

A Semi-Micro Method for the Quantitation of Sesquiterpenoid Stress Metabolites in Potato Tuber Tissue

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ABSTRACT

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A semi-micro method for quantitation of sesquiterpenoid stress metabolites in potato tuber tissue is described. The upper surfaces of two or three tuber slices, 3 cm in diameter and 5 mm thick, were inoculated with zoospores of an incompatible race of *Phytophthora infestans*. After incubation, the upper 1 mm of the slices was removed and two or three random samples from this tissue were extracted with methanol without homogenization. The residue remaining after evaporation of the methanol was partitioned in a

test tube between water and ethyl acetate, and the organic layer was dried. The residue was dissolved in methanol and terpenoids were determined by gas liquid chromatography. The method is applicable to samples as small as 0.1 g; yields recoveries of 85-95% of added standards of phytuberol, phytuberin, rishitin, and lubimin; and permits the testing of several treatments on tissue from a single tuber.

Additional key words: phytoalexins, *Phytophthora infestans*, *Solanum tuberosum*.

Since the first report on the identification of rishitin as a fungitoxic stress metabolite in potato tuber tissue (12), many procedures for the identification and quantitation of sesquiterpenoid stress compounds in potato have been described. Earlier methods of analysis involve separation and isolation of partially purified terpenoids by thin layer chromatography (TLC), followed by quantitation by a colorimetric method with concentrated sulfuric acid (2,7,10-12). More recently, gas-liquid chromatography (GLC) has been used (2-6,8,9,13,14). These methods are laborious, require large amounts of potato tissue, and terpenoid recoveries have not always been determined adequately. To overcome these shortcomings, a semimicro method for quantitative determination of terpenoids was devised. An earlier semimicro method yielded only 55-86% recovery of added terpenoids (4). The methods described by Currier (2) and Currier and Kuć (3) also yielded low recoveries. Because terpenoids were not detected in potato tissue remaining after extraction by earlier methods, the loss of terpenoids probably occurred subsequent to the extraction.

MATERIALS AND METHODS

Tissue and inoculation. Tubers of potato cultivar Kennebec (R-1), obtained locally and stored at 4 C for less than 10 mo, were used in all experiments. One day prior to use, tubers were brought to room temperature, rinsed in water, scrubbed with water and detergent, rinsed, and dried on paper towels. Healthy appearing, undamaged tubers were dipped in 70% ethanol shortly before use and dried in a transfer hood. The stolon end was removed and a core of tissue (3 cm diameter) was aseptically removed from the central parenchymatous tissue with a cylindrical stainless steel cutter. The core was extruded from the cutter with a plunger marked at 5-mm intervals and, by using the markings as a guide, slices of uniform thickness (5 mm or as indicated) were obtained. Slices were washed twice in 50 ml of sterile distilled water per 10 slices and randomly distributed in previously autoclaved 15 cm diameter petri dishes lined with Whatman No. 2 filter paper moistened with 4 ml of sterile distilled water. All procedures were performed aseptically.

Three to nine slices all kept in the same petri dish were used per treatment. Slices were aged for 6-8 hr at room temperature prior to inoculation with zoospores of an incompatible race of *Phytophthora infestans*; incubation was in the dark at 19 C for 72 hr.

Zoospore suspensions of *P. infestans* race 4 were prepared as described earlier (4). One-tenth milliliter of this suspension, containing approximately 5×10^4 zoospores, was added to each slice and distributed evenly over its surface with a 1-ml graduated pipette.

Extraction. After incubation, the upper 1 mm was sliced from each disk with a floating-blade peeler. The tissue slices of each treatment were stacked and cut into eight segments. The segments were mixed and weighed into 1 ± 0.05 -g samples unless otherwise indicated. Generally three samples per treatment were assayed. The tissue samples were transferred immediately to glass stoppered 50-ml Erlenmeyer flasks containing 20 ml of methanol. Solvents used in all experiments were analytical grade and redistilled. The flasks then were placed on a reciprocal shaker at room temperature for at least 1 hr. At the end of this period the methanol was decanted into a 30-ml test tube, 10 ml of methanol were added to the flask, and the flask was shaken for an additional 30 min. The methanol in the tube was removed under reduced pressure at 45-50 C in an "Evapo-Mix" (Buchler Instruments, Fort Lee, NJ 07024) equipped with teflon test tube adaptors (standard rubber adaptors released contaminants that interfered with subsequent GLC analysis). When approximately 1 ml remained, the second methanol extract was added to the residue. The remaining tissue was extracted a third time with 10 ml of methanol, and the extract was pooled with the two earlier extracts and concentrated as described previously. Five milliliters of distilled water and 5 ml of ethyl acetate were added to the yellow cloudy residue in the test tube, the phases were mixed thoroughly on a Vortex mixer (avoiding emulsification), and the layers were left to separate. The upper organic layer was siphoned off and transferred to another tube. The water layer was extracted two more times with 5 ml of ethyl acetate, and the ethyl acetate extracts were combined and evaporated to dryness as indicated above. Care was taken to avoid prolonged drying which could cause appreciable loss of terpenoids. Residues were dried further by storing the tubes for at least 4 hr in a desiccator contain-

