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Combination of TLC and HPLC-MS/MS Methods. Approach to a Rational Quality Control of Chinese Star Anise

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In this study, a methodological approach for an effective and reliable quality control of Chinese star anise (*Illicium verum* Hook. F.) is developed and validated. A combined method of TLC and HPLC-MS/MS was used for differentiation of various *Illicium* species, especially Chinese and Japanese star anise. Species can be distinguished by their TLC flavonoid pattern. A sensitive and selective HPLC/ESI-MS/MS method was developed for the detection and quantification of lower admixtures of *I. anisatum* and of further toxic *Illicium* species at a low concentration range using the sesquiterpene lactone anisatin as a marker. The proposed assay includes a solid-phase extraction cleanup procedure with a high recovery (>90%). Chromatographic separation of anisatin was carried out on a C18 column, followed by MS detection using ESI in negative mode. The precursor/product ion transitions *m*/*z* 327 → 127 (quantifier) and *m*/*z* 327 → 297 (qualifier) were monitored. Statistical evaluation of this multireaction-monitoring procedure reveals good linearity and intra- and interday precision. The limits of detection and quantification are 1.2 and 3.9 μg/kg, respectively.

KEYWORDS: Chinese star anise; Japanese star anise; *Illicium verum* Hook. F.; *Illicium anisatum* L.; Illiciaceae; anisatin; sesquiterpene lactones; TLC; HPLC-MS/MS

INTRODUCTION

Chinese star anise (*Illicium verum* Hook. F.) is a plant originally used in traditional medicine of eastern Asia, where it is also cultivated. Its use in phytotherapy as well as for aromatization of pharmaceutical products, foods, and cosmetics has long been a tradition worldwide. However, intoxications connected to the use of products that contain Chinese star anise have repeatedly been reported in the past (*1*−*5*). These intoxications had serious, sometimes fatal, consequences and led health authorities to the decision to regulate the import of star anise from third-world countries (*6*). Recently, the discussion about the toxicity of Chinese star anise was resumed when a case of neurotoxicity in infants after consumption of star anise tea was published (*7*). The authors suggest that traces of anisatin and related sesquiterpenes, which are present in authentic *I. verum* as well, could have been responsible for the toxic effects observed in the very young infant. However, because none of the tested samples had been determined to be without adulteration, it seems reasonable for the time being to maintain the generally regarded as safe (GRAS) status of star anise (*8*). In view of the discussion it is surprising that there is still no method available which conclusively excludes the presence of significant amounts of toxic adulterations in commercially available products containing Chinese star anise.

The toxicity of these products has been found to be caused by an adulteration or confusion of Chinese star anise with other toxic *Illicium* species. These species contain higher amounts of toxic of sesquiterpene lactones than Chinese star anise itself (*9*). Most of the reported intoxications were shown to have their origin in mixing Chinese star anise with Japanese star anise (*Illicium anisatum* L., syn. *I. religiosum* Sieb. et Zucc., syn. *Illicium japonicum* Sieb., shikimi, shikimmi, skimmi), which contains the highly toxic sesquiterpene lactone ansatin (*10*). As Japanese star anise is also used in traditional medicine of eastern Asia this may be the reason for the frequent mixing of these two species. Others of the more than 50 *Illicium* species are known for their toxicity (e.g., *I. majus* Hook f. and *I. simonsii* Maxim. from eastern Asia or *I. floridianum* Ellis from North America) and may be present in products containing Chinese star anise.

The unavailability of an efficient method for the detection of toxic adulterations in Chinese star anise is caused by several facts. Formerly described morphological differences (*11*), which are part of the monograph "Star Anise" in the *European Pharmacopoeia* (*12*), were not found to be sufficient for differentiation between various *Illicium* species because even in one batch of Chinese star anise the morphological appearance of single fruits differs strongly. Morphological or microscopic differences are no longer of any use when a complete batch in the ton scale of Chinese star anise has to be controlled for adulteration (*11*). Because of the origin of the commonly traded Chinese star anise, it is difficult to obtain authentic samples of certain *Illicium* species that could be used for detection of adulterations for comparison of typical attributes. The current method for quality control of Chinese star anise, the GC-MS

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analysis of the extracted essential oil for quantification of "typical" markers of Chinese star anise (*trans*-anethole), and Japanese star anise [myristicin and safrole (*13*, *14*), methoxyeugenol, eugenol, and 2,6-dimethoxy-4-allylphenol (*15*)] does not include determination of the toxic principle, the sesquiterpene lactones, and is therefore inadequate.

