Application of Chromatography in Determination of Micro Quantities of 3-(*p*-Chlorophenyl)-1,1-dimethylurea

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In the microdetermination of 3-(p-chlorophenyl)-1,1-dimethylurea in plant tissues by hydrolysis to p-chloroaniline and subsequent conversion to an azo dye, some interference has been caused by the formation of traces of aromatic amine from plant tissues in the course of hydrolysis. The principal interfering amine was o-aminoacetophenone, and a simple technique was developed for separating it from p-chloroaniline. Thus, it is no longer necessary to analyze a corresponding sample of an untreated plant in order to correct for interfering materials. The maximum absolute level of 3-(p-chlorophenyl)-1,1-dimethylurea can be determined on a single sample. The use of p-chloroaniline as an index of maximum 3-(p-chlorophenyl)-1,1-dimethylurea was cross checked on a sample of plant tissue containing radioactive 3-(p-chlorophenyl)-1,1-dimethylurea and found to be reliable.

In the determination of 3-(*p*-chlorophenyl) - 1,1 - dimethylurea residues, an interfering azo dye is formed from plant tissue (*1*). This dye is equivalent spectrophotometrically to the azo dye derived from *p*-chloroaniline, the hydrolysis product of 3-(*p*-chlorophenyl)-1,1-dimethylurea used for identification, and differs from it only in its rate of color development. The interference has been established as having its principal origin in *o*-aminoacetophenone and a simple technique has been developed for separating the azo dye derived from *p*-chloroaniline from that formed from *o*-aminoacetophenone. This separation increases the sensitivity of the analytical procedure and eliminates the need for a correction factor based on the analysis of untreated plant tissue.

A survey of the literature revealed that *o*-aminoacetophenone was a probable cause of the interference. Tabone and coworkers (*4*, *5*) demonstrated the presence of *o*-aminoacetophenone in an alkaline hydrolyzate of tryptophan and showed that the diazonium salt of this compound coupled with *N*-(1-naphthyl)-diethylpropylenediamine hydrochloride at a slow rate to produce a purple dye. *o*-Aminoacetophenone can be steam-distilled from an alkaline medium, in contrast to most biological aryl amines. The present work indicates that *o*-aminoacetophenone is always obtained on alkaline hydrolysis of plant tissue, and confirms an observation of Tabone that the amount present is increased by exposure to oxygen.

The principal interference was identified by paper partition chromatography and infrared analysis. *p*-Chloroaniline was then separated from its interferences by applying column chromatography to the azo dyes derived from the amines. A sample of 3-(*p*-chlorophenyl)-1,1-dimethylurea containing carbon-14 in the benzene nucleus was used to establish the efficiency of chromatographic resolution and to confirm in at least one plant species (tomato) that the determination of *p*-chloroaniline is a true index of the maximum amount of 3-(*p*-chlorophenyl) - 1,1 - dimethylurea which may be present.

Two types of chromatography were used in the present study: paper chromatography of the amines, and column chromatography of the azo dyes derived from the amines. The paper method was employed to determine the nature of the interfering material, while the column method was developed as a practical analytical procedure to eliminate the interference.

Identification of Interfering Material

Materials And Methods

The incorporation of a paper chromatographic separation into the original procedure for the determination of 3-(*p*-chlorophenyl)-1,1-dimethylurea (*1*) requires concentration of the aryl amines prior to their application to the chromatographic paper. This concentration can be carried out directly on the acid extract of the amines, but it is not convenient to apply aqueous solutions to chromatographic papers. The most convenient procedure for preparing plant tissue hydrolyzates for chromatographic resolution consists of extracting the ether charge from the distillation assembly with 1*N* hydrochloric acid (*1*) adjusting the pH of the acid solution to a value equal to or greater than 11 with sodium hydroxide; and re-extracting the amines into a small volume of ether. The solution of the aryl amines in ether can be dried with anhydrous potassium carbonate and concentrated in vacuo. Ordinarily, samples containing 1 to 10 γ of aromatic amine are applied to the paper.

