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Rapid determination of aristolochic acid I and II in *Aristolochia* plants from different regions by β-cyclodextrin-modified capillary zone electrophoresis

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Abstract

Chinese herbs nephropathy (CHN) is a kind of severe kidney disease caused by excessively taking aristolochic acid (AA). Hence, it is essential for health security and quality control of related herbal medicines to develop an efficient method for separation and determination of these two important components in Traditional Chinese Medicines. In this study, a rapid capillary zone electrophoresis (CZE) method using 120 mM sodium borate buffer containing 10 mM β-cyclodextrin (β-CD) as modifier was firstly developed for the analysis of AA-I and AA-II within 4 min in some medicinal plant samples. The separation conditions including pH of running buffer, CD content in the buffer system, applied voltage and capillary temperature were systematically optimized, and two kinds of aristolochic acids in 37 herbal samples of *Aristolochia* plants were successfully determined with high separation efficiency, satisfactory sensitivity, repeatability and recovery. The result indicated high variability in the contents of aristolochic acids due to different species and regions. The comparison of CZE method with high performance liquid chromatography (HPLC) was also discussed.

Keywords: Chinese herbs nephropathy; β-Cyclodextrin; Aristolochic acid

1. Introduction

Traditional Chinese Medicines has been used for clinic treatment for about two thousand of years in the world. Although they are indeed effective to lots of diseases, some of the toxic components in them have to be warned by people. In the latest decade, it was reported in many countries that some people have suffered from severe kidney disease since ingesting some medicine made of Chinese herbs [1]. This disease, so-called Chinese herbs nephropathy (CHN), is characterized by a progressive interstitial fibrosis leading to a severe atrophy of the proximal tubules [2]. CHN was firstly identified in Belgium where there have been more than 100 women who needed the therapy of renal replacement after they had followed a weight-reducing treatment and taken the same kind of Traditional Chinese Medicine, *Aristolochia fangchi* [1]. Such cases were also occurred in other areas including France, Spain, UK, Japan and China Taiwan [3].

It has been proved by several researches that CHN is related to the use of high doses of aristolochic acids (AA), the major toxic component extracted from *Aristolochia* plants [4,5]. AA, composed of AA-I and AA-II (Fig. 1), is a mixture of structurally related nitrophenanthrene carboxylic acids [1]. They are not only responsible for the tumour development but also for the destructive fibrotic process in the

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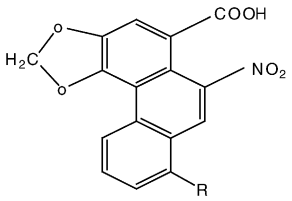

Aristolochic Acid I:R=OMe
Aristolochic Acid II: R = H

Fig. 1. Structures of aristolochic acid I and II.

kidney because of forming AA-DNA adducts, leading to permanent kidney damage in a short period [6]. It was indicated that AA-I is the major active component to result in renal failure [7].

With the growth of intoxication incidents, some health institutions, for example, Food and Drug Administration (FDA) and Medicines Control Agency (MCA), have warned people to pay attention to the safety information about botanical products containing aristolochic acid. Nay, many countries including UK, Canada, Australia, and Germany have announced banning to restrict the importation, sale and use of AA-containing medicines like *Aristolochia fangchi* or *Mutong* in past two years [1], so that further cases can be prevented. In the November 2003, five herbal drugs containing aristolochic acid, commonly used in Traditional Chinese Medicine, were officially banned in Taiwan district. Therefore, it is of great significance to quantify the content of AA in regular herbal medicines, or to find alternative herbals in which AA is at very low level. As to the clinic security, it is crucial to investigate the distribution of AA in different species [8] of herbal medicines from different regions.

