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PESTICIDE RESIDUES

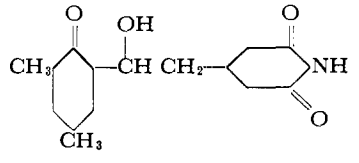
Determination of Cycloheximide (Acti-dione) Residues in Cherries

GEORGE C. PRESCOTT, HAROLD EMERSON, and JARED H. FORD

Department of Microbiology, The Upjohn Co., Kalamazoo, Mich.

The antibiotic cycloheximide has been found very effective in the control of cherry leaf spot. Because preharvest sprays are usually required, a method for determining cycloheximide residues on the fruit was desired. Chloroform extraction of the macerated fruit, followed by S. *pastorianus* bioassays of the extracts, detects as little as 0.04 p.p.m. in the fruit. When the ripe fruit on the tree was sprayed with cycloheximide, the residues had a half-life period of about 24 hours after the first day. This rate of inactivation is much greater than the rate in aqueous solutions of about the same pH and temperature and suggests that an enzyme system of the fruit may be involved.

CYCLOHEXIMIDE (Acti-dione), an antibiotic which was isolated in 1946 from streptomycin-producing strains of *Streptomyces griseus* (*3, 7, 13*), has been shown (*6*) to have the following structure:



It has been reported effective for control of cherry leaf spot at concentrations of 0.5 to 2.0 p.p.m. (*1, 2, 5, 8–11*). Because preharvest sprays are usually required in order to obtain satisfactory control of this disease, a method for determination of cycloheximide residues on the fruit was desired.

A bioassay method for cycloheximide which employs a yeast, *Saccharomyces pastorianus*, as the test organism has been described by Whiffen (*14*). As this method has a working range from about 1.5 to 8 p.p.m., a preliminary concentration step was required. It was found that a preliminary separation and concentration could be effected by extracting the cycloheximide from the macerated fruit with chloroform, evaporating the solvent from the extract, and preparing an alcohol-water suspension of the residue for the bioassay.

Procedure for Total Amount on Outside and Inside of Fruit

Grind a 500-gram sample of cherries (including pits and juice, when present) in a Waring Blendor for 2 to 3 minutes. Transfer the macerated product to a 2-liter flask containing 300 ml. of chloroform and boil the mixture under reflux for 45 minutes. Cool to room temperature and transfer to a separatory funnel. Draw off 250 ml. of the chloroform layer and evaporate to 10 to 20 ml. on a steam bath. Remove the last of the solvent by blowing air on it at room temperature. Suspend the dry residue in 1 ml. of S.D. 3A ethyl alcohol and dilute with 9 ml. of water. Bioassay the solution against *S. pastorianus* with a sufficient number of replicate assays to obtain the desired confidence limits.

Calculation of Results

The percentage of cycloheximide which theoretically could be recovered by this method, if the entire 300 ml. of chloroform extract were recovered, would be 92.6%. The calculation is as follows:

$$\frac{X}{100 - X} = \frac{21 \times 300}{500}$$

$$X = 92.6$$

where 21 = distribution coefficient of cycloheximide between chloroform and water

300 = volume of chloroform, ml.

500 = approximate volume of cherries, grams or ml.

As only 250 ml. of the chloroform extract is actually used, the theoretical recovery is $92.6 \times \frac{250}{300}$ or 77.2%. As the actual recovery is only 75% of theoretical (Table I), a further correction factor of 0.75 is added. In verifying the procedure, the actual recovery from macerated cherries was found to be from 60 to 84% of the theoretical recovery, with an average of about 75% (see Table I). This probably means that the distribution coefficient of cycloheximide between chloroform and macerated cherries is somewhat less than the distribution coefficient between chloroform and water. As the 10 ml. of solution used for the bioassay represents 500 grams of cherries, the cycloheximide content of the fruit is calculated by means of the following expression:

$$\text{Cycloheximide content, p.p.m.} = \frac{\text{bioassay result, } \gamma\text{/ml. (or p.p.m.)}}{50 \times 0.772 \times 0.75}$$

Table I. Recovery of Cycloheximide from Cherries

Type	P.P.M. Added	P.P.M. Recovered[a]	Av. % Recovery
Canned, sour (water pack)	0	0, 0, 0, 0	
	0.05	0.045, 0.040, 0.040, 0.040	82
	0.10	0.069, 0.069, 0.080, 0.078	74
	0.20	0.17, 0.15, 0.17, 0.15	80
Frozen, sour (sweetened)	0	0, 0, 0, 0	
	0.05	0.04, 0.04, 0.04, <0.04	
	0.10	0.066, 0.070, 0.072, 0.085	73
	0.20	0.12, 0.12, 0.10, 0.14	60
Fresh, sweet (picked about 10 days before it was ripe)	0	0, 0, 0, 0	
	0.053	0.04, <0.04, <0.04, <0.04	<75
	0.107	0.089, 0.094, 0.082, 0.094	84
	0.214	0.17, 0.18, 0.15, 0.19	81
Fresh, sour (picked about 5 days before it was ripe)	0	0, 0, 0, 0	
	0.053	<0.04, <0.04, <0.04, <0.04	<75
	0.107	0.052, 0.067, 0.080, 0.080	65
	0.214	0.14, 0.12, 0.19, 0.16	71

[a] Each cycloheximide extract from cherries was bioassayed (*13*) on 4 different days.

