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Resolution of *Penicillium roqueforti* Toxin and Eremofortins A, B, and C by High-Performance Liquid Chromatography

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A method for the quantitative analysis of *Penicillium roqueforti* toxin using a high-performance liquid chromatography system is proposed.

Penicillium roqueforti toxin (PRT) is a mycotoxin isolated from cultures of *P. roqueforti* (6), a fungal species used in the ripening of French Roquefort cheeses and also commonly found in fermenting silage (5). The toxin is lethal for rats and mice with a 50% lethal dose of about 7 mg/kg for male rats and mice after intraperitoneal injection (7). PRT also induces biochemical alterations in rat liver, including the inhibition of protein and RNA syntheses (3, 7). The aldehyde group of the molecule has been shown to be directly involved in the biochemical activity of this compound (4).

This report describes a method for the quantitative determination of PRT and other structurally related *P. roqueforti* metabolites by high-performance liquid chromatography (HPLC) (1, 2).

MATERIALS AND METHODS

Standards. Crystalline eremofortin A (EA), eremofortin B (EB), eremofortin C (EC), and PRT were prepared as described previously (1, 2). A concentrated standard solution of a mixture of EA, EB, and PRT was prepared containing 0.5 mg of each compound per ml in chloroform. A standard solution of EC was made by dissolving EC in chloroform at 0.5 mg/ml.

Solvents. Two solvent systems were used: solvent A contained *n*-hexane and tetrahydrofuran at 75/25, vol/vol (Solvant, Documentation Synthese, Valdonne, France); solvent B contained only chloroform (Merck). All solvents were of analytical grade.

HPLC. HPLC separations were carried out with a Waters Associates ALC 204 instrument equipped with an M-6000 A pump, a U6 K injector, a model 440 absorbance detector (0.005 to 2 absorbance units full scale [AUFS]). Chromatograms were recorded on an omniscribe 10-inch (25.4-cm) recorder (Houston Instruments). A micro Porasil 10-μm silica gel column (4 mm inner diameter by 30 cm long) was used. EA, EB, and PRT were analyzed with solvent A at a flow rate of 1.5 ml/min, and EC was analyzed with solvent B at a flow rate of 2 ml/min. Detection of these compounds after chromatographic separation was made at a wavelength of 254 nm. Sensitivities of 0.005 to 0.5 AUFS were used depending on the amount of compounds injected.

Peak heights (in millimeters) were corrected by the expression H = peak height × AUFS × 10 and drawn as a function of the amount of the compounds injected in nanograms.

RESULTS AND DISCUSSION

The validity of the method depends on the complete separation of the compounds produced by various *P. roqueforti* strains: EA, EB, EC, and PRT. Since these four compounds have ultraviolet absorption maxima in the region of 250 nm with molar extinction coefficients of about 15,000, a 254-nm filter was chosen for the detection of these compounds after chromatographic separation.

A small-particle (10-μm) porous silica gel column was used for chromatographic separation. Among numerous elution solvents tried, optimum resolution of the four compounds was obtained with *n*-hexane-tetrahydrofuran (75/25, vol/vol) at a flow rate of 1.5 ml/min. A typical chromatogram of the four substances is shown in Fig. 1. Quantitative analysis by peak height

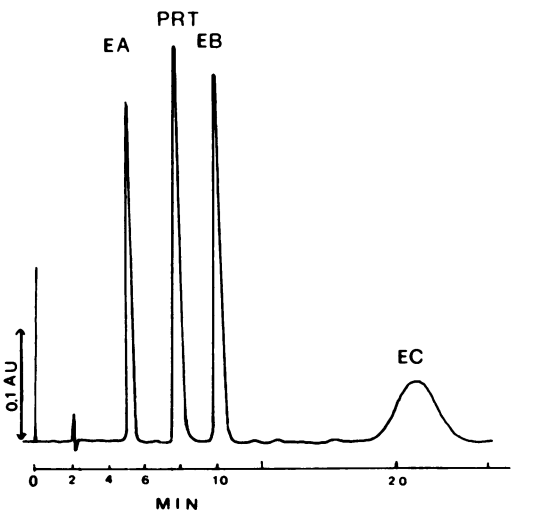

FIG. 1. *HPLC resolution of EA, PRT, EB, and EC. Retention time in minutes (MIN) and peak heights in absorbance units (AU) are shown. Elution solvent A: n-hexane-tetrahydrofuran, (75/25 vol/vol). Flow rate, 1.5 ml/min.*

TABLE 1. *Reproducibility of retention time*

Compound	Retention time (s)		Mean retention (min)	Standard deviation (s)	Variation coefficient (%)
	Range	Mean			
EA[a]	291–295	294	4.9	1.1	0.37
PRT[a]	443–449	447	7.4	1.5	0.33
EB[a]	605–625	617	10.3	3.19	0.5
EC[b]	395–414	406	6.7	5.2	1.3

[a] Based on 26 injections of mixed standard (EA, PRT, EB) at 10 to 5,000 ng.
[b] Based on 30 injections of EC standard at 50 to 5,000 ng.