In general, AA-I and AA-II are difficult to be separated due to their extremely similar structures and identical medicinal activities. There have been some studies that focus on analysis of the total amount of AA-I and AA-II or the major active component AA-I by thin layer chromatography (TLC) [9–11] or ultraviolet spectrophotometry [12]. High performance liquid chromatography (HPLC) has been used for the detection of AA-I and AA-II in recent years [13–20], and a C18 column was often utilized. In most cases, a mixture of acetonitrile and water was utilized as the mobile phase with some special modifiers, such as ammonium carbonate [1], TFA [21], or acetic acid [14,15] to control pH. In order to achieve satisfactory separation, gradient elution was necessary in some methods [11,21–23]. In most cases, the analytical times of these HPLC methods reported were usually above 15 min, even more than 40 min [17]. In our previous work, a fast HPLC method with the analysis time less than 5 min was achieved [24]. In order to further confirm the structural identification of compositions of Chinese herbs, LC–MS method was also performed in determination the content of AA, but the operation procedure is more complicated comparing with that of HPLC [23,25].

The obvious disadvantage of most HPLC methods in the analysis of AA is the high cost for magnitude of mobile phase and time-consuming, which would not meet the needs of high throughput analysis. Even the analytical time is shortened in our previous HPLC method [24], the separation efficiency need to be further improved, especially for the analysis of real samples. On the other hand, capillary electrophoresis (CE) has been proved to be an effective and convenient technique in various fields including the analysis of herbal medicines [26–33]. As to AAs, which are acidic compounds, CE is especially suitable for their analysis because they will ionize at high pH, and these components with negative charges can be easily separated by CE. However, no publication on the analysis of AA-I and AA-II by CE has been available up to now. In our work, a fast capillary zone electrophoresis (CZE) method with β-CD as modifier was firstly developed for the determination of the contents of AA-I and AA-II in herbal samples of *Aristolochia fangchi Wu*, *Caulis Aristolochiae Manshuriensis* or other *Aristolochia* plants from different regions. A baseline separation of the two kinds of AA was achieved within 4 minutes with high separation efficiency. Compared with HPLC separation, CZE was faster, cost-effective and more suitable for high throughput analysis. This study discloses the relationship of the content of AA and their cultivation region, which is very helpful for the safe use of herbal medicines.

2. Experimental

2.1. Apparatus and conditions

All experiments were performed with an Agilent 3D CE system with air-cooling and a diode array detector (Agilent Technologies, Palo Alto, CA, USA). A 48.5 cm (40.0 cm to the detector) × 50 μm i.d. uncoated fused-silica capillary (Ruifeng Inc., Hebei, China) was utilized. Other conditions are: capillary temperature 10 °C, applied voltage 30 kV, UV detection at 254 nm, samples injection at 50 mbar for 5 s.

The capillary was washed with 1.0 M sodium hydroxide (10 min), water (10 min) and the running buffer (10 min) in turn. Between consecutive analyses, the capillary was flushed with the running buffer for 5 min to guarantee good reproducibility.

2.2. Chemicals and reagents

The standard mixture sample containing AA-I (38%) and AA-II (59%) was obtained from Sigma, and the standard AA-I and cyclodextrins including α-CD, β-CD, γ-CD and HP-CD were kindly provided by Beijing Institute for Drug Control, and 37 kinds of medicinal plant samples of *Aristolochia* plants were identified by one of the authors, Professor Hu at China Academy of Traditional Chinese Medicine. All chemicals were of analytical-reagent grade: methanol from Beijing Chemical Factory (Beijing, China);

pure water was prepared by Aquapro Reverse Osmosis system (Aquapro, Chongqing, China).

2.3. Preparation of buffer and standard solutions for construction of calibration curve

Sodium borate buffers with different concentrations were adjusted to the desired pHs with 1.0 M NaOH. The mixed stock solution was prepared by dissolving 2.5 mg standard mixture of AA-I (38%) and AA-II (59%) in methanol into a 5 mL volumetric flask, and a stock solution of AA-I was also prepared by dissolving 4.4 mg standard AA-I in methanol (5 mL). Then the two stock solutions were mixed and diluted with methanol to obtain tested standard solutions for calibration and the concentration was ranged from 1.5×10^{-2} to 3.0×10^{-1} mg mL^{-1} for AA-I and AA-II. Both of the buffer and standard solutions were filtered through a 0.45 μm membrane filter and degassed by ultrasonication before use.

2.4. Preparation of sample solutions of medicinal plants

Each dried plant (0.5 g) was marinated in 5 mL anhydrous methanol for 16 h. After that, it was then extracted by ultrasonication and marinated at room temperature for 30 min, then centrifuged at 3000 rpm for 8 min. The extraction was repeated two times (1.5 mL × 2), and the extracts were combined and diluted to 5 mL with methanol, which was then passed through a 0.45 μm membrane filter before analysis.

