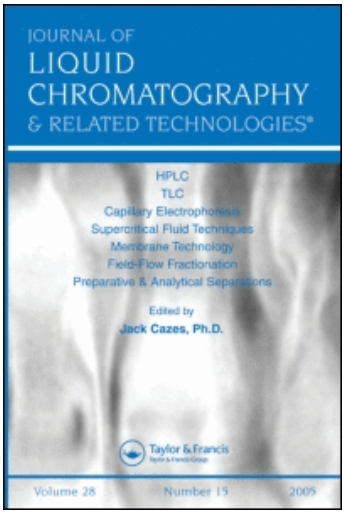

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Qualitative and Quantitative HPTLC Methods for Quality Control of *Stephania tetrandra*

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ABSTRACT

High performance thin-layer chromatography (HPTLC) can successfully be employed for quality control of *Stephania tetrandra*. This paper presents two newly developed methods that are superior to those included in the Chinese Pharmacopoeia. One method, featuring an optimized Soxhlet extraction procedure, is focused on the reproducible separation/detection of tetrandrine, the main alkaloid. Using the same mobile phase [toluene–ethyl acetate–methanol–ammonia 28% (10 : 10 : 5 : 0.3)], this method allows identification of *Stephania* with high certainty and quantitative evaluation of the tetrandrine content. Quantitation by scanning densitometry is performed at 210 nm. The calibration curve is linear for 50–112.5 ng of tetrandrine per zone. A method for the detection of aristolochic acids (AAs), first published as part of the German Drug Codex, was further improved and adapted for assessing the purity of

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Stephania raw material. Optimized for sensitivity, this method allows detection of 1 ppm of AA that may be present due to adulteration with toxic species. Complementary to the US food and drug administration's high performance liquid chromatography-mass spectrometry (HPLC-MS) method, HPTLC on silica gel with toluene–ethyl acetate–water–formic acid (20 : 10 : 1 : 1) and derivatization with tin(II) chloride is a rapid and very powerful screening tool helping to ensure the safety of *Stephania*.

Key Words: Han fangji; Fen fangji; *Stephania tetrandra*; Tetrandrine; *Aristolochia*; Aristolochic acid; Quality control; Fingerprint; Identification.

INTRODUCTION

While traditional Chinese medicine (TCM) is also gaining more and more acceptance in western countries, safety concerns with some plant species have caused authorities worldwide to look into analytical methods for proper identification and quality control of botanicals, including *Stephania tetrandra.* According to the Chinese Pharmacopoeia,[1] the root of *Stephania* is used to improve diuresis and relieve rheumatic conditions. The plant is easily confused with several other toxic species and has, thus, become a serious safety issue. Confusion can be caused by similar TCM (Pin Yin) names for very different botanical species. For example, Han fangji (or Fen fangji or Fanji) is the name of *S. tetrandra* and Guang fanji that of *Aristolochia fangji.* Another problem is the interchangeability of ingredients and the established practice of substitution of one plant species for another in TCMs. In many countries, the use of *Aristolochia* species as an herbal medicine is not permitted because the characteristic constituents of the family, the aristolochic acids (AAs), have been shown to be nephrotoxic, carcinogenic, and mutagenic. No restrictions apply in China, where TCM herbs containing AA are prescribed and traded.

In order to ensure the safety of the patient and enable manufacturers to establish a meaningful quality control, suitable analytical methods are needed that can establish the identity and quality of *Stephania.* High performance thin layer chromatography (HPTLC), a cost efficient, flexible, and rapid chromatographic technique, can be used in quality control at three levels:

A specific fingerprint of the plant focusing on the main alkaloid as chemical reference ensures identity of the plant material.

Quantitative determination of the marker tetrandrine establishes whether a given batch meets established acceptance criteria.

The safety of the material is ensured by proving the absence of common adulterants based on the detection of AAs at the low ppm level.