In this study, a pragmatic approach for routine quality control on Chinese star anise products, which combines the well-known principle of TLC testing and the possibilities of modern HPLC-MS/MS methods, is presented. The results are compared with GC-MS data obtained with commercial samples of Chinese star anise.

MATERIALS AND METHODS

Materials and Reagents. Anisatin was an addipharma reference substance from PhytoLab (Vestenbergsgreuth, Germany). Rutin, chlorogenic acid, and caffeic acid were purchased form Sigma-Aldrich (Taufkirchen, Germany). Reagents and solvents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Methanol of LC-MS grade was obtained from Sigma-Aldrich. Extrelut 3N cartridges and HPTLC glass plates, 20 × 10 cm, SiO_2 60 F_{254}, were from VWR. All samples of Chinese star anise (*I. verum*) were commercially traded products. Japanese star anise (*I. anisatum* L.) and *I. lanceolatum* A. C. Sm. were obtained from Meheco Herbs (China), Ximei Trading (China), and Hydrosa Trading Ltd. (China). Samples of *I. henryi* Diels, *I. micranthum* Dunn, and *I. simonsii* Maxim were provided by CAMAG Laboratory (Muttenz, Switzerland). One sample of *I. floridanum* was obtained from the Institute of Pharmaceutical Biology (University of Düsseldorf). All water used was doubly ionized water (Milli-Q water purification system) (Millipore Corp., Bedford, MA).

Preparation of Standard Solutions. Standard stock solution of anisatin (500 μg/mL) was prepared by weighing 5 mg of anisatin into a 10 mL volumetric flask, dissolving in methanol, and making up to volume. Working standard solutions and fortification solution for LC-MS/MS were diluted with a mixture of acetonitrile and water (10:90). Stock solution was stored at −20 °C. Reference solutions for TLC were prepared by dissolving 7 mg of rutin, 5 mg of chlorogenic acid, and 8 mg of caffeic acid in a separate volumetric flask and diluting with 30 mL of methanol. All working solutions and reference solutions were kept refrigerated, generally at 4 °C.

TLC Method. Two grams of the pulverized sample was dissolved in 10 mL of methanol and heated under reflux in a water bath (60 °C) for 5 min. After cooling to room temperature, an aliquot of the clear supernatant was applied onto a HPTLC plate using an ATS 4 automatic TLC sampler (CAMAG). Fifteen microliters of each sample solution was applied in bands of 2 cm. For reference, 15 μL of a solution of rutin, chlorogenic acid, and caffeic acid was also applied, bandwidth = 2 cm. The HPTLC plate was developed over a distance of 7.5 cm at room temperature (~35% relative humidity) in a 20 × 10 cm twin-trough chamber. The developing solvent was a mixture of ethyl acetate, formic acid, acetic acid, and water (100:11:11:26). Time for saturation of the chamber was 30 min. For derivatization, natural products reagent (Roth, Karlsruhe, Germany) and PEG 400 were used (time of development = 30 min; dried in a hot air stream). Detection was done at 365 nm UV light using a CAMAG Digistore video documentation system and Wincats software.

GC-MS Method. Sample preparation and quantitative analysis were carried out as described in the monograph "Star Anise" of the current *European Pharmacopoeia*.

