CE and CE/MS for the analysis of natural products

Application Note

Pharmaceutical

Authors

Yi Li
Peking University,
Beijing, PRC

Gordon Ross,
Carsten Buhlmann
Agilent Technologies,
Waldbronn, Germany

Introduction

Natural products have played a major role in human health care for centuries. The discovery that compounds from natural sources are highly effective against "modern" disease, such as breast cancer, in combination with the realization that there are thousands of uncharacterised and even unknown species still to be investigated, means that the analytical investigation of natural products is of great importance. Whether the source is animal or plant, it is frequently the case that the effective components are present in a rich matrix of other compounds. This presents specialized problems to the investigating scientist. Not only should the separation technique employed be capable of high resolution and high efficiency separations, the detection technique chosen should be highly selective and hopefully capable of providing as much identification data points as possible.

Capillary electrophoresis (CE) is a high efficiency, high resolution technique with the added benefits of being able to handle very complex matrices. Since CE is performed using an open tube of very small diameter, it is not prone to fouling as is an LC column, and can easily be washed by flushing to prevent carry-over. This makes the system inherently more robust, more reliable and reduces the sample preparation required. In this application note we show a number of applications demonstrating the utility of CE with UV and MS detection for the analysis, component identification and quantification, and characterization of traditional Chinese medicines.

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Experimental

Capillary Electrophoresis

All analyses were performed using the Agilent Capillary Electrophoresis system which is equipped with diode array detection and controlled by the Agilent ChemStation software. For CE/MS analyses the CE instrument was fitted with the CE/MS adapter kit (PN G1603A) which prepares the CE for coupling with the MS. This includes an MS cassette which allows the capillary to be threaded through the UV detector interface before leaving the instrument for MS connection thus providing tandem UV and MS detection.

Mass Spectrometry

An Agilent MSD single quadrupole mass spectrometer was used for all CE/MS analyses. An Agilent CE-ESI-MS Nebulizer kit G1607A) was used to couple the CE with MS using electrospray ionization (ESI). The sprayer was of a triple tube design accommodating the separation capillary, a tube for delivery of sheath liquid and an outer tube for delivery of the nebulizing gas and is arranged orthogonal to the MS entrance capillary in the ion source. The sheath liquid was delivered by an Agilent isocratic LC pump equipped with a 1:100 splitter.

The traditional medicines samples used in the described applications were the kind gift of Professor Liu, Peking University, Beijing- PRC.

1. Identification of Ephedrine in Mahuang

Mahuang (*Ephedra sinica stapf*) is a Chinese herbal medicine commonly used in the treatment of asthma and respiratorial infections. Its active ingredients (ephedrine, norephedrine, psudoephedrine and norpseudoephedrine) are strong central nervous system stimulants. Mahuang can be used alone or to potentiate the effects of other herbal medicines. In order to evaluate the herbal quality, HPLC may be used to determine the concentrations of these active ingredients. However, this is complicated by the long run time necessary (30 minutes) and contamination of the LC column. The LC method is also a gradient method which requires column re-equilibration and washing between runs. The CE method described here is simple and rapid requiring only a 4-minute capillary wash between runs.

Sample extraction

1 g herb extracted with 20 mL water and heated to 80 °C for 30 minutes then filtered through 0.2 µm pore and injected.

Results

Figure 1 shows the separation of Mahuang extract. The ephedrine peak appears after approximately 6 minutes but the rest of the sample shows an enormous complexity. If using LC, the remaining sample must be washed from the column after detection of the active ingredients. However in CE, the capillary is simply flushed through with fresh buffer. Figure 2 shows the analysis of a standard solution of ephedrine and a Mahuang extract and compares the spectra of the indicated peaks. Ephedrine could therefore be identified by its spectra and its migration time and confirmed by spiking with a known standard. For ephedrine the analysis was linear over the range 1 µg/mL to 1 mg/mL with $r^2 = 0.9995$.

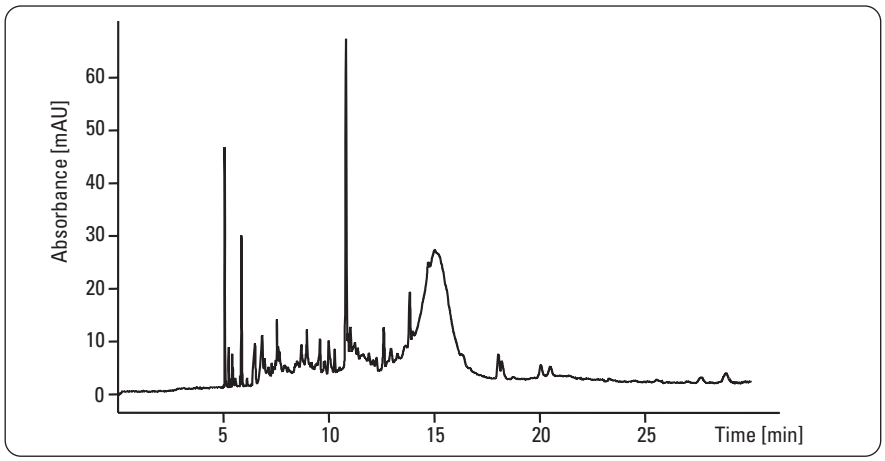

Figure 1
Analysis of a Muhuang extract.

