Note

High-performance liquid chromatographic determination of stress-induced sesquiterpenes of the potato (*Solanum tuberosum*)

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Under the stress of bacterial and fungal infection, potato tuber tissue produces a number of sesquiterpenes that are not native to uninfected tissue[1-8]. These compounds are of interest because of the possibility that such compounds are involved in the resistance of the tuber and the potato plant to infection. Qualitative and quantitative analytical methods are needed for determining the conditions under which sesquiterpenes are formed in the potato tuber. Gas–liquid chromatography (GLC) has been used to separate and quantitate some of these compounds[9-11].

The purpose of this investigation was to establish high-performance liquid chromatography (HPLC) parameters suitable for the separation and quantitation of the four main sesquiterpenes produced in infected potato tissue.

EXPERIMENTAL*

Phytuberin[12-14], katahdinone[15,16], rishitin[17], and lubimin[18-20] (Fig. 1) have been reported as the predominant sesquiterpenes produced in infected potato tissue.

PHYTUBERIN
(MW = 294)

KATAHDINONE
(MW = 218)

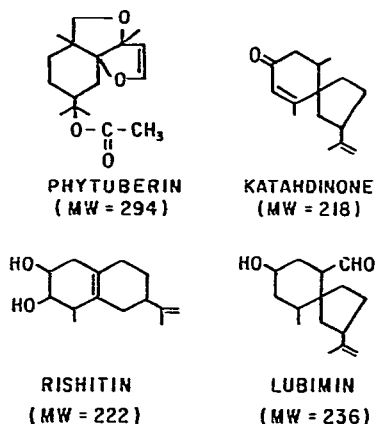RISHITIN
(MW = 222)

LUBIMIN
(MW = 236)

Fig. 1. Predominant sesquiterpenes produced in infected potato tubers. MW = Molecular weight.

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

For the purposes of this study these compounds were isolated from potato tubers infected with *Phytophthora infestans* and were purified by combinations of vacuum steam distillation and column and thin-layer chromatographic (TLC) techniques[6]. The identities of these four compounds were confirmed by mass spectrometry at this laboratory.

Standard solutions containing 0.1 $\mu g/\mu l$ of lubimin, rishitin, phytuberin, and katahdinone in methanol were prepared and used to evaluate HPLC separation parameters. The HPLC system was assembled from Waters Assoc. (Milford, MA, U.S.A.) components (solvent delivery system, Model 6000 A; injection system, Model U6K; and variable-wavelength UV detector, Model 450) and a column that was 30 cm × 3.9 mm I.D. and packed with 10-μm, reversed-phase, μBondapak C_{18} (monomolecular layer of organosilane bonded to porous silica particles). The system was operated isocratically with a binary solvent mixture consisting of methanol–water. The proportions of organic solvent and water were varied over a range of 9:1 to 6:4, in conjunction with a varying flow-rate of 0.4 to 2.0 ml/min, respectively, to determine optimum conditions for separation of the four sesquiterpenes. A wavelength scan was run with the Model 450 variable-wavlength UV detector, and a wavelength of 200 nm was found to be satisfactory for all four compounds. Katahdinone also can be detected at 255 nm.

RESULTS AND DISCUSSION

With a methanol–water (7:3) solvent system, at a flow-rate of 1.0 ml/min and a wavelength of 200 nm, good separation of authentic lubimin, rishitin, phytuberin, and katahdinone was obtained, the resolutions being 1.1, 1.5, and 3.4 for lubimin–rishitin, rishitin–phytuberin and phytuberin–katahdinone, respectively (Fig. 2). Other conditions were investigated, namely, a μBondapak phenyl column (30 cm × 3.9 mm I.D.) and an acetonitrile–water solvent system. In no case was the resolution as good as that obtained with the methanol–water system and the μBondapak C_{18} column as described above. The early peaks in the chromatogram do not represent impurities in the standards; they were present in the control (solvent only) injection. As expected for the four compounds, the capacity ratio (k') (based on unretained solvent peak) for each compound increased as the amount of water, the more polar solvent in the binary system, was increased (Fig. 3). In addition to tests on extracts spiked with authentic compounds, the identities of lubimin, rishitin, phytuberin, and katahdinone separated from extracts were substantiated further on the HPLC eluate collected in fractions corresponding to the retention volumes of the authentic compounds, then concentrated and spotted on TLC plates; TLC R_F values agreed with those of authentic compounds, and no extraneous compounds were found. Standard curves were developed with known amounts of authentic lubimin, rishitin, phytuberin, and katahdinone (Fig. 4); recorder response was linear throughout the range of weights used to establish the curves with the stated operating conditions. Calculated as peak height (mm) divided by weight of compound (μg) response factors per μg of compound for lubimin, rishitin, phytuberin, and katahdinone were 364, 1674, 698, and 926, respectively.

