Three $R_{50}$'s were obtained for trinitrobenzene-aniline on three groups of house mice trapped in the wild at three locations within a 70-mile radius of Denver, Colo. The $R_{50}$'s for the three groups of mice were 12.7, 13.8, and 15.9 mg. per sq. inch, all falling within the allowable confidence limits. This greatly increases confidence in the method for predicting results with wild rodent populations.

The results of tests with tetrakis(laurylammino)boronium chloride show that much less chemical was required to protect cotton than burlap (Table II). The $R_{50}$ of tetrakis(laurylammino)boronium chloride for house mice was 23.8 mg. per sq. inch for 10-ounce burlap and only 4.0 mg. per sq. inch for $4^1/_4$ ounces per yard cotton. In other tests with house mice not herein reported, paper (multiwall bags) required less repellent per square inch than cotton, and polyethylene (1.5 to 6 mil) required more than burlap. This difference is attributed to the natural resistance of these materials.

There did not appear to be a great difference between reactions of male and female mice to repellents. Differences in ages of male, female, and mixed sexes may have accounted for the slight, but not significantly different, results obtained, in tests with white mice. It was hoped that white mice, which are easier to obtain and handle, could be used interchangeably with house mice, but white mice obtained from two different sources reacted differently. The limits of the $R_{50}$ for tetrakis(laurylammino)boronium chloride varied from 2 to 6 mg. per sq. inch for mice from one source and 9 to 15 mg. per sq. inch for mice from another source.

**Relationships of Effectiveness to Other Factors.** Effectiveness is the first and most important consideration in the development of a chemical as a successful rodent repellent treatment for packaging, but other properties such as stability, use hazards, and cost must also be favorable. Thus, a compound that possesses only one-fifth or one-tenth the activity of the most effective rodent repellent may prove to be a more useful packaging protectant.

Results obtained show that the concentration-repellent effect technique can be used to measure reliably the comparative effectiveness of various repellents for protecting packaging materials against rodent damage.


### Acknowledgment

Important contributions to this study were made by coworkers Robert I. Starr and Ralph W. Dutton of the Denver Wildlife Research Center.


### Literature Cited


(1) Bellack, C., DeWitt, J. B., Triechler, R., "Relationship between Chemical Structure and Rat Repellency," Chem-Biological Coord. Center Rev. No. 5, 48–156 (1953).
(2) DeWitt, J. B., Besser, J. F., *Pest Control* **26,** No. 8, 22 (1958).
(3) Litchfield, J. T., Wilcoxon, F. W., *J. Pharmacol. Exptl. Therap.* **96,** No. 2, 99–113 (1949).
(4) Spector, W. S., ed., "Handbook of Toxicology," Vol. **5,** p. 84, Saunders, Boston, 1956.
(5) Tigner, J. R., Besser, J. F., "Method Used in Evaluating Rodent Repellents for Packaging Materials," U. S. Fish Wildlife Serv., Denver, unpublished rept. 1961.
(6) Weeks, J. R., J. AGR. FOOD CHEM. **7,** 193 (1959).
(7) Welch, J. F., *Ibid.*, **2,** 142 (1954).



*Received for review September 18, 1961. Accepted January 11, 1962. Work supported by Research and Development Command, U. S. Army Quartermaster Corps, Natick, Mass.*


**INSECTICIDE RESIDUES**

# Estimation of Insecticide Residues in Foods through Parallel Screening Methods


**W. F. PHILLIPS, M. C. BOWMAN, and R. J. SCHULTHEISZ**

**Campbell Soup Co., Camden, N. J.**



**A parallel screening system has been developed which provides a basis for detection, characterization, and estimation of most of the insecticides that inhibit cholinesterase or contain chlorine. The system consists of bioassay, organic chlorine, and acetylcholinesterase inhibition analyses of the same extract. Further information is obtained by mathematical treatment of the data which provides a high degree of selectivity. Data are presented for toxicants that are representative of the two major classes of insecticides.**


THE DETERMINATION of insecticide residues in food products presents a complex challenge because it is necessary to show that no tolerance is exceeded. The problem is greatly magnified when complete spray history information is unavailable, which often is the situation confronting the food processor. The processor is also faced with the further complication that the analytical work on many products must be completed within a short time so that quality and factory operation are unaffected. To expect specific methods to be applicable on a routine basis is impractical mainly because of the large number of compounds and variety of materials to be analyzed.