Chromatographic conditions

Capillary:	80.5 cm (72 cm) x 75 µgm
Buffer:	25 mM borate pH 9.3
Detection:	200/10 nm
Injection:	500 mbar x s
Voltage:	30 kV

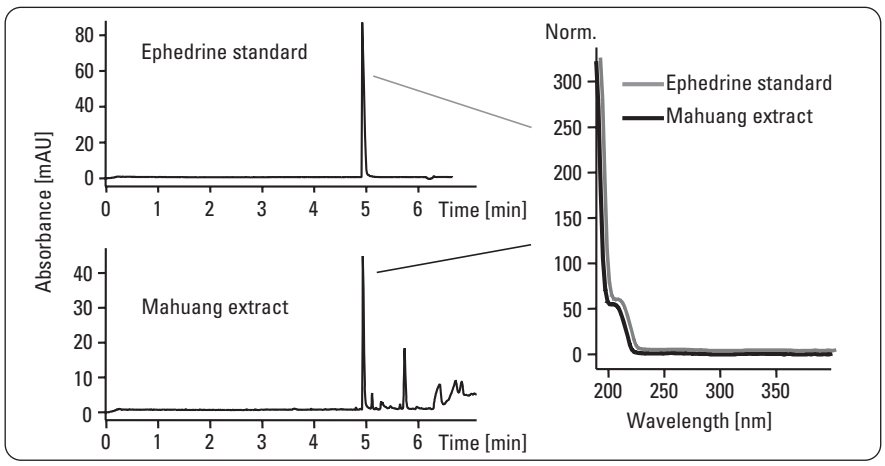

Figure 2
Comparing the spectra of ephedrine in Mahuang with that of a standard.

2. Identification and quantitation of the alkaloids berberine, palmatine and jatrorrhizine in Mahonia stem

Many species of the Mahonia plant are used in China as traditional medicines. Some of the active components have been identified as alkaloids and pharmacological research has determined that the plant has antibacterial, antioxidant, antifungal, anticancer and antiproliferative properties. Here we describe the quantitation of three alkaloids in the stem of various Mahonia species.

Sample extraction

2 g of pulverized Mahonia stem was ultrasonicated in 10 mL methanol for 30 minutes. This was repeated twice using 5 mL methanol and ultrasonicated for 20 minutes. The combined extracts were centrifuged for 10 minutes (4000 rpm) and filtered through a 0.45-µm membrane before injection. Prior to each run the capillary was flushed for 2 minutes with 0.1 M NaOH, 2 minutes with water and 5 minutes with buffer. Replenishment of buffer vials was performed every 6th run to achieve best reproducibility.

Results

A typical separation of a Mahonia stem is shown in figure 3. Detection at 265 nm confers some selectivity on the analysis and the three alkaloids berberine, palmatine and jatrorrhizine are well separated from other sample matrix components which absorb at that wavelength. Detection at 200 nm illustrates the complexity of the sample matrix. These three alkaloids can be automatically detected and identified by spectral library search using a library constructed from standards (figure 4). Detection of berberine, palmatine and jatrorrhizine was linear over the ranges 3.4–109 mg/mL, 1.4– 44 mg/mL and 1.1–37 mg/nL respectively with r^2 better than 0.999 in all cases.

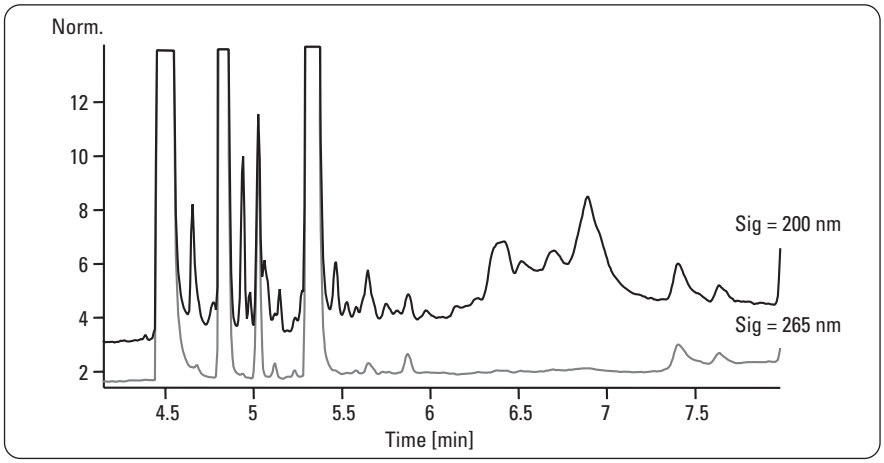

Figure 3
Analysis of Mahonia stem by CE with detection at 200 nm and 265 nm.

Chromatographic conditions

Capillary:	50 µm id x 48.5 cm (40 cm effect.)
Buffer:	50 mM phosphate, 50 mM borate containing 50 % methanol with apparant pH adjusted to pH 8
Detection:	200/10 nm and 265/20 nm
Injection:	500 mbar x s
Voltage:	30 kV
Temperature:	20 °C

