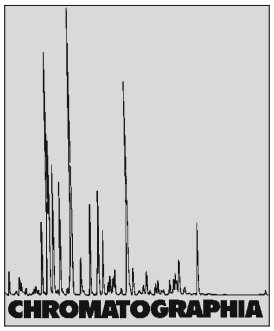
Analysis of (−)-Shikimic Acid in Chinese Star Anise by GC–MS with Selected Ion Monitoring



2009, *69*, 339–344

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Received: 21 June 2008 / Revised: 8 September 2008 / Accepted: 25 September 2008
Online publication: 21 January 2009

Abstract

A sensitive, specific, accurate, and reproducible GC–MS method with selected ion monitoring for quantitative determination of (−)-shikimic acid (SA) in Chinese star anise, using benzoic acid as internal standard, has been developed and validated. The homogeneity of the sample was evaluated by one way analysis of variance. The extraction efficiency of ultrasound-assisted and Soxhlet extraction was compared. Results showed that ultrasonic extraction was as efficient as Soxhlet extraction but was more rapid and simpler. Linearity and recovery were good. The method was used for analysis of (−)-SA in Chinese star anise from different areas in Wenshan state, Yunnan province. It was found that Chinese star anise collected from Pingbian and Funing counties contained more (−)-SA than other samples, with two plants from Pingbian county containing >15% (−)-SA.

Keywords

Gas chromatography-mass spectrometry
Selected ion monitoring
(−)-Shikimic acid
Chinese star anise (*Illicium verum* Hook. f.)

Introduction

Star anise (*Illicium verum* Hook. f.), the seed pod of an evergreen tree grown in southwestern China and in Japan, has a pungent, licorice-like flavor. It is used as a spice in cooking and for treatment of some skin problems in traditional Chinese medicine. This herb has recently been discovered to be a major source of (−)-shikimic acid (SA), a primary raw material used to produce the anti-influenza drug Tamiflu, the only medication effective against the deadly H5N1 strain of avian flu [1–3]. A global shortage of star anise has caused the price of (−)-SA to increase sharply to $40–60 g^{-1}, however [2].

Shikimic acid (3*R*,4*S*,5*R*-(−)-3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) was first isolated from the Japanese flower Shikimi. The compound is a precursor to many aromatic metabolites, for example the aromatic amino acids phenylalanine and tyrosine, indole derivatives, many alkaloids, tannins, flavonoids, and lignin. As an important biochemical intermediate in plants and micro-organisms, shikimic acid is present in most autotrophic organisms, although usually at low concentrations. Star anise has been reported to yield 3 to 7% shikimic acid, which is the highest yield from a plant [4]. The relatively low content of (−)-SA (< 10%) coupled with the challenge of no other agricultural resource has generated great interest in discovery of a star anise resource with a higher content of (−)-SA. Development of a simple and reliable method for analysis of (−)-SA is, therefore, necessary and crucial.

There are many published methods for analysis of carboxylic acids, including (−)-SA, in different matrices. Ion-exclusion chromatography has frequently been used to separate (−)-SA from wine and others matrices [4–6]. High-performance liquid chromatography (LC) on a reversed-phase C_{18} column [7–9], a

Full Short Communication
DOI: 10.1365/s10337-008-0898-6
0009-5893/09/02

sulfonylstyrene–divinylbenzene (S-DVB) column [11], and an NH_2 column [4], and capillary zone electrophoresis (CE) have usually been used [10, 12]. Because of the low UV absorbance of (−)-SA, however, indirect UV detection or derivatization is required for these methods. Other analytical methods, for example gas chromatography (GC) [13] and gas chromatography—mass spectrometry (GC–MS) [14–16] have been proposed, and GC–MS with selective ion monitoring (SIM) [17, 18] has emerged as one of the most efficient methods for quantitative analysis of plant composition. Jesús Ibarz et al. [18] have reported identification of carboxylic acids, including (−)-SA, in grapes by use of GC–SIMMS.

There are few publications on analysis of (−)-SA in Chinese star anise [4]. Here, we report a GC–SIMMS method, with benzoic acid as internal standard, for quantification of (−)-SA in Chinese star anise. Compared with previously published methods, this method is rapid, simple, sensitive, and reliable. In this method the homogeneity of the sample was evaluated by one-way analysis of variance, and two methods of extraction, ultrasonic and Soxhlet, were compared. The method has also been evaluated as a tool for determination of the (−)-SA content of Chinese star anise from different growing areas in Wenshan state, Yunnan province, China, a region suitable for production of large amounts of Chinese star anise.