In HPTLC, modern equipment and software allow the precise and accurate quantitation of UV-absorbing substances in situ (directly on the plate), which makes scraping-off of the zones unnecessary.

EXPERIMENTAL

Material

Authenticated *S. tetrandra* and *A. fangji* roots were provided by Roy Upton (American Herbal Pharmacopoeia, Scotts Valley, CA), Eric Wong (Nikyang Enterprise Ltd, Hong Kong, China), and AOAC International (Gaithersburg, MD). Tetrandrine was supplied by ChromaDex (Santa Ana, CA) and pure aristolochic acid A by PhytoLab (Hamburg, Germany). A mixture of AAs was purchased from Aldrich (Milwaukee, WI).

HPTLC plates silica gel 60 F_{254} were manufactured by Merck (Darmstadt, Germany) and all chromatographic equipment (twin trough chamber 20×10 cm, glass sprayer, immersion device, automatic TLC sampler 4, digital documentation system, scanner 3, winCATS 1.3.0 software) by CAMAG (Muttenz, Switzerland). Other equipment included a centrifuge, a rotatory evaporator, diverse glassware, and a Soxhlet extractor.

Preparation of Standards and Test Solutions

For qualitative identification and detection of adulteration, 0.2 g of powdered drug was sonicated with 10 mL of methanol–water–formic acid (20 : 4.5 : 0.5) for 10 min at 25°C. After centrifugation, the supernatant was used as the test solution. As reference, 1 mg of tetrandrine was dissolved in 1 mL of methanol. The marker for adulteration was 1 mg of AA (mixture of several acids) dissolved in 10 mL of methanol.

For quantitative determination of tetrandrine, the powdered drug was first dried at 80°C for 4 hr. Then, for exhaustive extraction, 50 mg of drug was weighed into the thimble of a Soxhlet extractor and wetted with 25 mL of methanol containing 5% concentrated ammonia. After standing for 15 min, an additional 25 mL of the same solvent was added in order to start the extraction, which was then continued for 2 hr. After cooling to room temperature, the volume of the extract was adjusted to 50 mL with methanol. As the calibration standard, a solution containing 0.0250 mg mL^{-1} of tetrandrine in methanol was used.

For the detection of very low amounts of adulteration with AAs in *Stephania*, the sample was prepared as in the first paragraph, but the amount of drug was doubled. Solutions of 4.0, 0.40, and 0.040 mg L^{-1} of pure aristolochic acid A in methanol were used for quantitation.

Preparation of Reagents

Iodine reagent: 0.05 g of iodine was dissolved in 10 mL ethanol 96%. Anisaldehyde–sulfuric acid reagent: 10 mL of sulfuric acid was carefully added to an ice-cooled mixture of 170 mL of methanol and 20 mL of acetic acid; to this solution, 1 mL of anisaldehyde was added. Tin(II) chloride reagent (to be prepared freshly): 1.5 mL of hydrochloric acid (36%) was diluted with 8 mL of water; 1 g of tin(II) chloride $\cdot 2H_2O$ was dissolved in this mixture.

Mobile Phases

Two mobile phases were used. For the separation of alkaloids (qualitative and quantitative), chromatography was performed with (A) toluene–ethyl acetate–methanol–ammonia 28% (10 : 10 : 5 : 0.3). For determination of AAs, the upper phase of the mixture (B) toluene–ethyl acetate–water–formic acid (20 : 10 : 1 : 1) was used.

Chromatography

Five to ten microliter of test solution and 2–5 μL of standard (qualitative), or varying amounts of test and standard solutions (quantitative), were applied as 8 mm bands, 8 mm from the lower edge of plate. All plates were developed over a distance of 70 mm from the lower edge of plate using a twin trough chamber, saturated for 30 min (alkaloids) or 20 min (AAs), with 10 mL of mobile phase per trough. Filter paper was placed in the trough not used for development. The developed plates were then dried with a hair dryer (cold air) for 5 min.

