residues in excess of 0.01 p.p.m. of metabolites in the milk of cows fed treated forage.

A study of the cows on the 2.8-p.p.m. feeding rate showed that 1 to 2 days after the feeding of Guthion ceased the residues in milk had disappeared.

Residue data for tissues from animals fed at the equivalent of 1.4 p.p.m. and sacrificed on the last day of treatment are presented in Table VII. In all cases the residues were less than 0.1 p.p.m. For ease of comparison, results for the tissues are expressed on the same basis as for milk, although no direct evidence is presented to show that the metabolites in tissue are the same as those in milk. In all probability they are.

Conclusions

The radioactive studies have established that the fluorometric method (*1*) is satisfactory for determining Guthion residues in milk and tissues. The residues observed in milk are due to four unidentified phosphorus-free metabolites which still contain the benzazimide moiety. One metabolite accounts for 85 to 90% of the residue observed. Comparison of radiochemical and fluorometric results for aliquots of the same extract indicates that the yield of anthranilic acid from the metabolites is at least 76% of theoretical. If Guthion is used according to label recommendations, the residue in the fresh forage at harvest will usually be less than 1 p.p.m. and always less than the tolerance level of 2 p.p.m. The residue in milk from feeding forage containing 2 p.p.m. of Guthion will be 0.008 p.p.m., expressed as mercaptomethylbenzazimide. Under the same conditions, tissue residues will not be in excess of 0.1 p.p.m.

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RESIDUE DETERMINATION

Spectrophotofluorometric Method for Guthion Residues in Milk and Animal Tissues

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A sensitive fluorometric method has been developed for the determination of Guthion [O,O-dimethyl *S*-4-oxo-1,2,3-benzotriazin-3(4*H*)-ylmethyl phosphorodithioate] residues in milk and animal tissues. The oxygen analog is also determined by the procedure described. The method involves an alkaline hydrolysis to give anthranilic acid which, after adjustment of the pH, is extracted into benzene and determined fluorometrically at an activating wavelength of 340 mμ and a fluorescence wavelength of 400 mμ. The sensitivity of the method is about 0.005 p.p.m. for milk, 0.02 p.p.m. for most animal tissues, and 0.03 p.p.m. for fat.

In residue analysis of crucial food products such as milk, greater sensitivity is required than for most food commodities. It was apparent that a residue method for Guthion, more sensitive than the colorimetric methods previously described (*1*, *3*), would be desirable for studies involving milk. Since previous work (*3*) has shown that Guthion is very easily hydrolyzed to anthranilic acid, a very fluorescent compound, the possibility of developing a fluorescence method suggested itself. Preliminary work indicated that the sensitivity of such a procedure would be excellent and dependent only on the degree to which the background fluorescence could be reduced.

A preliminary method was developed which gave background values for milk of about 0.03 p.p.m. (*2*). The procedure involved a column cleanup with Florisil, followed by fluorescence measurement in buffer solution. Some analyses were performed using the procedure, although it was realized that a better cleanup would eventually be required (*2*).

Consideration of the effect of hydrogen ion concentration on the fluorescence of anthranilic acid led to the hypothesis that pH 4.1 might represent the "isoelectric point" of the compound, and that at this pH it might be possible to extract anthranilic acid quantitatively from an aqueous solution with an immiscible nonpolar solvent, such as benzene, and thereby effect an additional measure of cleanup. This proved to be the case. It was found that the compound could be quantitatively extracted from a pH 4.1 buffer solution using an equal volume of benzene. There was essentially no difference in the fluorescence readings of samples buffered over the range of pH 4.0 to 4.2.

Application of the "isoelectric extraction" procedure to control milk samples indicated that the additional cleanup step, by itself, would not give control values of 0.01 p.p.m. or less. However, further work showed that a combination of chromatography on acid-washed aluminum using chloroform as solvent and isoelectric extraction would give the desired control values.

Apparatus

Chromatographic tubes, 20 × 400 mm., equipped with Ultramax stopcock and integral 300-ml. reservoir.

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Spectrophotofluorometer, Aminco-Bowman or equivalent.

Reagents

Alumina, acid-washed, chromatographic grade, Merck catalog No. 71695.