The analytical system described herein provides a basis for detection, characterization, and estimation of most of the insecticides that inhibit cholinesterase or contain chlorine. The system consists of bioassy, organic chlorine, and acetylcholinesterase inhibition tests on each extract; hence the term parallel screening. In addition to the data from each determination, two additional information factors are obtained that aid the analyst in estimating the significance and identity of the residue present: the product of the $LD_{50}$ value and parts per million organic chlorine, and the ratio of $LD_{50}$ to $AChEI_{50}$. These two factors relate the in vivo system with the chemical determinations. The $LD_{50}$ is the dose required to kill 50% of the fly population, and the $AChEI_{50}$ is the amount of toxicant required to decrease acetylcholinesterase enzyme activity 50%, hereafter designated as $I_{50}$.

### Procedures

**Sample Preparation.** The stripping procedure varies with the particular crop and its mechanical-physical properties. Maceration with 2 ml. of benzene per gram of sample followed by either filtration or centrifugation is preferred when a suitable volume of extract is recovered. In the event of poor extract recovery, benzene–2-propanol (2:1) is used at the rate of 3 ml. per gram of sample. The 2-propanol is removed by water-washing

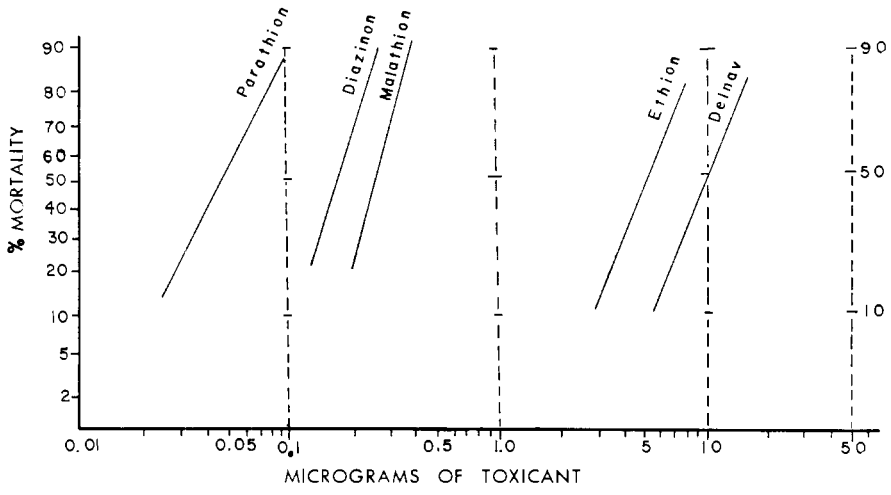


Figure 1. Mortality curves for five organophosphates

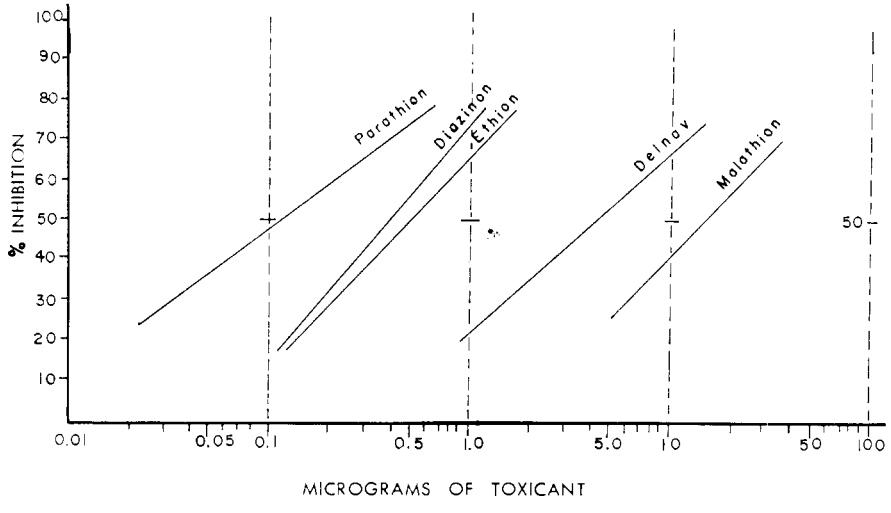


Figure 2. Per cent acetylcholinesterase inhibition for five organophosphates

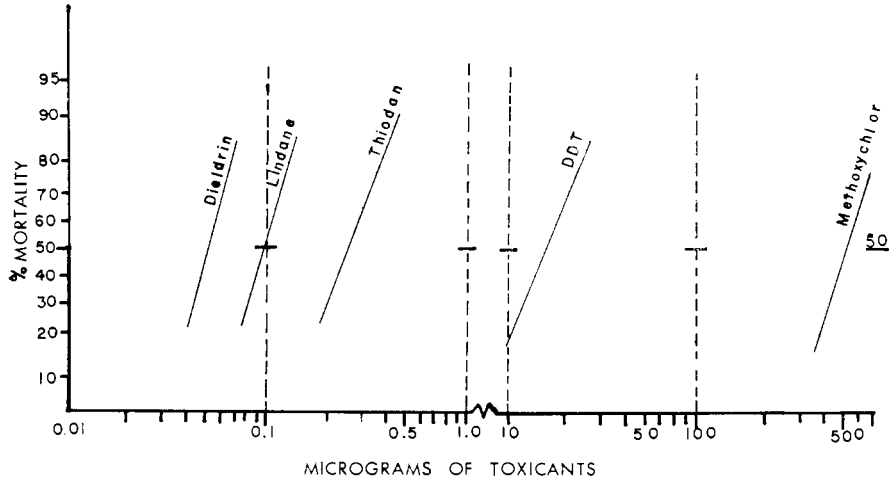


Figure 3. Mortality curves for five chlorine-containing toxicants

prior to cleanup. Petroleum ether and 2-propanol combinations were found unsatisfactory since several organophosphates are not recovered from the charcoal cleanup.