2.5. Recovery test for AA-I and AA-II

1.0 mg of standard mixture of AA-I and AA-II (AA-I: AA-II = 38:59) was added to the sample of *Caulis Aristolochiae Manshuriensis* from Dunhua, Jilin. The mixture was extracted and analyzed by the procedures described earlier, thus the recovery was calculated.

3. Result and discussion

3.1. Optimization of CZE conditions

In our study, CZE method was established for the determination of AA in the real samples, based on a series of optimizations. Several kinds of buffers, including sodium borate buffer (pH = 8–10), phosphate buffer (pH = 3–7) and citrate buffer (pH = 4–6), were tested at the beginning for the separation of AA-I and AA-II, and sodium borate buffer showed a better separation. Taking sodium borate buffer as a starting point, the separation conditions were systematically optimized by investigating the effects of buffer pH, the concentration of sodium borate, organic modifiers with different compositions and concentrations, capillary temperature and applied voltage.

3.1.1. Influence of pH

A series of buffers with pH ranging from 8.1 to 9.7 were investigated by using a 120 mM sodium borate buffer and the result showed that the influence of pH on separation was not significant. Because of little difference in pK_a values between AA-I and AA-II, it was difficult to find a suitable pH in this range to improve the separation without any modifier in the buffer. When 10 mM β-cyclodextrin (CD) was added into the buffer, the separation efficiency was greatly improved. Taking the stability and migration time into account, pH 8.8 was considered to be the best value for the running buffer.

3.1.2. Effect of sodium borate concentration

Sodium borate concentration was changed in the range of 40–120 mM in the buffer system containing 10 mM β-CD, at pH 8.8 under 10 °C, temperature and 25 kV applied voltage. It was demonstrated that with the increase of sodium borate concentration, the resolution (R_s) of AA-I and AA-II was obviously improved and no obvious change of migration time of AAs was observed. Sodium borate buffer at the concentration of 120 mM was confirmed to be the optimal due to the best resolution.

3.1.3. Effect of CD modifier

As mentioned above, the resolution between AA-I and AA-II was insufficient in 120 mM sodium borate buffer without any modifier, so different organic modifiers such as methanol or acetonitrile were separately added to the BGE. However, no obvious improvement on the resolution appeared, which can be explained by the minor difference in the mobilities of AA-I and AA-II, as shown in Fig. 2(a). With regard to the three-cyclic structure of AAs, their molecular size can match to the size of the cavum of cyclodextrins, different kinds of CD such as α-CD, β-CD, γ-CD and HP-CD were investigated as BGE modifiers, showing great improvement of the resolution. The only difference between the structures of AA-I and AA-II is in the methoxy group. It is the special hydrophobic interaction between this group and CD that greatly affected the separation, which is the key reason for improvement of separation. Concerned with the separation parameters including R_s, theoretic plate number (n), β-CD was proved to be the best modifier with most satisfactory resolution and theoretic plate number, as listed in Table 1. The reason for that α-CD cannot achieve good separation is its small cavity diameter, which is not in concordance of the molecular sizes of AA-I and AA-II. Several mixed buffer systems composed of different CD, such as sodium borate buffer containing 6 mM β-CD and 4 mM γ-CD or sodium borate buffer containing 3 mM β-CD and 2 mM γ-CD have also been tested respectively in the study, but no obvious improvement of separation efficiency was observed. Based on this, the concentration of β-CD was further optimized in the range of 5–15 mM with 120 mM sodium borate buffer (pH 9.2) under 10 °C temperature and 30 kV applied voltage, illustrating that the concentration of β-CD exhibited great effect on the separation. The separation of two analytes was