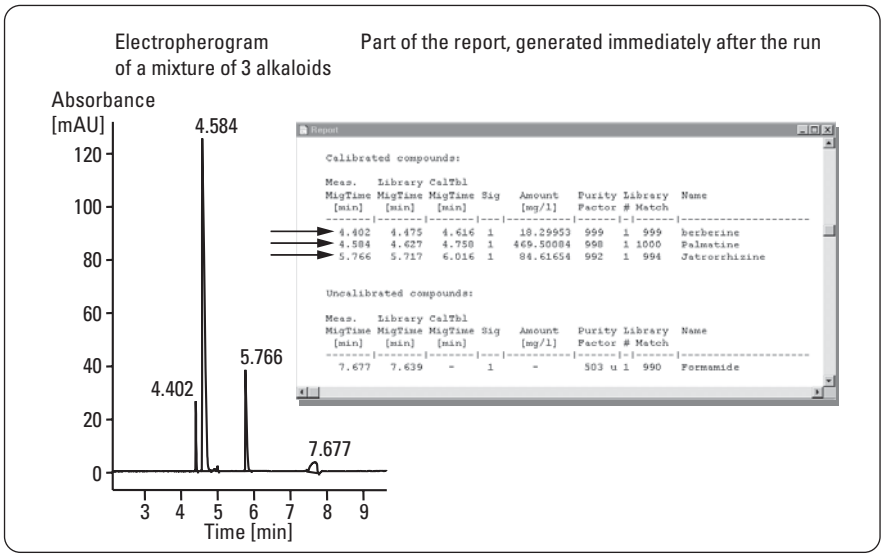

Figure 4
Library search from separation of alkaloids in Mahonia stem.

3. Quantitative analysis of tetrandrine and fangchinoline

Fangchinoline and tetrandine are two alkaloids which are present in Radix *Stephaniae tetradrae S. Moore*. These compounds have pain-relieving activities, can reduce blood pressure, have antineoplastic and antibiotic activity and are therefore of pharmaceutical interest. The plant is used in various Chinese herbal preparations. Here we describe application of a CE method to quantitative analysis of these alkaloids in some traditional Chinese medicines.

Sample extraction

2 g of each pulverized herbal drug were extracted with 7 mL 50 % ethanol by stirring for 30 minutes followed by centrifugation (4000 rpm, 10 minutes). The extraction was repeated two times and the combined extracts were filtered through 0.45-µm pore. For electrokinetic injection, a volume of 200 mM NaCl solution equivalent to one fifth of the sample volume was added to the sample to equalize the sample conductivity.

Results

The medicines were separated using a MEKC system with Tween- 20 as the surfactant[2]. This resulted in a very clean electropherogram where two peaks could easily be seen (figure 5). Due to their similar structure tetrandrine and fang-chinoline (figure 6) have very similar spectra and therefore were identified by spiking experiments with pure standards. After identification the two alkaloids were quantified in a number of traditional Chinese medicines. Linearity was determined for both compounds over the range of 5 to 250 µg /mL. Linearity was greater than 0.9999 for both analytes. Reproducibility of migration times was very good (< 0.4 %). For quantitation, the reproducibility of peak areas was acceptable (< 4 %) but depended on the medicine and therefore the sample matrix (table 1).

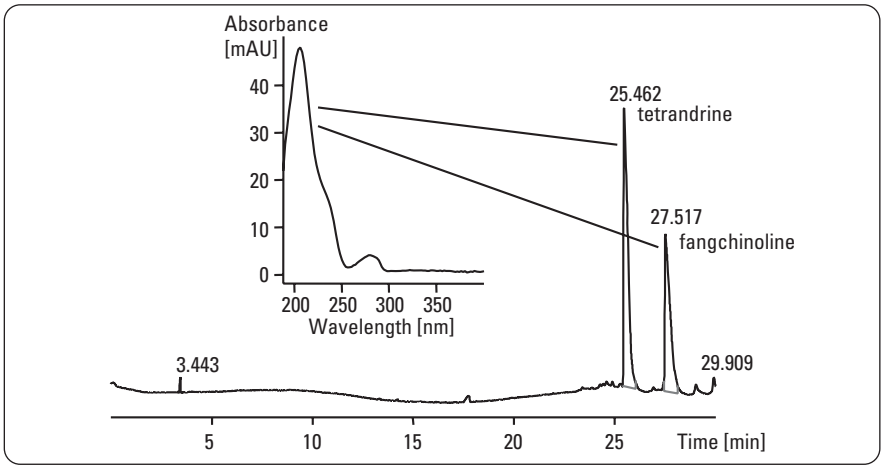

Figure 5
Analysis of tetrandrine and fangchinoline in Radix *Stephaniae tetradrae S. Moore*.

Chromatographic conditions

Capillary:	64,5 cm (56 cm) x 50 µm
Buffer:	60 mM phosphoric acid\TAE, 50 mM Tween-20, 20 % methanol, pH 2,5,
Detection:	214/10 nm
Injection:	4 kV x 23 sec
Voltage:	20.2 kV

Medicine	Tetrandrine mg/L	% RSD area	% RSD time	mg/g	Fangchinoline mg/L	% RS area	% RSD time	mg/g
Fang ji guan jie wan	28.41	2.44	0.32	0.41	17.03	3.45	0.36	0.25
Qu feng gu tong lu	6.96	1.89	0.08	0.07	3.88	2.15	0.07	0.04
Ling long gan mao jiao nang	16.52	1.08	0.23	0.17	9.79	1.16	0.22	0.10
Xi xian feng shi wan	7.47	5.58	0.28	0.07	3.54	2.11	0.29	0.04
Feng shi ton gao	7.14	0.05	0.15	0.08	3.93	1.16	0.15	0.04
Shen jin dan jiao nang	10.94	3.33	0.21	0.12	5.81	3.97	0.25	0.07
Radix Stephaniae tetrandrae	233.26	1.09	0.21	5.52	185.04	1.06	0.22	4.38

Table 1
Migration time and peak area reproducibility (n=3) and quantitation of tetrandrine and fangchinoline in various traditional Chinese medicines.