Derivatization

Iodine reagent: the dried plate was evenly sprayed until the background appeared yellow. After waiting until the plate background was white again, examination was performed under white light.

Anisaldehyde–sulfuric acid reagent: the plate was immersed in the reagent for 1 sec, then heated at 100°C for 2–5 min, followed by evaluation under 366 nm ultraviolet (UV) light.

Tin(II) chloride reagent: the plate was evenly sprayed until slightly wet, then heated at 100°C for 1 min. Examination of fluorescing zones was performed under 366 nm UV light.

Documentation

Digital images of each plate were captured in various illumination modes (UV 254 nm, UV 366 nm, and white light).

Densitometry

The quantitation of tetrandrine was performed by densitometric evaluation in the absorption mode at 210 nm using a deuterium lamp. The size of the scanning slit was adjusted to 6.00 mm × 0.45 mm and the scanning speed to 20 mm sec^{-1}, at a data resolution of 100 μm $step^{-1}$.

RESULTS AND DISCUSSION

Identification of *S. tetrandra*

The starting point of the investigation was the monograph for *Stephania* published in the Chinese Pharmacopoeia.[1] The chromatogram obtained by following the procedure for identification of raw material is not satisfactory (see Fig. 1). The required stationary phase "silica gel G" is difficult to obtain as a pre-coated layer. On HPTLC silica gel 60, tetrandrine shows tailing and its R_F value increases with higher concentration. It seems that two substances are migrating very closely. Derivatization with potassium iodobismuthate is not optimal, because the background of the plate appears yellow. Bismuth as a heavy metal can be considered as a safety risk.

After testing different published mobile phases, which provided no significant improvement, a new mobile phase was developed. The goal was to obtain a specific fingerprint with reproducible R_F values for all components and no tailing of zones. For safety reasons, the mobile phase should preferably not contain chloroform. The derivatization step was also simplified by replacing potassium iodobismuthate reagent with iodine solution. This reagent can be conveniently prepared, stored, used, and discarded.

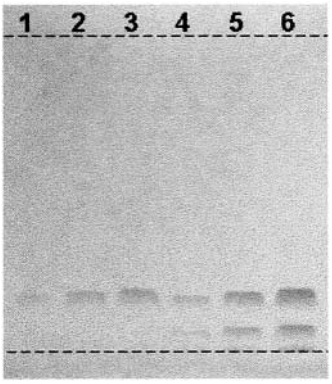

Figure 1. Chromatogram of *S. tetrandra* according to the Chinese Pharmacopoeia. Mobile phase: chloroform–acetone–methanol (6 : 1 : 1), derivatization with potassium iodobismuthate. Lanes 1–3: three increasing amounts of tetrandrine, 4–6: three increasing amounts of *S. tetrandra* sample. The R_F values of tetrandrine vary with the amount. (*View this art in color at www.dekker.com.*)

The proposed method allows quick, convenient, and specific identification of *S. tetrandra* based on the main alkaloid tetrandrine ($R_F \sim 0.5$). Tetrandrine is well separated from two other alkaloids, which can be detected just above and below it at $R_F \sim 0.6$ and $R_F \sim 0.35$, respectively. The compound at higher R_F is only present in low amounts. It is not seen in all samples without adjusting the applied sample amount. The three alkaloids absorb UV light [quenching of fluorescence indicator F_{254}, Fig. 2(a)], fluoresce under 366 nm UV light [Fig. 2(b)], and react with iodine to form derivatives visible under white light as yellowish zones [Fig. 2(c)]. If the plate is subsequently derivatized with anisaldehyde solution, the alkaloids show a strong blue fluorescence under 366 nm UV light. A red zone at $R_F \sim 0.2$ is then also seen in all samples [Fig. 2(d)].

The sample is stable during chromatography and no artifact is generated. This fact was established by two-dimensional (2-D) chromatography[2] (Fig. 3).

