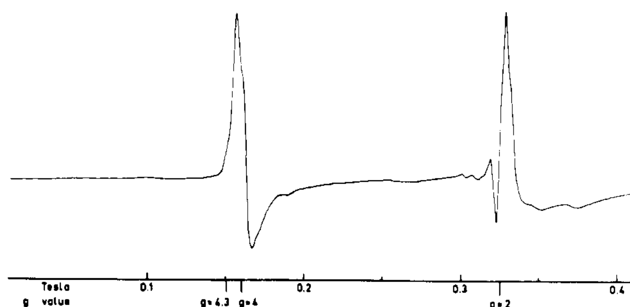

Figure 8. EPR spectrum after the addition of NO gas to anaerobic native lipoxygenase (85 mg/mL) in 0.1 M sodium phosphate (pH 7.0). Microwave frequency 9.105 GHz. Power 0.5 mW. Receiver gain 10^3, temperature 15.3 K.

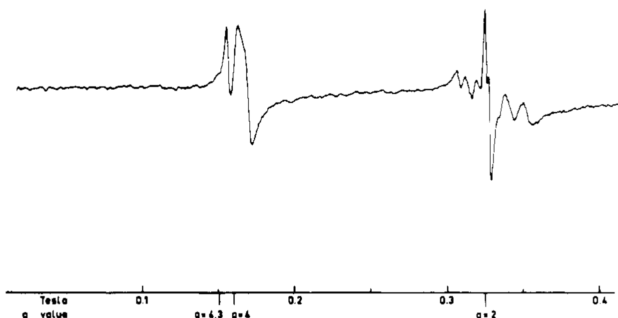

Figure 9. As in Figure 8 but with the addition of 13-hydroperoxylinoleic acid in a 2:1 ratio to enzyme. Conditions as in Figure 8 except receiver gain = 5 × 10^3 (Galpin et al., 1978).

of the activity in the normal aerobic reaction is recovered.

Addition of linoleic acid in large excess caused no significant change in the signal near $g = 4$. However, the addition of 13-L-hydroperoxide to the enzyme–NO complex results in a substantial decrease in the signal near g = 4 (Figure 9). If the 13-LOOH was added before treatment with NO the usual signal near $g = 6$ (De Groot et al., 1975) disappeared completely and only a small signal near $g = 4$ was observed. Apparently a ferric–NO complex is formed which is EPR silent. It seems therefore quite reasonable to conclude that (a) the anaerobic native enzyme is a ferrous species and (b) the aerobic species indeed contains an iron–oxygen complex.

LITERATURE CITED

Aoshima, H., *J. Biochem.* **82**, 569–573 (1977).
Axelrod, B., *Adv. Chem. Ser.* **No. 136**, 324–348 (1974).
De Groot, J. J. M. C., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., Wever, R., Van Gelder, B. F., *Biochim. Biophys. Acta* **377**, 71–79 (1975).
Egmond, M. R., Brunori, M., Fasella, P. M., *Eur. J. Biochem.* **61**, 93–100 (1975).
Egmond, M. R., Fasella, P. M., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., *Eur. J. Biochem.* **76**, 469–479 (1977).
Eskin, N. A. M., Grossman, S., Pinsky, A., *Crit. Rev. Food Sci. Nutr.* 1-40 (1977).
Finazzi-Agrõ, A., Avigliano, L., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., *Biochim. Biophys. Acta* **326**, 462–470 (1973).
Galpin, J. R., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., *Biochim. Biophys. Acta* **536**, 356–362 press (1978).
Garssen, G. J., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., *Eur. J. Biochem.* **62**, 33–36 (1976).
Garssen, G. J., Vliegenthart, J. F. G., Boldingh, J., *Biochem. J.* **122**, 327–322 (1971).
Garssen, G. J., Vliegenthart, J. F. G., Boldingh, J., *Biochem. J.* **130**, 435–442 (1972).
Gatt, S., Bartfai, T., *Biochim. Biophys. Acta* **488**, 1–12 (1977).
Hamberg, M., *Anal. Biochem.* **43**, 515–526 (1971).
Lagocki, J. W., Emken, E. A., Law, J. H., Kézdy, F. G., *J. Biol. Chem.* **251**, 6001–6006 (1976).
Van Os, C. P. A., Vente, M., Vliegenthart, J. F. G., unpublished data (1978).
Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., *Progr. Chem. Fats Other Lipids* **15**, 131–166 (1977).
Verhagen, J., Bouman, A. A., Vliegenthart, J. F. G., Boldingh, J., *Biochim. Biophys. Acta* **486**, 114–120 (1977).
Verhagen, J., Veldink, G. A., Egmond, M. R., Vliegenthart, J. F. G., Boldingh, J., Van der Star, J., *Biochim. Biophys. Acta* **529**, 369–379 (1978a).
Verhagen, J., Vliegenthart, J. F. G., Boldingh, J., *Chem. Phys. Lipids*, in press (1978b).
Vliegenthart, J. F. G., Veldink, G. A., *Ann. Technol. Agric.* **26**, 175–187 (1977)