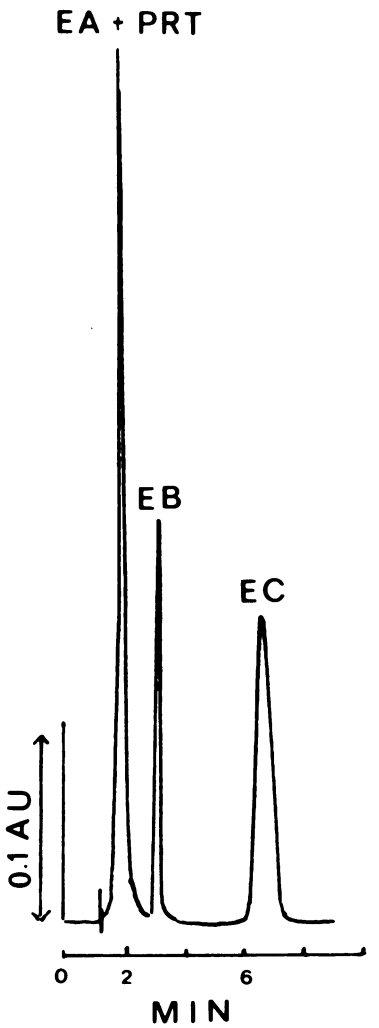

FIG. 2. *HPLC resolution of EA, EB, EC, and PRT. Retention time in minutes (MIN) and peak heights in absorbance units (AU) are shown. Elution solvent B: chloroform. Flow rate, 2 ml/min.*

measurements was possible for only the first three compounds with this solvent. EC was eluted with chloroform at a flow rate of 2 ml/min to obtain a sharp peak (Fig. 2) and to allow quantitative analysis.

Retention times for EA, EB, and PRT, which were analyzed together, were highly reproducible with the HPLC column and solvent used. A total of 26 injections of standards over a 2-day period gave mean retention times (in minutes) of 4.9 (EA), 7.4 (PRT), and 10.3 (EB) with coefficients of variation ranging from 0.3 to 0.5% (Table 1). EC analyzed with the second solvent gave a mean retention time of 6.7 min for 30 injections over a 2-day period. The coefficient of variation was 1.3% with this solvent.

The precision of the method was evaluated by injecting 500 ng of the mixed standard (EA, PRT, and EB) and EC 10 times over a 1-day period. Reproducibility of the peak height measurements was good, with coefficients of variation from 1.6 to 2.8% representing the combined errors of HPLC injection, resolution, and detection (Table 2). The coefficient of variation for EC was 0.8%.

The sensitivity of the method was evaluated by injecting 50 ng of the mixed standard (EA, PRT, and EB) and 50 ng of the EC standard at a maximum sensitivity of 0.005 AUFS. Peak heights (in millimeters) divided by the quantities (in nanograms) of compounds gave 4.7 (EA), 4.1 (PRT), 1.4 (EB), and 0.95 (EC). A 10-ng amount of EA and PRT could easily be measured, but 20 ng of EB and EC were necessary for precise determination. Figures 3 and 4 show the chromatograms obtained with 10 and 500 ng of compounds EA, PRT, and EB, and with 50 and 500 ng of EC.

TABLE 2. *Reproducibility of peak height*

Compound	Peak height (mm)		Standard deviation (mm)	Variation coefficient (%)
	Range	Mean		
EA[a]	222–243	235	6	2.5
PRT[a]	199–210	206	3.4	1.6
EB[a]	67–74	71	1.9	2.8
EC[b]	101–104	102	0.8	0.8

[a] Based on 10 injections of 500 ng of mixed standard (EA, PRT, EB) (0.05 AUFS).
[b] Based on 14 injections of 500 ng of EC standard (0.02 AUFS).

