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Analysis of Artemisinin in *Artemisia annua* L. by LC-MS with Selected Ion Monitoring

MINGFU WANG,* CHUNGHEON PARK, QINGLI WU, AND JAMES E. SIMON

New Use Agriculture and Natural Plant Products Program, Department of Plant Biology and Pathology, Rutgers University, 59 Dudley Road, New Brunswick, New Jersey 08901

A liquid chromatography−mass spectrometry (LC-MS) method with selected ion monitoring (SIM) was developed and validated for the analysis and standardization of artemisinin in *Artemisia annua* L. This method is simple and accurate and requires only an 11 min per sample running time for the direct detection and quantification of artemisinin, a sesquiterpene lactone with antimalarial activity. To accurately analyze artemisinin, SIM was used to record the abundance of the $[M - 18 + H]^+$ ion peak at *m*/*z* 265.3, with a scan range between *m*/*z* 250 and 270. Quantification was based on the LC-MS peak area of artemisinin, and the standard curve was used for calculation. This method was then validated and applied to several populations of *A. annua* to assess the population means and the diversity within a selected population. The distribution of artemisinin was found to vary by plant origin with population means ranging from 0.03 to 0.71% artemisinin dry leaf weight. Individual plants within the selected population that exhibited the highest artemisinin mean were then sampled, and the contents ranged from trace amounts to 1.5% artemisinin dry leaf weight basis, making these single plants accumulating artemisinin in concentrations >1.0% promising candidates for plant breeding and varietal development for high-yielding artemisinin.

KEYWORDS: Artemisinin; *Artemisia annua* L.; LC-MS; selected ion monitoring

INTRODUCTION

Malaria continues to be a major health problem in many areas of the world and was reported by the World Health Organization (WHO) to cause about 300 million illnesses and at least 1 million deaths a year, with 90% of them from tropical Africa and involving children, pregnant mothers, and other high-risk populations (*1*). With the increased resistance of *Plasmodium* strains to commonly used antimalarial drugs such as chloroquine and sulfadoxine/pyrimethamine, the WHO has recommended a major shift from monotherapies to artemisinin-based combination treatments (ACTs) (http://www.who.int/en). As a consequence, there is a huge demand for artemisinin-based products, but a shortage of supply. Artemisinin, a sesquiterpenoid lactone peroxide (structure shown in **Figure 1**), also known as qinghaosu, is a promising antimalarial agent from the Chinese medicinal herb *Artemisia annua* L. This compound and its derivatives such as artemether, dihydroartemisinin, arteether, and artesunate are each effective against both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, as well as cerebral malaria (*2*).

Although there are many groups now working toward artemisinin's total or near complete synthesis, at present, the procurement of artemisinin is entirely from the plant, and the relatively low content of artemisinin coupled with the challenges facing agricultural production and processing of artemisinin has generated great interest in the development of improved genetic lines yielding greater biomass and higher artemisinin. There have been many published methods for the analysis of artemisinin and other related sesquiterpenes in this species including dihydroartemisinin, arteannuin B, artemisinic acid, deoxyartemisinin, and artemisitene, but more rapid yet sensitive methods are still needed. Several analytical methods have been developed to determine artemisinin in *Artemisia* including HPLC, GC, GC-MS, TLC, LC-MS, UV, and immunoquantitative assay (*3*−*19*). Among these methods, the HPLC method was widely used, although HPLC-UV detection of artemisinin is not straightforward because it lacks a suitable chromophore. However, artemisinin can be converted to a reproducible UV-absorbing compound, Q260 in alkaline solution, which is therefore detectable (*16*). HPLC with reductive electrochemical detection

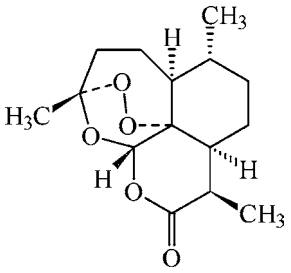

Figure 1. Structure of artemisinin.

* Author to whom correspondence should be addressed [current address: Department of Botany, The University of Hong Kong; tel: 852-22990338; fax: 852-28583477; e-mail: mfwang@hkusua.hku.hk].

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(ED) has been proven to be a sensitive and specific method for artemisinin analysis (*2*, *11*, *13*, *15*), but this method has a drawback in that it requires rigorous deoxygenation of the HPLC system and injection. Recently, an HPLC method with an evaporative light-scattering detector (ELSD) was reported to be one alternative way for analysis of artemisinin, and several methods based on ELSD detection have been published (*9*, *10*). In addition, Maillard and co-workers reported the first LC-MS method for the analysis of artemisinin in *A. annua* L., and this method was ideal for the detection of artemisinin using thermospray mass spectrometry (*18*). LC-MS with selective ion monitoring (SIM) was also proven to be a reliable method for the analysis of artemisinin in 1998 (*19*). Here, we report a modified LC-MS method with SIM that can be used in the quantitative analysis of artemisinin. This method is fast, simple, sensitive, and reliable. We developed this method to more rapidly screen *A. annua*-based extracts, products, and plants to quantitate artemisinin. We also discuss the variation of artemisinin content in different populations of *A. annua* using this modified method.