Buffer-Base Solution. Dissolve 325 grams of $Na_2HPO_4 . 7H_2O$, 191 grams of citric acid, and 145 grams of potassium hydroxide (85% minimum) in distilled water and dilute to 1 liter.

Guthion Blank Solution. Mix 30 ml. of benzene with 30 ml. of 0.5*N* potassium hydroxide in isopropyl alcohol. Continue through the remainder of the hydrolysis procedure, scaling up the entire procedure by a factor of 3.

Guthion Increment Solution. Prepare this in the same way as the blank solution, but start with 30 ml. of benzene containing 40 μg. of Guthion per ml.

Redistill all solvents in an all-glass apparatus.

Procedure

Extraction. Weigh 200 grams of milk (100 grams of tissue, 50 grams of fat) into a Waring Blendor jar and add 5 grams of Super-Cel. Add 400 ml. of acetone and blend for 5 minutes. Filter the sample, under suction, through Whatman No. 42 filter paper until the filter cake appears dry. Return the filter cake to the blender jar, add 200 ml. of benzene, and blend for 5 minutes. Repeat the filtration step and wash the filter cake with 100 ml. of fresh benzene. Transfer the combined filtrate to a 1000-ml. separatory funnel. Rinse the suction flask with 100 ml. of fresh benzene and add the rinsings to the contents of the separatory funnel. Shake the sample vigorously and allow the phases to separate. Draw off and discard the lower aqueous layer. Add 2 grams of Super-Cel to the contents of the separatory funnel, shake, and filter through a Whatman No. 12 fluted filter paper into a 1000-ml. beaker. Rinse the separatory funnel with 100 ml. of acetone and use the rinsings to rinse the filter paper. Evaporate the filtrate just to dryness on a steam bath under an air jet. Do not overheat.

Dissolve the residue in 100 ml. of Skellysolve B saturated with acetonitrile and transfer to a 250-ml. separatory funnel. Extract the Skellysolve B solution by shaking vigorously with 50 ml. of acetonitrile previously saturated with Skellysolve B. Allow the phases to separate and draw off the lower (acetonitrile) phase into a second 250-ml. separatory funnel containing 100 ml. of fresh Skellysolve B saturated with acetonitrile. Shake the second separatory funnel vigorously, allow the phases to separate, and draw off the lower (acetonitrile) phase into a 150-ml. beaker. Repeat the extraction sequence twice with 25-ml. portions of acetonitrile. Evaporate the combined acetonitrile extracts just to dryness on a steam bath under an air jet. Do not overheat.

Chromatography. Dissolve the residue from the acetonitrile evaporation in 15 ml. of chloroform and chromatograph on a column prepared as follows:

Tamp a pledget of glass wool into the bottom of a chromatographic tube. Pour in 5 grams of Super-Cel and tap the column on a wood block until the trapped air is liberated. Pour a slurry of 15 grams of alumina in chloroform into the column. Rinse the beaker and walls of the reservoir and column with 100 ml. of chloroform. After the alumina has settled, add a pledget of glass wool to the top of the alumina layer.

Pour the sample solution onto the column just as the last of the chloroform rinse passes into the glass wool, and change receivers. Rinse the sample beaker twice with 25 ml. of fresh chloroform and transfer each rinse to the column just as the last of the previous chloroform addition passes into the glass wool.

Elute the column with an additional 150 ml. of chloroform and collect a total of 200 ml. of effluent in a 250-ml. beaker. Evaporate the sample almost to dryness on a steam bath under an air jet. Continue evaporation to dryness under a jet of air at room temperature.

Hydrolysis and Separation of Anthranilic Acid. Transfer the residue into a 60-ml. separatory funnel with two 5-ml. portions of benzene. Add 10 ml. of 0.5*N* potassium hydroxide in isopropyl alcohol, and mix. Hydrolyze at room temperature for 30 minutes. Add 10 ml. of benzene to the sample in the separatory funnel. Extract the combined benzene–isopropyl alcohol solution with 10 ml. of 1.67*N* hydrochloric acid. After the solvent layers separate, draw off the lower (aqueous) layer into a 125-ml. separatory funnel. Repeat the extraction, using 5 ml. of the acid solution. Add 10 ml. of buffer-base solution and 20 ml. of benzene to the combined acid extracts in the separatory funnel and shake vigorously for 30 seconds to extract. Allow the solvent layers to separate and draw off and discard the bottom (aqueous) layer.