Experimental

Chemicals

(−)-Shikimic acid (> 99%) and benzoic acid (>99.5%) (Sigma–Aldrich, St Louis, MO, USA), and *N*,*O*-bis (trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (TMCS) (Fluka, Switzerland) were obtained commercially. All solvents used were analytical grade (The Fourth Chemistry Company, Shanghai, China).

Preparation of Standards for GC–MS Analysis

Accurately weighed (−)-SA (approx. 50 mg) was dissolved in methanol in a 25-mL volumetric flask. A set of calibration standards containing 1, 2.5, 5, 10, 25, 100, and 250 mg L^{-1} (−)-SA were prepared by dilution of the stock solution with methanol. A stock solution (100 mg L^{-1}) of internal standard was prepared by dissolving 10 mg benzoic acid in 100 mL methanol.

Sample Preparation

Plant Material

One kilogram of Chinese star anise was harvested in August and September, 2007, from different areas in Wenshan state, Yunnan province, China, (identified by Professor Shaoping Li, Yunnan Academy of Agriculture science). The sample was dried at room temperature for two weeks and then ground to a fine powder using a Waring (Hunan, China) HD100 blender at high speed (20,000 rpm), twice, each for a short time to avoid loss of sample components. The powder, size < 1 mm, was stored frozen at −20 °C.

Ultrasound-Assisted Extraction

Ground Chinese star anise samples (500 mg) were macerated with 15 mL 90:10 methanol–water and sonicated for 5 min. The extract solution was filtered and transferred to a 50-mL volumetric flask. The extraction was repeated two more times and the combined extracts were diluted to volume with methanol.

Soxhlet Extraction

Ground Chinese star anise samples (500 mg) were placed in a Soxhlet apparatus and extracted with 50 mL methanol at 75 °C for 16 h. The extract was transferred to a 50-mL volumetric flask and diluted to volume with methanol.

TMCS Derivatization

Crude extract (2 mL) was placed in a centrifuge tube, 2 mL *n*-hexane was added, and the sample was mixed and centrifuged. The *n*-hexane layer was discarded and the methanol phase was quantitatively transferred to a 10-mL volumetric flask and diluted to volume. This solution (100 µL) was mixed with 100 µL internal standard solution in a vial and the mixed solution was evaporated to dryness at 55 °C under a gentle stream of nitrogen. The residue was then derivatized with 50 µL TMCS at 70 °C for 30 min in the absence of air. The solution was then evaporated to dryness under nitrogen, reconstituted in 1 mL acetone, and this solution was analyzed by GC–MS.

GC–MS Analysis

GC–MS analysis was performed with a GC Trans Ultra/MS-DSQ system (Thermo Electron, San Jose, USA) equipped with a Transplus AS autosampler and split/splitless injector. Data acquisition and processing were performed by Thermo Technologies Xcalibur Software resident in a Dell computer.

Compounds were separated on a 30 m length × 0.25 mm i.d. × 0.25 µm film DB-5 MS capillary column (J&W Scientific, St Louis, MO, USA). The injector temperature was 220 °C, and 1-µL samples were injected in splitless mode. The split ratio was 10:1, the split flow was 50 mL min^{-1}, and the splitless time was 1 min. The oven temperature was maintained at 80 °C for 1 min after injection, then programmed at 10 °C min^{-1} to 150 °C, which was held for 1 min, and then at 6 °C min^{-1} to 250°C, which was held for 3 min. The gas save flow was set at 20 mL min^{-1} and the gas save time was 2 min in constant-flow mode at 1 mL min^{-1} (helium, 99.99%).

The transfer line and ion-source temperatures were 230 and 200 °C, respectively. Spectra were acquired in

electron-impact (EI) mode; the electron energy was 70 eV. In total-ion-current (TIC) mode, spectra were acquired between 45 and 500 amu. Compounds were identified by use of the NIST standard database of mass spectra (2005).