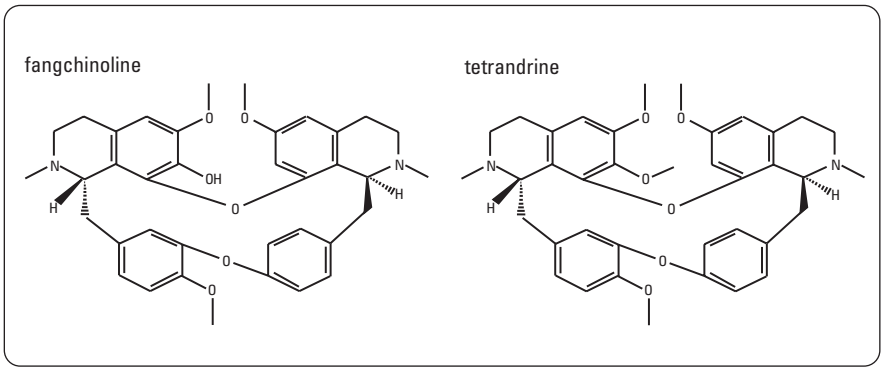

Figure 6
Structure of terandrine and fangchinoline.

4. Identification and quantification of chlorogenic acid

Chlorogenic acid (CA) (figure 7), is an ester of caffeic acid and quinic acid. All three of these substances naturally

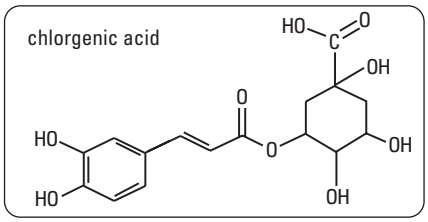

Figure 7
Structure of chlorogenic acid.

occur in many plants. For example, CA which is present in the surface skin of peaches, inhibits the cutin-digesting enzyme of the brown rot fungus, *Monilinia fructicola* demonstrating its antifungal activity. It has also been demonstrated to have antioxidant activity. CA is an active consituent of *Flos Lonicerae* and CZE has been used for its analysis in the plant and in some traditional medicines which contain this plant as a constituent 3. Here we describe how CA can be identified and quantified in some Chinese traditional medicines using capillary electrophoresis.

Sample extraction

Weighed amounts of pulverized samples were soaked with 7 mL 50 % ethanol/water overnight and extracted by stirring for 30 mintes. After centrifugation (4000 rpm, 10 minutes) the extraction was repeated two more times. The combined extraction volume was made up to 25 mL and filtered through 0.45 µm. Liquid samples were simply diluted and filtered before measurement.

Results

The analysis of extracts using the above method gives a complex electropherogram (figure 8). Identification of CA is made more problematic because when injected individually as a standard it migrates in an area occupied in the sample injection by three peaks, all of which have similar spectra. By spiking samples with pure CA it can be unequivocally identified in the prepared TCM extract (figure 9). Table 2 shows the calculated amounts of CA in *Flos Lonicerae*, some TCMs and in a coffee extract.

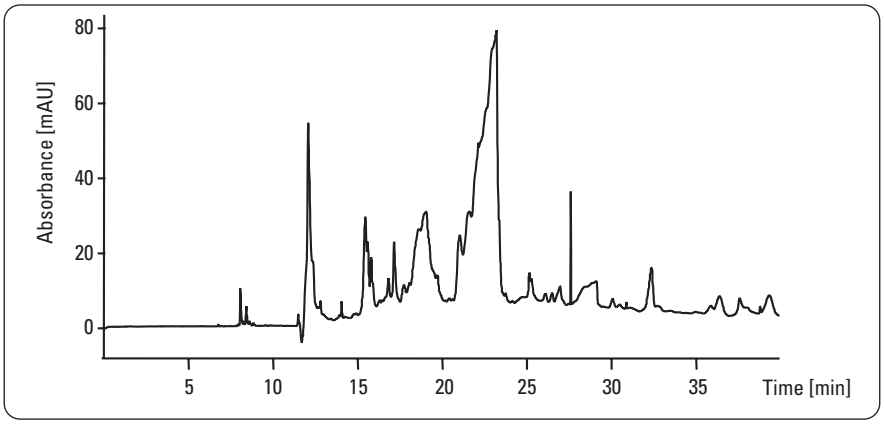

Figure 8
Analysis of an extract of the traditional Chinese medicine Zhi zi jin wan.

Chromatographic conditions

Capillary:	64.5 cm x 50 µm (56 cm effect.)
Buffer:	40 mM phosphate, 80 mM boric acid containing 5 % ethanol with apparant pH adjusted to 7.0
Detection:	254 nm
Injection:	500 mbar x s
Voltage:	20 kV