Fluorescence Measurement. Pipet 5-ml. aliquots of the benzene solution into each of two test tubes, labeling the tubes A and B. Pipet 2.0 ml. of the blank solution into tube A and 2.0 ml. of Guthion increment solution into tube B, and shake to mix thoroughly. The internal standard corrects for any quenching of the fluorescence resulting from the presence of sample extractives.

Transfer aliquots of samples A and B to spectrophotofluorometer cuvettes and measure the fluorescence at an activating wavelength of 340 mμ and fluorescence wavelength of 400 mμ. The instrument sensitivity is arbitrarily set so that a solution of quinine sulfate (2.5 μg. per ml.) in 0.1*N* sulfuric acid reads 0.50 fluorescence unit on the photometer. The fluorescence of the quinine solution decreases about 5% per week. Therefore, although changes in the fluorescence of the quinine standard will have no effect on the results of an individual analysis by the increment method, for comparative purposes, it is recommended that this solution be prepared fresh weekly.

A plot of the fluorescence reading *vs.* concentration of hydrolyzed Guthion (anthranilic acid) is linear through a concentration of 10 μg. per ml. Thus, the linear range extends from the limit of sensitivity through fluorescence values equivalent to 0.6 p.p.m. of Guthion for milk, 1.2 p.p.m. for meat, and 2.4 p.p.m. for fat.

Table I. Recovery of Guthion Added to Animal Products

Compound	Animal Product	Guthion Added, P.P.M.	Recovery, %
Guthion	Milk	0.10	82
		0.20	111
		0.20	112
		0.20	116
	Liver	0.20	89
	Ground chuck	0.20	83
	Heart	0.20	93
	Kidney	0.20	88
	Brain	0.20	106
	Fat	0.40	79
Guthion oxygen analog	Milk	0.10	67
		0.20	96
		0.20	103
		0.20	95
	Liver	0.20	71
	Ground chuck	0.20	77
	Heart	0.20	74
	Kidney	0.20	70
	Brain	0.20	94
	Fat	0.40	64

Calculations. Let A = fluorescence due to Guthion in 1/4 of original sample.

B = fluorescence due to Guthion in 1/4 of original sample plus fluorescence of 40 μg. of added Guthion.

$B - A$ = fluorescence due to 40 μg. of added Guthion.

C = sample weight—200 grams for milk, 100 grams for tissues except fat, and 50 grams for fat.

$$\text{Then, Guthion, p.p.m.} = \frac{(A)(40)(4)}{(B - A)(C)}$$

For milk this reduces to $0.8\ (A)/(B - A)$; for animal tissues, except fat, to $1.6\ (A)/(B - A)$; and for fat, to $3.2\ (A)/(B - A)$.

These calculations will introduce an error in the determination of Guthion residues if any appreciable quantity of the oxygen analog or any of several benzazimide moieties of Guthion are present. The error introduced will vary depending upon the relation of the molecular weight of the compound to the molecular weight of Guthion.

Discussion

Control values and consequently sensitivity vary with the animal product analyzed. Control values for milk ranged from 0.003 to 0.007 p.p.m. The mean for 21 determinations was 0.004, with an average deviation of 0.001. Only very limited control data are available for tissues. Values for animal tissues, except fat, were all less than 0.02 p.p.m. Controls for fat were 0.03 p.p.m.

In view of the control values encountered, and the common practice of considering as significant only net

residues equivalent to the control value, the sensitivity of the method when applied to milk is considered to be 0.005 p.p.m.; when applied to animal tissues, except fat, 0.02 p.p.m.; and when applied to fat, 0.03 p.p.m.

Recovery experiments were carried out by adding known amounts of Guthion or its oxygen analog at the initial blending step and processing by the procedure described above. The recoveries for various animal products given in Table I indicate that the method is satisfactory for both Guthion and its oxygen analog.