ing granular phosphorus pentoxide. Sixty to 75 μg of methylarachidate per gram of tissue extracted was added to the residues, and the mixture was taken up in 0.3 ml of methanol. The extracts were transferred to vials and stored at 4 C until analyzed by TLC (4) and by GLC. Each sample was analyzed at least three times.

Quantitation. GLC was performed on a Varian Series 1400 instrument equipped with a flame ionization detector and operated at a range of 10^{-11} A/amV. Oven, injector, and detector temperatures were maintained at 180, 235, and 255 C, respectively. The 2 mm (ID) \times 180 cm Pyrex glass column was packed with 3% OV225 on 100–120 Supelcoport (Supelco Corporation, Bellefonte, PA 16823). Air and hydrogen flow rates for the detector were optimized at 375 and 30 ml/min, respectively. Nitrogen carrier gas flow was regulated at 30 ml/min, which was based on the optimal separation of rishitin and lubimin from the internal standard (Fig. 1). The detection limit for the various terpenoids was approximately 1 ng, which corresponded to terpenoid concentrations of 0.3 $\mu\text{g/g}$ (fresh weight) of tissue.

Under the conditions described for GLC, the retention time for methylarachidate was 14.7 min and the relative retention times for phytuberol, phytuberin, solavetivone (katahdinone), rishitin, and lubimin were 0.32, 0.36, 0.57, 0.78, and 1.34 min, respectively. Terpenoids were quantitated by measuring peak heights relative to methylarachidate. Since response factors of the different terpenoids relative to methylarachidate were variable, depending on instrument and column age, these were recalculated continuously. A standard mixture, containing known amounts of the terpenoids, was assayed before and after assays of five to seven unknown samples. The mean response factors of the terpenoids to the internal standard were calculated for the two standard mixtures and used for subsequent quantitation of the five to seven unknown samples. All calculations and a statistical analysis of the results were performed with a computer program based upon SAS-76 (1).

Terpenoid standards, except solavetivone, were prepared from whole tuber slices of Kennebec and Russet Burbank potatoes treated with Ethrel, inoculated with *Helminthosporium carbonum*, and incubated at 19 C for 86 hr (4). Methods employed were those

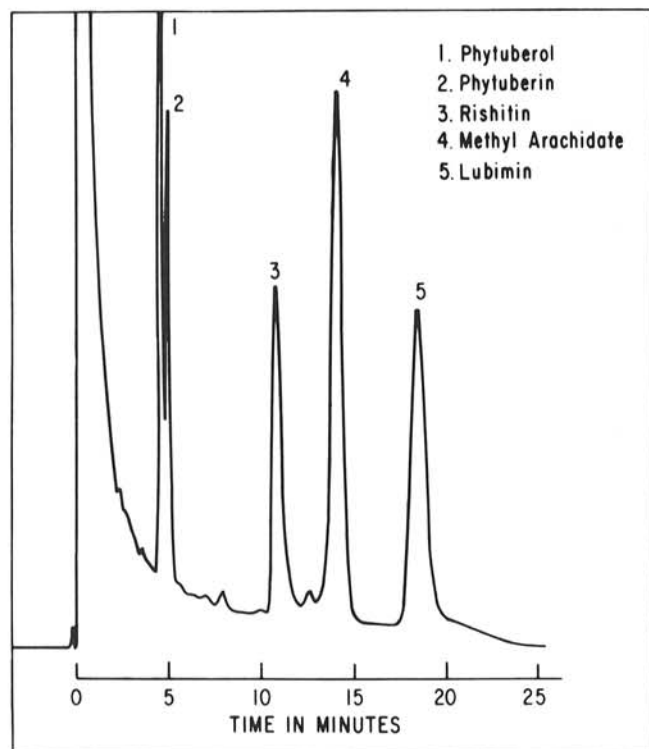


Fig. 1. Gas chromatogram of a mixture of four terpenoid stress metabolites from potato tuber tissue. Conditions for chromatography are given in Materials and Methods.

