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GERMPLASM VARIATION IN ARTEMISININ CONTENT OF *ARTEMISIA ANNUA* USING AN ALTERNATIVE METHOD OF ARTEMISININ ANALYSIS FROM CRUDE PLANT EXTRACTS[1]

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ABSTRACT.—Artemisinin content was evaluated in a germplasm collection of *Artemisia annua* using an HPLC-EC detector with a glassy carbon electrode. The highest artemisinin content (41.7%) was found in the leaves from the top 50-cm portion of the plant. The artemisinin content among accessions ranged from 0.003% to 0.21%, and individual plants within accessions ranged in artemisinin content from 0% to 0.39%.

The annual herb *Artemisia annua* L. (Asteraceae), is a potential crop in the United States for the production of the antimalarial drug artemisinin, a sesquiterpenoid lactone peroxide. Artemisinin (qinghaosu), isolated from the Chinese traditional medicinal plant *A. annua* (1,2) is of increasing clinical interest as it is effective against both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, as well as against cerebral malaria (3,4). Artemisinin has also been found to be a potent plant inhibitor with potential as a natural herbicide (5,6). The relatively low content of artemisinin in cultivated European and New World types of *A. annua* has been the limiting factor for the isolation of the substance on a large scale. As the synthesis of the molecule does not appear to be economical (7,8), a rapid, reproducible, and sensitive analytical method to determine artemisinin content in crude plant extracts is critical for plant breeding and production research.

Tlc has been used to estimate the artemisinin content (9), but because of the poor staining characteristics of the intact molecule and interference with contaminating constituents of the plant, this method is not very reliable. Hplc with uv monitoring at 210 nm has been used (10), but the presence of constituents that absorb at 210 nm completely obliterates the peak of artemisinin. Liersch *et al.* (11) were able to monitor the level of artemisinin by utilizing the reproducible rearrangement of artemisinin in alkaline solutions to a product that has a uv maximum at 260 nm and was therefore detectable. However, because other compounds in plant extracts absorb at 260 nm, this method is not satisfactory. Acton *et al.* (12) reported an hplc with electrochemical detection method using EDTA in the mobile phase with a gold/mercury electrode which was very selective for artemisinin. Artemisinin was the only constituent in the crude plant extract to give a peak in the chromatogram. We report in this paper an alternative to the method of Acton *et al.* (12) using a glassy carbon electrode without EDTA in the mobile phase, which also gives good sensitivity for artemisinin, and report both the relative distribution of artemisinin in the plant and the range of genetic

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variation in artemisinin within a germplasm collection of *A. annua*.

MATERIALS AND METHODS

CHEMICALS.—Purified artemisinin was provided by Dr. Klayman (Walter Reed Army Institute of Research, Washington, DC). Reagent-grade NH_4OAc came from J.T. Baker (Phillipsburg, NJ) and MeOH, Me_2CO, and MeCN (hplc grade) from Fisher Scientific (Itasca, IL).

PLANT MATERIAL.—Seeds of *A. annua* were sown in the greenhouse and transplanted into the field at the Purdue University Vegetable Research Farm (Oakley-silt loam soil) during the summer of 1987. Entire plants were harvested 8 weeks and 13 weeks after planting. Immediately after harvest the foliage was divided into 3 parts: upper 50 cm, middle 50 cm and lower 50 cm. The air-dried leaves, stem, seeds, and roots were used for artemisinin extraction. The air-dried seeds were separated from other plant parts with the use of several seed-cleaning wire-mesh sieves. Leaves from the top 50-cm portion of the foliage were sampled from each accession for artemisinin content.

ARTEMISININ EXTRACTION.—Plant material (5–10 g) was placed in a 1-liter round bottom flask with 200–400 ml petroleum ether (30–60°) and refluxed for 48 h at 45°. Alternatively, 5 g of plant material was extracted continuously for 10 h in a Soxhlet apparatus with 200 ml of petroleum ether (30–60°). After filtering, the solvent was evaporated under reduced pressure. The residue was treated with 10–15 ml of MeCN, and the solution was again filtered. Before injection into the hplc, the solution was filtered through a 0.2 m Nylon 66 membrane filter (MF-5621, Bioanalytical Systems [BAS], West Lafayette, IN) in a microcentrifuge.

Hplc APPARATUS WITH REDUCTIVE ELECTROCHEMICAL DETECTOR.—The chromatographic system consisted of a Model 6000 A pump (Waters Associate, Milford, MA), an SP 8750 injector (Spectra Physics, San Jose, CA) with a 10 ml sample loop, and an LC-4B amperometric electronic detector (BAS), Fisher Recordall Series 5000 (Fisher, Lexington, MA). Separation was accomplished on a 200 × 4.6 mm i.d. 5 mm Biophase column (BAS, West Lafayette, IN), preceded by a 30 × 4.6 mm i.d. 5 mm guard column (BAS, West Lafayette, IN).

The mobile phase was aqueous 0.1 M NH_4OAc-MeCN (80:20), and the flow rate was 1.5 ml/min. Samples were reconstituted in MeCN, and 10 ml of each sample was injected after deoxygenation. The electrochemical detector was operated in the reductive mode at an applied potential of −1.0 V using a thin layer glassy carbon electrode (Model MF-1000, BAS) and an Ag/AgCl reference electrode (Model MW-2021, BAS) at a sensitivity of 100 nA f.s.

