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A RADIOLIGAND ASSAY OF TOMATINE

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Abstract—A novel method for the assay of tomatine in plant extracts is described. It is based on precipitation of tomatine with cholesterol-4-^{14}C and determination of the radioactivity remaining in solution by scintillation counting. Pigments that cause quenching are removed by chromatography on Sephadex LH20.

INTRODUCTION

IN A previous paper we have traced the degradation of tomatine to allopregnenolone.[1] Further work on practical methods of removing tomatine from harvested tomato vines requires a fairly accurate and convenient assay method. The most reliable method of tomatine analysis is the precipitation by cholesterol and gravimetric determination of cholesterol tomatide, according to Schulz and Sander.[2] To make this method suitable for small quantities of tomatine in tomato root cultures Bruske[3] has introduced the use of a modified anthrone reagent, which indicates the sugar content of the precipitated complex. Because that method is still laborious, Sočič[4] has sacrificed specificity for speed and convenience by omitting the cholesterol precipitation. Another approach to increasing specificity has been proposed by Roddick and Butcher.[5] These authors have estimated tomatine in tomato cultures colorimetrically with sulfuric acid after elution from TLCs.

Combining the specificity of the cholesterol precipitation with the sensitivity and convenience of tracer methodology, we have developed a novel micro technique, which does not require the purification of the precipitated complex. Radioactive cholesterol is added to a crude plant extract in excess of the tomatine present. The radioactivity remaining in solution after precipitation gives a measure of the amount of tomatine precipitated.

RESULTS AND DISCUSSION

Fresh tomato seedlings and a freeze-dried tomato leaf powder were used for testing the accuracy and precision of the method. The efficiency of the extraction procedure was tested by addition of tomatine-4-^{14}C.[1] Continuous extraction for 16 hr of a fresh tomato seedling, weighing 4·3 g, in a Soxhlet with 500 ml methanol gave a 77% yield of radioactive tomatine. When lyophilized tomato leaves were first extracted for 2 hr with ether, 2·3% of the radioactivity was recovered from the ether. Subsequent extraction for 6 hr with methanol yielded 79% of the radioactive tomatine. For serial determinations lyophilized material is preferred.

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The dry weight of a tomato seedling harvested above the soil line averages 15·8%. Although ether extraction does not remove appreciable quantities of tomatine, it constitutes an extra step, which can be omitted unless the plant material contains large amounts of relatively nonpolar pigments.

For recovery experiments on leaf powder a standard solution of tomatine was first applied to an inverted extraction thimble and, after evaporation of the solvent, the leaf powder was weighed into the thimble. After extraction with methanol, the extract was evaporated and the residue was dissolved in 96% ethanol. Originally, the precipitating reagent of cholesterol-4-^{14}C in 96% ethanol was added to this extract and, after precipitation of the tomatine, an aliquot of the supernatant solution was counted in a planchet counter.[1] However, self-absorption introduced large errors in the assay, because the specific activities were quite low. Experiments with scintillation counting also initially showed large errors due to quenching, because the solutions were quite dark. Solutions suitable for scintillation counting were ultimately obtained by eluting thin-layer chromatograms in good yield (87·6% average), but a more efficient and less laborious method for removing the interfering dark pigments was found. When the residue was applied to a column of Sephadex LH20, most of the pigments were eluted by ethyl acetate before tomatine was recovered, in over 90% yield, with an eluent of ethyl acetate–methanol (1:1).

TABLE 1. ASSAY OF TOMATO LEAF POWDER AND RECOVERY OF TOMATINE

No.	Leaf powder (mg)	Tomatine (mg)	cpm	Tomatine (μg) Calcd.	Tomatine (μg) Found	Error (%)
1	37·8	0	1745	236	262	+11·0
2	215·4	0·5	516	1846	2006	+8·7
3	141·3	0	1348	883	825	−6·6
4	156·3	0·5	889	1477	1477	0
5	190·4	0	1074	1190	1214	+2·0
6	68·6	1·0	950	1429	1390	+2·7
7	290·6	0	475	1816	2064	+13·3
8	123·7	1·0	600	1773	1886	+6·4
9	255·2	0	859	1595	1519	−4·8
10	297·1	0	522	1857	1997	+7·6
11	0	1·0	1285	1000	915	−8·5
12		0	1929			
13		0·5	1580			
14		1·0	1223			
15		1·5	871			
16		2·0	495			

The final assay, which is described in the Experimental, gave recoveries of 91·5 to 113·3% when aliquots of 0·5 or 1 mg of pure tomatine were added to lyophilized tomato leaf samples, ranging in weight from 37·8 to 297·1 mg, prior to Soxhlet extraction (Table 1). The calculations are based on a tomatine content of the leaf powder of 0·625%, as determined by quadruple analysis. When known amounts of tomatine up to 2 mg are precipitated with the radiocholesterol reagent and the radioactivity of the supernatant solution is determined, a linear relation between micrograms of tomatine and counts per minute is obtained. The amount of tomatine in a sample precipitated with the same reagent can then be read off a graph or calculated by substituting in the equation for the calibration line. For

the results in Table 1 the μg tomatine in a given sample was obtained by multiplying the difference between the counts for 0 μg tomatine and for the sample by the slope of the calibration line, 1·42 μg/cpm.

EXPERIMENTAL

The plant material, lyophilized by freezing in liq. N_2 and drying at room temp. under high vacuum, was weighed into single-thickness extraction thimbles, 10 × 50 mm. This was extracted in a Soxhlet micro extraction apparatus with 20 ml MeOH for 6 hr. The extract was evaporated in a stream of N_2 at 60°. All solvents used were spectrochemically pure.

A chromatography tube was prepared by plugging the tip of a 10-ml Mohr pipette with cotton and mounting it vertically. 1 g Sephadex LH20 was slurried in EtOAc and the slurry was allowed to settle in the pipette. The extraction residue was applied to this column and eluted with 10 ml EtOAc. The dark-green eluate was discarded. Next, tomatine was eluted with 10 ml EtOAc–MeOH (1:1). The yellowish eluate was concentrated and evaporated to dryness in a 3·5-ml polypropylene Sorvall centrifuge tube. The residue was dissolved in 2·0 ml 96% EtOH.

The precipitating reagent was prepared by dissolving 2·22 × 10^6 dpm cholesterol-4-^{14}C, 375 mg carrier cholesterol, and 5 mg BHT in 50 ml 96% EtOH. The tomatine standard contained 1 mg tomatine per ml 96% EtOH. Both solutions keep indefinitely at room temp. The precipitating reagent was calibrated by adding 100-μl portions to 2 ml 96% EtOH containing various amounts of tomatine (0–2·0 mg) in polypropylene centrifuge tubes. At least one blank and one standard tube should be included with every batch of analyses.

A 100-μl portion of precipitating reagent was added to each of the sample tubes. All tubes were briefly heated in a boiling H_2O bath and then their contents were mixed with a glass stirring rod. After standing at room temp. for 1 hr, the tubes were kept in the rotor of a refrigerated centrifuge overnight. The next morning the tubes were centrifuged at 15 000 rpm for 40 min. A 1-ml aliquot of each supernatant solution was transferred to 10 ml Scintisol Complete and counted in a Packard Tricarb Model 3003 scintillation counter with an efficiency of 77% and a background counting rate of 11 cpm.