LC-MS/MS Method. Dried plant samples were pulverized with a centrifugal grinder. A homogeneous sample (10 g) was blended with 250 mL of *n*-hexane. The resulting suspension was filtered through a funnel, containing a filter paper. Residue and filter were dried for 30 min in a drying oven at 100 °C. Two grams of dried and defatted sample was weighed into a 50 mL volumetric flask. After the addition of 20 mL of methanol, the mixture was extracted by sonication for 30 min. Then, the sample was centrifuged at 4000 rpm for 10 min. Four milliliters of the clear supernatant was evaporated to dryness at 50 °C in a gentle stream of nitrogen. The residue was dissolved in 3 mL of water and centrifuged for 5 min. The solution was applied to the Extrelut 3 cartridge. After a residence time of 10 min, the analyte was eluted with 4 × 3 mL of MTBE. The eluate was evaporated to dryness at 50 °C in a gentle stream of nitrogen and dissolved with 5 mL of 10% acetonitrile in water. Finally, 20 μL of the solution was injected into the LC-MS/MS system. In the case of pure Japanese star anise or *I. floridanum* the final test solution was diluted 1:500 with 10% acetonitrile in water.

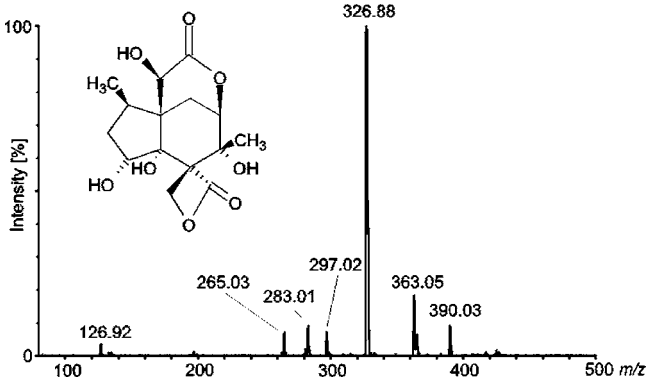

Figure 1. Mass spectrum and chemical structure of anisatin.

An Alliance 2695 HPLC system (Waters, Milford, MA) coupled with a Micromass QuattroUltima triple-quadrupole mass spectrometer was used for all analyses. The 150 × 2.0 mm i.d., 5 μm Prodigy ODS (2) HPLC column (Phenomenex, Aschaffenburg, Germany) was used preceded by a 4 × 3.0 mm i.d. C18 ODS precolumn [and a particle filter (Security Guard, Phenomenex, Aschaffenburg, Germany]. A flow rate of 0.3 mL/min was maintained while all analyses were conducted at ambient temperature. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). The gradient program was as follows: 90% A isocratic (2 min), 90−85% A (10 min), 85−75% A (2 min), 75−10% A (6 min), 10−0% A (1 min), 0% A isocratic (4 min), 0−90% A (1 min). The column was allowed to reequilibrate for 9 min of 90% A before another injection was made. Total analysis time, including reequilibration, was 35 min. Retention time for anisatin was 8.8 min. Electrospray ionization (ESI) in negative mode was used. Mass calibration was performed as per the manufacturer's recommended procedures, using reserpine and PPG. Full-scan mass spectrum in the negative-ion mode was obtained by scanning the first quadrupole (Q1 scan) in the 80−1000 amu region with a scan time of 1 s. The turboion spray source was operated at 130 °C, −2.7 kV capillary voltage, −30 V cone voltage, and 400 °C desolvation temperature. The flow rates for cone gas and desolvation gas were 100 and 800 L/h, respectively. The collision energies for MS/MS were 14 eV (quantifier) and 12 eV (qualifier) with argon as collisionally induced dissociation (CID) gas. The collision cell pressure was maintained at 2.2 mbar. Optimization of ion source and MS/MS parameters for data acquisition was performed by infusion of a 10 μg/mL solution of anisatin (25 μL/min) with a syringe pump (Harvard Apparatus, Holliston, MA) coupled with a tee-union to the outlet of HPLC system. Analysis was carried out by multireaction monitoring, using *m*/*z* 327 → 127 for quantifier and *m*/*z* 327 → 297 for qualifier (dwell times were 400 ms for each transitions) (**Figure 1**). The LC-MS/MS analysis was controlled by MassLynx. Qualitative identification in samples was performed by comparison of MS/MS spectra and LC retention times with standards. Gaussian smoothing (provided by the instrument software) was used for chromatographic displays.