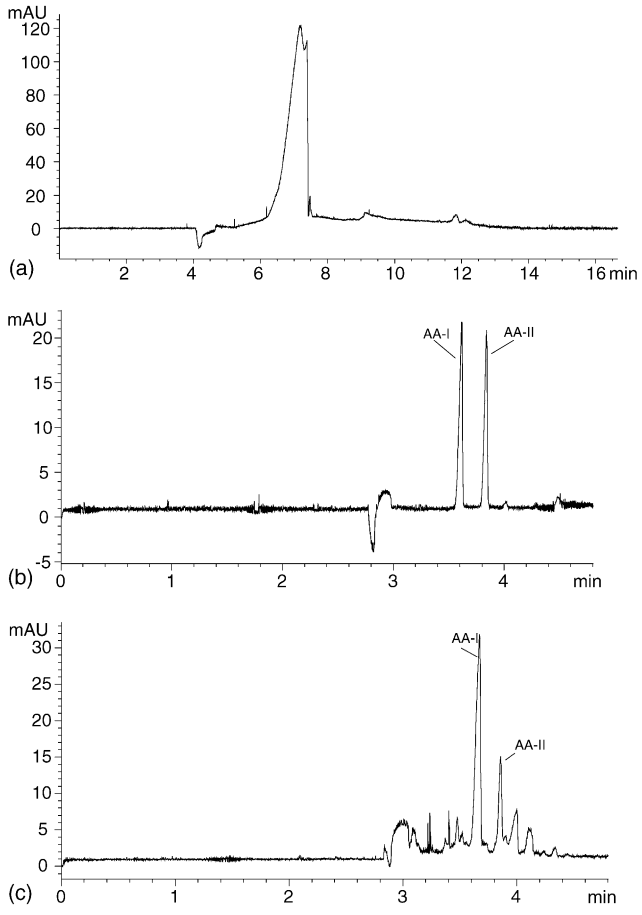

Fig. 2. Typical electropherograms of AA-I and AA-II by CZE: (a) standard sample, without β-CD modifier; (b) standard sample, 10 mM β-CD; (c) a real sample of *Caulis Aristolochiae Manshuriensis* from Dunhua, Jilin region, 10 mM β-CD. Other conditions: 120 mM sodium borate buffer (pH 8.8), 50 μm i.d. × 48.5 cm (40.0 cm effective) fused-silica capillary, applied voltage of 30 kV, capillary temperature of 10 °C, UV detection at 254 nm, injection at 50 mbar for 5 s.

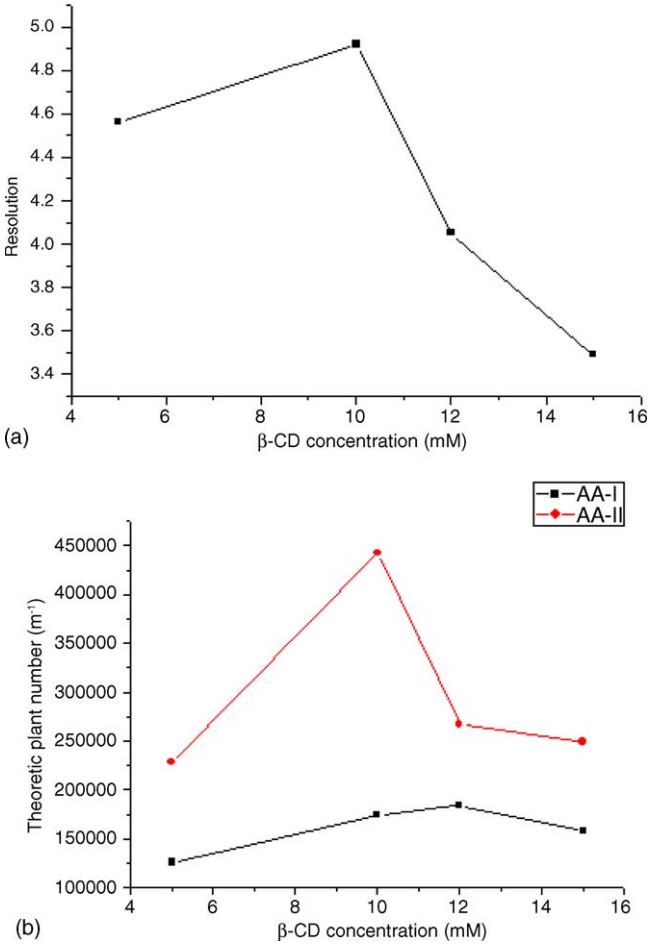

Fig. 3. Effect of β-CD concentration on resolution (a) and theoretical plate number (b) of AAs. Separation conditions: borate buffer (pH 9.2), 50 μm i.d. × 48.5 cm (40.0 cm effective) fused-silica capillary, applied voltage of 30 kV, capillary temperature of 10 °C, UV detection at 254 nm, injection at 50 mbar for 5 s, β-CD concentration varied in a range of 5–15 mM.

