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Separation and detection of tropane alkaloids in *Anisodus tanguticus* by capillary electrophoresis-electrochemiluminescence

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A $Ru(bpy)_3^{2+}$-based electrochemiluminescence method coupling with capillary electrophoresis has been developed for the separation and detection of two tropane alkaloids, anisodamine and anisodine within 7 min, with a platinum microelectrode modified with europium (III)-doped Prussian blue analog film as the working electrode. Under the optimized conditions, the ECL intensity was in proportion to the log values of anisodine and anisodamine concentrations in the range 5.0×10^{-6}-1.0×10^{-3} mol/L (r=0.9992) for anisodine and 1.0×10^{-6}-1.0×10^{-3} mol/L (r=0.9991) for anisodamine, with the detection limits of 1.2×10^{-7} mol/L for anisodine and 4.3×10^{-8} mol/L for anisodamine, respectively. The proposed method has been applied to identify and detect the two alkaloids in the different parts (seeds, roots and leaves) of *Anisodus tanguticus*. The results showed that anisodine existed in all samples from seeds, roots and leaves of the plant, and its content was highest in seeds while lowest in leaves. However, anisodamine was not found in the samples mentioned above due to the low level in the plant and maybe the interreaction between anisodmine and some components in the plant. Finally, the average recoveries from 98.0 to 107.0 % were obtained.

Introduction

The medicinal plant *Anisodus tanguticus (Maxim.) Pascher* is locally known as "Zhangliushen" in Qinghai, Xizang, Sichuan and Gansu Provinces of China. It has often been used as a common Tibetan medicine for the treatments of anesthesia, analgesia, bacteremic shock, septic shock and motion sickness.[1-3] A large number of studies show that tropane alkaloids, especially anisodamine and anisodine are principally the efficacious biochemical compositions in the *Anisodus tanguticus*.[4] Anisodamine and anisodine have similar chemical structures, as shown in Fig. 1. They are extensively used in ophthalmic diagnosis as mydriatic as well as anticholinergic, antispasmodic and preanesthesic agents.[5] However, these alkaloids are extremely toxic so that unintentional ingestion of plants containing them or abuse of these alkaloid drugs may cause serious illness, injury, or even death.[6,7] Therefore, it is important to develop rapid, sensitive, and accurate methods for the identification and determination of these alkaloid components in *Anisodus tanguticus.*

Up to now, the common methods for the detection of anisodamine or anisodine are chromatographic methods, including liquid chromatography-mass spectrometry[8-12] and thin-layer chromatography (TLC)[13]. In addition, some spectroscopic methods including atomic absorption spectrometry[14], atomic emission spectrometry[15], resonance light scattering[16] and UV spectrophotometry[17] have also been developed. However, the drawback of the methods mentioned above appears to be time-consuming, low sensitivity, the complex procedure or the expensive instrument.

Increasingly, capillary electrophoresis (CE) has been proved to be a powerful separation technique for analysis of alkaloids because of its high efficiency, high resolution and minimal sample volume.[18,19] Especially, CE combined with electrochemiluminescence (ECL) takes advantages of high efficiency and high sensitivity, and has been widely used for the determination of some alkaloids.[20-26] Thus, a $Ru(bpy)_3^{2+}$-based CE-ECL method has been established for the separation and determination of anisodine and anisodamine in seeds, roots and leaves of *Anisodus tanguticus* in this work, using a platinum microelectrode modified with europium (III)-doped Prussian blue analog film (Eu-PB) as the working electrode, which was proved to help to avoid poisoning effect of complex matrices in real samples,[21,22] and to greatly improve the reliability and sensitivity of a CE-ECL method.[21,23]

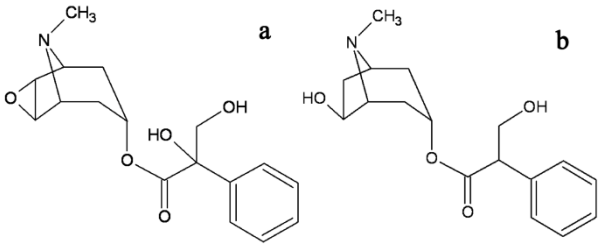

Fig.1 Structures of anisodine (a) and anisodamine (b).

