Fluorometric Determination of Antibiotic Residues in Citrus Trees Injected with Tetracycline Hydrochloride

J. N. Moll

The use of tetracyclines as chemotherapeutants for the treatment of mycoplasma diseases in plants is well established (*1, 3, 6, 8, 9*). It is anticipated that these antibiotics will be put to use in commercial agriculture. Before tetracycline can be recommended for general use in agriculture, however, a sensitive and reliable method must be developed for the detection of antibiotic residues in plant material.

The technique currently used for such analysis is a bioassay. This technique is unsuited to the assay of antibiotics in citrus tissue because it lacks precision, and because bacteriostatic flavones are present in citrus tissue (*7*). Fluorometric techniques have been extensively used in the assay of antibiotics in human tissue and pharmaceutical preparations, and several are available for the extraction and fluorometric determination of tetracyclines (*2, 4, 5*). Such determination is possible because a chemically modified form of a tetracycline that is excited by light of 390-nm wavelength emits a fluorescence at 520 nm.

Attempts were made to screen the various fluorometric techniques and develop one that could be used for plant material.

EXPERIMENTS AND RESULTS

One of the principal difficulties associated with fluorimetry is the removal of extraneous materials that could cause background fluorescence. This is especially important in plant material, which contains more extraneous substances than does animal material. The extraction procedure found most suitable for plant materials was that used by Ibsen *et al.* (*4*). In this procedure, the problem of background fluorescence is largely overcome by using an extraction method that is relatively specific for tetracyclines, and by making two separate fluorescence readings. The first reading is taken when the tetracycline is present in the form of fluorescing magnesium chelate. The second is taken after this chelate has been disrupted by ethylene diaminetetra acetate (EDTA), which liberates the non-fluorescing, free form of the tetracycline. As the EDTA specifically splits the tetracycline-magnesium chelate (*4*), the difference between the two fluorescence readings reflects the concentration of tetracycline present.

Since trials to test the efficiency of different tetracyclines in controlling greening disease showed that tetracycline HCl gave the best results (*8*), it is likely that this form of the antibiotic will be recommended for the control of greening disease. For this reason the extraction procedure was tested more specifically for the detection of tetracycline HCl residues, with preliminary tests to evaluate its use in determining chlortetracycline HCl and oxytetracycline HCl residues.

The practicality of the extraction and analytical procedure was evaluated by testing fruit from citrus trees injected with tetracycline HCl one to nine months previously.

All measurements were made on a Ferrand Spectrofluorometer Mk-1, using an excitation wavelength of 390 nm and an emission wavelength of 520 nm. The tetracycline HCl was supplied by Cyanamid (Pty) Ltd. All chemicals used were of AR grade.

One ml of orange juice was made up to 5 ml by addition of 10 per cent

trichloroacetic acid (TCA). The precipitated protein was removed by centrifugation at 5,000 *g* for 10 minutes. Four ml of the supernatant were pipetted into 4 ml of amyl alcohol. The mixture was shaken well for 2 minutes and then centrifuged for 5 minutes at 3,000 rpm. Four ml of the alcohol layer were placed in a test tube with 4 ml of 7N NH_4OH and 1 ml of hexane fraction. The mixture was shaken and centrifuged as before. Four ml of the basic aqueous layer were pipetted into a cuvette, and 0.5 ml of $MgCl_2$ (4 μmoles) solution was added. The cuvettes were inverted against clean parafilm to obtain mixing, and allowed to stand for 15 minutes before reading. After reading, 0.1 ml of 0.1 M EDTA solution was added to each cuvette. The cuvettes were inverted against clean parafilm and allowed to stand for 30 minutes. They were then read and reread after a few minutes to ensure that stability had been reached. The differences between $MgCl_2$ and EDTA readings were compared with the differences obtained with standards that were similarly extracted.

Standards and blanks were extracted as described above. Standards were prepared by adding known amounts of tetracycline HCl to the fruit juice, and blanks by adding 1 ml of distilled water.

Two standard curves for known concentrations of tetracycline HCl were prepared. The first curve (table 1) was obtained by adding known concentrations of tetracycline HCl to the solution after extraction had been completed, thus obtaining the data for the full fluorescence of 100 per cent of the tetracycline HCl. The second curve (table 2) was prepared by adding known concentrations of tetracycline to the orange juice before extraction. These curves

TABLE 1

FLUORESCENCE OF VARIOUS CONCENTRATIONS OF TETRACYCLINE HCl ADDED TO ORANGE JUICE AFTER EXTRACTION

Conc. tetracycline HCl (μgm/ml juice)	Fluorescence		
	$MgCl_2$	EDTA	Difference
0.0	0.10	0.07	0.03
0.1	0.21	0.08	0.13
0.2	0.29	0.07	0.22
0.5	0.55	0.08	0.47