poor when β-CD was below 5 mM, while the analytes were very difficult to be resolved even with long time when 15 mM β-CD was used. From Fig. 3, it can be seen that the resolution and theoretic plate number fluctuated with the increase in β-CD concentration. It was concluded that 10 mM β-CD was the obvious optimal point with highest resolution, and as the theoretic plate numbers of AA-I and AA-II were comprehensively concerned, this concentration was also the best choice.

3.1.4. Applied voltage and temperature

The effect of applied voltage in the range of 15–30 kV on the separation was tested in 120 mM sodium borate buffer at pH 8.8, containing 10 mM β-CD under 10 °C, indicating that

Table 1
The theoretic plate number and resolution (R_s) of aristolochic acids in a real sample of *Caulis Aristolochiae Manshuriensis* from Shanghai when different kinds of CD were used as modifiers in the buffer

	β-CD	α-CD	γ-CD	β-CD + γ-CD	β-CD + γ-CD
C (mmol L^{-1})	10	10	10	6 + 4	3 + 2
$N1$[a] (m^{-1})	174357	35882	116110	43842	74322
$N2$[b] (m^{-1})	442647	241910	227050	129105	139052
R_s	4.92	1.43	3.61	2.09	3.02

Other separation conditions: 120 mM borate buffer (pH 8.8), 50 μm i.d. × 48.5 cm (40.0 cm effective) fused-silica capillary, applied voltage of 30 kV, capillary temperature of 10 °C, UV detection at 254 nm, injection at 50 mbar for 5 s.

[a] The theoretical plate number of aristolochic acid I peak.

[b] The theoretical plate number of aristolochic acid II peak.

high applied voltage can not only bring higher separation efficiency but also improve the peak shape. Therefore, the highest voltage of 30 kV allowed by the instrument was finally used.

Finally, the optimal capillary temperature was studied in the range of 10–25 °C. The results illustrated that the separation efficiency was significantly higher at lower temperature. Considering the high R_s that is preferred for the analysis of real herbal samples and proper migration time, the capillary temperature of 10 °C was chosen as operation temperature, under which the analytical time was less than 4 min.

3.2. Method evaluation

In most HPLC or HPLC–MS methods, vast amount of organic solvent have to be used as mobile phase and long analytical time is usually a boring problem to be resolved. Comparing with HPLC, the proposed CZE method is simple, fast, and greatly practical for the determination of AA-I and AA-II under following conditions: 120 mM borate buffer (pH 8.8) containing 10 mM β-CD under 10 °C temperature and 30 kV applied voltage. In this study, a little amount of β-CD was used, and the whole analysis was achieved within 4 minutes. The resolution between two components is as high as 4.17, and theoretic plate numbers greater than 170,000 m^{-1} for AA-I and 230,000 m^{-1} for AA-II were obtained, which has not been reported in HPLC. A typical electropherogram of standard mixture of AA-I and AA-II is illustrated in Fig. 2(b).

Under optimal conditions, a series of standard samples were analyzed and the calibration curves of the peak area (A) versus amount of AA (c) are as following:

$$\text{AA-I}: \quad A = 381.23 \times c - 1.40 \quad (r = 0.9956)$$

$$\text{AA-II}: \quad A = 517.64 \times c - 1.54 \quad (r = 0.9952)$$

It can be seen that the linearity was satisfactory with a correlation coefficient (r) greater than 0.99. As shown in Fig. 4, the detection limits for both compounds were found to be 1.0 μg mL^{-1}, that was equivalent to 0.02 ng of each AA on the capillary (20 nL injection volume). Obviously, it can meet the requirement of detection of AAs in *Aristolochia* plants, though it is comparatively higher than that of HPLC method, which is ranged from 0.015 μg mL^{-1} [23] to 1.0 μg mL^{-1} [17], and the detection limits of AA-I and AA-II in real samples of *Aristolochia* plants were both 10 μg g^{-1}.