The most convenient developing solvent for *p*-chloroaniline in food crop hydrolyzates is a mixture of 35 ml. of

methanol, 17.5 ml. of isoamyl alcohol, 35 ml. of benzene, and 12.5 ml. of 2N hydrochloric acid prepared according to Ekman (*3*). This mixture gives compact spots and good resolution in short running time, and possesses excellent wettability toward the waxy coating that invariably results when the hydrolyzate of a food crop is concentrated to a single spot on the paper. The papers are prewashed with the solvent mixture and dried over a steam plate (dry surface) prior to the application of the ether concentrates of the plant materials and standard reference substances.

p-Chloroaniline is conveniently located with the Ehrlich reagent (*p*-dimethylaminobenzaldehyde in hydrochloric acid) and by diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride. The Ehrlich reagent forms a brilliant yellow anil with *p*-chloroaniline. The anil exhibits a striking fluorescence under ultraviolet light. Diazotization and coupling produce the familiar magenta colored dye. Each reagent will detect less than 1 γ of *p*-chloroaniline.

o-Aminoacetophenone produces an orange anil with the Ehrlich reagent and a magenta dye upon diazotization and coupling. It can be detected with the same sensitivity as *p*-chloroaniline. The orange anil, in contrast to the analogous product of *p*-chloroaniline, exhibits only a weak fluorescence under ultraviolet light.

The ascending boundary technique was employed throughout. Sheets of Whatman No. 1 filter paper ($11 \times 18^1/_4$ inches) were stapled into the form of self-supporting cylinders and placed within insulated battery jars maintained at room temperature. The ether concentrates of the plant tissues under examination were applied to the paper with a micropipet. The solutions were applied layer upon layer at such a rate that the over-all diameter of the spots never exceeded 5 to 6 mm.

Standard levels of *p*-chloroaniline (stock solution in ether) were applied to the base line adjacent to each of the unknowns. The samples were applied on a line parallel to the 18-inch axis 1 inch from the edge of the papers and care was taken that the paper cylinders were always placed in contact with the same volume of freshly prepared developing solvent. Running time averaged 4.5 to 5 hours over a 10-inch path of development. After development was complete, the papers were air dried and sprayed with the reagents for colorimetric identification. The Ehrlich reagent was prepared according to Block (*2*). The spray reagents for diazotization and coupling consisted of 1% sodium nitrite in 1N hydrochloric acid (weight/volume) and 0.2% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in absolute ethyl alcohol (weight/volume). The papers were not dried between sprays in the case of diazotization and coupling.

Experimental Results

Identification of Interfering Material. Hydrolyzates of ten plant species were characterized by paper partition chromatography. In every case a principal amine was found which gave rise to an orange anil with the Ehrlich reagent and/or to a magenta colored dye upon diazotization and coupling with *N* - (1 - naphthyl) - ethylenediamine dihydrochloride. Table I lists the plant tissues and gives the R_f value of the principal spot. It is estimated that the accuracy of these values is within ± 0.03.

The R_f values of synthetic *o*-aminoacetophenone and of tryptophan that have been subjected to alkaline hydrolysis are included in the same table. Table I indicates that a common interference exists in each of the plant tissues listed and establishes that the R_f and color reactions of that interference are the same as those of *o*-aminoacetophenone and of hydrolyzed tryptophan for the solvent system involved in the resolution.

In order to remove any elements of doubt regarding the resolving power of the solvent system or the specificity of the reagents used in the chromatographic comparisons cited in Table I, it was decided to prove the presence of *o*-aminoacetophenone in the hydrolyzate of a typical crop. A large sample of alfalfa was carried through the usual separation and concentrated for chromatography.

Table I. Paper Chromatographic Identification of Interference

Crop	R_f
Alfalfa	0.50
Asparagus	0.51
Beans (Lima)	0.50
Beets	0.48
Broccoli	0.53
Carrots	0.50
Cottonseed meal	0.49
Pineapple	0.50
Spinach	0.53
Tomato plant	0.49
Tryptophan (hydrolyzed)	0.48
o-Aminoacetophenone	0.50

A paper separation was made on a small aliquot of this concentrate. The chromatogram showed that a single amine was present and that it had an R_f equal to that of *o*-aminoacetophenone. The bulk of the alfalfa concentrate was then transferred into carbon tetrachloride and concentrated to a single drop. The infrared absorption spectrum of this drop was obtained and compared with that of synthetic *o*-aminoacetophenone. All 14 peaks of the synthetic were present in the concentrate, confirming the presence of *o*-aminoacetophenone in alfalfa.

