Quantitative Determination of 3-Amino-1, 2, 4-triazole

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A simple, reproducible method has been developed for the separation and quantitative determination of 3-amino-1,2,4-triazole in biological systems. The method consists of diazotization, followed by coupling with 8-amino-1-naphthol-3,6-disulfonic acid (H-acid) in the cold. The method is sensitive in the range of approximately 0.1 to 3.3 p.p.m.

THE COMPOUND, 3-amino-1,2,4-triazole (amitrole), has received much attention recently because of its growth-regulating properties (*3*) and its effect on catalase activity (*4, 5*). To study the nature of these reactions, methods must be devised which are sensitive and specific for the compound in question. Quantitative methods reported previously did not prove satisfactory for subsequent work on Canada thistle [*Cirsium arvense* (L.) Scop.].

One method (*1*) using nitroprusside resulted in high and variable blank values from material which had not been treated with amitrole. A second method (*2*) did not follow Beer's law, and lacked the required sensitivity. A paper chromatographic procedure using the principle of diazotization followed by coupling with 8-amino-1-naphthol-3,6-disulfonic acid (H-acid) has been reported (*6*). The present method uses the H-acid reagent in a diazotization and coupling procedure for the quantitative determination of amitrole in extracts from biological systems freed of contaminating metabolites derived from amitrole itself.

Experimental

Preparation of Test Samples from Biological Systems. Amitrole is metabolized by plant tissues, forming at least two metabolities which react with H-acid to produce a color similar to that obtained with pure amitrole (*4, 6*). These materials, if present, interfere with the determination of pure amitrole in the extract. These contaminating metabolites can be removed, with quantitative recovery of amitrole, by the use of a weakly acid, carboxylic (polymethacrylic-type), cation exchange resin.

The plant tissue to be examined is ground in a Waring Blendor with suitable solvent, such as water, methanol, or aqueous ethyl alcohol. The solvent used and the time of extraction depend upon the particular tissue involved. With thistle, a 2-hour aqueous extraction at room temperature is satisfactory. The insoluble material is filtered, and the filtrate is passed through a strongly acidic cation resin column, such as Dowex 50W-X8 or Amberlite IR-120 (10 × 1.1 cm. resin bed) in the H^+ form. The column is then washed with distilled water (200 ml.) and eluted with NH_4OH (100 ml., 1.0*N*). The NH_4OH effluent is concentrated to remove ammonia. This can be done in a hood over a steam bath. The samples should not be taken to complete dryness. The concentrated eluate is then passed onto a column of weakly acidic cation exchange resin such as Amberlite IRC-50 in the H^+ form (10 × 1.1 cm. resin bed), washed with water (200 ml.), and eluted with HCl (50 ml., 0.6*N*). The amount of resin, the size of columns necessary, the amount of washing required, and the amount of eluent are dependent upon the amount and type of tissue involved and the amount of amitrole and its metabolites present. These should be evaluated for each particular situation.

Amitrole Determination. A 3-ml. test solution containing amitrole is placed in an ice bath. After cooling the solution, 0.5 ml. of 1% $NaNO_2$ in water is added followed by 0.5 ml. of 20% concentrated HCl in water and the solution is mixed by swirling. Then 0.2 ml. of 0.25% H-acid in 50% acetone-water is added to the reaction mixture and the tube is again swirled to mix the reagents thoroughly. The color is allowed to develop for 30 minutes in the ice bath and then the solution is transferred to matched cuvettes (17 × 10 mm.). The color is read within 3 minutes of removal from the ice bath at 538 mμ in a Coleman, Jr., spectrophotometer using a blank solution.

Results

Preparation of Test Samples. Quantitative recovery of pure amitrole from the weakly acidic resin is shown in Table I. This table also shows that amitrole is recovered quantitatively in the presence of contaminating metabolites when a mixture of the metabolites and amitrole are added together to the weakly acidic cation column. The metabolites, unknowns I and II (*4*), were isolated from amitrole-treated Canada thistle plants by paper chromatography and added (the combined amount of unknowns I and II was approximately 120 μg. of amitrole equivalents or approximately 40% of the amount of amitrole applied) to a known amount of pure amitrole. This was passed through the column, followed by distilled water. All the contaminating materials were removed after 200 ml. of water had passed through the columns,

Table I. Recovery of Amitrole from Solutions Containing Pure Amitrole, Amitrole and Metabolites, and Extracts Containing Amitrole

(Each figure represents an average of at least two determinations)

Application Procedure	Amount, μg. Applied	Amount, μg. Recovered	Recovery, %
Pure amitrole to IRC-50	151	152	100.7
	284	284	100.0
	410	411	100.3
Pure amitrole with unk. I and II to IRC-50	284	287	101.0
	295	300	101.7
Pure amitrole with thistle ext. to Dowex 50W, conc., and applied to IRC-50	285	281	98.6