The benzene strippings are cleaned up by shaking an appropriate aliquot with activated charcoal (0.05 gram of Darco KB per gram of sample) for 3 minutes and then filtering. The residue is washed with two separate 20-ml. portions of benzene saturated with water, and the filtrates are combined. Aliquots of this solution equivalent to 0.2 and 2.5 grams of sample are taken for enzyme inhibition. Appropriate aliquots for bioassay and organic chlorine are evaporated to dryness on a steam bath under a gentle current of air. These residues are dissolved in petroleum ether for the bioassay and organic chlorine determination.

**Table I. Estimation of $LD_{50}$ Value[a]**

| Sample Weight (Grams) | $D_1$ | $A_1$ | $A_2$ | $D_2$ | Total | % Mortality |
|---|---|---|---|---|---|---|
| 0.25 | 5 | 15 | 35 | 5 | 40 | 12.5 |
| 0.50 | 8 | 12 | 20 | 13 | 33 | 39 |
| 1.0 | 14 | 6 | 8 | 27 | 35 | 77 |
| 2.5 | 18 | 2 | 2 | 45 | 47 | 96 |
| 5.0 | 20 | 0 | 0 | 65 | 65 | 100 |

[a] $LD_{50} \cong 0.6$ gram.

**Bioassay.** The bioassay procedure is essentially that described by Dewey (*2*, *3*) employing *Drosophila melanogaster*. Flies 7 to 31 hours old are exposed to a dry film of extract in tubes (25 × 200 mm.) for 24 hours. This technique was modified with respect to dosage, number of tubes, and computation of $LD_{50}$ values.

A series of aliquots representing 0.25, 0.5, 1.0, 2.5, and 5.0 grams of unfortified sample is bioassayed without replication, and the $LD_{50}$ value is estimated by an adaptation of the method of Reed and Muench (*7*). In brief, this method is based on the assumption that flies surviving a given dosage would survive a lesser dose, and those that die at a given dosage would not survive a greater dose. An example of this is shown in Table I. Column $A_1$ is added from the bottom up, and the subtotal for each concentration is entered in column $A_2$; column $D_1$ is added from the top down, and the subtotals are entered in column $D_2$. The per cent mortality for each concentration is calculated from the values in columns $A_2$ and $D_2$. The mortality values are plotted on the probability scale of log-probit paper and the dosage on the log scale. The $LD_{50}$ value is estimated from the best straight line connecting the plotted points. This method generally produces points from which a straight line can be more readily drawn and usually produces slightly more conservative values than the conventional methods.

