analog of any essential sulfur metabolite would be toxic to some system, but there is no proof that such toxicity is related to the problem of selenium toxicity as it affects the livestock industry.

When a mechanism acquires general acceptance, such acceptance will possibly affect the design of experiments in the field. Much that has in the past been interpreted as evidence concerning the nature of selenium toxicity may simply be evidence concerning a step in the metabolism of selenate.

In the practical control of the selenium problem, ion antagonisms show considerable promise. It will be necessary to learn more about the nature of selenium in the soil solution, but from the wide variety of antagonisms discussed in this and an earlier paper (*1*), it should be possible to regulate the uptake of selenium by plants. Whether such regulation would be complete or inexpensive enough to be practical remains to be seen.

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RESISTANCE FACTOR DETERMINATION

Quantitative Estimation of the Resistance Factor, 6-Methoxybenzoxazolinone, in Corn Plant Tissue

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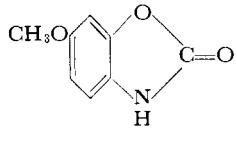
A method is described for estimating the concentration of 6-methoxybenzoxazolinone in corn plant tissues. The plant tissue sample is extracted with diethyl ether, and the ether extract is purified, chromatographically, with aluminum oxide. The compound is eluted from the aluminum oxide by an ether-alcohol solvent mixture, and its concentration is estimated by its extinction at 285 mμ.

An organic compound, $C_8H_7O_3N$, was recently isolated from the tissues of the corn plant by Loomis (*6*). Independently and nearly simultaneously, the same substance was isolated from wheat and corn by Virtanen, Hietala, and Wahlroos (*11*), who tentatively identified it as 6-methoxybenzoxazolinone (I). Subsequent work by Virtanen and coworkers (*5*, *12*) and the authors' group (*8*, *9*) has established the validity of this characterization. The demethoxy analog was also isolated by Virtanen and Hietala (*10*) from rye plants. Virtanen and coworkers discovered these substances during the course of a study of antifungal chemicals present in plants of the grass family. The work of Loomis came as an outgrowth of the earlier finding of Beck (*3*), that young corn plants contain substances inhibiting the growth of European corn borer larvae, *Pyrausta nubilalis* (Hbn.). 6-Methoxybenzoxazolinone, termed resistance factor A (RFA), acts as a growth inhibitor toward a variety of bacteria, fungi, and insects in addition to the European corn borer (*1*, *7*).

The role of chemical factors in the resistance of corn plants to successful attack by the European corn borer has been studied in some detail (*1*, *2*, *4*, *7*). In addition to the ether-soluble resistance factor A, a water-soluble borer and fungus inhibitor has been detected, but not yet isolated, which has been designated as resistance factor B (RFB) (*4*, *7*). The existence of a third factor (RFC) was found after the analytical method described below was developed (*2*). Like factor A, factor C is ether-soluble, but it has not yet been isolated and characterized. Factor A is primarily responsible for the resistance of young corn plants to the leaf-feeding stage of the borer (*2*, *7*). Tissue concentrations of factor A tend to be high in young plants, but decline rapidly as the plant matures (*2*, *7*). Corn which has tasseled no longer contains factor A in amounts which are effective against either fungi or European corn borer (*1*, *2*). Resistance manifested by corn plants, which have reached the tassel growth stage, appears to be causally related to the concentrations of factors B and C in those tissues attacked by the insect (*2*).

The importance of these chemical factors in plant resistance to insects and disease makes them of considerable biological and chemical interest. The only methods available for estimating the presence and concentration of the resistance factors in plant tissues have been bioassays employing either fungi or insects (*4*). The bioassay techniques require relatively large samples of plant tissues, and are not sufficiently specific for many purposes. Such difficulties hamper detailed physiological and genetic studies. As 6-methoxybenzoxazolinone has been shown to play an important role in the defense of young corn to disease and insects, a simple quantitative method of analysis should be of value in agronomic screening of genetic lines of corn and in studies of the biochemistry of plant metabolism.