of Currier (2). Purity of individual terpenoid standards was determined by TLC, with several systems and reagents for detection, and by GLC. Identity of each terpenoid was confirmed by combined GLC/mass spectrometry with a Finnigan Automated gas chromatograph/mass spectrometer system, Model 3300-6100. Solavetivone was kindly provided by E. B. Kalan, U.S. Department of Agriculture, Eastern Regional Research Center, Philadelphia, PA 19118.

RESULTS

Fractionation between water and selected organic solvents. Pure standards dissolved in methanol were pipetted into test tubes each containing 1 ml of distilled water. The contents of the tubes were mixed with a Vortex mixer, 5 ml of water was added to each tube followed by 5 ml of an organic solvent or mixture of solvents. The contents of the tubes were mixed with a Vortex mixer, and the organic phase was removed, dried, and analyzed as described. Extractions were performed once. Each solvent was tested on three samples in two experiments.

Of the solvents tested, hexanes (the solvent used in an earlier semimicro assay [4]) was a poor choice for extraction of the potato tuber terpenoids (except for phytuberin), whereas chloroform or ethyl acetate each removed 79% or more of all terpenoids in one extraction (Table 1). Tissue debris settled in the lower phase, thus there was no need to filter extracts when ethyl acetate was used as the organic phase.

Recovery of terpenoids from potato tuber tissue. Uninoculated potato tuber slices were aged for 72 hr at 19 C. The upper millimeter of each disk was harvested, a mixture of the four terpenoids was added to 1-g samples of tuber tissue, and the slices were extracted as described in Materials and Methods. For the controls, the terpenoid standards were added to methanol containing methylarachidate and the samples were analyzed by GLC. The average recovery for each of the four terpenoids in two experiments was approximately 90% (Table 2). In another experiment, not reported in Table 2, recovery was much lower due to excessive drying of the ethyl acetate extract under reduced pressure. The loss of phytuberin under such conditions confirms earlier observations by Currier (2).

Effect of tuber slice thickness on accumulation of terpenoids. Total accumulated terpenoids varied widely from experiment to experiment but not the relative amount accumulated as a function of slice thickness; results of a representative experiment are shown (Table 3). A disk thickness of approximately 5 mm appears satisfactory for determination of terpenoid accumulation.

TABLE 1. Distribution of the terpenoids rishitin, lubimin, phytuberol, and phytuberin between water and organic solvents

Organic solvent ^a	Recovery of terpenoids from the organic solvent ^b			
	Rishitin (%)	Lubimin (%)	Phytuberol (%)	Phytuberin (%)
Ethyl acetate	91 A ^c	90 A	81 A	79 B
Chloroform	89 A	99 A	85 A	83 B
Hexane/ethyl acetate (1/1, v:v)	82 B	84 B
Cyclohexane	71 C	98 A
Ethyl ether	61 D	79 B
Hexanes	62 D	77 B	56 B	95 A

^aFive milliliters of distilled water and 5 ml of the organic solvent were added to a mixture of terpenoids suspended in 1 ml of water in a test tube. After mixing the phases, the organic layer was removed, concentrated and terpenoids were quantitated.

^bResults given are the average of three replicates in two experiments. Actual quantities added in each experiment were 8 and 11 μg of phytuberol and phytuberin, respectively, and 48 and 28 μg of rishitin and lubimin in the first and 13 and 19 μg of these compounds in the second experiment.

^cRecovery percentages followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

^dThree dots indicate that recovery was not determined.

Effect of sample size on the quantitation of terpenoids. Potato tuber slices inoculated with zoospores of *P. infestans* were harvested and the tissue was distributed randomly in 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 g samples and extracted as described. Results obtained in three experiments with three replicates of each sample size in each experiment showed no significant sample-size differences in terpenoid accumulation. Moreover, there were no significant differences when tissue in a sample was derived from three whole slices rather than as a random sample from the total tissue. The coefficient of variation for each sample size also was not correlated with the sample size, suggesting that decreasing sample size to 0.1 g did not enhance the variation.