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Experimental

Apparatus

A MPI-A multi-parameter chemiluminescence capillary electrophoresis analysis system with self-compiled CE-ECL software (Xi'an Remax Electronic and Technological Co., China) was used. Uncoated fused-silica capillary (50 cm×25 μm i.d.) was obtained from Yongnian Optical Fiber Factory (Hebei, China). The end-column ECL detection was installed with a three-electrode configuration, which was made up of a self-prepared Eu-PB modified platinum disk (Φ=0.5 mm) as a working electrode, a platinum wire as an auxiliary electrode, and an Ag/AgCl filled with saturated KCl as a reference electrode. The capillary-to-working electrode distance was adjusted to about 150 μm. The schematic diagram of the CE-ECL detection system is the same as in our previous work.[22]

Reagents

All the reagents were of analytical grade except for specific statements and were used as received without further purification. Tris(2,2'-bipyridyl) ruthenium(II) chloride hexahydrate was obtained from Aldrich (Milwaukee, WI, USA) and prepared with doubly deionized water. Anisodine hydrobromide was purchased from Sigma-Aldrich (St.Louis, MO, USA). Anisodamine was purchased from National Institutes for Food and Drug Control (Beijing, China, The Batch number is 100249-199501). Both anisodine and anisodamine were freshly prepared with 10% methanol (Spectrum analytical grade) just before use. The plant *Anisodus tanguticus (Maxim.) Pascher* was obtained from Gucheng tree farm, Tianzhu, Gansu province. The buffers used in this work were sodium dihydrogen phosphate and disodium hydrogen phosphate (G.R.). All solutions were stored at 4℃ in a refrigerator and were filtered through a membrane of 0.45 μm prior to injection into the system.

Electrophoresis conditions

During the experiment, a solution of 5 mmoL/L $Ru(bpy)_3^{2+}$ in 60 mmoL/L phosphate buffer (pH 8.0) was directly injected into the reaction reservoir. Running buffer solution contained 30 mmol/L (pH 8.0) NaH_2PO_4-Na_2HPO_4 and 10% (v/v) methanol. Samples were injected in an electrokinetic mode at 10 kV for 10 s. The separation voltage was 15 kV. The photomultiplier tube (PMT) was biased at -800 V. The detection potential applied at the working electrode was fixed at 1.15 V. Prior to experiments every day, the capillary was sequentially rinsed with 0.01 moL/L NaOH for 3 min at first, then with doubly deionized water for 3 min and finally equilibrated with the running buffer for 5 min so as to maintain an active and reproducible inner surface. Fresh $Ru(bpy)_3^{2+}$ was replaced every 3 h in order to obtain good reproducibility. Capillary was rinsed with the running buffer between two sample injections until the baseline was stable. The sample concentrations were quantified by ECL peak intensities.

Preparation of sample solution

The samples from seeds, roots and leaves of *anisodus tanguticus (Maxim.) Pascher* were prepared according to the reported work with a modification.[22] They were washed and dried. The powder of them (3.0 mg) was extracted separately with 5 mL methanol for 30 min in an ultrasonic bath. The extraction was repeated twice. Finally, all the extracts were combined and diluted with methanol to 10 mL. Before use, the sample solution was diluted to the desired concentrations with 10 % methanol, following by passing through a 0.45 μm membrane and being directly injected into the capillary electrophoresis system and analyzed.

Results and discussion

CE–ECL behavior of the analytes

The typical electropherograms for anisodamine and anisodine were shown and compared in Fig.2. It was seen that the ECL intensity of anisodine was lower than anisodamine with the same concentration, suggesting that the present method be more sensitive for anisodine than for anisodamine. The possible reasons could be attributed to two factors. One factor is the different substituents attached to the β-carbon atom bonding with nitrogen atom in the tertiary amine group.[27] As seen in Fig.2, the electron-withdrawing effect of epoxy group in the structure of anisodine is greater than that of hydroxyl group in the structure of anisodamine, decreasing the stability of positive nitrogen radical ion ($-N^{+\cdot}$), and hence leading to a weaker ECL intensity. Another factor is dimensional conformation surrounding nitrogen atom.[28] The perpendicular configuration of N-CH_3 in the structure of anisodine increases dimensional block, interfering with stability of positive nitrogen radical ion, and leading to a lower ECL intensity. Contrarily, the prostrated conformation of N-CH_3 in the structure of anisodamine decreases the ionization energy of nitrogen atom, stabilizing the positive nitrogen radical ion, and resulting in a stronger ECL peak.[29]

On the other hand, it was also found in Fig.2 that the migration time of anisodine is longer than that of anisodamine mainly because of a bigger molecular size and a less thorough dissociation from dimensional block, and consequently a slower electrophoresis rate.