Results and Discussion

Formation of (−)-SA-TMS was confirmed by GC–MS in total-ion-current mode. A typical chromatogram is shown in Fig. 1a. The mass spectrum of the peak at $t = 13.91$ min (Fig. 1b) showed it was (−)-SA-TMS with a molecular ion ($[M]^+$) at m/z 462). The base peak at m/z 204 ($[TMSO–CH = CH–OTMS]^+$) corresponding to the product from retro Diels–Alder cleavage of the cyclohexene ring, and other major peaks at m/z 372 ($[M - TMSOH]^+$) from loss neutral trimethylsilanols, m/z 255 ($[M - TMSOCO - TMSOH]^+$), and m/z 357 ($[M - CH_2 - TMSOH]^+$). The peak in the chromatogram at $t = 5.52$ min was that of the TMS derivative of benzoic acid, the internal standard.

To enhance selectivity for plant analysis it was necessary to perform quantification by selected-ion monitoring (SIM). On the basis of previous experience we knew the spectrum of (−)-SA contained an odd-electron base peak at $m/z = 204$, the molecular ion peak at $m/z = 462$, and an abundant fragment at $m/z = 357$. We selected $m/z = 204$ as the ion for quantification. The spectrum of the internal standard contained the molecular ion at $m/z = 194$, the base peak at $m/z = 179$, and an abundant fragment at $m/z = 105$. We selected $m/z = 179$ for quantification.

Chromatograms obtained from a standard solution and from a Chinese star anise sample are shown in Fig. 2.

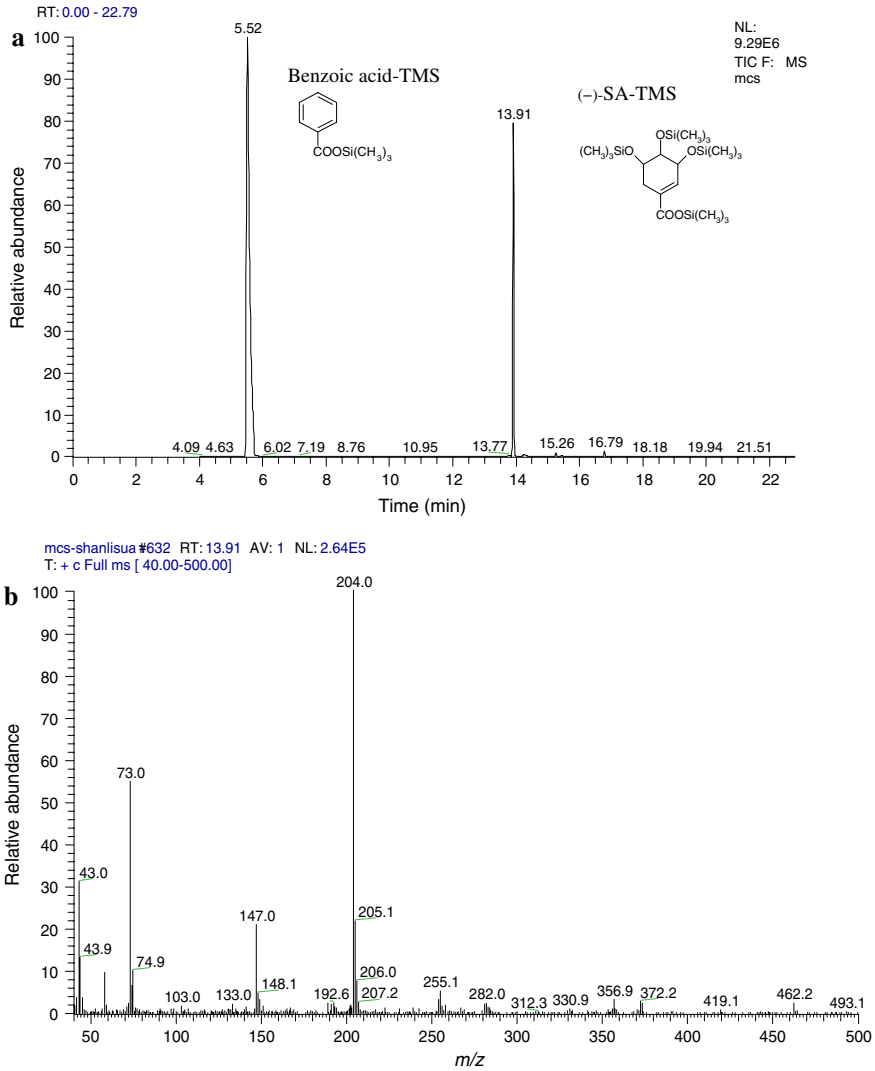

Fig. 1. (**a**) Total-ion-current (TIC) chromatogram obtained from (−)-SA-TMS (250 mg L^{-1} solution) and benzoic acid-TMS (internal standard). (**b**) Mass spectrum of (−)-SA-TMS