**Total Organic Chlorine.** The organic chlorine procedure is a modification of the methods described by Lisk (*4*) and Bergman and Sanik (*1*). The combustion is performed in an F & M, Model 141 Schoniger oxygen flask assembly, and a 2-liter flask equipped with a side arm and rubber balloon is employed. The sample carrier is a cylindrical plug of dental cotton approximately 10 mm. in diameter and 20 mm. in length. The cotton plugs prior to use are extracted in a Soxhlet apparatus with acetone for 20 hours, washed with distilled water, re-extracted with distilled water for 30 hours, and

**Table II. Toxicity and Product Values for 16 Chlorine-Containing Insecticides**

| Compound | $LD_{50}$ (μg.) | Chlorine Conversion Factor | Product ($LD_{50}$, μg. Cl) |
|---|---|---|---|
| Ronnel | 0.02 | 0.333 | 0.0066 |
| DDVP | 0.05 | 0.322 | 0.0166 |
| Dieldrin | 0.05 | 0.559 | 0.0279 |
| Aldrin | 0.05 | 0.583 | 0.0293 |
| Dibrom | 0.05 | 0.605 | 0.0305 |
| Lindane | 0.10 | 0.731 | 0.0731 |
| Chlordane | 0.20 | 0.693 | 0.1386 |
| Thiodan | 0.27 | 0.52 | 0.1404 |
| Endrin | 0.30 | 0.559 | 0.1677 |
| Heptachlor | 0.30 | 0.665 | 0.1995 |
| BHC | 1.0 | 0.731 | 0.731 |
| Toxaphene | 10 | 0.67 | 6.7 |
| Chlorobenzilate | 32 | 0.218 | 6.72 |
| DDT | 15 | 0.50 | 7.5 |
| TDE | 18 | 0.442 | 7.56 |
| Methoxychlor | 500 | 0.308 | 154 |

**Table III. Toxicity, $I_{50}$, and Ratio of $LD_{50}$ to $I_{50}$ for 16 Organophosphates**

| Compound | $LD_{50}$, μg. | $I_{50}$, μg. | Ratio |
|---|---|---|---|
| Schradan | 0.01 | 500 | 0.00002 |
| Phosdrin | 0.01 | 4 | 0.0025 |
| Methyl parathion | 0.05 | 10 | 0.005 |
| Dipterex | 0.04 | 4 | 0.01 |
| Malathion | 0.3 | 16 | 0.019 |
| Demeton | 0.75 | 30 | 0.025 |
| Ronnel | 0.02 | 0.7 | 0.029 |
| Dibrom | 0.05 | 1.0 | 0.05 |
| DDVP | 0.05 | 1.0 | 0.05 |
| Guthion | 1.0 | 14 | 0.071 |
| Diazinon | 0.18 | 0.4 | 0.45 |
| Parathion | 0.05 | 0.1 | 0.5 |
| Trithion | 0.8 | 1.0 | 0.8 |
| Delnav | 10 | 4 | 2.5 |
| Ethion | 5.6 | 0.5 | 11.2 |
| Co-Ral | 300 | 0.1 | 3000 |

then dried in an oven at 60° to 70° C. The cotton, thereafter, is carefully handled with clean forceps.

The center of one end of a cotton plug is pulled out to form a thin fuse about 40 mm. in length. The plug is then placed in the platinum basket of the ignition head, and the end of the fuse is wrapped around the ignition wire.

Solvent extracts following cleanup are concentrated, and a suitable portion is applied to the positioned cotton plug. Combustion of the sample should not be undertaken until all of the solvent has evaporated. The volume of solvent used in the transfer should be held to a minimum; less than 1 ml. is usually sufficient.

The combustion head with sample is placed in position in a clean 2-liter flask that contains 15 ml. of distilled water and that has been purged with oxygen. The sample is electrically ignited. After combustion and cooling, the flask is removed from the assembly and shaken vigorously for 3 minutes. The aqueous solution in the ignition flask is removed, and the flask is washed twice with 5-ml. portions of distilled water. The solutions are combined; the volume is adjusted to 25 ml. and transferred to a 125-ml. glass-stoppered flask. Five milliliters of concentrated $HNO_3$, 1.0 ml. of aqueous ferric ammonium sulfate solution, 0.3 gram of $[FeNH_4(SO_4)_2 \cdot 12\ H_2O]$ per ml., and 3 ml. of absolute ethanol saturated with $Hg(SCN)_2$ are added to the flask, and the contents are shaken. The absorbance is determined immediately in a 2-cm. cell at 460 mμ with a Beckman DU spectrophotometer. Distilled water is used as a reference. Ten micrograms of chloride ion gave an absorbance of about 0.035, and sensitivity is approximately 3 μg. when the cotton blank is used.