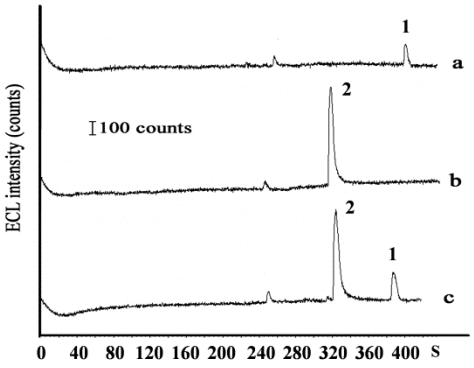

Fig.2 Electropherograms of (a) anisodine standard solution; (b) anisodamine stardard solution; (c) the mixture of anisodine and anisodamine. 1. anisodine ,2. anisodamine. Separation capillary: 25 μm i.d., 50 cm length; sample injection: 10 s at 10 kV; separation voltage: 15 kV; running buffer: 30 mM sodium phosphate with 10 % methnol (pH 8.0); phosphate in the detection cell: 60 mM contained 5 mmol/L $Ru(bpy)_3^{2+}$ at pH 8.0; anisodine: 1.0×10^{-5} mol/L; anisodamine: 1.0×10^{-5} mol/L.

Effect of Eu-PB modified Pt electrode

The use of Eu-PB modified Pt electrode helped to avoid poisoning effect of complex matrices in real samples, and to greatly improve the sensitivity and reliability of the detection. As reported in our previous work,[22] a higher current response and a slight negative shift for the direct oxidation peak of $Ru(bpy)_3^{2+}$ on the Eu-PB modified Pt electrode could be obtained. It indicated that the prepared electrode could improve the electro-oxidation efficiency of $Ru(bpy)_3^{2+}$ and consequently enhance the ECL intensity of a $Ru(bpy)_3^{2+}$-based ECL system since the excited state $Ru(bpy)_3^{2+}$ was considered as a luminescent intermediate.[21] On the other hand, some complex matrices in real samples were subjected to chemically oxidized by $Ru(bpy)_3^{3+}$ produced in the system. As a result, the poisoning effect of such compounds on a bare Pt electrode were reduced and avoided, resulting in a good stability and reliability for the detection. It was also shown that the modified electrode was stable enough for repetitive use in the present system within four weeks with no need for electrode replacement. The results further proved the electrode gave less interfering signals from other electroactive substances in real samples.

Optimization of CE-ECL system

Effect of $Ru(bpy)_3^{2+}$ concentration. $Ru(bpy)_3^{2+}$ is a luminous reagent and its concentration has an important influence on ECL intensity. Increasing the concentration of $Ru(bpy)_3^{2+}$ could increase the ECL intensity. However, the background noise also increased with the increase in the concentration of $Ru(bpy)_3^{2+}$. In this work, 5 mmol/L $Ru(bpy)_3^{2+}$ was adopted due to concerned over sensitivity and economy in use of reagent.

Effect of phosphate concentration in $Ru(bpy)_3^{2+}$ solution. Because the concentration of phosphate in the ECL cell had an effect on the oxidation efficiency of reactants on the electrode, and then affecting the ECL intensity, we investigated the effect of phosphate concentration on the ECL intensity of two alkaloids. As illustrated in Fig.3, when the concentration of phosphate added into $Ru(bpy)_3^{2+}$ solution changed from 30 to 100 mmol/L, the ECL intensity for two alkaloids both reached the highest at 60 mmol/L. Thus, 60 mmol/L phosphate concentration in $Ru(bpy)_3^{2+}$ was selected.

Effect of detection potential. Detection potential had a great effect on the E CL intensity. The result in Fig.4 showed that ECL intensities

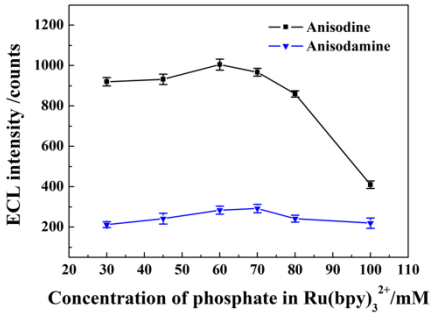

Fig.3 Effect of phosphate concentration in $Ru(bpy)_3^{2+}$ on ECL intensity. Other conditions are the same as in Fig.2.