The oxygen effect reported by Tabone (*4*) is worthy of elaboration. If a plant material is subjected to alkaline hydrolysis under a sparge of oxygen, and then transferred to the automatic steam distillation–ether extraction assembly according to the usual procedure, there is a striking increase in the level of material capable of diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride. This same effect is apparent when food crops are compared under widely different states of physical subdivision. The levels of blank are generally lower when food crops are charged intact into a hydrolysis mixture than when they are subjected to prior drying and grinding. This difference is the effect of exposure to oxygen with increased surface due to grinding and not the result of failure of the hydrolysis mixture to penetrate cell structure. This can be demonstrated on an aliquot of an air-dried plant material by replacing oxygen bound at the surface with nitrogen before hydrolysis operations. Fortunately, from a practical standpoint, the steam distillation–ether extraction assemblies provide a reproducible environment for hydrolysis. It is necessary only that crops be handled uniformly.

It is possible to eliminate the formation of *o*-aminoacetophenone during the hydrolysis of synthetic tryptophan by adding an antioxidant, such as ferrous sulfate, to the hydrolysis medium. Thus far, however, attempts to utilize this method in the presence of plant tissue have not been successful.

Identification of Radioactive 3-(*p*-Chlorophenyl) - 1,1 - dimethylurea in Plant Tissue. The paper chromatographic procedure was applied to a tomato plant grown hydroponically in the presence of radioactive 3-(*p*-chlorophenyl)-1,1-dimethylurea tagged in the benzene ring with carbon-14. The spot corresponding to *p*-chloroaniline was located with the Ehrlich reagent and then was cut out of the paper for carbon-14 assay. Using a radiotracer method, 97% of the activity present in the original sample could be accounted for in terms of the *p*-chloroaniline spot. This chromatographic experiment on the tomato plant containing labeled 3-(*p* - chlorophenyl) - 1,1 - dimethylurea affords excellent confirmation of the efficiency of the modified analytical procedure and provides independent proof on at least one food crop, that the method for determining 3-(*p*-chlorophenyl)-1,1-dimethylurea by measuring the *p*-chloroaniline formed during the caustic digestion of the treated tissue is a true index of the maximum residual herbicide.

Elimination of Interference

Materials And Methods

A convenient method has been found for separating the azo dye derived from *p*-chloroaniline from the azo

dye derived from *o*-aminoacetophenone. The separation can be combined with the standard procedure for the determination of 3-(*p*-chlorophenyl)-1,1-dimethylurea (*1*) in order to isolate the color produced from *p*-chloroaniline, and involves no changes in the standard procedure other than the addition of a separation on a cellulose column.

The azo dyes of *o*-aminoacetophenone and *p*-chloroaniline can be concentrated from 1*N* hydrochloric acid by making the system alkaline with ammonia, extracting the dyes into a small volume of chloroform, and partitioning the dyes back into 1*N* hydrochloric acid. This series of steps makes it possible to concentrate diazonium dyes preparatory to column separation and, also, to recover components from large volumes of eluate.

Table II. Efficiency of Column Chromatographic Separation

Source of Interference	*Level of Interference Expressed as p-Chloroaniline, γ*	*p-Chloroaniline Added, γ*	*p-Chloroaniline Recovered, γ*	*%[a] Recovery*
Cottonseed	10	21.6	22.5	104
Flax seed	20	21.6	20.0	93
Tomato plant tissue	15	19.0	18.0	95
Corn leaves	5	5.0	5.1	102
	5	30.0	31.2	104
Alfalfa	30	5.0	5.3	106
	30	30.0	28.0	93.5

[a] No corrections for blanks required.

The separation is accomplished by pouring the mixture of dyes onto a dry column of packed cellulose (Whatman No. 1 filter paper powder, standard grade). The column is developed by adding 1*N* hydrochloric acid (the same medium used for diazotization and coupling). Separation starts almost immediately. The *o*-aminoacetophenone dye moves ahead of the *p*-chloroaniline dye, and in a short time two distinct zones are apparent. The zones continue to separate until the *o*-aminoacetophenone dye has passed completely through the column, leaving the *p*-chloroaniline dye free from interference.