Recieved for review August 7, 1978. Accepted January 2, 1979. Presented at the 175th National Meeting of the American Chemical Society, Division of Agricultural and Food Chemistry, Anaheim, CA, March 1978.

Determination of Phorate and Its Metabolites by Mixed-Phase Gas Chromatography

Philip R. Boshoff* and Victor Pretorius

Recent advances in mixed-phase gas chromatography are developed and applied to the determination of phorate and its metabolites. Phases which were taken into account are OV-101, OV-17, OV-225, and Apolar-5CP.

Phorate (*O*,*O*-diethyl *S*-[(ethylthio)methyl] phosphorodithioate) (I) is used extensively as an insecticide for the protection of a wide variety of crops. Due to its high toxicity to mammals (LD_{50} to rats 1.5 mg/kg) there is a particular need for the monitoring of its residues. This is especially true where such crops as tomatoes, lettuce, carrots, and cabbages are concerned, which are consumable in their raw state and where their residues are not therefore destroyed through boiling. It has become evident that such monitoring should not be confined to phorate, as is often done, but extended to include its three major (II–IV) and two minor (V–VI) metabolites as well (Figure 1). While, for example, the conversion of phorate to its sulfoxide in plant tissue (Saunders and Getzin, 1973), by soil bacteria (Higgins and Burns, 1975) or even during the normal methods of extraction (Brown, 1975), is relatively rapid, its subsequent oxidation and breakdown is known to be

Institute for Chromatography, University of Pretoria, Pretoria 0002, Republic of South Africa.

0021-8561/79/1427-0626$01.00/0 © 1979 American Chemical Society

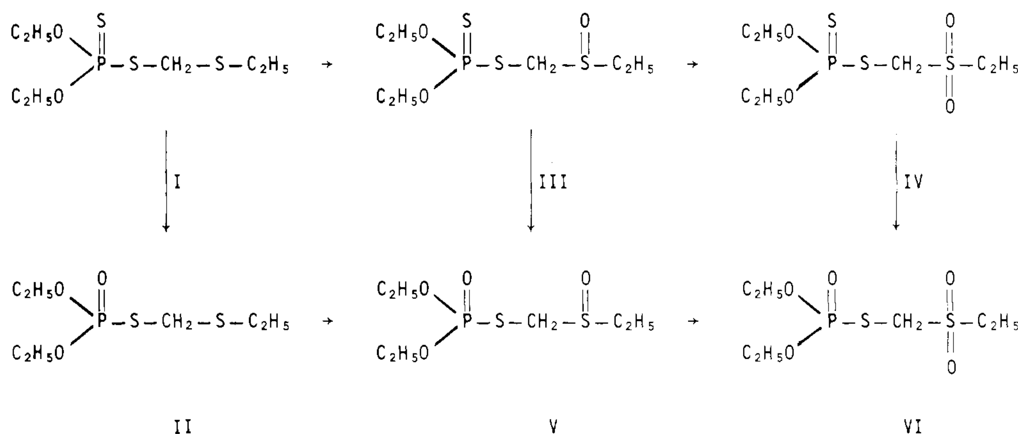

Figure 1. Phorate and its metabolites: (I) phorate, (II) phorate oxygen analogue, (III) phorate sulfoxide, (IV) phorate sulfone, (V) phorate sulfoxide oxygen analogue, (VI) phorate sulfone oxygen analogue.

