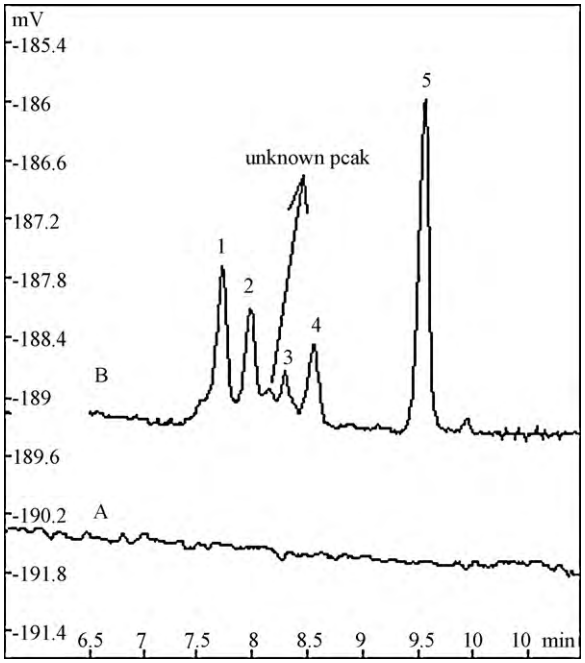

Fig. 6. Electropherograms of CZE. (1) GA-T; (2) GA-Mk; (3) GA-Me; (4) GA-S; (5) GTA. (A) Blank sample; (B) real sample and 20 μg/mL internal standard. Conditions: the optimized experimental conditions, 69 cm total length (60 cm to the detector) other conditions were the same as those in Fig. 2.

blank sample were 0.5, 1, 5, 10, 20, 30, 50, 60, 80 and 100 μg/mL, and 20 μg/mL GTA as internal standard. LOD (S/N = 3) reflected the sensitivity of the method and equipment while the LOQ (S/N = 10) reflected the reliability of the method when used for the analysis if low concentration sample. The regression equations of the curves, the correlation coefficients, linear ranges, LOD and LOQ for the samples were all given in Table 1.

3.2.3. Precision

To test the reproducibility of the developed method, both intra-day and inter-day R.S.D. of peak areas ratio between analytes and internal standard were examined. The intra-day values of R.S.D. were performed by analyzing samples with the interval of 2 h in a day for five times at three different concentrations of low (5 μg/mL), medium (30 μg/mL) and high (80 μg/mL), while the inter-day values of R.S.D. were performed in five different days with the same three concentrations. As shown in Table 2, all of the R.S.D. values were less than 5%. This implied that the experimental conditions selected above could provide a stable background with quite good repeatability.

3.2.4. Recovery

The recovery experiments were performed at three different concentrations as precision values. The recovery was calculated using the regression equation showed in Table 1 from calibration and the testing results. The recovery data in Table 2 were all within the acceptable range. It was obvious that all of the recoveries were higher than 90% and the R.S.D. values were less than 5%. These results further demonstrated that the developed method was feasible and reliable.

3.2.5. Analysis of real samples

In order to evaluate the applicability of the developed method for a real analysis of GAs, we used the method for the detection of GAs in dried fermentation mycelia powder. The contents of this four GAs were 3.5 mg g^{-1}, 3.3 mg g^{-1}, 1.8 mg g^{-1} and 2.3 mg g^{-1} for GA-T, GA-Mk, GA-Me and GA-S, respectively.

4. Conclusions

This paper developed a novel, simple and applicable capillary zone electrophoresis method using sodium borate buffer as BGE with 57%ACN (v/v) for the analysis of four bioactive triterpenes in dried *G. lucidum* mycelia powder. Under the optimized conditions, the four GAs reached the baseline separation in 9 min and the developed method had wide linear range of determination, quite well LOD and LOQ, good precision and recovery. In conclusion, the newly established CZE method was fit for the analysis of the four GAs extracted from dried fermentation mycelia powder and the quality control of the Chinese herbs. This was a promising feature in terms of the identification and analysis of bioactive triterpenes in Chinese herbs.

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