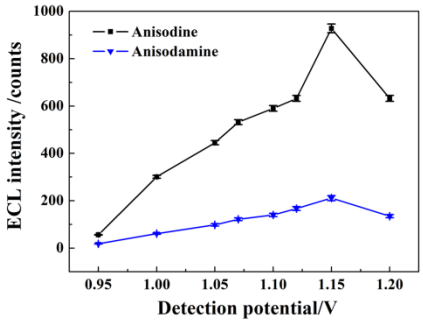

Fig.4 Effect of detection potential on ECL intensity. Other conditions are the same as in Fig.2.

of the two alkaloids were both enhanced when potential increased (*vs*.Ag/AgCl) and reached the highest at 1.15 V. When the potential exceeded 1.15 V, the ECL response weakened. Hence, the applied potential was set at 1.15 V.

Choice of running buffer. Running buffer played an important role in the CE-ECL detection. It affected the electroosmotic flow (EOF) and the electrophoretic behavior of solute. In this work, separation and determination of alkaloids were studied in different buffer systems including Tris-H_3PO_4, Tris-Citric Acid, Tris-HCl, acetate, phosphate, and borate buffers. Finally, NaH_2PO_4-Na_2HPO_4 was chosen in terms of the stable baseline, lower noise, shorter analysis time, and better peak shape.

pH effect of running buffer. The pH effect of phosphate was investigated in the pH range of 3.5-9.5 at intervals of 0.5 pH units. As illustrated in Fig.5, the highest ECL intensity of anisodine and anisodamine were observed at pH 8.5 and 8.0, respectively.

At the same time, the pH value of running buffer also influenced the resolution (R). The R was calculated using the following equation: $R=2(t_{R2}-t_{R1})/(W_1+W_2)$, where t_{R1} and t_{R2} are the migration times of two analytes and W_1 and W_2 are the peak widths of two analytes measured at the baseline. It was seen in Fig.5 that the resolution reached the maximum at pH 8.0 and then decreased with the increase in the pH. As a result, pH 8.0 was selected in the experiments.

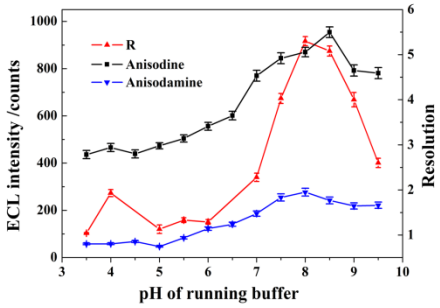

Fig.5 pH Effect of running buffer on ECL intensity and resolution. Other conditions are the same as in Fig.2.

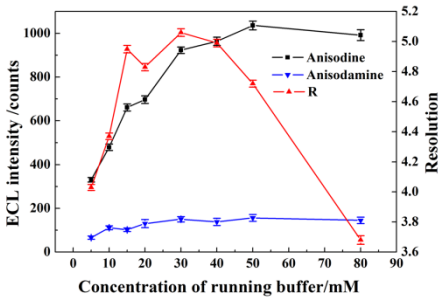

Fig.6 Effect of running buffer concentration on ECL intensity and resolution. Other conditions are the same as in Fig.2.

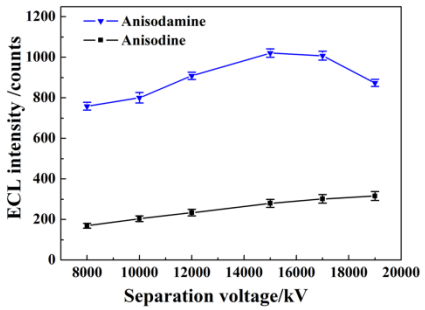

Fig.7 Effect of separation voltage on ECL intensity. Other conditions are the same as in Fig.2.

Effect of running buffer concentration. The buffer concentration affected the migration time and resolution. It was found that the migration time of alkaloids increased with the increase of the buffer concentration, which helped to improve the resolution of them. However, the ECL peak broadened and the baseline became unstable at higher concentration due to excess Joule heat, decreasing the separation efficiency. As seen in Fig.6, the highest resolution was observed at 30 mmol/L. On the other hand, the ECL intensities of anisodine and anisodamine both gradually enhanced with increasing phosphate concentration until 30 mmol/L. Above 30 mmol/L, the ECL intensity changed slightly. Hence, 30 mmol/L phosphate was preferred as the optimum condition.

