Early in the study, it was realized that in many instances, a cream-over-cream separation had occurred that was difficult to detect. By viewing the emulsion with transmitted light, the cream line was easily defined. For this type observation, a diameter of 1 inch appeared to be maximum with about $^3/_4$ inch as optimum. Thin walls for the container reduced reflection along with a suitably designed light source and rack for the tubes. The optimum tube was found in present laboratory equipment as a 50-ml. Nessler tube (tall form), (*2, 5*).

Emulsion Depth. The depth of emulsion influences results as shown in Figures 9 and 10, illustrating the necessity of standardization and a minimum depth of 7 to 8 inches.

A method of evaluating emulsifiable concentrates has been devised using the above data and has been published (*1*). It has been in use in our laboratory for about 4 years with gratifying results.

Ingredients

The ingredients of the emulsion influence the results; one of the principal ingredients, the water, is sufficiently common to be disregarded often, and yet it is of paramount importance. Particularly is this true with emulsifiable concentrates that will be emulsified by the consumer, with water of unknown hardness (*3*). A brief study of the effect of a change in water hardness has been made, and the results are presented in Figure 11.

For a given formulation, the emulsification efficiency usually goes through a maximum at one hardness. It is possible to formulate concentrates that are most efficient at low hardness, or at medium, or high hardness. With a truly satisfactory product, efficiency is not markedly affected by varying considerably the hardness, but a measurable difference is obtained. This allows formulation for the hardness that is most likely to be encountered, and evaluation under these conditions.

For special conditions of high or low pH, or unusual salt concentrations, an evaluation test should be carefully scrutinized to make sure that the conditions imposed yield the proper degree of critical conditions.

Summary and Conclusions

Experimental results have been presented on the effect of a number of variables that influence the design of an emulsion test procedure.

The preparation of the test emulsion has been found to be critical, with the amount and vigor of agitation, test temperature, and mode of addition and ratio of ingredients influencing results. Regulation of agitation was accomplished by shaking 100 ml. of emulsion in 4-ounce, wide-mouth, screw cap jars with a commercial shaker operated at 292 r.p.m. with a 1-inch stroke for an interval of 30 seconds.

The observation of emulsions was studied with the finding that the depth of the emulsion and the shape and size of the container produced effects on the creaming rate. Standardization was obtained using a commercial emulsion viewer employing 50-ml. Nessler tubes filled to a depth of $7^3/_4$ inches, and equipped with a shaded light to permit examination of the test emulsion by uniform transmitted light.

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TRICHLOROACETIC ACID

Colorimetric Method for Quantitative Determination in Plant Tissue

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A method is described for the separation of trichloroacetic acid from plant tissue and the colorimetric estimation of the amount of the chemical recovered. The plant tissue is homogenized with 0.1 *N* acetic acid and the fat-soluble pigments (chlorophylls and carotenoids) and the insoluble cellular components are effectively separated from the chemical by filtration through asbestos. An aliquot of this filtrate is added to a tube containing pyridine and sodium hydroxide. The solution is heated to develop a red-violet color which is measured colorimetrically. The procedure has been used effectively to determine the quantity of chemical in the roots and above ground portions of several different plants.

ACCURATE DETERMINATION of an herbicidal chemical within plant tissue may often facilitate an understanding of the differential response to the chemical by plants of various species. Further, a quantitative estimate of the amount of herbicide accumulated in various plant parts may give information concerning the center of activity of that chemical and thus provide a clue to the mechanism by which it brings about its particular plant response. With the advent of each promising new herbicidal compound a diligent search is made for a suitable method of determination. Biological assays have been described for use with some herbicides but are often limited in scope because of variability

and low sensitivity. Chemical methods are frequently more acceptable since they may be refined to greater sensitivity and may lend themselves more readily to rigid control. A difficulty common to all such chemical methods of determination, however, is that of isolating the herbicidal compound from whole or processed plant tissue.