Quantitation of Tetrandrine in *S. tetrandra*

The Chinese Pharmacopoeia[1] includes an assay of tetrandrine in *Stephania.* It is based on spectrophotometry, following separation of the sample on a TLC plate, scraping-off the tetrandrine zones, and clean-up on a column. The method shows several weak points:

Although, the extraction of the raw material is very time-consuming (7 hr each), it is not exhaustive (see Table 1).

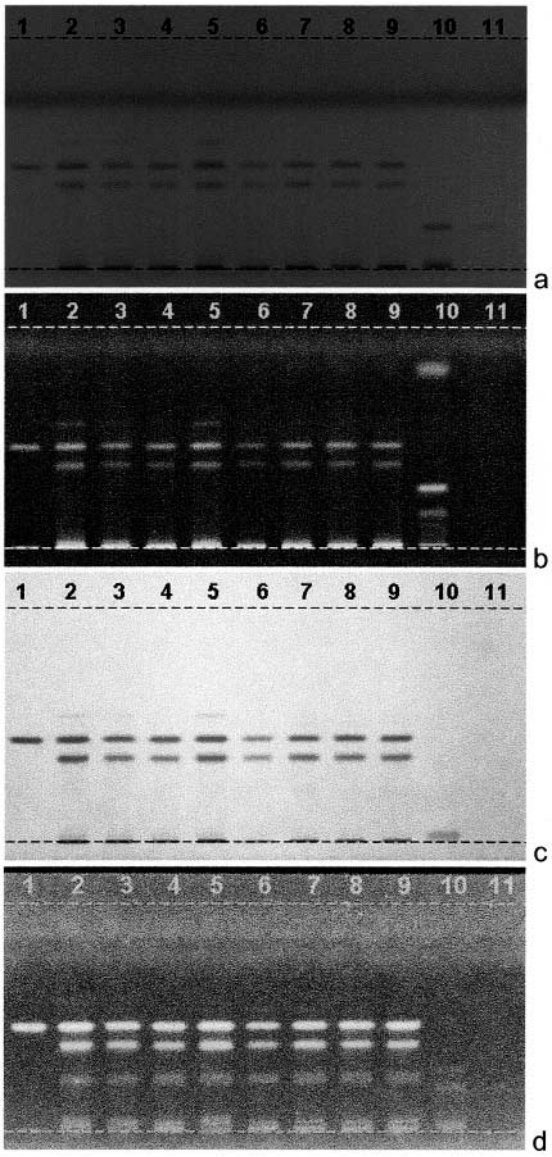

Figure 2. Identification of *S. tetrandra* root. Image under 254 nm UV light (a), 366 nm UV light (b), after derivatization with iodine under white light (c), and subsequent derivatization with anisaldehyde, under 366 nm UV light (d). Mobile phase (A) lane 1: tetrandrine ($R_F \sim 0.5$), 2–9: different *S. tetrandra* root samples, 10: *A. fangji* root, 11: AAs mixture (only a faint band seen under 254 nm UV light at $R_F \sim 0.2$). (*View this art in color at www.dekker.com.*)

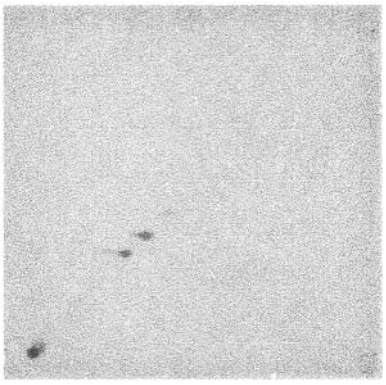

Figure 3. 2D Chromatogram of *Stephania.* The sample is stable during chromatography. Mobile phase (A); derivatization with iodine. (*View this art in color at www.dekker.com.*)

A baseline separation of the compounds during the TLC step is not achieved.

The quantitation process is tedious and complex. Most steps are sources of uncertainty.

Table 1. Comparison of extraction procedures.