Effect of organic additives. Under optimized conditions mentioned above, anisodamine and anisodine were still not separated by baseline. So some organic additives such as acetonitrile, β-cyclodextrin, hydroxypropyl β-cyclodextrinon and methanol were added into the running buffer solution to improve the separation efficiency and enhance the ECL intensity of the analytes. The results showed that the addition of acetonitrile helped to enhance the ECL intensity while did not improve separation efficiency; the addition of β-cyclodextrin had little effect on the detection; the addition of methanol and hydroxypropyl β-cyclodextrinon both improved the resolution and enhanced the ECL intensity, but the separation efficiency of methanol was better. So methanol was selected and the amount of methanol in the buffer solution was studied in the following experiment. The results indicated that the migration time increased and the resolution was improved with increasing the amount of methanol. And the baseline separation could be obtained using a phosphate buffer solution containing 10 % methanol with a shorter analysis time and a better stability. Therefore, 10 % methanol was used as a running buffer additive in this work.

Effect of separation voltage. Separation voltage simultaneously impacted on the ECL intensity and resolution. It was found that resolution decreased linearly with increasing separation voltage. On the other hand, although the ECL intensity of anisodine gradually increased with increasing the separation voltage, it decreased for anisodamine when the separation voltage was beyond 15 kV because of excessive heating caused by Joule effect, as shown in Fig.7. Therefore, generally considering ECL intensity and resolution, the best choice for separation voltage was 15 kV.

Effect of injection voltage and injection time. The effects of were studied. The results showed that increasing injection voltage or injection time enhanced the ECL intensity while reduced the resolution and damaged the reproducibility when an excessive sample volume was introduced. So as a compromise of the high ECL intensity and the improved column efficiency, the injection parameters of 10 s at 10 kV were recommended.

Effect of extracting solvent

The extracting solvent was chosen from ethanol and methanol. Finally methanol was found to give a better resolution and a higher ECL intensity for two alkaloids. Therefore, methanol was selected for the extraction of anisodine and anisodamine in *Anisodus tanguticus* in order to obtain a higher extraction yield. Besides, the concentration of methanol solution was optimized, too. The results indicated that the ECL intensity reached maximum when methanol in samples was 5-10 %, and the greater precision of the method was obtained with 10 % methanol solution as the extracting solvent. Thus, the methanol solution of 10 % was adopted.

Linearity, detection limit and reproducibility

Under the optimum conditions established above, the ECL intensity was in proportion to the log values of anisodine and anisodamine concentrations in the range 5.0×10^{-6}-1.0×10^{-3} mol/L for anisodine and 1.0×10^{-6}-1.0×10^{-3} mol/L for anisodamine, respectively. The calibration curve of them and the correlation coefficients had been fitted, as shown in Fig. 8. The detection limits, which were obtained at the signal to noise ratio of 3 (S/N=3), were 1.2×10^{-7} mol/L for anisodine and 4.3×10^{-8} mol/L for anisodamine, respectively.

The precision of the proposed method was determined by reduplicate injections (n=6) of a standard mixture solution containing 1.0×10^{-5} mol/L anisodine and anisodamine. The relative standard deviations (R.S.D.) of the migration time and ECL intensity were 0.98 % and 2.98 % for anisodamine, 1.21 % and 4.57 % for anisodine, respectively.

Separation and detection of anisodine and anisodamine in *Anisodus tanguticus*

It was reported that the alkaloids existed in *Anisodus tanguticus* might be different in different plants, and the contents of two main components, anisodine and anisodamine, also might be quite different in the plants from different area.[30] In this work, the proposed method has been successfully applied to separate and determine anisodine and anisodamine in seeds, roots and leaves of