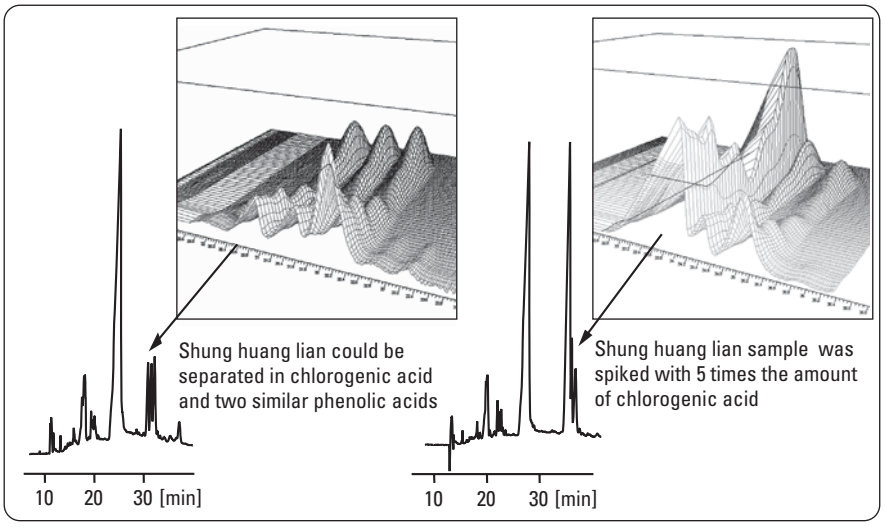

Figure 9
Identification of cholorogenic acid in a traditional Chinese medicine by spiking with a pure standard.

Medicine	Chlorogenic acid (mg/L)	Powder weight (g)	Content (mg/g) or (mg/L)
Flos Lonicerae	670	0.946	17.83
Zhi I jin hua wan	61.18	3.34	0.49
Vc yin qiao pain	104.1	2.83	0.85
Yin qiao jie du pain	542.9	2.9	3.5
Xiao er qing re jie du kou fu ye	31.14	liquid	0.39
Shung huang lian	187.8	liquid	2.1
Café (hot water extract)	197.23	liquid	0.2

Table 2
Calculated amounts of CA in *Flos Lonicerae*, some TCMs and in a coffee extract.

5. Analysis of tetrandrine and fangchinoline by CE/MS

Although CE can provide good resolution of these compounds in complex matrices (see section 3) one of its main limitations, when used with UV detection, is its relatively low sensitivity due to the short optical path length which is the capillary bore. These two alkaloids have extremely similar UV absorption spectra making their unequivocal identification via UV-spectral library search alone problematic. The online coupling of CE with mass spectrometry (MS) is a promising combination. While CE provides high separation efficiency, MS affords high sensitivity and selectivity, as well as molecular structural information. MS detection can provide unequivocal identification of the analytes by their masses.

Successful coupling of CE to MS is dependent upon the efficiency of ions' transfer from the aqueous to the gaseous phase. This, in turn, depends upon the volatility of the running buffer and therefore places some restrictions on the types and /or concentrations of buffer which can be used for CE/MS.

The previous separation of tetrandrine and fangchinoline was only obtained using MEKC conditions, with a buffer solution of 60 mM triethylamine-phosphate containing 50 mM Tween-20 and 20 % methanol, where the analytes were separated according to differences in their hydrophobicity. However, such a running buffer is not suitable for ESI- CE/MS because its non-volatile components can hinder the electrospray process by reducing the spray efficiency and can cause intense background signals resulting in a decreased sensitivity in the TIC[4]. Further, these buffer components can contaminate the ESI interface and the MS ion optics, especially where the spray is "in-line" with the MS entrance capillary, although the orthogonal arrangement of the sprayer used in this study can help to mitigate this effect[5]. In this application a method was developed for the CE/MS analysis of tetrandrine and fangchinoline in Chinese herbal medicines, and their concentrations in some Chinese herbal medicines were determined.

Experimental
The preparations analyzed were:

1) Fang ji guan jie wan:
Radix *Stepahniae tetrandrae* (1.80 g), *Poria* (*Poria cocos* (Schw.) Wolf) (1.80 g), *Rhizoma atractylodis macrocepahalae (Atractylodes macrocephala* Koidz) (1.2 g), Radix *Aconiti* (0.60 g), Radix *Glycyrrhizae* (*Glycyrrhiza uralensis* Fisch.) (0.60 g), Radix *Codonopsis pilosulae* (*Codonopsis pilosula* (Franch.) Nannf.) (0.6 g).

2) Qu feng gu tong lu:
Caulis *Piperis futokadsurae* (*Piper fotokadsura* Sinb. et Zucc.) (3.4 g), Radix *Clematis* (2.8 g) Caulis *spatholobi* (*Spatholobus suberectus* Dunn) (2.8 g), *lignum sapan* (*Caesalpinia sappan L.*) (2.0 g), Radix *Stepahniae tetrandrae* (2.0 g), Cortex *periplocae radicis* (*Periploca sepium* Bge.) (2.0 g), *Herba Sieges-beckiae* (2.0 g), Radix *Aconiti kusnezoffi* (*aconitum kusnezoffii* Rchb.) (1.4 g).

3) Shen jin dan jiao nang:
Lumbricus (*Pheretima aspergillum* Perrier) (8.3 g), *Flos Carthami* (*Carthamus tinctorius L.*) (5.83 g), *Olibanum* (*Boswellia carterii* Bierdw.) (2.5 g), *Myrrha* (*Commiphora myrha* Engl.) (2.5 g), Radix *Stephaniae terandrae* (2.5 g), Cortex *periplocae radicis* (2.5 g) *Rhizoma drynariae* (*Drynaria fortunei* (Kunz) J. Sm.) (2.5 g).

Sample preparation
Different concentrations of tetrandrine and fangchinoline ranging from 0.2 µg/mL to 200 µg/mL were prepared by dissolving the standard in 50 % aqueous ethanol solution. Other samples were prepared as previously described.