For calibration curves, appropriate volumes of anisatin standard stock solution (500 μg/mL) were diluted with 10% acetonitrile in water, and seven concentration levels ranging between 0.5 and 300 ng/mL were analyzed. The calibration curve was estimated by a weighted least-squares regression procedure, using $1/x^2$ as weighting factor.

Chinese star anise samples ($n = 4$ for each concentration level) were adjusted to concentrations of 600 and 3000 μg/kg anisatin, respectively, and then analyzed using the mentioned procedure to determine absolute recovery. Recovery was calculated by comparing the peak area for

Table 1. Results of GC-MS Analyses of Essential Oil Components in Different *Illicium* Species

Illicium species	essential oil (%)	*trans*-anethol (%)	safrole (%)	myristicin (%)
I. verum Hook. f. (Chinese star anise), $n = 17$[a]	6.5–11.5	78.7–89.8	nd[b]	nd
I. anisatum L. (Japanese star anise), $n = 4$	1.1–1.7	0.2–1.6	nd–2.4	nd–0.3
I. lanceolatum A. C. Sm., $n = 4$	<1	0.5–14.1	nd–0.02	0.002–0.1
I. henryi Diels, $n = 2$	0.6/1.3	0.2/1.0	up to 0.01	0.02
I. micranthum Dunn, $n = 1$	0.4	1.2	0.004	0.02
I. simonsii Maxim., $n = 1$	1.2	1.3	0.02	0.004

[a] *n* is the number of samples. [b] Not detected.

transition *m*/*z* 327 → 127 of directly injected anisatin standard solution with the ones obtained in the Chinese star anise samples. Reproducibility was determined by analyzing anisatin in Japanese star anise samples on the same day (intraday $n = 6$) and on two different days (interday $n = 9$). Blank samples (solvent) were extracted and analyzed for potential interfering peaks coeluting with anisatin. The limits of detection (LOD) and quantification (LOQ) were calculated using S/N ratios from 3 to 1 for LOD and from 10 to 1 for LOQ, respectively. Model mixtures of Chinese star anise with Japanese star anise adulterations in a range from 0.1 to 5% were produced and analyzed.

RESULTS AND DISCUSSION

In a first approach, a series of different *Illicium* species were analyzed using the GC-MS method for their content of the three markers *trans*-anethole, safrole, and myristicin in the essential oil to evaluate a potential contamination with Japanese star anise. The results are shown in **Table 1**. As expected, the essential oil content of samples of Chinese star anise was found to be significantly higher than in other *Illicium* species. In accordance with literature data the main component of the essential oil of Chinese star anise is *trans*-anethole, with relative contents ranging from approximately 79 to 90% (*13*). Safrole and myristicin were not detected. It is reported that Japanese star anise contains 0.5−1% essential oil and, besides others, 3.5% myristicin and 6.6% safrole (*12*). However, only one of the four tested samples of Japanese star anise was found to contain appreciable amounts of safrole (2.4%) and myristicin (0.3%). In these samples *trans*-anethole was found in a range from 0.2 to 1.6%. However, the low content of *trans*-anethole is characteristic not only for Japanese star anise but also for other *Illicium* species. For these, traces of myristicin and safrole were detected, also. In mixtures of the essential oil of Chinese star anise and Japanese star anise, containing 2.4% safrole and 0.3% myristicin, the two components were detectable down to the spiked concentration of 1% of the Japanese star anise oil.

As already stated, the high content of *trans*-anethole is characteristic for the essential oil of Chinese star anise. Although the two marker substances for Japanese star anise, safrole and myristicin, are also found in other *Illicium* species, their presence could be a hint for contamination with such species, because no safrole or myristicin is present in the essential oil of Chinese star anise itself. For that reason, the German food control authority demanded the absence of the two markers myristicin and safrole as a proof for the absence of contamination with Japanese star anise (*6*). However, in view of the fact that the amount of essential oil in Chinese star anise can be 20-fold higher than in other *Illicium* species, a dilution effect has to be considered in the ratio of the oil contents. This means that a content of 1% of essential oil, which may result from *I. henryi* and can be detected via safrole and myristicin, equals an amount of nearly 20% of the plant material. This clearly demonstrates that neither the content of total essential oil nor the anethole content itself is a valid criterion for the evaluation of a possible contamination of Chinese star anise with low amounts of other *Illicium* species.