The *p*-chloroaniline dye is easily recovered from the column by adding a 1 to 1 mixture of 1*N* hydrochloric acid and glacial acetic acid (v.v.). This combination compresses the magenta band of *p*-chloroaniline and quickly "sweeps" the column clean. The compressing action of the solvent and the intense color of the *p*-chloroaniline azo dye allow very sharp cuts on the eluate to be made. Usually, all of the dye can be collected in 10 to 15 ml. of the mixed acid. It is a simple matter to adjust the color intensity of the eluted dye to a value in the range of the calibration curve for *p*-chloroaniline (*1*) and to make a quantitative determination. The presence of acetic acid in the eluate from the column does not affect the hue or intensity of the *p*-chloroaniline dye.

It is not necessary to use an elaborate column in the foregoing procedure. Almost any type of packed cellulose will accomplish a separat on. It is convenient, however, to use 15-mm. glass tubing and to prepare columns 20 to 25 cm. long by hand packing. Penicillin assay disks provide an ideal packing support in tubing of this diameter, and the tubes are kept simple enough to be expendable.

Experimental Results

The column chromatography procedure was established using synthetic mixtures of diazotized and coupled *p*-chloroaniline and *o*-aminoacetophenone. The separation of the magenta colored dyes on cellulose was so rapid and so complete that there could be little question as to the efficiency of resolution. It remained only to demonstrate that the azo dye derived from *p*-chloroaniline could be recovered quantitatively from a column of cellulose. Visual examination of the column and the eluate is a very sensitive test in the case of the azo dyes and it was soon apparent that a small volume of a 1 to 1 mixture of 1*N* hydrochloric acid and glacial acetic acid (v.v.) was capable of eluting the color completely. This was verified by carrying one half of a *p*-chloroaniline dye solution through the steps of a chromatographic resolution, eluting the dye, and comparing the intensity of color with that of the control. The per cent transmittance readings before and after column resolution were identical within the limits of the colorimetric procedure.

The column chromatographic procedure was then tested on five different untreated plant tissue hydrolyzates to verify the conclusions reached on the basis of paper chromatography and the quantitative recovery of *p*-chloroaniline dye. The experimental results using column chromatography are summarized in Table II. The untreated plant tissues were hydrolyzed and carried through the complete procedure for the determination of 3-(*p*-chlorophenyl)-1,1-dimethylurea in plant tissue (*1*). In the case of each of the crops a magenta colored dye was formed which was indistinguishable from *p*-chloroaniline dye, except in its rate of color development.

Standard levels of *p*-chloroaniline azo dye were mixed with each of the crop blanks and the synthetic mixtures were subjected to column chromatography. The interfering substance from each of the plants behaved in the same fashion on a column of cellulose and the behavior pattern was the same as that observed with the azo dye of synthetic *o*-aminoacetophenone. The azo dye of *p*-chloroaniline was recovered by elution and compared to the original level of *p*-chloroaniline. The recovery values are listed in the last column of Table II. The recovery was essentially quantitative in the presence of these levels of interference. The experiment was carried out with levels of interference higher than those normally encountered in plant tissue. In the case of those samples where the blank was equal to or greater than the level of *p*-chloroaniline under consideration, it would not be possible to attach significance to residues of 3-(*p*-chlorophenyl) - 1,1 - dimethylurea determined in the absence of chromatographic resolution.

The addition of this simple column separation to the original procedure for determining 3-(*p*-chlorophenyl)-1,1-dimethylurea in plant tissue increases the sensitivity of the method, eliminates any assumptions based on the proper choice of biological controls, and ensures against extraneous effects such as that due to excess oxygen. Column separation can be included in the original procedure or omitted, as a situation demands. An entire separation and recovery can be accomplished in 1 hour.

Indo e is one of the trace interferences in the analytical procedure involving diazotization for the determination of 3-(*p*-chlorophenyl)-1,1-dimethylurea residues in plant tissue. Column chromatography removes any interfering color due to this compound.

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