Several biological and chemical tests have been reported for the quantitative determination of 2,4-dichlorophenoxyacetic acid, an effective agent for use on broad-leaved weeds. The compound, trichloroacetic acid (TCA), has recently been shown to be highly toxic to grasses. This paper is concerned with the development of a colorimetric method for the determination of trichloroacetic acid in plant tissue.

Fujiwara, in 1916 (*4*), described a qualitative colorimetric method for identifying chloroform extracted from animal tissue. The extract was added to a tube containing pyridine and saturated sodium hydroxide. After boiling, the mixture developed a distinctive red-violet color. More recently, Cole (*2*) has standardized the method for quantitative determinations, and Daroga and Pollard (*3*) have reduced the proportion of sodium hydroxide so that the color is more stable. Hummer and Barrons (*1*, *5*) have successfully used the procedure to determine the concentration of trichloroacetic acid in plant sap and in extracts from treated soils.

Procedure

This paper presents a procedure for the rapid determination of trichloroacetic acid in fresh plant tissues.

Reagents

Standard Trichloroacetic Acid Solution. One gram of U.S.P. trichloroacetic acid is diluted to 1000 ml. with distilled water and further dilutions are made to obtain solutions with trichloroacetic acid contents ranging from 10 to 100 micrograms per milliliter.

Satured Sodium Hydroxide Solution. One hundred grams of ACS reagent grade sodium hydroxide is diluted to 100 ml. with distilled water.

Approximately 1 N Sodium Hydroxide Solution. ACS reagent grade sodium hydroxide (4.00 grams) is diluted to 100 ml. with distilled water.

Approximately 0.1 N Acetic Acid Solution. Glacial acetic acid (5.7 ml.) is diluted to 1,000 ml. with distilled water.

Pyridine. Reagent grade.

Preparation of Sample

Preparation of Homogenate. Weigh the tissue and cut it into approximately 2 mm. squares with a razor blade. Place in an homogenizer test tube and add 2 ml. of 0.1 N acetic acid for each 500 mg. of tissue. Homogenize with the Potter-Elvehjem (*6*) ground-glass tube and pestle until the tissue is ground to a creamy consistency.

Preparation of Plant Extract. Prepare an asbestos pad in a 42-mm. Hirsch funnel and dry the inside of the funnel stem with a cotton swab to minimize dilution of the sample. Filter the homogenate with moderate suction and save the filtrate which should be clear and free from all chlorophylls. Pipet a 1-ml. aliquot of the filtrate into a clean test tube and add 1 N sodium hydroxide (about 0.1 ml.) until the pH is between 8 and 10. A flocculent precipitate of proteinaceous material will indicate a slight alkalinity (see discussion). Refilter through an asbestos pad. The resulting filtrate should be clear. This final filtrate may be stored in a refrigerator overnight if necessary.

Colorimetry

Add 0.2 ml. of final filtrate and a small amount (about 0.025 ml.) of saturated sodium hydroxide to an 18 by 110 mm. test tube containing 3 ml. of pyridine. Stopper with a cork and shake vigorously 10 times. Remove the cork and place the tube in boiling water for 5 minutes to develop the characteristic red-violet color which is produced if trichloroacetic acid is present. Place in water at 25° C. for 3 minutes. Decant the pyridine layer and determine the light absorption after setting the colorimeter or spectrophotometer at zero with a blank. The blank contains all of the reagents and an aliquot of a filtrate from an untreated plant. The maximum absorption is at 510 mμ (*4*).

Standard Curve

Weigh 1.000 gram of USP grade trichloroacetic acid and make to 1 liter with 0.1 N acetic acid. Dilute with 0.1 N acetic acid to obtain a range of concentrations from about 10 to 100 mg. per liter. Adjust the pH of each solution with 1 N sodium hydroxide to a pH of 9.0. The quantity of base required for neutralization must be measured accurately in order to determine the final concentration of trichloroacetic acid in the solution for color development. Pipet 0.2 ml. of each corrected standard solution into the pyridine and develop the color as with the plant tissue extracts. Measure the intensity of the color against a blank which is similar to the standard solutions except that an aliquot of 0.1 N acetic acid is added instead of the aliquot of the appropriate trichloroacetic acid solutions. See Figure 1.