Extraction procedure	Amount of tetrandrine per gram of raw material after first extraction (mg)	% Tetrandrine found in 2nd extraction compared with first extraction	Completion of extraction
Chinese Pharmacopoeia: 1 hr moistening with ammonia, 6 hr Soxhlet extraction with chloroform	7.5	>7%	No
Sonication with methanol for 10 min	3.5	>22%	No
15 min moistening with ammonia–methanol, 2 hr Soxhlet extraction with ammonia–methanol	11.2	<0.5%	Yes

Therefore, a new method for quantitation of tetrandrine is proposed. Because the mobile phase developed for identification purpose achieves baseline resolution for tetrandrine (Fig. 4), the same system was also used for quantitative measurements. However, instead of removing the target compound from the plate, it was directly measured using a scanning densitometer.

The practical applicability of the method for solving problems of quality control was demonstrated through validation of several parameters. It should be noted that at this point no acceptance criteria have been established and, therefore, further validation for individual products may be necessary. One of the principal requirements of any quantitative analysis is its specificity, which ensures that no component of the samples interferes with detection of the target compound. Specificity of the proposed method was established by comparison of the UV spectra of the reference substance and that of the corresponding zone in the extract (Fig. 5). For densitometric measurements evaluating peak heights, the absorption maximum of tetrandrine at 210 nm was selected.

Linear regression was used to fit a set of six calibration points. Linearity was established in the range from 50 to 112.5 ng. The target amount of tetrandrine to be quantified was set to 75 ng absolute. This represents the amount contained in 6–7 μL of an extract made from 50 mg raw material in 50 mL, assuming

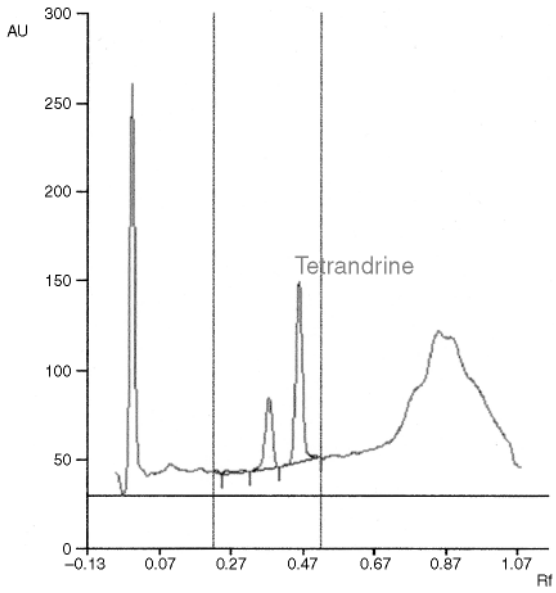

Figure 4. Densitogram of a *S. tetrandra* sample; Mobile phase (A). (*View this art in color at www.dekker.com.*)

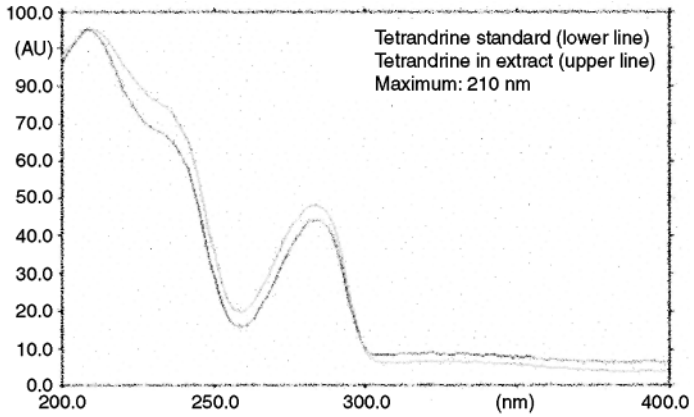

Figure 5. Comparison of UV spectra of tetrandrine standard solution and the corresponding band of a *Stephania* extract. (*View this art in color at www.dekker.com.*)

that the raw material contains about 1% tetrandrine. The required working range of 60–90 ng (80–120% of target) is well within the linear range of the method (Fig. 6). The relative standard deviation (RSD) for six replicates in the middle of the working range (75 ng) is 0.45%. At the lower and upper limits, the RSD values are higher: 2.4% and 5.2%, respectively. RSD measuring six replicates of the same sample on one plate varies between 1.6% and 3.7%, while the RSD of the means from three different plates (six measurements each) is 1.2%.