Assessment of Sample Homogeneity

The method used for sample preparation can be a source of substantial systematic and random errors, which can be estimated. Production of a very well-mixed sample of the plant material was, therefore, an essential stage of the analysis. To check the homogeneity of the milled sample one-way analysis of variance was used compare the variance of sample processing. The results revealed agreement was good—at a significance level, α, of 0.05 there were no significant differences. Samples were sufficiently homogeneous after being ground twice.

Ultrasound-Assisted Extraction, and Extraction Solvents

Ultrasound-assisted extraction of (−)-SA was optimized. For this purpose, extraction of spiked star anise (0.1 mg fortification level) was performed with different mixtures of methanol, ethanol, acetone, and water, previously used for extraction of carboxylic acids from plants [19, 20]. Samples were sonicated for times from 1 to 30 min. Three consecutive extractions were performed for each set of conditions. These tests showed that extraction of 0.5 g sample for 5 min with 3 × 15 mL 90:10 (v/v) methanol–water enabled quantitative extraction of (−)-SA (mean recovery was very close to 100%). When 90:10 (v/v) ethanol–water was used for extraction more than 30 min was required and when acetone and water were used for extraction recovery was as low as 30%.

Table 1. Amounts of (−)-SA (%) measured in a sample of Chinese star anise after use of different methods of extraction ($n = 5$)

Sample	Soxhlet extraction	Ultrasound-assisted extraction
1	8.73 ± 0.30	8.32 ± 0.34
2	5.78 ± 0.27	5.98 ± 0.23
3	7.65 ± 0.30	7.02 ± 0.25
4	5.58 ± 0.28	4.32 ± 0.21
5	8.11 ± 0.27	7.62 ± 0.31