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METHOD OF ESTIMATION

Estimation of Malathion by Oxidation with Chloramine-T

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Recently a titrimetric method for the estimation of parathion by oxidation with chloramine-T was described. A rapid titrimetric method based on the oxidation of O,O-dimethyl dithiophosphoric acid by chloramine-T has been worked out for the estimation of malathion.

MALATHION [*S*-(1,2-dicarbethoxyethyl) *O,O*-dimethyl dithiophosphate] is an important pesticide widely used in plant protection. Its determination is therefore of great interest.

Polarographic (*2*) and colorimetric (*4*) methods are available for the estimation of malathion. The colorimetric method is based on its decomposition in carbon tetrachloride–ethyl alcohol solution by alkali to give sodium dimethyl dithiophosphate and sodium fumarate. The sodium dimethyl dithiophosphate is extracted into water and converted into the copper complex of dimethyl dithiophosphoric acid, which is then extracted into carbon tetrachloride and determined colorimetrically at 418 mμ.

A rapid titrimetric method based on the oxidation of *O,O*-dimethyl dithiophosphoric acid by chloramine-T has been worked out for the estimation of malathion, similar to the one for parathion (*3*).

The thio compounds of various oxy acids of phosphorus react with water to give hydrogen sulfide by a process in which sulfur is replaced by the oxygen of the water (*5*). This hydrogen sulfide can be oxidized quantitatively to sulfuric acid in acid medium (*6*) by an excess of chloramine-T, which can then be estimated iodometrically.

Reagents

Malathion. Samples of 95% and 99.50% purity supplied by the American Cyanamid Co., New York, N. Y.

Chloramine-T (0.1*N*). About 15 grams of recrystallized sample of pure chloramine-T is dissolved in 1 liter of water and stocked in an amber-colored bottle. The solution is standardized iodometrically in an acid medium (*1, 5*).

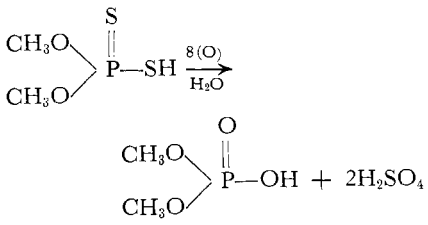
Sodium thiosulfate, 0.1*N*.
Dilute sulfuric acid, 2*N*.
Potassium iodide, 5% (w./v.)
Ethyl alcohol (aldehyde-free)
Alcoholic potassium hydroxide, 1*N*.

Procedure

Fifty to 80 mg. of the malathion sample are accurately weighed into a 250-ml. round-bottomed flask, and 30 ml. of alcoholic potassium hydroxide are added and refluxed gently on a water bath for 30 minutes. After cooling, the alkali is neutralized by dilute sulfuric acid and 25 ml. of acid are added in excess. Then 50 ml. of chloramine-T are added to the flask, which is well shaken and kept aside for about 30 minutes. At the end of this period, 25 ml. of potassium iodide solution are added and the liberated iodine is titrated with sodium thiosulfate using starch as the indicator. The amount of chloramine-T consumed by the malathion is obtained from the titer value and the number of equivalents of the oxidant required per mole of malathion is calculated. No blank correction is necessary, as it is less than 0.05 ml. of thiosulfate.

The analytical values are reproducible and the results of a few experiments are given in Table I.

It appears that 16 equivalents of chloramine-T react with 1 mole of *O,O*-dimethyl dithiophosphoric acid formed from 1 mole of malathion as indicated by the equation



Therefore 1 ml. of 0.1*N* chloramine-T is equivalent to 2.064 mg. of pure malathion.

When malathion is estimated in commercial formulations, impurities may consume chloramine-T and the value reported may be too high. This limitation has to be kept in mind before applying this method of analysis for such samples.

Table I. Moles of Chloramine-T Required per Mole of Malathion

Moles of Malathion Taken for Experiment × 10^4	Equivalents of Chloramine-T Required, × 10^4	Equivalents Required per Mole of Malathion
1.248	20.09	16.10
2.300	36.98	16.08
1.656	26.68	16.11
1.069	17.32	16.20
1.254	20.30	16.19
2.313	37.35	16.14
2.124	34.20	16.10
1.342	21.50	16.03
1.753	28.35	16.12
0.925	14.85	16.06