The extraction step is a key element affecting the accuracy of quantitation. However, it is difficult to assess the completion of the extraction when the true content of the target compound is not known in a plant material. Even if a recovery study using a placebo would be employed, it is impossible to predict extraction efficiency because the behavior of the target compound that was simply added to the plant material could be significantly different from that of bio-chemically produced material, which is present within the cell structures of the plant. Nevertheless, different extraction procedures were evaluated. They gave significantly different results (Table 1).

The proposed extraction procedure is exhaustive. When comparing the results of an extraction of three portions of the same plant material (mean of three measurements each), the RSD was 5.2%.

Detection of Adulteration

For safety reasons, any product based on *Stephania* must be tested for absence of contamination or adulteration with toxic species. As raw plant bulks are not homogenous materials, mix-up is very likely to appear. In

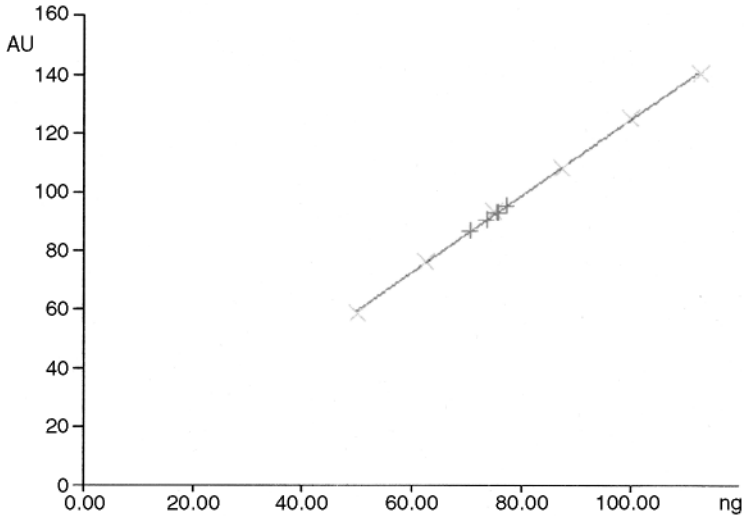

Figure 6. Calibration function for tetrandrine measured at 210 nm (peak height). Small crosses: samples. Linearity: 50–112.5 ng absolute. $y = -5.769 + 1.302x$; $r = 0.9997$, sdv 0.84%. Working range: 60–90 ng absolute (target 75 ng). (*View this art in color at www.dekker.com.*)

the case of *S. tetrandra*, the primary adulterant is *A. fangji.* The Chinese names of both species are very similar, and the drugs are commonly interchanged. The FDA has published a sophisticated method for quantitation of AAs in TCM using HPLC-DAD or high-performance liquid chromatography-mass spectrometry (HPLC-MS),[3] which are very sensitive but time-consuming and costly. A rapid screening method for detection of AAs by TLC was first published in the German Drug Codex.[4] AAs are markers for adulteration and can be selectively derivatized with tin(II) chloride. By changing the stationary phase to HPTLC material and fine-tuning several parameters, this method was optimized for reproducibility and sensitivity. During validation, the limit of detection for the main marker aristolochic acid A was found at 0.2 ng absolute, which (in theory) would equal adulteration at the 0.5 ppm level with pure marker substance. In real samples, the LOD is slightly higher due to interference of the complex plant matrix. However, spiking experiments (1 μg aristolochic acid A per gram *S. tetrandra*) have shown that contamination at the 1 ppm is clearly detectable (Fig. 7). Although, it is not possible to determine the exact values at this level, no false negatives were found in a collaborative study with AOAC International and the FDA.