Results
The run buffer was chosen for its compatibility with electrospray ionization. The separation achieved was not optimal however using the second dimension of MS detection ensured that the analytes could be selectively detected and identified.

Linearity and detection limits
Linearity and limits of detection were determined for tetrandrine and fangchinoline. The low detection limit and linear range of CE/MS indicate the possibility of trace-level identification and quantitative determination of tetrandrine and fangchinoline in Chinese herbal medicine. Detection was linear over the range 0.2 to 200 µg/mL for tetrandrine and fangchinoline with r^2 values of 0.9991 and 0.9986 respectively. The limits of detection were 3 µg/mL in TIC and 0.05 µg/mL in SIM.

Repeatability
The % relative standard deviation was caculated for peak areas for five replicate injections of standard solution of 50 µg/mL. Reproducibility for fangchinoline was 6.1 % while that for tetrandrine was 5.9 %. The relatively poor repeatability of peak area by SIM compared to the results of the UV detection, which showed % RSD of ca. 3.1, was most probably due to variation in the efficiency of the ionization process. The crude drug of Radix *Stephaniae tetrandrae* and three Chinese herbal preparations which contain Radix *Stephaniae tetrandrae* were analyzed.

Figure 10 shows that the presence of fangchinoline and tetrandrine in the herbal medicine "Qu feng gu tong lu" extract is unclear using CE-UV where the electropherogram contains a number of large and very small peaks. A number of other peaks are present in the area from two to four minutes in the UV trace, where the alkaloids' peaks are expected. However, using the SIM mode of MS the two alkaloids could be detected unambiguously. The repeatability (% RSD, n=5) of peak area of fanchinoline and tetrandrine in "Qu feng gu tong lu" were 3.5 % and 4.3 % respectively. Results from quantitation tetrandrine and fangchinoline in Radix *Stephaniae tetrandrae* and three other Chinese herbal preparations are shown in table 3

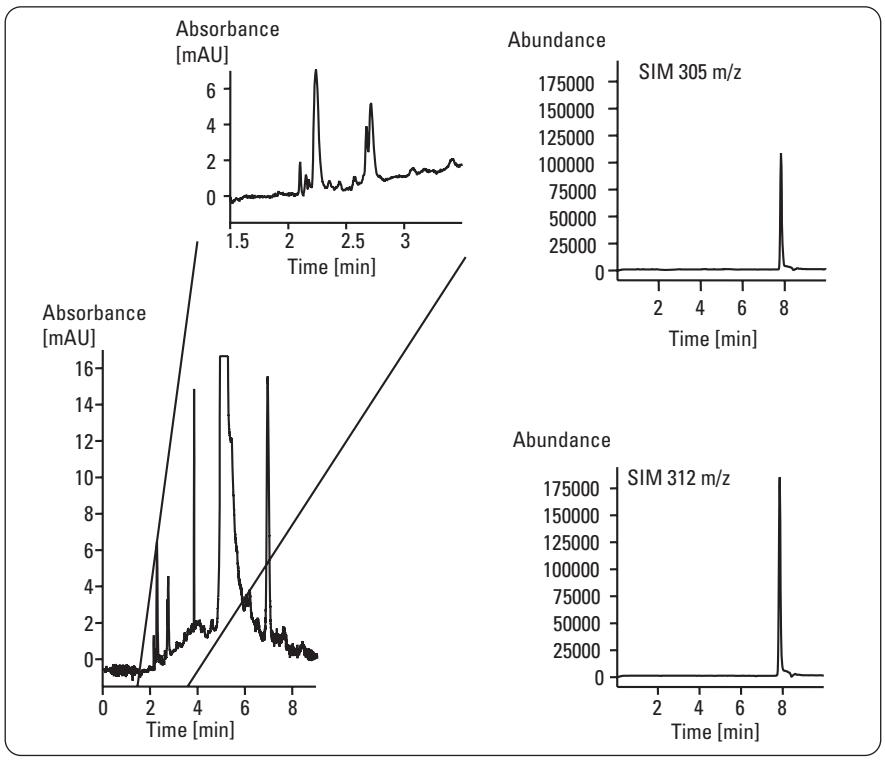

Figure 10
Analysis of Tetrandrine and fangchinoline in Qu feng gu tong lu by CE with UV and MS detection.

Chromatographic conditions

CE

Capillary:	80 cm (22 cm UV) x 50 µm id
Buffer:	100 mM formic acid pH 2.5
Detection:	210 nm/16 nm
Injection:	200 mbar x s
Voltage:	27 kV
Temperature:	20 °C

MS conditions

Sheath liquid:	5 mM ammonium formate in 50 % methanol
Flow rate:	5 µL/min
Nebulization gas pressure:	10 psi
Electrospray voltage:	-4.0 kV (positive ion mode)
Drying gas temperature:	250 °C
MS Scan:	m/z 300–m/z 650 at the rate of 0.85 s/cycle
MS SIM:	m/z=305.2 and m/z=312.1

	Fangchinoline (mg/g)	Tetrandrine (mg/g)
Radix Stephaniae tetrandrae	5.74	7.19
Fang ji guan jie wan	0.736	1.26
Qu feng gu tong lu	0.252	0.513
Shen jin dan jiao nang	0.470	0.833

Table 3
Determined values (mg/g) of fanchinoline and tetrandrine in Chinese herbal medicines by CE/MS.