**Table IV. Application of Parallel Screening System to 10 Insecticides Added at Tolerance Levels to Benzene Extract of Peas**

| Insecticide | Concn., P.P.M. | (A) $LD_{50}$, Grams | (B) Chlorine, P.P.M. | (C) $I_{50}$, Grams | Product A × B | MF | Corrected Product or Ratio |
|---|---|---|---|---|---|---|---|
| DDT | 7 | 10 | 3.8 | Negative | 38 | 4.67 | 8.1 |
| Dieldrin | 0.25 | 5.8 | 0.03 | Negative | 0.17 | 6.2 | 0.027 |
| Lindane | 10 | 0.1 | 6.6 | Negative | 0.66 | 9 | 0.073 |
| Methoxychlor | 14 | 40 | 4.2 | Negative | 168 | 1 | 168 |
| Thiodan | 2 | 1.0 | 1.2 | Negative | 1.2 | 8.6 | 0.14 |
| | | | | | Ratio A/C | | |
| Parathion | 1 | 0.15 | Negative | 0.13 | 1.15 | 1.9 | 0.6 |
| Malathion | 8 | 0.07 | Negative | 2.10 | 0.03 | 1.8 | 0.016 |
| Diazinon | 0.75 | 0.82 | Negative | 0.53 | 1.51 | 3.4 | 0.45 |
| Ethion | 1.0 | 21 | Negative | 1.30 | 15.4 | 3.75 | 4.1 |
| Delnav | 2.8 | 22 | Negative | 1.9 | 11.5 | 6.1 | 1.89 |

**Enzyme Inhibition.** The acetylcholinesterase method is essentially the same as described by Patchett and Batchelder (*6*) for the determination of Trithion. The modifications were made to provide response to a large number of phosphates at tolerance levels on sample sizes ranging from 0.2 to 2.5 grams. Reagents and apparatus are the same as described by Patchett and Batchelder (*6*). The major differences are as follows: 3 ml. of the oxidized benzene solution were evaporated to dryness in a 50-ml. beaker. Twenty milliliters of distilled water and approximately 5 ml. of pooled human blood plasma were then added, and the mixture was adjusted to pH 7 for the first incubation period. The second incubation period was 105 minutes. The procedure was standardized to produce 50% inhibition with 0.1 μg. of parathion. The concentration of the plasma was adjusted, if necessary, to maintain this level of inhibition.

## Results and Discussion

The broad application of bioassay has been limited because it does not directly reflect absolute concentration of toxicants, particularly in larger aliquot sizes. This factor, referred to as masking effect, reduces the availability of the toxicant to the test organism. Others have employed only small aliquots (0.25 to 0.5 gram) to avoid or reduce masking effect. However, masking is comparatively constant within each crop and depends on the type of crop, the toxicant present, and the effectiveness of the cleanup. In this method, the masking factors (*MF*) are calculated by dividing the actual $LD_{50}$ (gram) value by the theoretical $LD_{50}$ (gram) amount. The actual value is determined by bioassaying a residue-free crop that is fortified with an insecticide at its tolerance level after cleanup; the theoretical value is calculated by dividing the $LD_{50}$ (μg.) of the insecticide alone by its tolerance. Theoretical values represent the weight of crop required to contain the $LD_{50}$ (μg.) amount and are the values that would be obtained if crop extracts caused no masking and there was no loss of insecticide in the analytical procedure.

In general, the sensitivity of the test organism to toxicants is directly related to tolerance quantities, and the majority of toxicants either contain chlorine or are anticholinergic. Figures 1 and 2 show that the response of bioassay and enzyme inhibition to the organophosphates tested is over a range of 4 log cycles. The response of the bioassay to organic chlorine compounds covers a range of 5 log cycles (Figure 3). A series of $LD_{50}$ values obtained for 16 chlorine-containing insecticides, deposited from petroleum ether or benzene solution, is presented in Table II. The toxicity of these compounds ranged from a high sensitivity of 0.02 μg. for a low tolerance compound to a low sensitivity of 500 μg. for a high tolerance compound. The product values of $LD_{50}$ (μg.) × the chlorine conversion factor relate toxicity to chlorine content, and are also shown in Table II. These values are used to identify unknown residues when the toxicity is attributed to an organic chlorine compound.