Influence of the duration of the methanol extraction on recovery of terpenoids. Tuber disks were inoculated with race 4 of *P. infestans*. The upper 1 mm slices of the disks were harvested 72 hr after inoculation, and the tissue was divided into 1-g samples. Samples were extracted with methanol for various periods (5 min to 24 hr). The terpenoids were determined in this first extract and in a second extract of the same sample. The duration of the second extraction was the time between the first extraction and 24 hr. It appeared that a first extraction of 30–60 min was almost as effective as a 24-hr extraction. A second short extraction of 30–60 min with methanol removed terpenoids remaining in the samples. Terpenoids seldom were detected in a third extract.

Influence of the position of tissue in the tuber on terpenoid accumulation. Four tubers were washed, surface-sterilized, and a 3-cm diameter core was obtained from the tuber through the stolon end. Two 5 mm thick slices were obtained from the stolon end, the center and the distal end of the tuber. These slices were rinsed individually in 20 ml of sterile water. Each pair of slices was placed in a large petri dish and inoculated, incubated, and extracted as

described. Variation among samples in some tubers was quite large, but an analysis of variance showed that these variations could not be attributed to differences in the position of tissue in the tuber or to differences between tubers.

DISCUSSION

We conclude that the semimicro method is dependable and useful for the quantitation of terpenoids. Previous methods used in our laboratory required 20–40 g of tissue obtained from the upper 1 mm of 1-cm-thick tuber slices. In addition to the large amount of tissue, the older methods required large quantities of solvents and were time-consuming. The use of small, uniform tuber slices facilitates the uniform application of inoculum or “elicitor” preparations (5) and permits the testing of many treatments on tissue from a single potato tuber. The small number of slices used per treatment requires small amounts of inoculum or “elicitor”, a serious limiting factor in previous work (2,5). Two to three slices of 3 cm diameter and 5 mm thickness provide adequate amounts of tissue for the reliable elicitation, extraction, and quantitation of terpenoids. Extraction of tissue without homogenization in methanol, followed by partition of the terpenoids into ethyl acetate, yields recoveries of 85–95% for rishitin, lubimin, phytuberin, and phytuberol. Extraction without homogenization reduces both the number of minor unidentified peaks which interfere with separation by GLC, and the deposition of residue in the injection port. It also substantially lengthens the time a column can be kept in use. The GLC procedure does not require prior separation of the sample by column (9) or thin layer chromatography (2,3,6,8,13,14) and does not require temperature programming (9). Spectrophotometric methods described in other reports (7,10–12) are only dependable for rather purified samples (TLC is required), are very susceptible to interference by minor contaminants and light, and have rather low limits of detection.

LITERATURE CITED

TABLE 2. Recovery of added terpenoids from potato tuber tissue extracted with the micromethod and analyzed by gas liquid chromatography^a

Terpenoid	Amount of terpenoid added ($\mu\text{g/g}$ tissue)		Recovery ^b (%)	
	Exp. I	Exp. II ^c	Exp. I	Exp. II
Phytuberol	60	60	89	92
Phytuberin	83	72	89	95
Rishitin	217	244	92	85*
Lubimin ^c	...	369	...	90

^aPure standards of the various terpenoids were added to 1-g samples of aged (6–8 hr) tuber slices. The tissue was extracted immediately as described in Materials and Methods.

^bValues given are the average of three samples per experiment. Except for the value followed by an asterisk ($P = 0.05$) the values were not significantly different from quantities determined in controls containing the terpenoid standards in methanol. The terpenoid in controls was added directly to vials, dissolved in 0.3 ml methanol, and quantitated. Statistical analyses were based on Duncan's multiple range test.

^cRecovery of lubimin was determined only once.

TABLE 3. Effect of potato tuber slice thickness on the elicitation of terpenoids by an incompatible race of *Phytophthora infestans*^a

Slice thickness (mm)	Terpenoids elicited in tuber tissue slices ^b	
	Rishitin ($\mu\text{g/g}$ fr wt)	Lubimin ($\mu\text{g/g}$ fr wt)
2.5	78 AB	20 C
5.0	90 A	30 B
7.5	62 B	35 A
10.0	40 C	18 C

^aSlices of various thickness were obtained from the same cores of tuber tissue (see Materials and Methods) by cutting off successive slices 2.5, 5.0, 7.5, and 10.0 mm thick. Slices of different thickness were kept and washed separately.

^bMeans of three replicate samples within one experiment. Data followed by the same letter are not significantly different, according to Duncan's multiple range test ($P = 0.05$).

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