As for the repeatability, the R.S.D. (n = 6) of migration time was 0.27% for AA-I and 0.28% for AA-II, and the R.S.D. (n = 6) of peak area was 4.2 and 3.5%, respectively. For real samples, R.S.D. (n = 5) of migration time and peak area was less than 0.3 and 4.7%, respectively. In addition, to test the repeatability of the extraction method, two dried medicinal plant samples were prepared in this experiment. By determining the contents of AA-I and AA-II, it was observed that the stability of sample solution and method reproducibility are all acceptable. R.S.D. of the contents AA-I and AA-II in these two samples were 3.7 and 4.0%, respectively. The analysis of the same sample at different time also showed

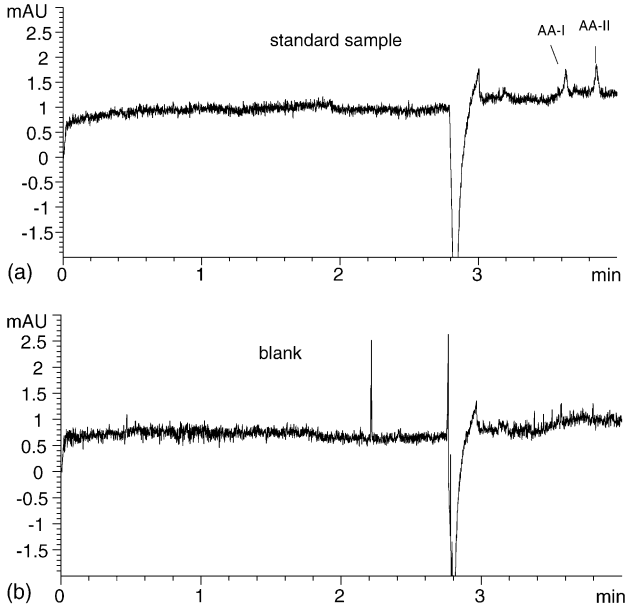

Fig. 4. Detection limits of AA-I and AA-II: (a) standard sample; (b) blank sample. Separation conditions: 120 mM borate buffer (pH 8.8) containing 10 mM β-CD, 50 μm i.d. × 48.5 cm (40.0 cm effective) fused-silica capillary, applied voltage of 30 kV, capillary temperature of 10 °C, UV detection at 254 nm, injection at 50 mbar for 5 s. The concentration of each AA was 1.0 μg mL^{-1}.

satisfactory reproducibility, and the R.S.D. of the contents of AA-I and AA-II is less than 1%.

The recoveries of the AA-I and AA-II were 96.7 and 77.8% (n = 3) with the R.S.D. of 3.0% for AA-I and 1.0% for AA-II, respectively, showing that the sample preparation method could provide acceptable extraction efficiency in a short period.

3.3. Analysis of real samples

In our work, 10 kinds of *Aristolochia fangchi Wu*, 21 kinds of *Caulis Aristolochiae Manshuriensis* and 6 kinds of other *Aristolochia* medicinal plant samples collected from various regions in China have been analyzed by the CZE method, and some of the results which can present the distribution of AA-I and AA-II among different samples were listed in Table 2, other results were omitted due to the limit of article length. Fig. 2(c) shows a typical electropherogram of a real sample, indicating great separation performance. The identifications of AA-I and AA-II peaks in real sample solutions were proved by the comparison of their migration times and on-line UV spectra with those of the standard samples (Fig. 5).

As well known, AA-related components are complicated in real plant samples, so the detection of AA would be disturbed by the analogues of AA extracted from herbal medicines if the resolution is insufficient. In present CZE method, not only the AA-I and AA-II but also the other AA-related components were separated completely, so the contents of AAs could be exactly determined without interference of other components.