Verification of Procedure

Increments of 0.05, 0.10, and 0.20 p.p.m. of cycloheximide were added to various lots of cherries and the percentage of theoretical recovery was determined, using the procedure described above. The results are listed in Table I. The estimated standard error of the average of four assays of one extract from one sample was 12%. The estimated average recovery was 75%, with a 95% confidence interval from 62 to 87%.

Procedure for Amount on Outside of Fruit

Place a 500-gram sample of cherries in a 1-liter beaker, cover with water (about 300 ml.), and allow to stand overnight at room temperature. Decant the water and rinse with two 100-ml. portions of fresh water. Combine the aqueous solutions and extract four times with chloroform, using 200, 100, 50, and 50 ml. Combine the chloroform extracts. The remainder of the procedure, except for the calculation of results, is the same as for determining the total amount on the outside and inside of the fruit. The cycloheximide content of the fruit is calculated by means of the following expression:

$$\text{Cycloheximide content, p.p.m.} = \frac{\text{bioassay result, } \gamma\text{/ml. (or p.p.m.)}}{50}$$

Results on Sprayed Cherries

Although the method was found to be capable of detecting 0.05 p.p.m. of cycloheximide added to the picked fruit, it was not sensitive enough to detect residues on fruit that had been sprayed according to the recommended schedule. This involved a maximum spray concentration of 2 p.p.m. and intervals of at least 2 weeks between applications. In order to determine how long the residues would persist, two experiments were performed in which the trees with ripe fruit were sprayed with 30 p.p.m. solutions.

A Montmorency tree (Calhoun Orchards, Oshtemo, Mich.) was sprayed thoroughly with 30 p.p.m. of cycloheximide solution at 200 pounds per square inch pressure. The tree had been sprayed four times previously using 1.5 pounds of fixed copper and 3.0 pounds of lead arsenate per 100 gallons, applied at 2- and 2.5-week intervals, with the last application 2 weeks before the cycloheximide was applied.

The fruit was sprayed at 11:30 A.M. (86° F.) and the first sample was picked at 1:00 P.M. (90° F.). After 3 days another sample was picked and assayed. A control sample was picked before spraying to check for the presence of any materials which might interfere with the bioassays. The maximum temperatures during the days of the experiment were 92°, 87°, 92°, and 77° F. The results are listed in Table II.

A Montmorency tree (C. N. Manhoff, Williamsburg, Mich.) was sprayed thoroughly with 30 p.p.m. of cycloheximide solution at 200 pounds per square inch pressure. The previous sprays were as follows:

Date	Concentration, Lb./100 Gal.
June 7	1.5 ferbam + 3.0 lead arsenate
June 12	1.5 ferbam + 3.0 lead arsenate
June 23	1.5 ferbam + 3.0 lead arsenate
July 5	3.0 copper sulfate + 3.0 lime

It was reported that there had been no rainfall from July 5 to July 31. The fruit was sprayed about 4:30 P.M. and the first sample was picked as soon as it had dried. A control sample was picked before spraying to check for the presence of any materials which might interfere with the assays for cycloheximide.

The temperature during the experiment as reported by the Traverse City Airport was as follows:

	7:30 a.m.	12:30 p.m.	7:30 p.m.
July 21	61	67	69
Aug. 1	64	77	75
Aug. 2	66	76	72
Aug. 3	65	75	74

The weather was partly cloudy during this period, but there was no rainfall.

Samples of the fruit were picked at about 12-hour intervals and frozen with solid carbon dioxide. At the end of the third day the samples were brought back to Kalamazoo. The frozen samples were placed in beakers, covered with water, and allowed to stand overnight. The fruit and the water in which it had been soaked were assayed separately for cycloheximide, using the method above. The results are given in Table III.

Table II. Persistence of Cycloheximide Residues on Ripe Cherries

(Oshtemo, Mich. Sprayed with 30 p.p.m. solution July 12, 1954)

Time Elapsed after Spraying, Hours	Amount of Cycloheximide Found, P.P.M. Surface	Inside	Total[a]
1.5	0.09		0.20
1.5[b]	0.08	0.14	0.22
67	<0.04	<0.05	<0.05
Unsprayed control			<0.05
Unsprayed control + 0.10 ppm.[c]			0.09

[a] Determined by direct extraction of macerated whole fruit without previous soaking.
[b] Duplicate of first 1.5-hour sample held 7 days at 8° C. before extraction.
[c] Added in laboratory.