MATERIALS AND METHODS

Plant Materials. Six distinct seed populations of *A. annua* L. were procured from both the research community including (1) CPQBA Campinas SP, Brazil, (2) Embrapa, Brasilia DF, Brazil, and commercial seed companies including (3) Johnny's Selected Seeds (1700.11 Artemisia Sweet Annie), Albio, MA, (4) Horizon Herbs LLC (Sweet Annie), Williams, OR, (5) Richters (S6983 Chinese "Sweet Wormwood" Sweet Annie), Goodwood, ON, Canada, and (6) Horizon Herbs LLC (Artemisia herba-alba), Williams, OR. These six populations of *A. annua* were greenhouse sown at Rutgers University on June 2, 2004, in a mist-house to improve germination and emergence, and seedlings later were transferred individually into 1.6 gal size (22 cm height and 23 cm top diameter) pots on August 11, 2004. On September 30, a 1.5 m standing pole was added to each pot to keep the plants upright. The greenhouse environment was kept at a growing temperature of 24 °C daytime and 23 °C night with a 14 h photoperiod. Leaves from each plant were manually harvested on November 9, 2004, after 5 months of growth. Plant height varied from 120 to 200 cm, and the average height was 159 ± 26.53 cm, whereas fresh weight per plant ranged from 62 to 255 g and the average was 117.3 ± 54.31 g. Leaves were manually harvested at 50 cm above the ground for each individual plant. Each treatment harvested from 40 to 180 g of fresh weight depending on the parts. Immediately following leaf collection and the placing of all leaves from individual plants into separate paper bags, leaves were dried under uniform conditions by placing all samples in a forced heated-air oven set at 30 °C for 5 days and stored in paper bags for 2 weeks before instrumental analysis. For the purpose of field evaluation to compare with greenhouse condition, three genetic sources of *A. annua* seedlings (CPQBA, Embrapa, and Horizon Herbs) were evaluated. Young plants that had been growing for 50 days were transplanted on July 22, 2004, and then harvested on October 18, 2004 at Walden, NY.

Chemicals and General Procedures. Artemisinin (98%) was purchased from Sigma-Aldrich (Milwaukee, WI). All solvents used in this research were of HPLC grade and purchased from Fisher Scientific (Springfield, NJ). LC-MS analyses were performed on an Agilent 1100 system equipped with an autosampler, a quaternary pump system, a photodiode array and multiple-wavelength detector, a thermostated column compartment, a degasser, and Chemstation software. A prepacked 250 mm × 4.6 mm (5 μM particle size) Prodigy ODS3 column (Phenomenex, Torrance, CA) was selected for HPLC separation. Positive ESI-MS were applied on an Agilent 1100 LC/MSD system equipped with an electrospray ion source, Bruker Daltonics 4.0, and Data Analysis 4.0 software.

Preparation of Standards for HPLC Analysis. About 15 mg of artemisinin was accurately weighed and placed into a 25 mL volumetric flask. Twenty milliliters of methanol was added, and the solution was sonicated for 15 min. The flasks were allowed to cool to room temperature and filled to full volume with methanol. Calibration curve was established on nine data points covering a concentration range from 1 to 128 times dilution.

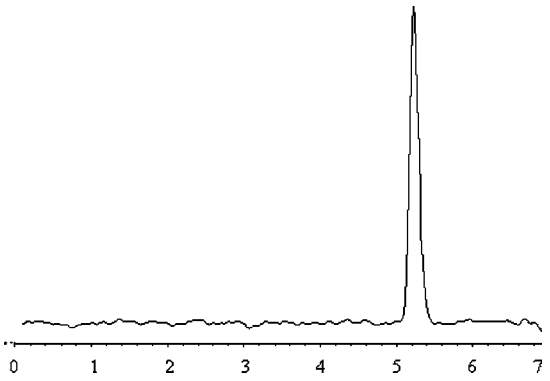

Figure 2. Representative SIM chromatogram of *A. annua* L. extract with ion monitored at *m*/*z* 265.3.

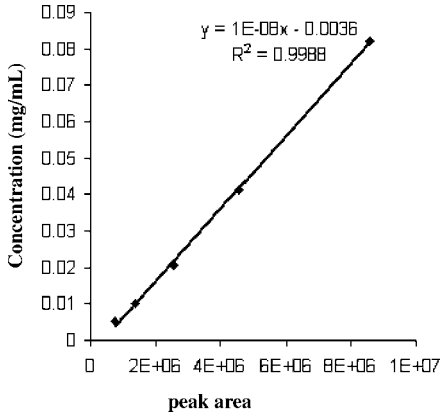

Figure 3. Calibration curve of artemisinin.