Detection limits of contamination of a *Stephania* raw material with *A. fangji* root were found at 1% (Fig. 8). Comparison of results obtained by

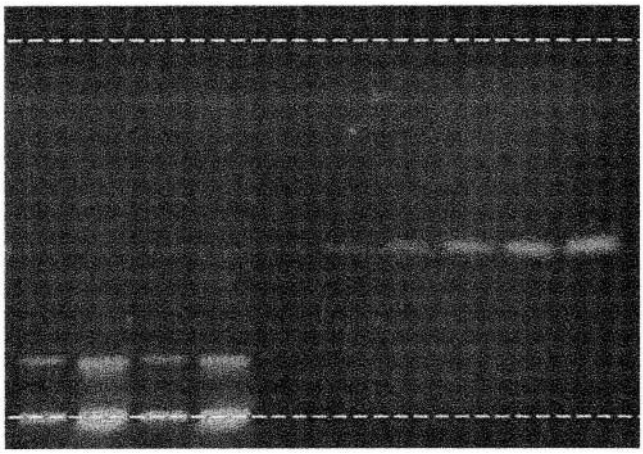

Figure 7. Detection of 1 ppm adulteration with aristolochic acid A in *Stephania* [derivatization with tin(II) chloride, 366 nm UV light], mobile phase (B). From left to right: *S. tetrandra* pure (10 and 30 μL), *Stephania* adulterated with 1 $\mu g\, g^{-1}$ aristolochic acid A (10 and 30 μL), increasing amounts of aristolochic acid A (400 pg to 8 ng absolute). (*View this art in color at www.dekker.com.*)

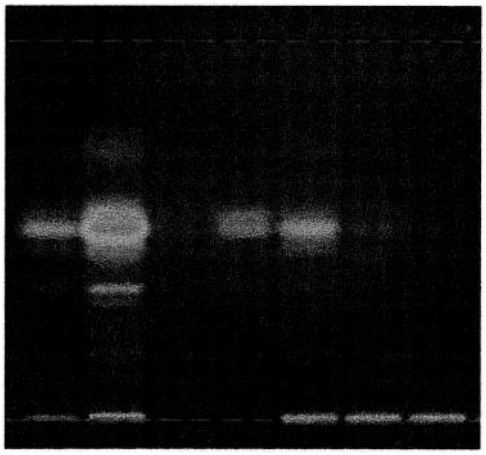

Figure 8. HPTLC screening of TCM samples for the presence of AAs [derivatization with tin(II) chloride, 366 nm UV light], mobile phase (B). From left to right: *A. fangji*, 1 and 10 μL; AAs mixture 10 and 50 ng (absolute); *S. tetrandra* adulterated with 10% and 1% *A. fangji*, 10 μL each; pure *S. tetrandra* 10 μL (shows no zone). (*View this art in color at www.dekker.com.*)

Table 2. Comparison of HPTLC and HPLC results.

Parameter	HPTLC	HPLC[3]
Detection mode	Densitometry after derivatization, 366 nm	DAD 390 nm, then MS (ion trap or triple quad)
Target level for aristolochic acid A	>400 pg (application volume: max 50 μL for samples w. high matrix, "unlimited" for standards)	2–15 ng (injection volume 25 μL)
Detection level	1 ppm (based on experimental spiking data, no quantitation possible)	0.5 ppm (estimation based on low-level standard injection)
Analysis time/ sample	<10 min (application, development, derivatization, densitometry, 12 samples per plate)	30 min + 10 min post-time

HPTLC and HPLC (Table 2) shows similar performance of both methods. While HPLC is more suitable for low-level quantitation, HPTLC allows rapid detection of AA presence. In comparison to HPLC-MS, the proposed method is extremely cost-efficient and time-saving. This makes it a very powerful alternative for screening large numbers of samples.

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