Table III. Persistence of Cycloheximide Residues on Ripe Cherries

(Williamsburg, Mich. Sprayed with 30 p.p.m. solution July 30, 1954)

Time Elapsed after Spraying, Hours	Amount of Cycloheximide Found, P.P.M. Surface	Inside	Total
1.5	0.08	0.13	0.22
15.5	0.07	0.16	0.23
26.5	0.05	0.19	0.24
38.5	0.03	0.12	0.15
48	<0.03	0.11	0.11–0.14
72.5	<0.03	0.06	0.06–0.09
Unsprayed control	<0.03	<0.05	<0.08
Unsprayed control + 0.15 p.p.m.[a]			0.15

[a] Added in laboratory.

Discussion

In both field experiments with 30 p.p.m. spray solutions the samples which

were taken at 1.5 hours were found to contain about 0.2 to 0.25 p.p.m. of cycloheximide. This indicates that the fruit cannot hold more than about 1% of its weight in spray solution. From Figure 1 it may be noted that the amount of

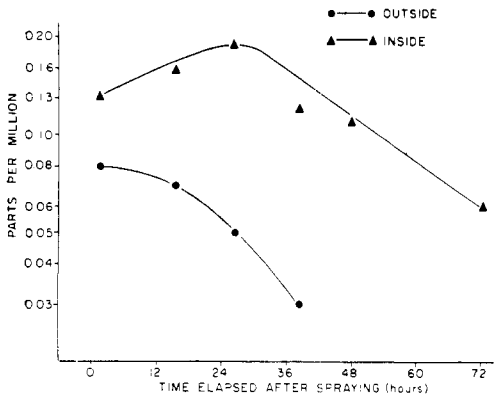


Figure 1. Persistence of cycloheximide spray residue on cherries sprayed with 30 p.p.m. solution

Average temperature 68° F.

antibiotic on the outside of the fruit which was removed by soaking in water decreased steadily, while the amount on the inside increased during the first 24 hours and then decreased. The relatively high proportion of the antibiotic found inside the fruit 1.5 hours after spraying may indicate a rapid absorption of the antibiotic during this period. Another possible explanation is that the antibiotic was absorbed by the fruit during the overnight soaking in water.

At the temperature of the experiment (61° to 77° F.) the half-life period of the cycloheximide residue was about 24 hours after the first day. This was estimated from the slope of the lines in Figure 1. As solutions of cycloheximide in dilute acetic acid (pH 3.3) have been found (*4*) to retain about two thirds of their activity after 3.5 months at 25° C., the rate of inactivation in the ripe cherries is much more rapid and may be due to the presence of an enzyme system in the fruit. The enzyme hypothesis receives further support from the fact that the antibiotic in canned cherries stored at room temperature has been found to lose about one fourth of its activity in 6 months (*12*).

Interfering Substances

Several products that are frequently applied to cherry trees for the control of other diseases were diluted with water to give the concentrations recommended by the manufacturer. The resulting suspensions were extracted with chloroform, the solvent was removed from the extracts, and the residues were bioassayed by the procedure used for the cycloheximide residues. Dieldrin, methoxychlor, lead arsenate, basic copper, and glyodin all failed to give inhibition zones on the *S. pastorianus* assay plates. The ferbam and captan both gave zones of inhibition and these substances would interfere with the assays for cycloheximide if they were present.

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TASTE PERCEPTION

Reliability of Phenylthiocarbamide–Sodium Benzoate Method of Determining Taste Classifications

EDWARD F. HOOVER
Wise Potato Chip Co., Berwick, Pa.

The phenylthiocarbamide–sodium benzoate method of determining taste classifications of individuals is unreliable, because most persons do not give reproducible taste reactions with at least one of the two chemical compounds involved. One of the principal factors affecting the reproducibility is the inability of some individuals to define standard tastes correctly. Therefore the problem of classifying individuals into groups according to their taste perceptions remains unsolved and is much more complex than might be inferred from methods proposed earlier.

In 1954 Fox (*3*) proposed a method for determining taste classifications of individuals based on interpretation of the flavor produced by two chemical compounds, phenylthiocarbamide (PTC) and sodium benzoate. The former is described by some people as intensely bitter, while others find it absolutely tasteless. This condition is known as "taste blindness" (*4*) and has been adequately shown to be caused by hereditary differences in individuals (*5, 7, 8*). Sodium benzoate, on the other hand, has been found either to be tasteless or to produce any of the four primary taste sensations—sweet, bitter, sour, or salty—depending upon the individual taster. This compound is utilized to divide each of the two classes obtained with phenylthiocarbamide (bitter or tasteless) into five separate subgroups. Thus, the