6. CE/MS for alkaloid fingerprinting of Mahonia species

The pharmaceutically relevant compounds in Mahonia species are alkaloids, including berberine, palmatine, jatrorrhizhine, columbamine, isotetrandrine, berbamine, oxyacanthine, and magnoflorine amongst others.

Experimental

Two species of Mahonia: *M. fortunei* and *M. japonica* were collected in China and identified by Beijing University of Chinese Medicine.

Sample preparation

A 2.0 g sample of pulverised dried stem was extracted with 10 mL methanol by ultrasonication for 30 minutes. Extraction was repeated twice with 5 mL methanol for 20 minutes. All methanol extracts were combined and centrifuged at 4000 rpm for 15 min, then filtered through a 0.45 µm filter.

Results

The electropherograms of the extracts are shown in figure 11. The total ion electropherogram (TIE) is shown in the lower part and the UV signal in the upper part of the figures. The main peaks 1, 2, and 5 in the electropherogram could be identified as berberine, palmatine and jatrorrhizine. Peak assignment was performed by comparison of migration time, UV spectrum and m/z with those of standards. Compound 4 showed a dominant signal at m/z 338 and was tentatively assigned as columbamine, which has been reported to exist in the genus of Mahonia. Compound 9 showed a major signal at m/z 312 and a minor one at m/z 623 indicating that it is an alkaloid with two nitrogens which exists both as a single protonated ion (m/z 623) and a double protonated ion (m/z 312). Therefore, it was tentatively assigned as isotetrandrine, which has also been reported to exist in the genus of Mahonia. Peaks 3, 6, 7 and 8 gave m/z values of 314, 305,

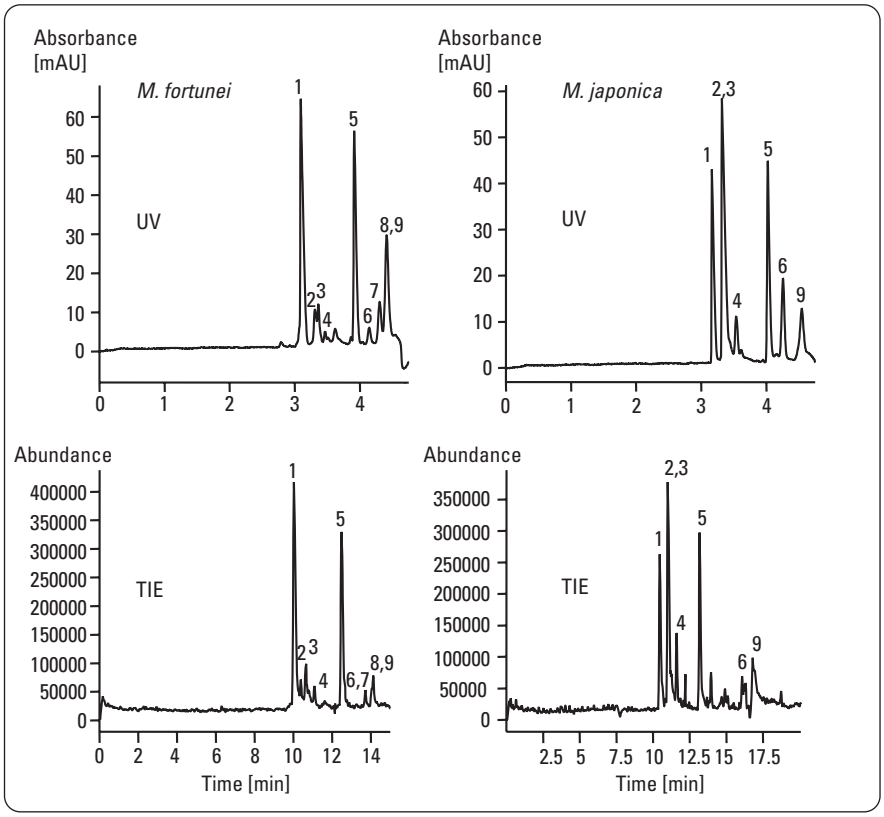

Figure 11
CE/MS of two Mahonia species.

Chromatographic conditions

CE

Capillary:	70 cm (22 cm UV) x 50 µm id
Buffer:	50 mM amonium acetate pH 9.0, 40 % methanol
Detection:	200 nm/16 nm
Injection:	250 mbar x s
Voltage:	25 kV
Temperature:	20 °C

MS conditions

Sheath liquid:	0.5 % formic acid in 50 % methanol
Flow rate:	5 µL/min
Nebulization gas pressure:	10 psi
Electrospray voltage:	-4.0 kV (Positive ion mode)
Drying gas Temperature:	200 °C
MS scan:	m/z 300–m/z 650 at the rate of 0.85 s/cycle

305 and 312 respectively. Since both berbamine and oxyacanthine have m/z of 305 and have been reported as present in Mahonia, peaks 6 and 7 could be assigned either as berbamine or oxyacanthine. Peak 3 and 8 could not be identified but due to their almost ubiquitous presence in a number of the species they were also monitored. Peaks 3, 6, 7, and 8 could not be unequivocally identified but they could be specifically characterized by their m/z and migration times. SIM mode was employed to detect all the putative alkaloid peaks which appeared in the TIC of the eight species of Mahonia.

Figure 12 shows the SIM signals obtained from analysis of these two Mahonia species. Since CE/MS is a two-dimensional separation technique, compounds with different μe could be resolved by electrophoresis, while compounds with similar μe but different m/z could be selectively detected by using SIM mode. Therefore, although compounds 4 and 5, 6 and 7, and 8 and 9 have the same m/z, they could be separated based on differences in their various electrophoretic mobilities prior to SIM detection.