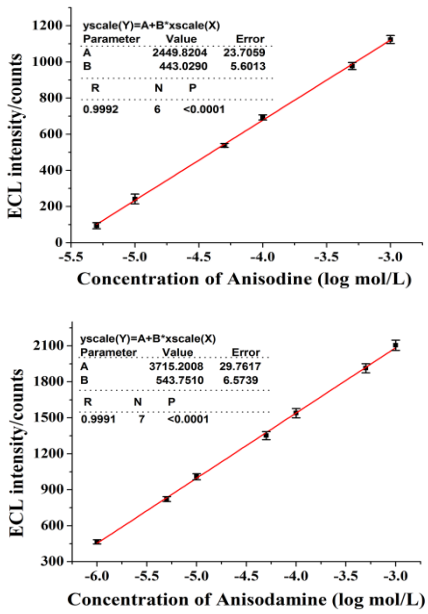

Fig.8 Calibration curve of anisodine and anisodamine

Anisodus tanguticus from a local tree farm. The peaks were confirmed by spiking with the standard solutions. The results, as listed in Tab.1, showed that anisodine existed in all samples and its content was highest in seeds while lowest in leaves. And the overall average contents of anisodine in seeds, roots and leaves of *Anisodus tanguticus* were estimated as 12.4 mg/g, 9.05mg/g and 2.01 mg/g, respectively. However, anisodamine was not found in the samples mentioned above due to low level in the plant and maybe the decreased ECL efficiency by the interreaction between anisodmine and some components in the plant. The results were also consistent with the fact that the content of anisodine in any parts of the plant is more than that of other alkaloids.[31,32] The typical electropherograms of the seed sample were shown in Fig.9. The recoveries were calculated and listed in Tab.1. However, it is a pity that we could not obtain the related data of anisodine for the spiked sample from leaves. The reason still remains in suspense.

Tab.1 Results for the determination of anisodine (AD) and anisodamine (ADM) in *Anisodus tanguticus*

Sample		Found ($\times10^{-6}$ mol/L)	Added ($\times10^{-5}$ mol/L)	Recovered ($\times10^{-5}$ mol/L)	Recovery % (n=5)
[a] AD	Seeds	7.4±0.09	1.0	1.78±0.06	104.0±6.0
	Roots	5.4±0.05	1.0	1.53±0.05	99.0±5.0
	Leaves	1.2±0.03	–	–	–
[b] ADM	Seeds	–	1.0	1.07±0.07	107.0±7.0
	Roots	–	1.0	1.01±0.10	101.0±10.0
	Leaves	–	0.5	0.49±0.02	98.0±4.0

[a] ADM: anisodamine; [b] AD: anisodine

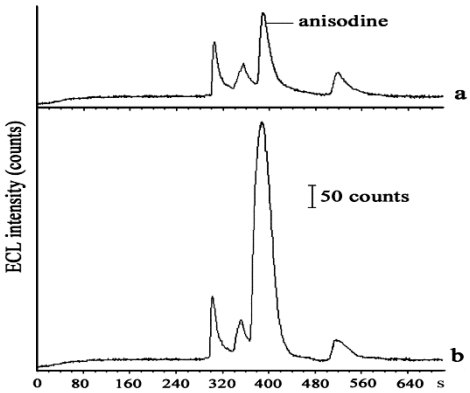

Fig.9 Electropherograms of (a) the seed sample of *Anisodus tanguticus*; (b) the seed sample solution spiked with 1.0×10^{-5} mol/L anisodine standard solution.

Conclusions

A capillary electrophoresis-electrochemiluminescence method has been developed for the separation and detection of anisodine and anisodamine by using a platinum microelectrode modified with europium (III)-doped Prussian blue analog film as the working electrode. Anisodine was separated and determined in different parts of the plant *Anisodus tanguticus (Maxim.) Pascher* within 7 min and the content of anisodine was highest in seeds while lowest in leaves, while anisodamine was not found in the plant. The results provide a medicinal reference for the application of the plant as a kind of Chinese traditional herbs. On the other hand, the proposed method shows the merits with rapid analysis speed, wide linear range, good accuracy, small consumption of reagents and samples, and has good prospects with respect to performance for the preliminary investigation of other quinolizidine alkaloids in Chinese traditional herbs.

Acknowledgements

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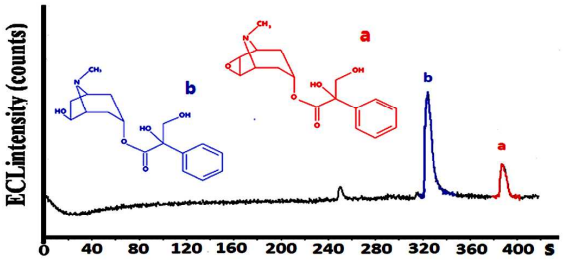

Anisodine and anisodamine in different part of *Anisodus tanguticus* are separated and determined using a chemically modified Pt electrode.