Reductive mode electrochemical detection requires rigorous deoxygenation procedures. All plumbing connections were of stainless steel to prevent infusion of oxygen. The outlet tube from the column to the detector was replaced with a special steel connector (BAS, P/N MF 1029). Mobile phase was filtered through a 0.2 μ Nylon 66 membrane filter (BAS, MF-5621). The mobile phase was kept in a 2-liter reflux apparatus, heated to 40° for 2 h. After the mobile phase cooled to 30°, argon was bubbled through for 2 h. The mobile phase was then pumped through the system and recycled back into the flask. Deoxygenation of samples was accomplished by purging the sample for 2 min with argon saturated with distilled, deionized H_2O.

All standard stock solutions of artemisinin were prepared in hplc grade MeCN. A standard curve can be generated using 10–50 pg artemisinin, but only a standard curve of 1–10 ng of pure artemisinin was used to calculate the content in the plant samples.

RESULTS AND DISCUSSION

The structure of artemisinin contains a peroxide bridge that undergoes an irreversible electrochemical two-electron reduction (12) that can be monitored by controlled potential coulometry (9). Because EDTA in the mobile phase (12) can react with the gold/mercury electrode surface, we used in this study a glassy carbon electrode which is inert to EDTA. The glassy carbon electrode has a number of advantages. It requires no preparation, it is much easier to use, and it offers better long-term stability than a mercury/gold electrode. Because in our experiments the presence of EDTA was not found to have any beneficial effects, we excluded EDTA from the mobile phase.

In agreement with Acton *et al.* (12), artemisinin is the only constituent that produces a significant peak in the electrochemical chromatogram. In this study artemisinin was found to elute at approximately 13.8 min with a flow rate of 1.5 ml/min. The fraction eluting after 13 min was collected, lyophilized, and shown by isobutane cims (Finnigan 4000) to match the complex cims spec-

trum of pure artemisinin. Furthermore, by enriching the sample with standard pure artemisinin we were able to show that the peak with a retention time of 13.8 min co-eluted with artemisinin. Because of the low signal-to-noise ratio at a high gain, the quantitation at levels lower than 10 pg becomes difficult.

The relative distribution of artemisinin is shown in Table 1. Leaves were found to have 89% of the total artemisinin in the plant. The highest artemisinin content was found to be in the leaves from the top 50 cm portion of the plant (41.7%), almost double of that in the leaves of the other two portions of the plant (Table 1). The side stem and the seeds had a measurable amount of artemisinin. However, artemisinin was absent in the roots and found only in trace amounts in the main stem. These results are in agreement with those of Acton *et al.* (12), who showed that the aerial parts of the plant had the most artemisinin with very little artemisinin in the stems, and the findings of Kelsey and Shafizadeh (13) who have reported that 35% of the mature leaf surface of *Artemisia nova* is covered with capitate glands which contain most of the monoterpenes and virtually all of the sesquiterpene lactones. No difference in the artemisinin content was observed using the petroleum ether reflux or Soxhlet extraction.

Artemisia accessions, place of seed origin, and artemisinin content are listed in Table 2. The artemisinin content among accessions ranged from 0.003% to 0.21%, and individual plants within accessions ranged in artemisinin content from 0.00% to 0.39%. Reported yields of extracted artemisinin from the aerial portions of the plant range from a poor yield of 0.01% to a high yield of 0.5% (w/w) in the People's Republic of China (2). Croom (14) reported in preliminary work a yield variation in artemisinin (0.02–0.8%) from *A. annua*. Singh *et al.* (15) have reported an artemisinin content ranging from 0.012% to 0.094% in three different strains of *A. annua* grown in India.

In summary, we report an alternate method for the quantification of artemisinin from crude plant extracts using hplc electrochemical detection with a glassy carbon electrode. The wide variation in artemisinin content of *A. annua* suggests a genetic basis for variation. This germplasm collection could be a useful source of genetic material for plant selection and breeding based upon artemisinin content.

TABLE 1. Relative Distribution of Artemisinin in *Artemisia annua*.[a]

Plant part sampled	Percent yield (g/100 g)	Total artemisinin/plant (%)
Upper leaves (0–50 cm)	0.15	41.7
Middle leaves (50–100 cm)	0.09	25.0
Lower leaves (100–150 cm)	0.08	22.2
Side shoots	0.04	11.1
Main stem	tr[c]	—
Roots	a[d]	—
Seeds[b]	0.04	—

[a]8 weeks after transplanting prior to flowering.
[b]Seeds collected from 13-week-old plants after transplanting.
[c]tr = trace amounts.
[d]a = absent.

TABLE 2. Accession Number, Artemisinin Content, and Seed Source of *Artemisia annua*.

Accession no.	Artemisinin content % (w/w)			Geographical source
	Population mean[a]	Individual plants		
		Low	High	
001	0.21 a	0.07	0.35	North Dakota
003	0.06 abc	0.05	0.08	Connecticut
004	0.05 bc	0.00	0.09	Washington, D.C.
005	0.11 abc	0.00	0.30	Washington, D.C.
006	0.08 abc	0.03	0.17	Kentucky
011	0.15 ab	0.02	0.23	Purdue[b]
012	0.14 abc	0.03	0.30	People's Republic of China
013	0.17 ab	0.00	0.39	New Hampshire
014	0.09 abc	0.00	0.22	Michigan
015	0.15 ab	0.00	0.35	Purdue[b]
016	0.06 bc	0.00	0.17	Purdue[b]
017	0.05 bc	0.04	0.07	Purdue[b]
018	0.003 c	0.00	0.01	Purdue[b]

[a]Mean = Means with the same letter are not significantly different at $P = 0.05$ using Duncan's multiple range test.

[b]Purdue = All lines originally derived from Accession 012.

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