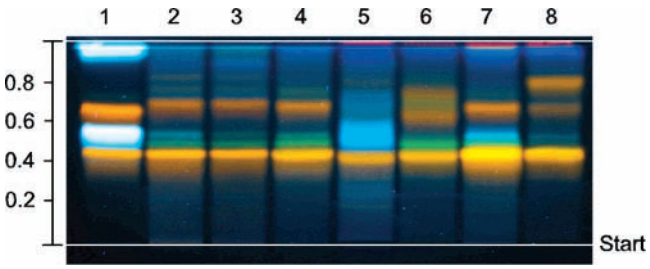

Figure 2. TLC fingerprint of different *Illicium* species: (1) reference solution (rutin, chlorogenic acid, hyperoside, caffeic acid); (2) *I. henryi* Diels; (3) *I. micranthum* Dunn; (4) *I. simonsii* Maxim; (5) *I. floridamun* Ellis; (6) *I. verum* Hook f.; (7) *I. lanceolatum* A. C. Sm.; (8) *I. anisatum* L.

Facing the problem of a fast and high-throughput quality control of samples from Chinese star anise, a TLC method was developed, which allows the comparison of the flavonoid spectra of *Illicium* species. With this method, samples of multiple commercial batches of Chinese star anise were compared with samples of other *Illicium* species obtained from different sources. In these experiments Chinese star anise showed a characteristic fingerprint in comparison with all other analyzed *Illicium* species (**Figure 2**). Therefore, the fingerprint analysis can be regarded as a useful tool for the rapid identification of Chinese star anise in the quality control of commercial batches. However, although this test allows the identification of different species, several disadvantages do not favor the TLC method as the only tool for a quality control of Chinese star anise. One of these disadvantages is, for example, that the toxic principle of possible adulterations is not detected. For that reason, the goal of this study was to develop and validate a fast, selective, and sensitive method, which combines the possibility of the determination of low adulterations of toxic *Illicium* species on the one hand with the detection of the toxic sesquiterpene lactones on the other hand. For this purpose, the feasibility of liquid chromatography/electrospray tandem mass spectrometry (HPLC/ESI-MS/MS) to quantify the major toxic sesquiterpene lactones, anisatin, in Chinese star anise or herbal mixtures containing Chinese star anise was assessed. The calibration curve for anisatin (0.5−300 ng/mL) was obtained using weighted least-squares regression of the peak area of transition *m*/*z* 327 → 127 versus the concentration. A weighting factor of $1/x^2$ was applied to the standard curve. Good linearity was observed for peak intensity within the specified anisatin concentration range. The correlation coefficient was 0.999. The mean absolute recovery at a concentration of 600 or 3000 μg/kg anisatin was 98.7 ± 0.3%. To estimate the influence of endogenous anisatin as a possible source of anisatin, Chinese star anise samples without detectable anisatin contents were used. The LOD and LOQ were 1.2 and 3.9 μg/kg, respectively. Intra- and interday precisions (defined as the RSD of replicate samples) were evaluated for one Japanese star anise sample with a mean anisatin concentration of 1154.4 mg/kg. The intra- and interday precision values were 3.0 and 6.6%, respectively. The validation