TABLE 2

FLUORESCENCE OF VARIOUS CONCENTRATIONS OF TETRACYCLINE HCl ADDED TO ORANGE JUICE PRIOR TO EXTRACTION

Conc. tetracycline HCl (μgm/ml juice)	Fluorescence		
	$MgCl_2$	EDTA	Difference
0.0	0.10	0.07	0.03
0.1	0.15	0.07	0.08
0.2	0.20	0.07	0.13
0.5	0.37	0.08	0.29
1.0	0.62	0.09	0.53

TABLE 3

MEAN FLUORESCENCES (n = 3) IN APRIL, 1972, OF FRUIT JUICES FROM TREES INJECTED WITH TETRACYCLINE HCl

Sample*	Fluorescence		
	$MgCl_2$	EDTA	Difference
1	0.16	0.14	0.02
2	0.16	0.14	0.02
3	0.16	0.14	0.02
4	0.15	0.13	0.02
5	0.15	0.14	0.01
6	0.16	0.14	0.02
7	0.15	0.14	0.01

* Treatments as follows: (1) Trees received no tetracycline HCl injection. (2) 1 liter distilled water per tree injected November, 1971. (3) 1 liter of 1,000-ppm tetracycline HCl per tree injected July, 1971. (4) 1 liter of 750-ppm tetracycline HCl per tree injected September, 1971. (5) 1 liter of 1,000-ppm tetracycline HCl per tree injected September, 1971. (6) 1 liter of 1,000-ppm tetracycline HCl per tree injected November, 1971. (7) 1 liter of 500-ppm tetracycline HCl per tree injected March, 1972.

can be used to determine the concentration of tetracycline HCl extracted from plant material.

When the juice of fruit from trees injected with tetracycline HCl was tested, the fluorescences obtained (table 3) were all on or below the threshold fluorescence of pure orange juice. As this method is accurate to about 0.05 mu mole of tetracycline it can be assumed that no tetracycline HCl residues were present in the materials tested.

CONCLUSIONS

No tetracycline HCl residues could be detected in trees one month after treatment. Some of the trees had received the dose that would normally be recommended as treatment of disease (*8*). The time recommended for injection is during the September flush period, which is about nine months before harvest (*8*). It would have been surprising if any residues had been detected in these trees, since even in pure aqueous solution, tetracyclines have a very short period of stability. For this reason, treatment 7, which was injected one month earlier, was included. Even in this case, no tetracycline residues could be detected. Thus tetracycline HCl injected during the September flush period for control of greening presents no health hazard as a result of residues.

The extraction and assay method based on that proposed by Ibsen *et al.* (*4*) is satisfactory for the purpose of tetracycline analysis in plant material. The extraction method removed most, but not all, other fluorescing materials. Because removal of extraneous fluorescing material is variable, it is recommended that the change in fluorescence caused by conversion of the Mg-tetracycline chelate to the non-fluorescing, free form be used as an index of concentration. Under the conditions employed, this change in fluorescence is apparently specific for tetracyclines. Nevertheless, extraction is necessary to reduce the background absorption, which interferes with the fluorescence of the tetracyclines.

This method was successful for the quantitative analysis of tetracycline HCl and oxytetracycline HCl, but not of chlortetracycline HCl. About 40 per cent of the original concentration of tetracycline HCl is lost during extraction. This method of analysis provides an accurate, sensitive means of analysing tetracycline HCl residues in plant tissue without the disadvantages associated with bioassay.

LITERATURE CITED

1. Frederick, R. J., M. Clein, and K. Maramorosch
1971. Acquisition and retention of tetracycline-hydrochloride by plants. Plant Dis. Reptr. **55**: 223–26.

2. Hayes, J. E., and H. G. Du Buy
1964. A simple method for quantitative estimation of tetracycline antibiotics. Analytical Biochem. **7**: 322–27.

3. Huhtanen, D. P., and R. H. Converse
1971. Temporary symptom remission of strawberry June yellows and witches-broom by use of oxytetracycline. Phytopathology **61**: 1137–39.

4. Ibsen, K. H., R. L. Saunders, and M. R. Urist
1963. Fluorometric determination of oxytetracycline in biological material. Analytical Biochem. **5**: 505–14.

5. Kelly, R. G., L. M. Peets, and K. D. Hoyt
1969. A fluorometric method of analysis for tetracycline. Analytical Biochem. **28**: 222–29.

6. Maramorosch, K., R. R. Granados, and H. Hirumi
1970. Mycoplasma diseases of plants and insects. Advan. Virus Res. **16**: 135–93.

7. PIATELLI, M., AND G. IMPELLIZZERI
1971. Fungistatic flavones in the leaves of citrus species resistant and susceptible to *Deuterophoma tracheiphila*. Phytopathology **10:** 2657–59.
8. SCHWARZ, R. E., J. N. MOLL, AND S. P. VAN VUUREN
1974. Control of citrus greening and its psylla vector by trunk injections of tetracyclines and insecticides, pp. 26–29, this volume.
9. WHITCOMB, R. F., AND R. E. DAVIS
1970. Mycoplasma and phytarboviruses as plant pathogens persistently transmitted by insects. Ann. Rev. Entomol. **15:** 405–64.