Table 2
Determined contents of AA-I and AA-II in some real samples of herbal medicinal plants (%, w/w, dry plants)

Species	Cultivation region	AA-I (%)	AA-II (%)
Aristolochia fangchi Wu	Zhaoqing, Guangdong	–*	–
	Zhongshan, Guangdong	–	–
	Luoding, Guangdong	–	–
	Qujiang, Guangdong	–	–
	Gaoyao, Guangdong	–	–
	Cangwu, Guangxi	0.072	0.018
	Cenxi, Guangxi	0.12	–
Caulis Aristolochiae Manshuriensis	Tumen, Jilin	0.30	0.063
	Antu, Jilin	0.073	0.022
	Dunhua, Jilin	0.17	0.043
	Huanren, Liaoning	–	–
	Dandong, Liaoning	0.049	0.021
	Shanghai	0.20	0.069
	Mudanjiang, Heilongjiang	0.13	0.031
Stephania tetrandra S. Moore	Zhaotong, Yunnan	0.065	0.014

Separation conditions see the optimal conditions in Section 2, and the detection limits of AA-I and AA-II in real samples of *Aristolochia* plants were both 10 $\mu g\,g^{-1}$ (0.001%).

* Means that the content of AA is lower than the detection limit.

As indicated by the data in Table 2, the content of AA-I is much higher than that of AA-II in most cases. It was also demonstrated that the species of medicinal plant samples has an important impact on the distribution of the contents of AAs, and the contents of AA-I and AA-II in some samples of *Caulis Aristolochiae Manshuriensis* were comparatively higher than those of the samples of *Aristolochia fangchi Wu*.

Furthermore, the results revealed high variability in the contents of AAs collected in many regions, and *Aristolochia fangchi Wu* from Cenxi and Mengshan, as well as the *Caulis Aristolochiae Manshuriensis* from Tumen and Shanghai have the highest concentration of AA among all samples. In accordance with the analysis result by our HPLC method [24], AA-I and AA-II were not detected in the medicinal plants from Zhongshan, Luoding, Qujiang, Gaoyao, and Zhaoqing in Guangdong province, which further approved that the contents of AAs are related to the cultivation region to a great extent. This result is instructive for the selecting of Traditional Chinese Medicine in clinic treatment. On the contrary, the contents of AAs in *Caulis Aristolochiae Manshuriensis* from Jilin and Heilongjiang provinces are comparatively high. Of course, these results cannot guarantee the complete safety of using the herbal medicines because some undetectable components in *Aristolochia* plants may be harmful to patients even if their contents are very low. Other factors such as the intaking method should be also investigated in future work to insure the complete safety of using the herbal medicines.

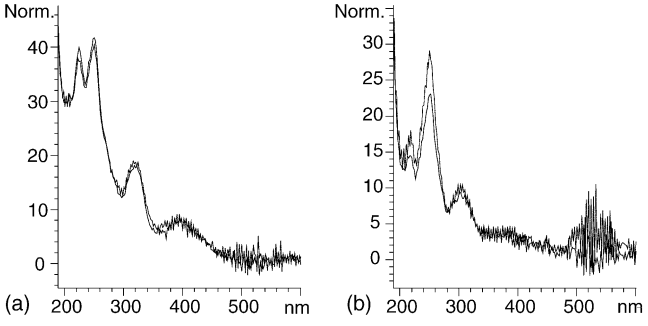

Fig. 5. Comparison of UV spectra between standard sample and a real sample of *Caulis Aristolochiae Manshuriensis* from Dunhua, Jilin region (spectra of the peak of standard sample and real sample were overlapped): (a) spectra of AA-I; (b) spectra of AA-II.

4. Conclusion

In this work, a CZE method using 120 mM sodium borate buffer (pH 8.8) containing 10 mM β-CD under 10 °C temperature and 30 kV applied voltage was firstly developed for the separation of AA-I and AA-II. The contents of AAs in 37 medicinal plants were successfully determined with satisfactory recoveries, sensitivities and reproducibility. This method is effective, fast and economic, with higher separation performance than that of HPLC. It was also demonstrated that the species of medicinal plant samples has an important impact on the distribution of the contents of AAs, and the contents of AAs vary significantly with the regions where the herbal medicines are collected. In order to further improve the detection limits of this CZE method, proper on-line concentration methods such as on-line sweeping is under investigation. The analysis of other neutral AA-related components including aristoloactam by MEKC or MEEKC is in progress too.

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