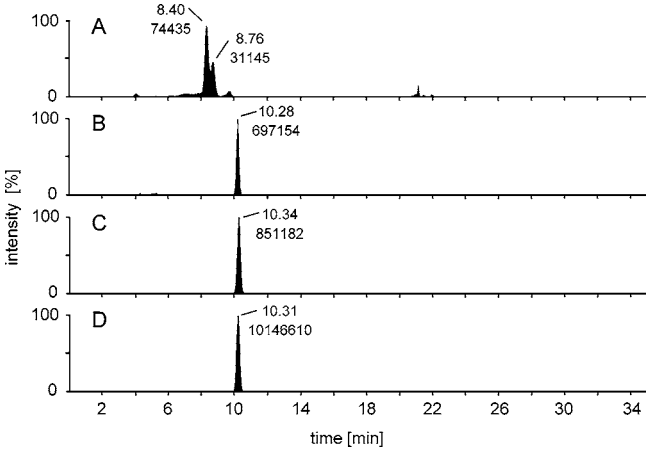

Figure 3. LC/ESI-MS/MS chromatograms of transition *m*/*z* 327−127 of (**A**) Chinese star anise (0.005 mg/kg anisatin), (**B**) star anise with 0.1% Japanese star anise (0.850 mg/kg anisatin), (**C**) anisatin standard solution (510 ng/mL), and (**D**) pure Japanese star anise (1061 mg/kg anisatin).

Table 2. Content of Anisatin in Different *Illicium* Species

Illicium species	mean content of anisatin (mg/kg)	SD[b] (mg/kg)
I. verum Hook. f. (Chinese star anise), $n = 40$[a]	0.094	0.082
I. anisatum L. (Japanese star anise), $n = 2$	1205	300
I. lanceolatum A. C. Sm., $n = 2$	230	76
I. henryi Diels, $n = 15$	129	60
I. micranthum Dunn, $n = 2$	76	9
I. simonsii Maxim., $n = 4$	728	24
I. floridanum Ellis, $n = 1$	9584	

[a] *n* is the number of samples. [b] Standard deviation.

data show a satisfactory intra- and interday precision and high linearity over the tested concentration range. In the case of an adulteration of 0.1% Japanese star anise in Chinese star anise, 850 μg/kg of anisatin was determined. Generally, pure Japanese star anise was found to have an anisatin content of ~1000 mg/kg. On the basis of these data the assay allows the determination of adulterations of Japanese star anise in Chinese star anise of <0.05% (**Figure 3**).

To evaluate the anisatin content of different *Illicium* species, 40 available samples of Chinese star anise and at least one sample of six other species were analyzed (**Table 2**). In all samples of Chinese star anise anisatin was determined in a concentration range from 2 to 312 μg/kg with median and mean values of 94 and 82 μg/kg, respectively. Although most other *Illicium* species contain anisatin in significantly lower amounts than those found in Japanese star anise, their total anisatin concentrations still exceed the levels present in Chinese star anise by at least 1000-fold. Therefore, the presented HPLC/ESI-MS/MS method allows Chinese star anise to be differentiated from other *Illicium* species and adulterations of Chinese star anise with even very low amounts of these toxic *Illicium* species to be detected.

The presented study shows that HPLC-MS/MS, associated with TLC, is a powerful technique to classify the different *Illicium* species and to determine adulterations of toxic *Illicium* species in Chinese star anise using anisatin as a marker substance. With the described HPLC/ESI-MS/MS method anisatin can be quantified to a minimal concentration of 4 μg/kg (LOQ). Taking into account that Chinese star anise contains a certain amount of anisatin itself (up to 0.3 mg/kg), it seems reasonable to recommend a maximum anisatin content of 1 mg/kg for release of the product in quality control, which ensures that the batch of Chinese star anise contains <0.1% of Japanese star anise and <1% of *Illicium lanceolatum*, respectively. The method also allows for an enlargement of the number of detected markers, which will be useful when further discussions about the toxicity of Chinese star anise will focus on other sesquiterpene lactones, such as the naturally occurring veranisatins (*9*).

Therefore, the combination of TLC and HPLC-MS/MS shown here can be regarded as a considerable contribution to an effective and reliable quality control of raw material and products containing Chinese star anise. In contrast to the test procedures of the current *European Pharmacopoeia* method, which focus exclusively on one possible adulterant of Chinese star anise, that is, Japanese star anise, the methodology described here allows toxicologically relevant adulterations with other *Illicium* species containing significant amounts of the toxic principle anisatin to be excluded, which would not be found with the *European Pharmacopoeia* method.

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