The $LD_{50}$ and $I_{50}$ values for residues of 16 organophosphates deposited from benzene solutions are presented in Table III. The ratio of $LD_{50}$ to $I_{50}$ for each toxicant compares in vivo to in vitro inhibition. These values are

used for the identification of unknown residues whose toxicity is attributed to an organophosphate.

The masking factors are applied in either of two ways. The appropriate product or ratio for an insecticide in Table II or III may be multiplied by *MF* to approximate the value expected from a crop containing that insecticide; or, the product or ratio result obtained on the crop may be divided by the *MF* for comparison with standard values in Table II or III. The first approach is usually employed when spray history information indicates a particular insecticide was used, and the latter when spray history is not available.

In applying the system to unknowns, the three tests are performed on the same strip solution, the product or ratio is employed to identify the residue, and either the organic chlorine or inhibition result used to estimate the quantity of the suspect chemical. To confirm the estimation, an appropriate specific method or paper chromatographic (5) system may then be applied.

In the analysis of a food sample containing a chlorinated organic compound, the bioassay result is expressed as $LD_{50}$ (grams) and the organic chlorine as parts per million. These values are inversely proportional; therefore, their product is constant and is independent of the concentration of toxicant. The product expresses $LD_{50}$ in terms of micrograms of chlorine and may be compared directly with the standard products for chlorinated insecticides in Table II for indentification of the compound. In the case of organophosphates, the $LD_{50}$ and $I_{50}$ values are directly proportional; therefore, their ratios are constant and independent of the concentration of toxicant. This permits the use of the ratio for tentative identification of organophosphates.

To demonstrate the application of this screening system, tolerance quantities of 10 insecticides were added to benzene extracts of peas prior to cleanup, and analyses were performed as previously described (Table IV). These data are characteristic of the results obtained in practice and are needed to determine the effect of masking and recovery. For example, the *MF* for DDT is calculated as follows:

$$MF = \frac{\text{Actual } LD_{50} \text{ (}\mu\text{g. DDT)}}{\text{Theoretical } LD_{50} \text{ (}\mu\text{g. DDT)}} = \frac{10 \text{ gm.} \times 7 \text{ p.p.m.}}{15 \ \mu\text{g. DDT}} = 4.67$$

Other *MF* values were calculated in the same manner, and are shown in Table IV.

The insecticides in Table IV were also analyzed at the same concentration without a crop to evaluate the benzene-charcoal cleanup. These results indicated that the system introduced a two-fold *MF* in the case of most organic chlorine insecticides while the organophosphates were relatively unaffected. The recovery of dieldrin from the charcoal cleanup was very low.

In general, the masking effect due to peas accounts for a fivefold decrease of sensitivity to organic chlorine compounds and a two- to threefold decrease to organophosphates. Table IV contains data representing the many types of compounds among the two largest classes of insecticides in a crop containing a high magnitude of extraneous extractives. However, the analytical system is equally applicable to other crops and insecticides, if results are adjusted for masking effect. When control crops are not available or crop standards—as shown in Table IV—have not been developed, general *MF* values of 5 for chlorinated organics and 3 for organophosphates have been found widely applicable. The product for DDT in peas, 38, is divided by the general *MF*, 5, and the result, 7.6, compares favorably with the product for DDT, 7.5, in Table II.

The analysis of an unknown sample may reveal the following information: $LD_{50} \cong 15$ grams, $I_{50}$ negative, and chlorine $\cong 2.5$ parts per million. Since the $I_{50}$ is negative and the organic chlorine positive, the toxicity is due only to a chlorine toxicant. The product, 37.5, is divided by the general *MF*, 5, and the result, 7.5, is compared with standard products in Table II and is identified as DDT. The organic chlorine result (2.5 p.p.m.) is then used to calculate the concentration of DDT (5.0 p.p.m.) in the unknown sample.