The precision of the HPLC method was determined with a crude extract of potato tissue that had been infected with *P. infestans* and incubated for 72 h at 20°C
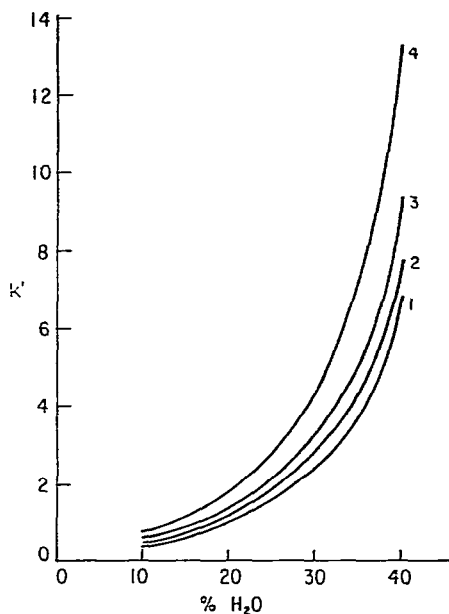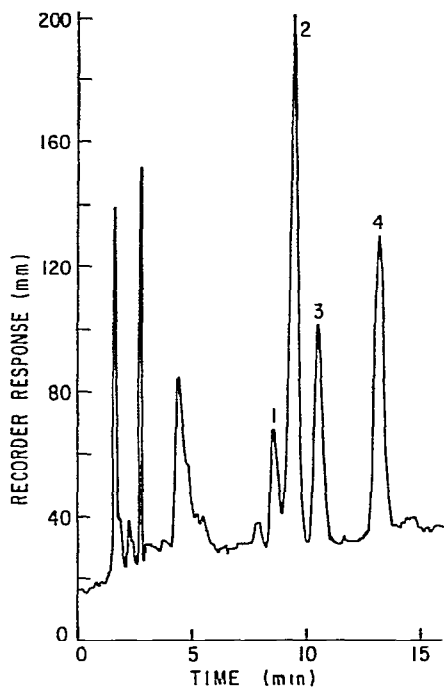
Fig. 2. High-performance liquid chromatogram of authentic sesquiterpenes: lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4) (0.2 μg of each compound). Methanol–water (70:30), 1.0 ml/min, wavelength 200 nm, 0.02 a.u.f.s., reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D.).

Fig. 3. k' values obtained for lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4). Methanol–water system, reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D.) $k' = (t_R - t_{R_0})/t_{R_0}$, where t_{R_0} is the retention time of the solvent front, and t_{R_1} is the retention time of the compound.
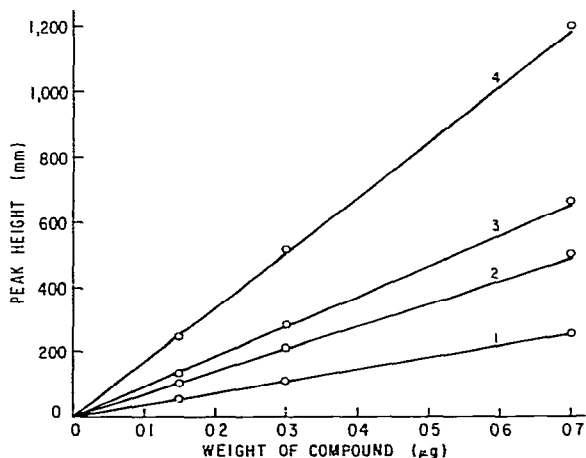

Fig. 4. Standard curves, lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4). HPLC, methanol–water (70:30), 1.0 ml/min, reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D.).
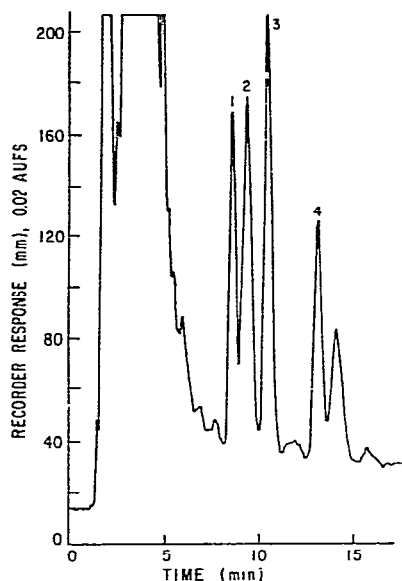
Fig. 5. High-performance liquid chromatogram of sesquiterpenes in a crude extract of potato tissue infected with *Phytophthora infestans:* lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4); 3.0-μl introduction of diluted (30 ×) crude extract. Column and operating conditions as in Fig. 2.

in an ethylene–oxygen atmosphere[7]. The initial extract was too concentrated and had to be diluted 30-fold. A 3.0-μl injection of the diluted extract was used for the precision study. An example of the chromatogram obtained is presented in Fig. 5. A series of 11 determinations for lubimin, rishitin, phytuberin, and katahdinone were made; the standard deviation of the values obtained for the concentration of each compound was calculated. The concentration values shown were calculated back to the original (undiluted) crude extract (Table I).

TABLE I

STANDARD DEVIATIONS OBTAINED FROM REPLICATE (n = 11) HPLC ANALYSES OF A CRUDE EXTRACT OF POTATO TISSUE INFECTED WITH *PHYTOPHTHORA INFESTANS*

Compound	Average concentration found (μg/μl)*	S.D.	Rel. S.D. (%)
Lubimin	7.48	0.321	4.29
Rishitin	1.40	0.218	15.57
Phytuberin	4.50	0.308	6.84
Katahdione	2.07	0.056	2.69

* Orig. (undiluted) crude extract.

The percent recoveries of the four compounds were determined by spiking the crude extract with known amounts of each compound (0.15 μg). Five determinations were made with the spiked and unspiked extracts. The average recoveries for lubimin,

rishitin, phytuberin, and katahdinone were, 99.8, 92.0, 116.2, and 98.8%, respectively.

As described herein, a reversed-phase, μBondapak C_{18} HPLC column eluted with methanol–water (70:30), at a flow-rate of 1.0 ml/min, can be used routinely to separate and determine the quantities of lubimin, rishitin, phytoberin, and katahdinone in crude extracts of potato tissue.

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