Table II. Effect of Acetone on Color Development in Coupling Diazotized Amitrole with H-Acid

Acetone, %	Absorbance, 538 Mμ
0.0	0.042
25.0	0.432
37.5	0.480
50.0	0.500
62.5	0.500
75.0	0.498

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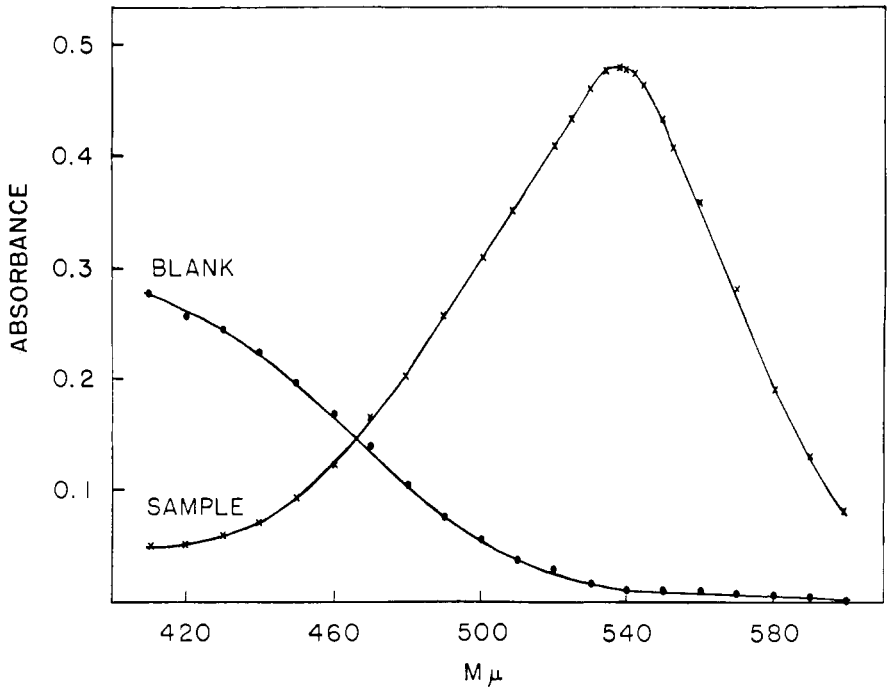

Figure 1. Absorption spectra of diazotized amitrole coupled with H-acid, and a blank system with amitrole absent

as indicated by blank values for the eluate. HCl (50 ml., 0.6*N*) was added to the column and the amount of amitrole eluted was determined (Table I). No apparent chemical changes of unknowns I and II occurred during the procedure as indicated by paper chromatography. Amitrole was recovered quantitatively within the limits of accuracy of detection when a known amount of amitrole was mixed with a thistle extract (representing 70 grams fresh weight of stem and leaf tissue) and carried through the entire procedure (Table I).

Using this procedure, a value of 0.63 μg. equivalent of amitrole was obtained per gram fresh weight of untreated thistle.

Amitrole Determinations. The H-acid appeared to be a more sensitive coupling agent than either phenol or 4,5-dihydroxynaphthalene - 2,7 - disulfonic acid and resulted in a more satisfactory absorption spectra (Figure 1) than reported for the latter reagent (*2*). The plot of absorbance *vs.* concentration was linear over the concentration range used.

Discussion

Reagent Stability. In the chromatographic procedure (*6*), the reagents were made up in acetone solutions. The 20% concentrated HCl solution, when made up with acetone, darkened in 8 hours at room temperature. Substitution of water for acetone increased the sensitivity of the method and resulted in a stable reagent. Substitution of water for acetone in the $NaNO_2$ reagent also increased the sensitivity of the test. The H-acid used in this procedure required recrystallization from water by the addition of ethyl alcohol. A 50% acetone-water solution of H-acid darkens at room temperature, but is stable when stored at 2° C. and can be used after several weeks. Various concentrations of acetone in the H-acid reagent indicated the necessity of acetone for the maximum development of color (Table II).

Concentration Range. The usable concentration range is approximately 0.5 to 10 μg. of amitrole in the final volume of solution. With more than 10 μg. of amitrole, the plot of absorbance *vs.* concentration does not maintain linearity; the deviation apparently is similar to that found previously (*2*).

Reproducibility. With three different standards solutions of amitrole, the reproducibility of the standard curve was approximately 3% over the range of concentrations.

Color Development. The development of a maximum color was dependent upon time and temperature. Development at room temperature was rapid, but did not reach the maximum attained by the same solution when the color was developed in an ice bath. The color developed in an ice bath reached a maximum in approximately 30 minutes and had to be developed entirely in the cold, as solutions removed before the maximum was reached did not give maximum values. The color remains constant up to 3 minutes following removal from the ice bath and should be read within this time.

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