(I)

The analytical method developed is based on a chromatographic purification of resistance factor recovered from plant tissue and an estimation of its concentration from the absorbance observed at a wave length of 285 mμ. Loomis (*6*) found that purified 6-methoxybenzoxazolinone displayed two absorption peaks in the ultraviolet; one peak was at 228 mμ and the other at 285 mμ. Absorption was at a minimum at 255 mμ. The data presented in Figure 1 show that extinction at either 228 or 285 mμ (Beckman DU spectrophotometer) was linear with concentration of highly purified 6-methoxybenzoxazolinone. In

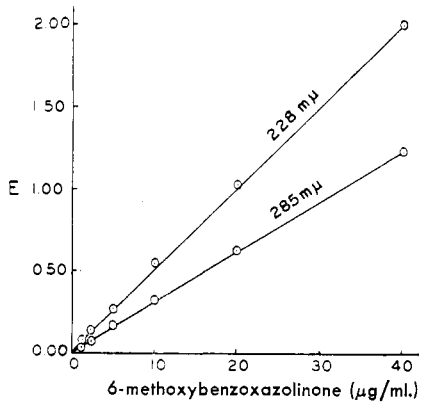


Figure 1. Demonstration of linearity between extinction and concentration of 6-methoxybenzoxazolinone

this study, the 285-mμ wave length was used in preference to 228 mμ, because of the smaller effect of interfering substances at that setting. In pure solution, as little as 2 γ of factor A per ml. was easily measurable. Using 3-ml. cuvettes, a total of 6 γ was detectable. This is in striking contrast to the bioassay technique of Beck and Stauffer (*4*) which required a concentration of 70 γ per ml. and a volume of 50 ml. for a total of 3.5 mg. for detectable mold growth inhibition.

Collection and Extraction of Plant Samples

Samples of different tissues of corn plants are collected, weighed, chopped, and quick-frozen in small sealed containers. The size of sample required to provide an adequate factor A level for analysis was found to vary according to the age of the plant and the type of tissue being sampled. One-gram samples (fresh weight) were adequate in most instances, and much smaller samples were feasible when the sampled tissue was leaves of corn seedlings. The tissues were routinely frozen before further processing, as subsequent extraction was facilitated by the cellular disruption that accompanies freezing and thawing.

The frozen plant samples may be extracted without further treatment, or dried prior to extraction. Drying must be accomplished at a relatively low temperature, however (40° to 50° C.), as exposure to higher temperatures results in a lowered recovery of factor A. The plant samples are extracted with diethyl ether (ACS specifications) in a micro-Soxhlet apparatus for 3 hours. The ether extracts are evaporated to dryness at room temperature under a gentle air stream. The residue is taken up in 5.0 ml. of anhydrous ether and purified by elution from an aluminum oxide column, as described below.

Aqueous extracts of large samples, such as those used in bioassays of resistance factor activity, may also be analyzed. A convenient volume of the aqueous preparation is acidified (approximately pH 2) with hydrochloric acid and extracted in a separatory funnel with eight to 10 small volumes of ether. The combined ether washes are dried with anhydrous sodium sulfate (granular form), evaporated down, and the residue is taken up in 5.0 ml. of anhydrous ether.

Preparation of Columns

Ten-inch columns of acid-washed aluminum oxide (Merck) are prepared. Glass tubing, 26 mm. in diameter and 14 inches in length, fitted with a coarse sintered-glass disk above a stopcock at the lower end and a 150-ml. reservoir bulb at the upper end, is satisfactory. With the stopcock closed, the tube is filled to the base of the reservoir with absolute ethyl alcohol. A 0.5-inch layer of thoroughly washed white sand is placed in the column. One hundred grams of aluminum oxide is mixed with 200 ml. of absolute ethyl alcohol in a beaker, and the mixture is stirred until bubble formation stops. About 50 ml. of the mixture is poured into the column; as it settles, the stopcock is opened. Packing the column is completed by adding the rest of the aluminum oxide–ethyl alcohol in small portions with careful stirring to ensure an evenly packed column. With glass of the size described, this procedure yielded a well-packed column 10 inches in length (± 1/4 inch).