Fingerprints of the two species of Mahonia were constructed by calculating the area of each alkaloid peak as a percentage of the total area of all nine peaks detected in SIM mode (figure 13). The profile of these nine peaks is quite different in these two species. Clearly the alkaloids berberine, palmatine and jatrorrhizine (peaks 1, 2 and 5) are the most predominant, however their proportions are quite different and levels of the other analytes help to further differentiate these two species.

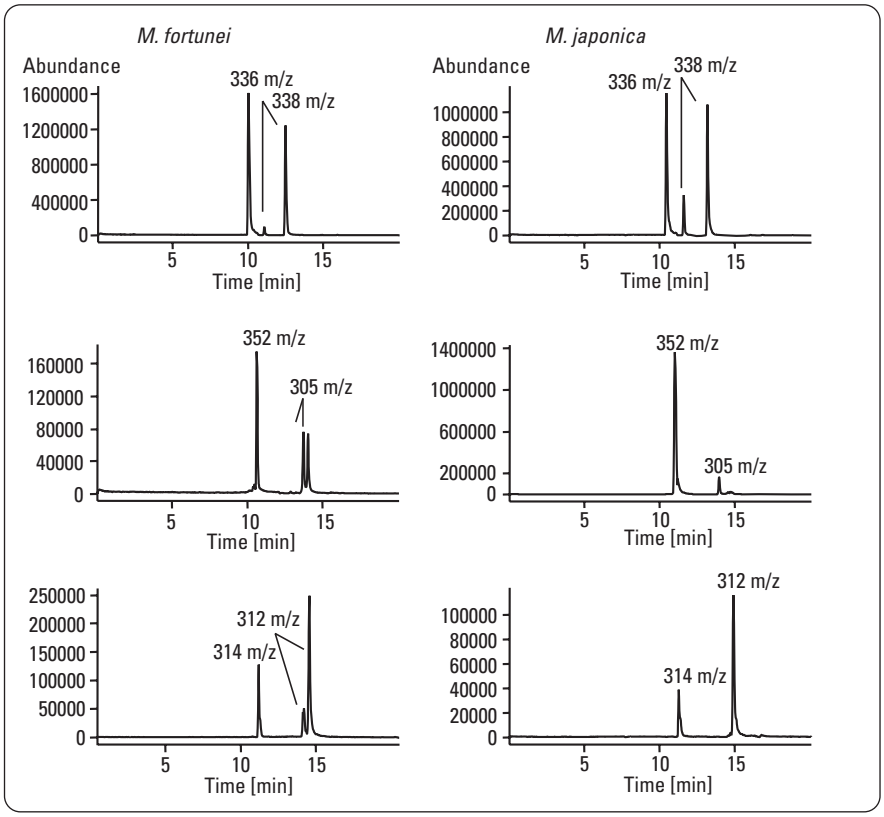

Figure 12
SIM traces from CE/MS of two species of Mahonia.

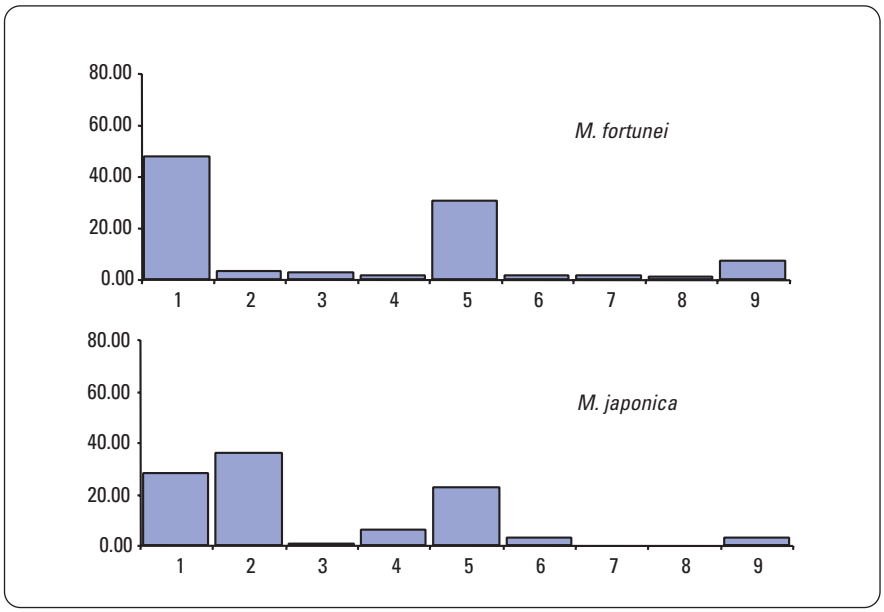

Figure 13
Fingerprint of components of Mahonia species detected by CE/MS. Blocks represent the peak.

Conclusion

CE has been shown to be useful for the separation of traditional Chinese medicines. In combination with DAD UV or MS detection, components of these sample types can be clearly identified using a variety of techniques, spectral analysis, spiking with known standards, m/z determination. The open tubular arrangement of CE makes it very suitable to the complex matrices which are encountered in the analysis of natural products. Coupling of CE with MS adds a second dimension of separation where analytes which have similar mobilities may be separated by the MS while isobaric analytes require separation prior to their detection in the MS.

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