The pure trichloroacetic acid is very hygroscopic so that weighings must be made within a closed weighing bottle.

Calculation

Micrograms of trichloroacetic acid per gram of fresh tissue =

$$\frac{Y \times (R + S) \times (X + Q)}{T \times R \times X}$$

When:

Q = Volume (ml.) of acetic acid for homogenation

R = Volume (ml.) of initial filtrate to be neutralized

S = Volume (ml.) of 1 N sodium hydroxide required for neutralization

T = Volume (ml.) of final filtrate used for color development

X = Weight of tissue in grams

Y = Micrograms of trichloroacetic acid from colorimeter reading

The error introduced in the above calculation by using weight and volume interchangeably has been shown by direct measurement to be less than 0.4%.

Discussion

Clarification Of Extract

A pH of about 3.5, produced by the acetic acid extracting solution, is required to precipitate the chlorophyll and carotenoid pigments which are removed from the homogenate in the initial filtration. Further, the low dissociation con-

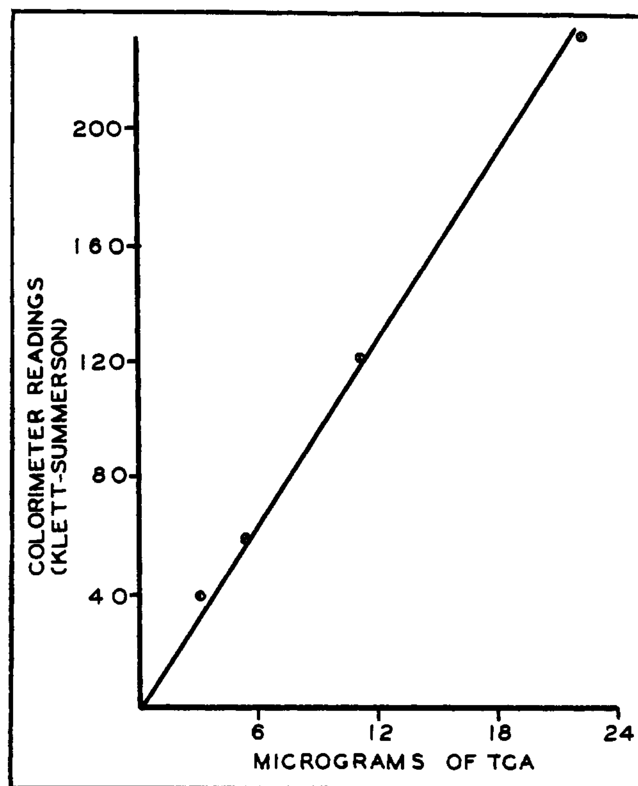

Figure 1. Standard curve indicating light absorption using known amounts of trichloroacetic acid

stant of acetic acid serves to minimize the differences in the acidity of the various plant tissues so that a relatively constant pH is maintained for all analyses. The filtrate resulting from the separation of the above pigments may at times be cloudy if an insufficient amount of asbestos has been used in making the filter pad, however, the second filtration in the procedure should serve to complete the clarification.

Adjustment of pH Of Solution

Initial Filtrate. One normal sodium hydroxide is added to the initial filtrate until the solution is above neutrality (between pH 8 and 10). It is well to make a direct determination of the acidity (with a pH meter or indicator solution) to determine the amount of sodium hydroxide needed the first time a new plant species or plant organ is studied. As the pH is raised above 8, a flocculent precipitate of proteinaceous material is produced, and the color of some of the water soluble pigments may be altered as well. Thus for repeated determinations upon similar tissue, these effects may be used to indicate the end point of neutralization. The rise in pH changes the flavones from a colorless to a bright yellow color, and shifts the anthocyanins from a purple-red to a yellow-brown color. An excess of sodium hydroxide will produce cloudiness when this final filtrate is added to the pyridine for color development, while insufficient sodium hydroxide makes it impossible to obtain the maximum color in the prescribed boiling period.