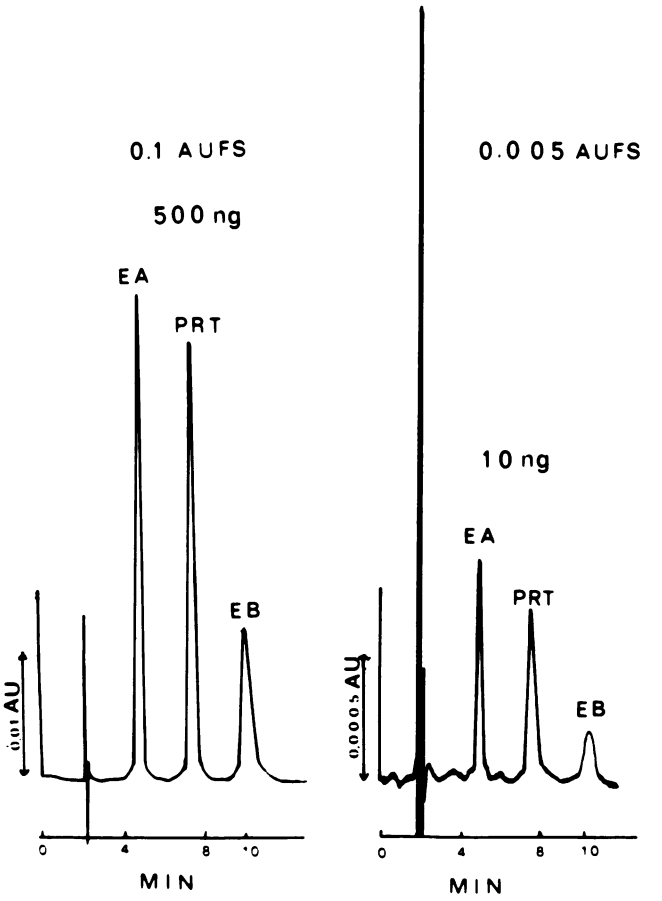

FIG. 3. *HPLC resolution of EA, EB, and PRT, 10 and 500 ng each. Elution solvent A: n-hexane-tetrahydrofuran (75/25 vol/vol). Flow rate, 1.5 ml/min (0.005 AUFS and 0.1 AUFS).*

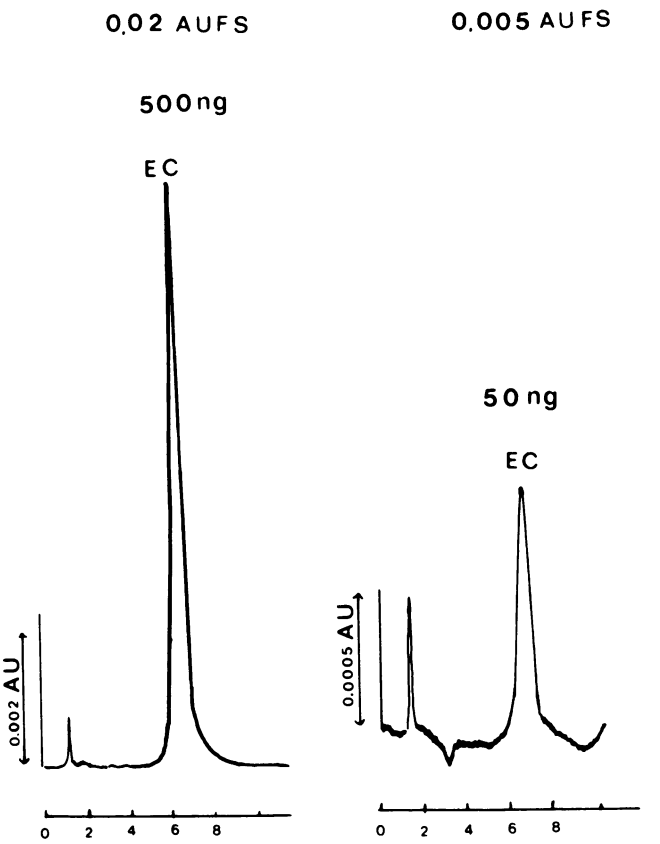

FIG. 4. *HPLC resolution of EC, 50 and 500 ng. Elution solvent B: chloroform. Flow rate, 2 ml/min (0.005 AUFS and 0.02 AUFS).*

The relationship between peak heights and the amount of compound injected was determined by injecting 10 μl of increasing concentrations of the mixed standards (EA, PRT, EB) and the EC standard. The quantities tested which ranged from 10 to 5,000 ng/10 μl were prepared by suitable dilution of the concentrated standards to include portions of 10, 50, 100, 200, 500, 1,000, 2,000, and 5,000 ng. The injections of each concentration were made over a 2-day period. Plots of the data demonstrated a linear relationship over a 10- to 5,000-ng range tested for EA, PRT, and EB and a 50- to 5,000-ng range for EC.

The data obtained in this study clearly indicate that HPLC resolution of *P. roqueforti* metabolite is a precise analytical method. Quantitative analysis is good for quantities above 10 ng of EA and PRT, and a qualitative analysis of about 2 ng is possible. EB and EC required 50 ng for precise analytical measurements, but quantities of about 20 ng could be detected.

This method is to be used on determinations of the various metabolites during *P. roqueforti* growth.

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