As the ethyl alcohol level reaches a point about 0.25 inch above the top of the aluminum oxide column, about 10 ml. of ether-alcohol is added. This solvent mixture—80 parts of anhydrous diethyl ether (ACS specifications) and 20 parts of absolute ethyl alcohol by volume—should be kept dry over anhydrous sodium sulfate. As the liquid level again approaches the top of the packed column, ether-alcohol is added until the reservoir bulb is filled. Washed white sand is introduced to cover the column with a 0.25-inch layer. Washing the column with ether-alcohol is continued until at least 450 ml. of the solvent has passed through the column. It is then ready for the introduction of the ether extract of a plant sample. Six such columns can be handled simultaneously by one technician without difficulty.

The stopcocks may be a source of

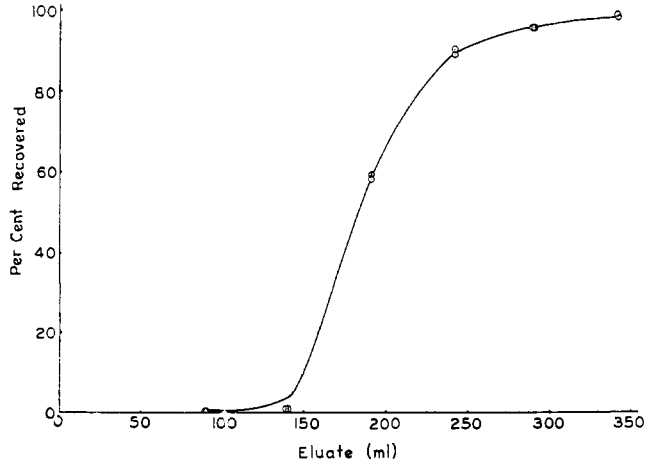


Figure 2. Elution of 6-methoxybenzoxazolinone from 10-inch columns of acid-washed aluminum oxide (Merck)

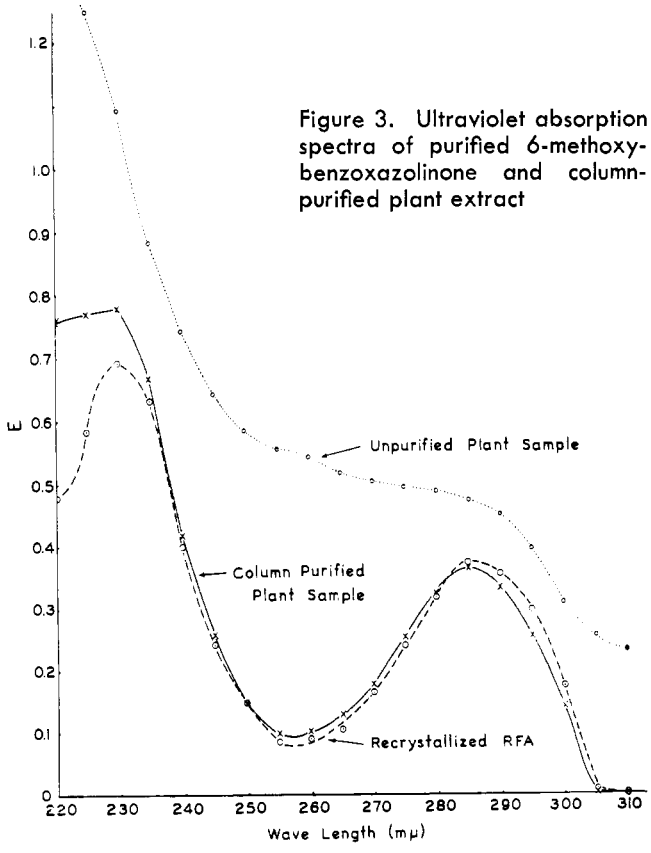


Figure 3. Ultraviolet absorption spectra of purified 6-methoxybenzoxazolinone and column-purified plant extract

trouble, as the use of ordinary stopcock grease sometimes results in the presence of interfering substances in the final solutions. Glycerol was used as a stopcock lubricant, and although it produced no interference, it left much to be desired. Although they were not tested in this work, Teflon stopcocks may be more satisfactory.