Preparation of Samples for HPLC Analysis and LC-MS Analysis. About 200 mg of dried *A. annua* L. powder was separately weighed into 50 mL volumetric flasks, and 35 mL of methanol was added to the flasks. Samples were sonicated for 45 min and then allowed to cool to room temperature and filled to volume with methanol. The extraction solution was transferred to the centrifuge tube and centrifuged at 12 000 rpm for 3 min to obtain a clear solution for HPLC analysis.

LC-MS Analysis of *A. annua* L. The LC-MS was run on the Agilent 1100 system with an ODS3 column. The mobile phase was water (0.1% formic acid) and acetonitrile (mobile phase B) in gradient. The mobile phase started with 72% B, was isocratic at 72% B for 6 min, and was then increased to 100% B in 1 min. The flow rate was set at 1.2 mL/min, injection volume was 2 μL, and the postrunning time was 5 min. The electrospray mass spectrometer (ESI-MS) was operated under positive ion mode and an optimized collision energy level of 80%, scanned from *m*/*z* 100 to 600 to get the total ion chromatogram and MS spectrum of artemisinin for qualitative identification. ESI was conducted using a needle voltage of 3.5 kV. High-purity nitrogen (99.999%) was used as dry gas, the flow rate was 12 mL/min, the capillary temperature was 350 °C, and helium was used as nebulizer at 60 psi. For quantitative analysis of artemisinin, SIM was used to record the abundance of the $[M - H_2O + H]^+$ molecular ion peaks at *m*/*z* 265.3 for arteminisin (the scan range was between *m*/*z* 250 and 270 instead of between 100 and 600). Quantification was based on the LC-MS peak area of artemisinin, and standard curve was used for calculation. A representative chromatogram of SIM at 265.3 is shown in **Figure 2**.

Validation of LC-MS Method with SIM. The precision of this LC-MS method with SIM under positive mode was performed by injecting one standard solution (0.0411 mg/mL) 10 times. The relative standard deviation (RSD) was found to be 4.78%, suggesting that the method was suitable for artemisinin analysis. The calibration curve (**Figure 3**) using this method was then constructed by injecting the

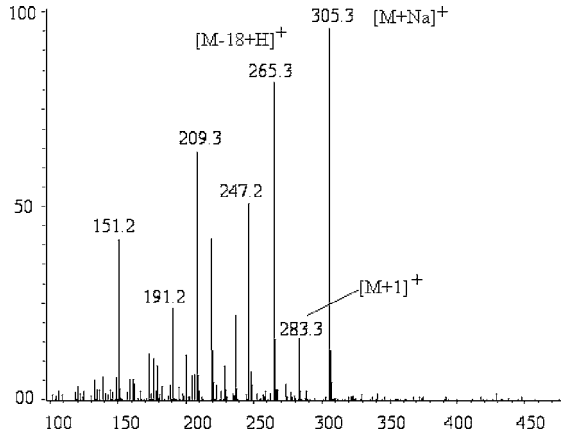

Figure 4. Mass spectrum of artemisinin under positive ion mode.

standard solution across nine different concentrations (0.005141−0.329 mg/mL for artemisinin).

RESULTS AND DISCUSSION

HPLC-MS with SIM has been shown to be a suitable method to analyze targeted phytochemicals in complex plant materials or extracts. We have recently used this technology to analyze proanthocyanidins in grapes and saponins in asparagus, and it has been sensitive, robust, and reliable over a wide concentration range of targeted compounds (*20*, *21*). In this research, we used LC-MS with SIM to analyze artemisinin only, one of the newer phytomedicines of great commercial interest for its antimalarial activity. The use of LC-MS has been proven to be effective in analyzing artemisinin from *A. annua* herbs by Sahai and co-workers, who published the first LC-MS method for the quantitative analysis of artemisinin by SIM of the molecular ion peak $[M + H]^+$, and this method is still the only published LC-MS method for the quantitative analysis of artemisinin in *A. annua* L. (*19*). However, we found that $[M + H]^+$ is not a significant ion peak for monitoring because it is not a dominant ion peak under positive mode (**Figure 4**). Also, the published sample preparation was very complex and time-consuming and therefore not suitable for rapid scanning of many samples in a short time. As a consequence, we developed a LC-MS method using a Phenomonex Prodigy ODS3 column (4.6 × 250 mm, 5 μM), with the mobile phase of acetonitrile and water (0.1% formic acid) in gradient. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. We tried many different conditions to identify a reliable and accurate method of analysis including LC-MS with SIM and LC-MS/MS with selected daughter ion monitoring under positive ion mode and negative ion mode and, finally, SIM was selected. The SIM was performed on ion peak 265.3 $[M - 18 + H]^+$ under positive mode. By monitoring this peak, the sensitivity of LC-MS was dramatically improved (5 times). In addition, the sample preparation described is simple and inexpensive and allows for the direct extraction of *Artemisia* leaf powders by methanol and then the direct injection of the clear methanol solution into the LC-MS for artemisinin analysis. Using this method, we can run five samples per hour, a considerable improvement over our prior methods (*2*, *15*) and those in the literature (*9*−*11*, *13*). The linearity range of detection was found to be 0.005141−0.08225 mg/mL for artemisinin, suggesting that this protocol is a very sensitive method for the quantitative analysis of artemisinin. The validation studies show that the recommended method is reliable and sensitive, allowing for the accurate analysis of artemisinin.