Final Solution. The quantity of saturated sodium hydroxide added to each tube of pyridine and final filtrate has been calculated to be in excess of the amount needed for color development. A small-bore medicine dropper has proved satisfactory for the addition of the small quantity of sodium hydroxide which is required.

Development of Color. It is important to control the length of time that the solution is boiled because the color intensity decreases as the heating is continued. The immediate cooling prevents any further rapid color change, and it has been established that the color will remain sufficiently stable for at least 15 minutes. After cooling, the colored solution is decanted into a colorimeter tube to separate it from the sodium hydroxide solution of higher specific gravity.

It is suggested that triplicate determinations of each extract of plant tissue be made because the values obtained for trichloroacetic acid from plant tissue were found to deviate about 3.24% from the average value. However, with standard trichloroacetic acid solutions the deviations were only 1.29%.

The color should be developed under a hood because pyridine is very volatile and the vapors are toxic. Pyridine and trichloroacetic acid contamination may be washed from the tubes with soap and water.

Measurement of Color. A Klett-Summerson photoelectric colorimeter was used with a green filter having a maximum transmission between 500 and 570 mμ. The scale reading of this instrument is directly proportional to the absorbance of the colored solution as measured by the photoelectric cell.

The colorimeter was set at zero with a blank prepared from untreated tissue, which was handled in the same manner as the treated tissue. This blank has a light yellow color which absorbs a small amount of light at 510 mμ. The untreated tissue from carrots, peas, snap beans, millet, spinach, and beets has not developed the characteristic pink color present in the extracts from the treated plants, thus indicating an absence of compounds within the plants which might interfere with the test.

TCA in Snap Beans

Trichloroacetic acid is particularly toxic to many monocotyledonous plants and to some specific dicotyledonous plants. The plant chosen for the analysis was snap bean (*Phaseolus vulgaris* var. Topcrop). This plant is sensitive to trichloroacetic acid and exhibits characteristic symptoms of injury at extremely low levels of the compound. The information presently available indicates that the most important avenue of entry of trichloroacetic acid is through the root system of the plant, although small amounts may enter through the leaves and stems.

This method has been used successfully for the determination of trichloroacetic acid in several plant tissues; among them, carrots, peas, snap beans, millet, spinach, and beets. For the study reported below, snapbeans were grown in 5 × 10 × 3 inch glass dishes containing 6.5 pounds of sterile sand. The plants were watered with a complete nutrient solution as required. They were held at a constant temperature and illuminated with fluorescent lights. Thirteen days after planting the beans, 15 mg. of commercial grade 80% trichloroacetic acid, as the sodium salt, was mixed with 150 ml. of the nutrient solution, and applied to the sand in a uniform manner. The unifoliate leaves of the plants were analyzed at successive periods following treatment to determine the rate of accumulation of trichloroacetic acid. Three unifoliate leaves from different plants were analyzed at each date, and three aliquots of each extract were taken for color development. The average amounts of trichloroacetic acid recovered from the leaves of beans in three different experiments are shown in Table I.

Table I. Rate of Accumulation of Trichloroacetic Acid in Bean Leaves

Days after Treatment	Micrograms per Gram of Fresh Tissue A	B	C	Average
1	106	159	124	130
2	321	275	270	289
3	478	467	388	444
5	536	500	421	486

Table II sets forth the quantities of trichloroacetic acid recovered from untreated tissue which was homogenized with acetic acid containing a known amount of trichloroacetic acid. The data presented were obtained from the analyses of three separate bean leaf samples and indicate that nearly complete recovery was obtained.

Table II. Recovery of Trichloroacetic Acid from Bean Leaves

Leaf Sample	TCA Added, γ	TCA Recovered, γ	% Recovery
1	367	368	100.3
2	367	357	97.3
3	367	362	98.6

Conclusion

A rapid, precise method is described for the determination of the concentration of trichloroacetic acid in plant tissue. Quantities of trichloroacetic acid as low as 25 micrograms per gram of fresh tissue may be extracted from plant tissue and measured colorimetrically. Data are presented to show the rate of accumulation of trichloroacetic acid in young bean plants.

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