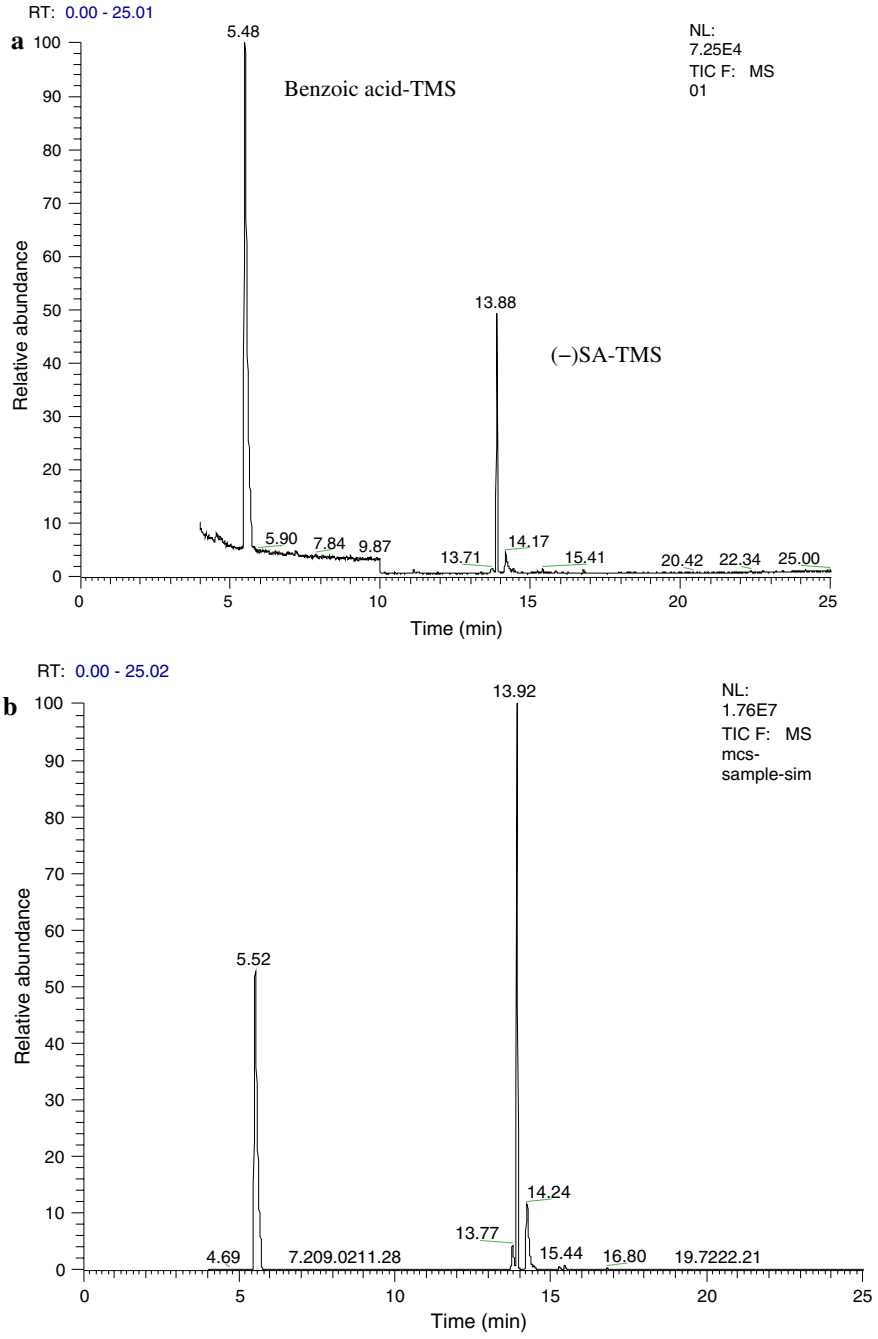

Fig. 2. Chromatograms obtained by selected ion monitoring (SIM): (**a**) standard solution; (**b**) extract of Chinese star anise

In general, addition of small amounts water to either methanol or ethanol significantly improved the efficiency of extraction.

The efficiency of ultrasound-assisted and Soxhlet extraction was compared. Analysis was performed five times. Average values and standard deviations are shown in Table 1. A *t*-test of paired measurements at a significance level of $\alpha = 0.05$ showed there was no significant difference between the methods ($t_{calc} = 2.19$ was lower than the corresponding critical value $t_{crit} = 2.78$ for four degrees of freedom). These results showed that ultrasound-assisted extraction was as efficient as Soxhlet extraction but was simpler and more rapid.

Conditions Used for Derivatization

The derivatization reaction was studied at temperatures from 30 to 80 °C. When the temperature was below 50 °C, little reaction occurred. When the temperature was 70 °C reaction was complete and the chromatographic signal was almost constant. The optimized conditions were reaction with 50 µL TMCS at 70 °C for 30 min.

Precision, Recovery, and Linear Range

Calibration standards (1, 2.5, 5, 10, 25, 100, and 250 mg L^{-1}; 1 µL) were analyzed by GC–MS to furnish a seven-point calibration plot of analyte-to-internal standard peak-area ratio (y) against concentration (x). In this range response was a linear function of concentration. The regression equation of the calibration plot, obtained by linear regression analysis, was $y = -1.95693 \times 10^6 + 964964x$ (correlation coefficient $R^2 = 0.9976$).

Sixteen homogenized samples of Chinese star anise were prepared; five were spiked with 0.1 mg (−)-SA (by addition of standard solution), five were spiked with 0.5 mg (−)-SA, five were spiked with 1 mg (−)-SA, and the other was not spiked. Accuracy was expressed

as recovery, and precision as coefficient of variation (CV). Within the range of the calibration plot, recovery was between 73.1 and 90.5% and CV was between 3.7 and 8.7%.

Inter-day precision and accuracy were assessed by constructing different calibration plots ($n = 7$) at a interval of two weeks. Two sets of quality-control samples ($n = 5$) were analyzed using each of the calibration plots. Recovery was between 82.1 and 110.5% and CV was between 3.5 and 7.4%.

Stability was tested by analysis of derivatized standards (10 mg L^{-1}). The stability of (−)-SA-TMS in a vial was determined by analysis after 1, 2, 6, 8, 12, 24, 72, and 96 h. The precision was 6.7%, indicating (−)-SA-TMS in a vial was stable for at least 4 days.

The LOD, defined as the smallest observable peak response for an analyte above the background noise, was regarded as three times the system noise from the matrix and was found, by trial, to be 0.01 mg L^{-1}.