If mixed residues of both chlorine and phosphate are encountered, all of the toxicity produced by the sample ($LD_{50}$) is attributed to each class separately. After all of the data is evaluated, the most feasible specific method is applied to verify the interpretation. For example, the analysis of a sample of peas containing DDT (3.5 p.p.m.) and parathion (0.5 p.p.m.) gave the following results: $LD_{50} \cong 0.3$ gram, organic chlorine $\cong 1.8$ p.p.m., and $I_{50} \cong 0.25$ gram. The product value is $(0.3 \times 1.8) \cong 0.54$, and the ratio value is $(0.3/0.25) \cong 1.2$. When the sample is treated as an unknown, the general *MF* values for organic chlorides, 5, and organophosphates, 3, are used. The product and ratio values adjusted for masking become 0.11 and 0.4. These values are compared with theoretical values in Tables II and III. The ratio value compares closely with that of parathion (0.4 *vs.* 0.5) and therefore tentatively identifies the significantly toxic portion of the residue as parathion. The inhibition result is used to estimate the concentration and is equivalent to:

$$\frac{I_{50} \text{ of parathion } (\mu\text{g.})}{I_{50} \text{ of sample (grams)}} \cong \frac{0.1}{0.25} \cong 0.4 \text{ p.p.m. parathion.}$$

This identity and concentration fits all of the data well and can readily be confirmed by specific determination. If all of the toxicity were attributed to the chlorine value, it suggests the possible presence of chlordane at a concentration of 5 parts per million. However, the combination of these two insecticides at the concentrations indicated are is compatable with the bioassay result.

Usually the presence of an organophosphate will obscure the presence of a chlorinated organic of the DDT type because of the greater sensitivity of *Drosophila* to the phosphate insecticides. This is particularly true at concentrations normally encountered in residue work. Therefore, if mixtures of the two classes are present, most frequently, the ratio value will provide the key to resolving the mixture. In the example above and in similar situations, it is necessary to identify one of the toxicants by chemical means.

Mixed residues within the same class are indicated when the individual results limit the residue to one class, and the product or ratio value of the unknown does not correspond to any standard value. In this case, compounds whose products or ratios approximate the unknown are determined by specific tests. In many instances, the data may show that only one insecticide could be over tolerance and a specific determination for it will resolve the question.

One of the major advantages of this approach is that its screening utility is not drastically affected by low recovery. The main purpose of the screening system is to single out samples that may be over tolerance. Good recovery, although desirable in any analytical method, is not considered as essential in this system as in others, since the product or the ratio value provides the basis for residue classification and when a sample is indicated to contain a residue of near or above tolerance level, specific analyses must follow. For example, the recovery of dieldrin by the chlorine analysis of pea extracts as shown in Table IV amounted to only 20%, but the toxicity × chloride product (0.17) in light of the $LD_{50}$ value, places the sample in the suspect class and would identify the residue as dieldrin or aldrin. However, good recovery is essential in order to assign an analytical value for any specific insecticide by the organic chloride or inhibition procedures. Therefore, in order to assign an analytically significant value for products of that magnitude, it is necessary to determine the chlorine content of the extract prior to cleanup. In the case of bioassay, the assignment of an analytical value can be made only when the masking effect is eliminated or accounted for and good analytical recovery demonstrated.

### Acknowledgment

The authors express their appreciation to J. E. Dewey, Cornell University, for advice regarding the application of bioassay techniques.

### Literature Cited

(1) Bergman, J. G., Sanik, J., Jr., *Anal. Chem.* **29,** 241 (1957).
(2) Dewey, J. E., "*Drosophila* Bioassay—Methods of Extraction and Testing Techniques," Department of Entomology, Cornell University, Ithaca, N. Y., unpublished.
(3) Dewey, J. E., "The Rearing and Culture of *Drosophila melanogaster* for Bioassay," Department of Entomology, Cornell University, Ithaca, N. Y., unpublished.
(4) Lisk, D. J., J. AGR. FOOD CHEM. **8,** 1921 (1960).
(5) Mills, P. A., *J. Assoc. Offic. Agr. Chemists* **42,** 734 (1959).
(6) Patchett, G. G., Batchelder, G. H., J. AGR. FOOD CHEM. **8,** 54 (1960).
(7) Reed, L. J., Muench, H., *Am. J. Hyg.* **27,** 493 (1938).