Adsorption and Elution of Plant Extracts

After the columns are washed, 5.0 ml. of plant extract or appropriate ether blanks and standards are introduced. Three separate ether rinses of 3 ml. each are added as the last of the rinses goes into the sand layer; 10 ml. of ether-alcohol is added. Collection of eluate is now begun. As this last portion of solvent passes into the column, the reservoir bulb is filled with the solvent. If a group of columns are being worked, their rates of flow should be made to be approximately equal.

Under the conditions employed, elution of factor A from the column occurred between 140 and 240 ml. of eluate volume (Figure 2). Collecting a somewhat broader range did not result in an increase of interfering impurities; therefore, the fraction was taken routinely from 90 to 290 ml. The rate of movement of factor A on the column was dependent on relative proportions of ether and alcohol in the eluent. Ether and alcohol in a 1 to 1 mixture accomplished elution in the 90- to 160-ml. fraction, but purity was unsatisfactory. Ether alone resulted in prohibitively slow movement of the compound on the column.

Determination of Concentration and Purity

The eluates collected from the columns are evaporated to dryness. This may be done under reduced pressure or with the aid of warming and an air stream, but care must be taken to avoid overheating the residue (40° C. is satisfactory). The dried residues are taken up in 10.0 ml. of distilled water, with warming. The absorbance of the samples, a reagent blank, and a standard solution of 6-methoxybenzoxazolinone are read in a spectrophotometer against a distilled water blank at wave lengths of 255 and 285 mμ. From these data, the factor A contents of the samples are calculated. The pure compound shows an absorption minimum at 255 mμ, and readings of absorbance at this wave length are of value in that they give an indication of the optical purity of the samples. The ultraviolet absorption spectra of a plant extract before and after column purification are shown in Figure 3, where they are compared to the spectrum of recrystallized highly purified 6-methoxybenzoxazolinone. In the unpurified plant sample, interfering substances showed an absorption spectrum with a broad peak between 250 and 270 mμ which obscured the factor A minimum at 255 mμ. The column purification gave a satisfactory resolution of the 255-mμ minimum and the 285-mμ peak, but not of the absorption peak at 230 mμ.

The efficiency of the analytical procedure was determined by a series of recovery experiments. In one group of experiments, different amounts of the pure compound were put through the procedure and the percentage recovery determined. Table I shows the results obtained. Recovery experiments in which known amounts of the pure compound were added to plant extracts prior to column purification were also run. These experiments yielded an average recovery of 91%.

Table I. Recovery of Purified 6-Methoxybenzoxazolinone from Aluminum Oxide Chromatographic Columns

Amounted Added, γ	Average Amount Recovered	
	γ	%
500	460	92
400	340	85
206	182	91
100	93	93
50	41	82
25	23	90

The analytical method described above has been used to estimate the factor A content of different tissues of corn plants of different ages and varieties. Good consistency among replicate samples was obtained. No significant optical interference was encountered in any of the plant samples. Leaves, tassels, stems, leaf sheaths, husks, silks, and pollen samples have been used. Factor A concentrations varied from 3 to 260 γ per gram of fresh weight of tissue among the plant parts, varieties, and ages tested.

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PHOSPHORUS AVAILABILITY

Solubility Characteristics of Nitric Phosphate Fertilizers in Calcareous Soils, and Comparative Effectiveness in Greenhouse Pot Cultures

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NITRIC PHOSPHATE FERTILIZERS are produced by reaction of rock phosphate and nitric acid, either alone or in combination with sulfuric or phosphoric acid. This reaction is followed by ammoniation to neutralize excess acid and to increase the nitrogen content, and if desired to give a complete fertilizer product, by potassium addition. The cost advantage of the nitric phosphate process is based partly on the action of nitric acid in dissolving rock phosphate and partly on the contribution nitric acid makes to the nitrogen content of the final fertilizer grade. The cost of both nitric acid and ammonia used is chargeable to fertilizer nitrogen rather than production of available phosphorus.

The TVA began producing "nitraphosphates" on a pilot plant basis in 1948 (*8*). The preferred designation of