Analysis of the (−)-SA content of Chinese Star Anise Grown in Wenshan State, Yunnan Province, China

The method was used determine the (−)-SA content of Chinese star anise from different growing areas in Wenshan state, Yunnan province, an area which is highly suitable for production of large quantities of Chinese star anise. We refer to these as populations and genetic sources, rather than varieties, because it was unclear whether they represented stable uniform varieties per selection. This study found significant variation among samples from the different areas, with the (−)-SA content ranging from 2.2 to 8.0% (average per population; $n = 15$). In Chinese star anise from four of the growing areas (Xichou, Ma lipo, Ma guan, and Wenshan) the (−)-SA content was, on average <3.5%. It was found that plants collected from Pingbian and Funing counties contained greater amounts of (−)-SA. This was expected because plants in the most suitable growing areas are selected for high (−)-SA content.

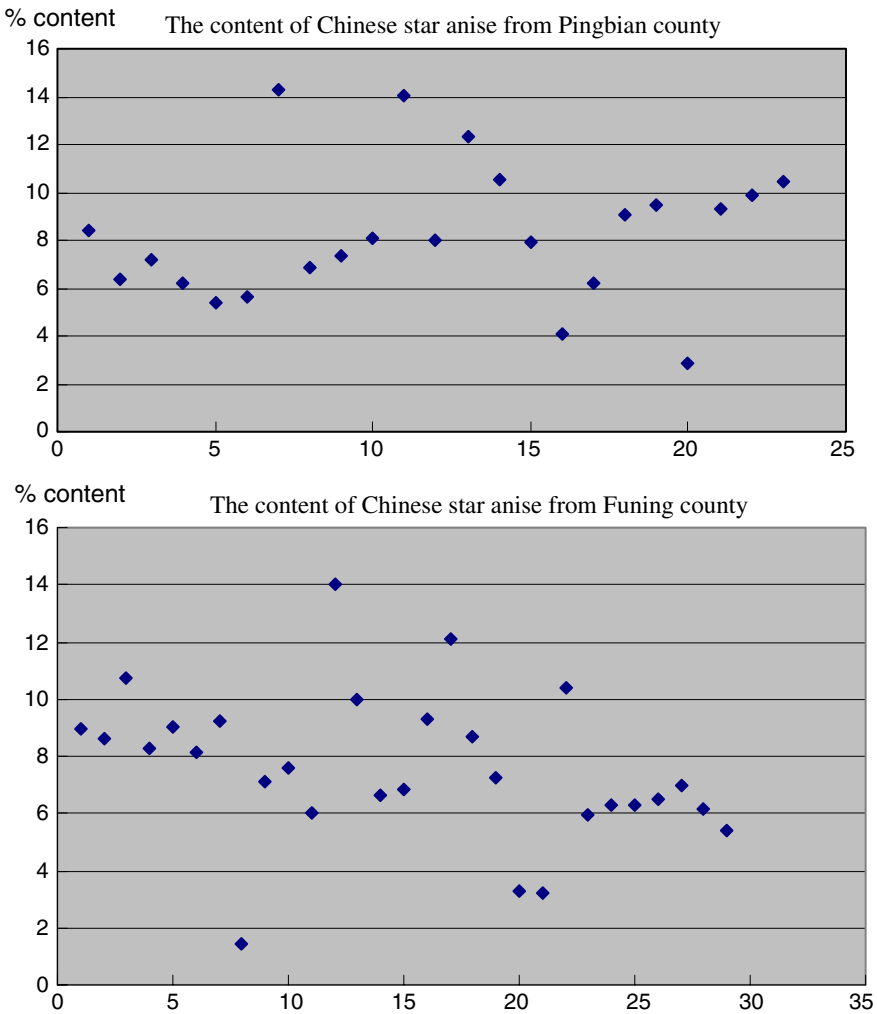

Fig. 3. Distribution of the (−)-SA content of individual samples of Chinese star anise ($n = 50$) from Pingbian county and Funing county, Yunnan province

Figure 3 shows the distribution of the amounts of (−)-SA in 50 individual plants collected from Pingbian and Funing counties. Many individual plants contained more than 10% (−)-SA, with levels reaching as high as 15% in two individual plants from Pingbian county. This research is continuing.

Acknowledgments

We thank Mo Shunyan and Dong Lin-Lin, now working on natural products at the University of Illinois at Chicago, for their discussion and revision of this paper.

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