*Received for review October 24, 1961. Accepted March 5, 1962. Division of Agricultural and Food Chemistry, 140th Meeting, ACS, Chicago, September 1961.*

**ANTIOXIDANT MEASUREMENT**

# Determination of 2,6-Di-*tert*-Butyl-4-Hydroxytoluene (BHT): Application to Edible Fats and Oils

**C. R. SZALKOWSKI and J. B. GARBER**

**Merck Chemical Division, Merck & Co., Inc., Rahway, N. J.**

BHT is a very effective antioxidant for many organic substances such as edible fats and oils. Its wide use either alone or in combination with other antioxidants has indicated a need for a specific and effective method for its quantitative determination to maintain effective control over processing operations and to ensure adherence to regulatory requirements. The method presented is based on separation of BHT from fat or oil by steam distillation and colorimetric determination with a dianisidine–nitrous acid reagent. The method is capable of determining 10 to 200 p.p.m. BHT in the presence of other allowable antioxidants.

THE COMPOUND 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT) is widely used as an effective antioxidant for a wide variety of organic substances, such as Vitamin A oils, edible fats and oils, rubber, petroleum, and plastics. The use of this compound has been permitted in the U. S. (*9*) and Canada (*2*).

There is a need for a specific and quantitative method for analysis of commercial products to maintain effective production control over processing operations employing this antioxidant and to ensure compliance to regulatory requirements. Butylated hydroxytoluene is used either alone or in combination with other permitted antioxidants; therefore, the method should be capable of determining 10 to 200 p.p.m. in the presence of other allowable antioxidants.

A survey of the literature showed that previous methods for the determination of BHT were based on colorimetry with ferric chloride-1,1′-bipyridine (*6*), ferric chloride and *o*-phenanthroline (*1*), and phosphomolybdate reagent (*8*).

Anglin, Mahon, and Chapman (*1*) separate butylated hydroxytoluene and butylated hydroxyanisole (BHA) from fats and oils by steam distillation. The distillate is analyzed for the sum of butylated hydroxyanisole and butylated hydroxytoluene with ferric chloride-2,2′-bipyridine and for butylated hydroxyanisole with 2,6-dichloroquinonechloroimide, thereby permitting butylated hydroxytoluene to be determined by difference. This method has been studied collaboratively (*3*). Phillips and Henkel (*7*) reported a method for the estimation of butylated hydroxytoluene by ultraviolet spectrophotometry after chromatographic separation on silicic acid, but tocopherol interferes in this analysis. A specific and rapid method by gas-liquid partition chromatography has also been reported (*5*). The colorimetric methods were not applicable in the presence of ascorbyl palmitate and natural tocopherols.

This study was initiated to develop a specific and rapid color reaction for the determination of BHT in the presence of butylated hydroxyanisole, ascorbyl palmitate, tocopherols, and other antioxidants. Preliminary experiments indicated that BHT treated with an alcoholic solution of dianisidine and nitrous acid formed a chromogen which could be extracted with a chlorinated solvent, such as chloroform or tetrachloroethane. This novel color reaction was studied intensively so that it could be applied to the quantitative determination of BHT.

Since water and alcohol are required for the reaction between the butylated hydroxytoluene and the dianisidine–nitrous acid reagent, the analysis cannot be carried out directly on an anhydrous fat or oil. Therefore, some means is necessary to separate the antioxidant from the fat or oil prior to final analysis. Various solvent extractions were studied and found to be unsatisfactory. The distillation technique of Anglin, Mahon, and Chapman (*1*) was modified and found to be applicable to corn, cottonseed, peanut, and soya oils, and to hydrogenated oil and lard.

### Reagents

Unless otherwise indicated all reagents are reagent grade.

Magnesium Chloride Solution. Dissolve 100 grams of magnesium chloride, $MgCl_2 \cdot 6H_2O$, in 50 ml. of water.

Dianisidine Solution. Dissolve 250 mg. of dianisidine (3-3′-dimethoxybenzidine) in 50 ml. of methanol. Add 100 mg. of activated charcoal, shake for 5 minutes, and filter. Mix 40 ml. of the clear filtrate with 60 ml. of 1*N* hydrochloric acid. Prepare daily and protect from light.

Sodium Nitrite Solution. A 0.3% aqueous solution.

Reference Standard Solution of 2,6-di-*tert*-butyl-4-hydroxytoluene. Dissolve 50.0 mg. of pure (99.8%) 2,6-di-*tert*-butyl-4-hydroxytoluene in sufficient methanol to make 100.0 ml. Prepare working standards containing 1 to 5 μg. per ml. by diluting the reference standard stock solution with 50% (v./v.) methanol.

### Apparatus

Distilling Apparatus. A microsteam distillation apparatus of the Jenden-Taylor (*4*) type, with a spiral coil con-