We further applied this method to evaluate the content of artemisinin from six different populations of *A. annua*, each

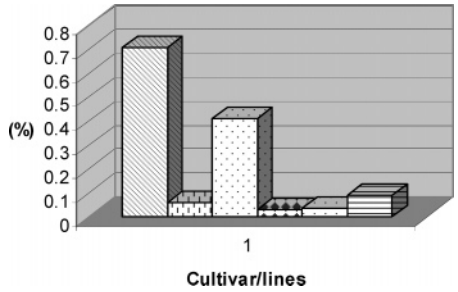

Figure 5. Artemisinin contents of different populations of *A. annua* under controlled greenhouse conditions, Rutgers University, New Brunswick, NJ. Bars represent, from left to right, CPQBA, Johnny's, Embrapa, Horizons, Cheko, and Richters.

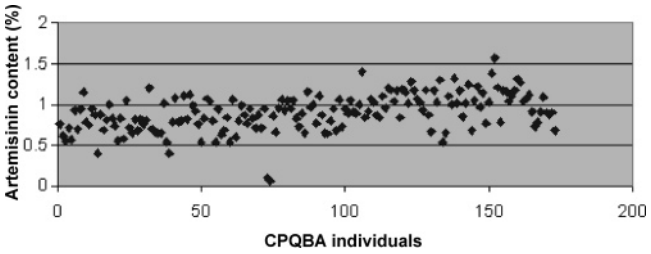

Figure 6. Population distribution of artemisinin in individual *A. annua* plants grown under controlled greenhouse conditions, Rutgers University, New Brunswick, NJ.

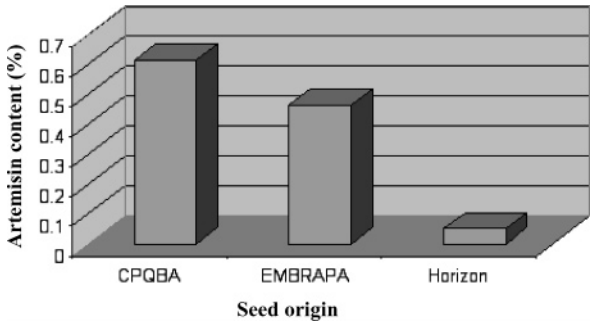

Figure 7. Artemisinin contents from three populations of *A. annua* grown under field conditions to assess the relative performance of each source to each other and to the population's performance in the greenhouse (see **Figure 5**).

grown under controlled conditions at the Rutgers University greenhouse; the greenhouse results were then validated with the field performance of three of the six populations as a secondary check. We refer to these as populations and genetic sources, rather than varieties, as it is unclear whether they represent stable uniform varieties per selection. In the main greenhouse study, we found significant variation among the six populations or genetic sources, with the artemisinin content ranging from 0.03 to 0.71% (average/population). Four of the populations were found to contain on average <0.1% artemisinin (**Figure 5**), and the seeds from all four sources were these that were available commercially on the open market. Such seed sources may lead to acceptable biomass yields but would not be nearly as promising as sources of artemisinin. We found the plants from CPQBA and from Embrapa to have higher artemisinin contents. This was expected as both populations had been selected for a variety of traits including high artemisinin content. We then used this analytical method to ascertain the variation within both populations. We report here the distribution of artemisinin within 173 CPQBA individual plants grown in the Rutgers greenhouse (**Figure 6**). We found many individual plants containing >1.0% artemisinin, with one plant reaching as high as 1.5% artemisinin

on a dry leaf weight basis. In the small field study, we compared the relative artemisinin content of the Horizon seed with the two Brazilian populations as a comparative check and found that indeed the artemisinin content was highest in the CPQBA, followed by Embrapa, and lowest in Horizon seedstock, in agreement with our greenhouse study (**Figure 7**), thus confirming both the method and the genetic evaluations.

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