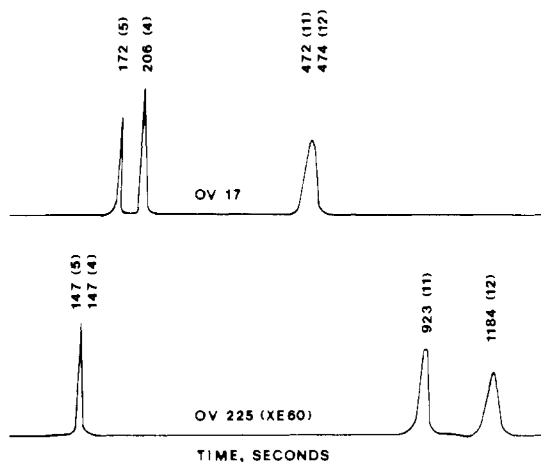

Figure 2. The separation of phorate (5), phorate oxygen analogue (4), phorate sulfoxide (11), and phorate sulfone (12) on OV-17 and OV-225 (for peak identity, refer to Table I).

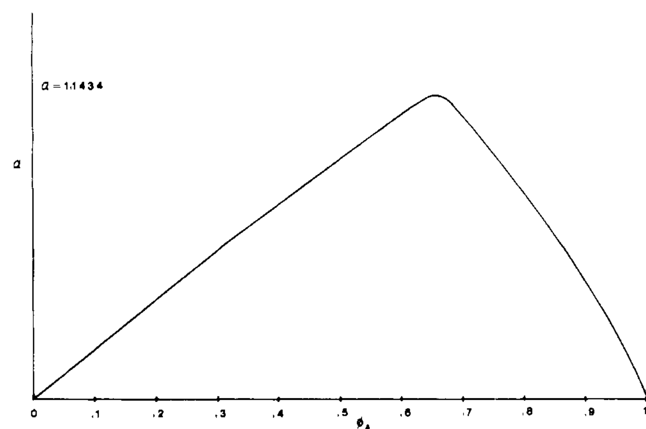

Figure 3. "α plot" for phorate, phorate oxygen analogue, phorate sulfoxide, and phorate sulfone.

protracted (Saunders and Getzin, 1973). The amount of precursor material remaining at the time of analysis may therefore be expected to be relatively small. Indeed, it is the oxidation products, the sulfoxides and sulfones, which constitute the insecticidal properties rather than phorate itself (Higgins and Burns, 1975).

Failure to include these metabolites in residue analysis may largely be attributed to the difficulty experienced with their determination by gas chromatography (GC). A way around this difficulty has been to convert phorate and its metabolites to the sulfone oxygen analogue (VI) prior to

Table I. Retention Times of Phorate, Its Metabolites, and Some Commonly Used Organophosphorus Pesticides

		OV-101	OV-17	OV-225	Apolar-5CP
1.	dichlorvos	59	67	64	82
2.	phosdrin	89	97	113	175
3.	demeton-*s*-methyl	148	166	206	373
4.	phorate	160	206	147	251
5.	phorate O.A.[a]		172	147	251
6.	dimethoate	182	224	648	1430
7.	diazinon	206	266	146	250
8.	disulfoton	220	287	213	390
9.	phorate sulfone O.A.	285	423	1247	2768
10.	malathion	345	461	510	1092
11.	phorate sulfoxide	405	472	923	2077
12.	phorate sulfone	375	474	1184	2746
13.	parathion	370	496	665	1335
14.	bromophos	420	556	406	818
15.	phorate sulfoxide O.A.		440	861	2240

[a] O.A. refers to oxygen analogue.

determination by GC, but even the best available oxidation agent, *m*-chloroperbenzoic acid, only produces a moderate yield (Blinn, 1964) of this product.

To date no single GC stationary phase has been found which would satisfactorily separate phorate and its three major metabolites (I–IV). Reports of success with diethyl glycol succinate (DEGS) have been shown to be optimistic (Brown, 1975). Saunders and Getzin (1973) used OV-17 to separate phorate and its oxygen analogue, while Brown (1975) used SE 30. Both used XE 60 for the detection of phorate sulfoxide and sulfone (Figure 2). For the determination of the oxygen analogues of the sulfoxide (V) and the sulfone (VI), Saunders and Getzin (1973) resorted to thin-layer chromatography.

In looking for a solution to this problem it was decided to investigate mixed liquid phases, an approach which in the past has been of considerable value in GC analysis of pesticides. This task was facilitated by recent advances made by Laub and Purnell (1975, 1976) in the method for the calculation of mixed phase ratios in GC from retention data obtained from individual phases.

In the approach of Laub and Purnell a plot is obtained between liquid phase ratio and α values (separation factors), which provides an indication of potentially suitable liquid phase ratios. Referring to Figure 5, the plot shows four "windows" (enclosed curves), each representing the α value for the most difficult separable pair of peaks at particular phase ratios. The best phase ratio can be expected to correspond to the highest α value; in this instance

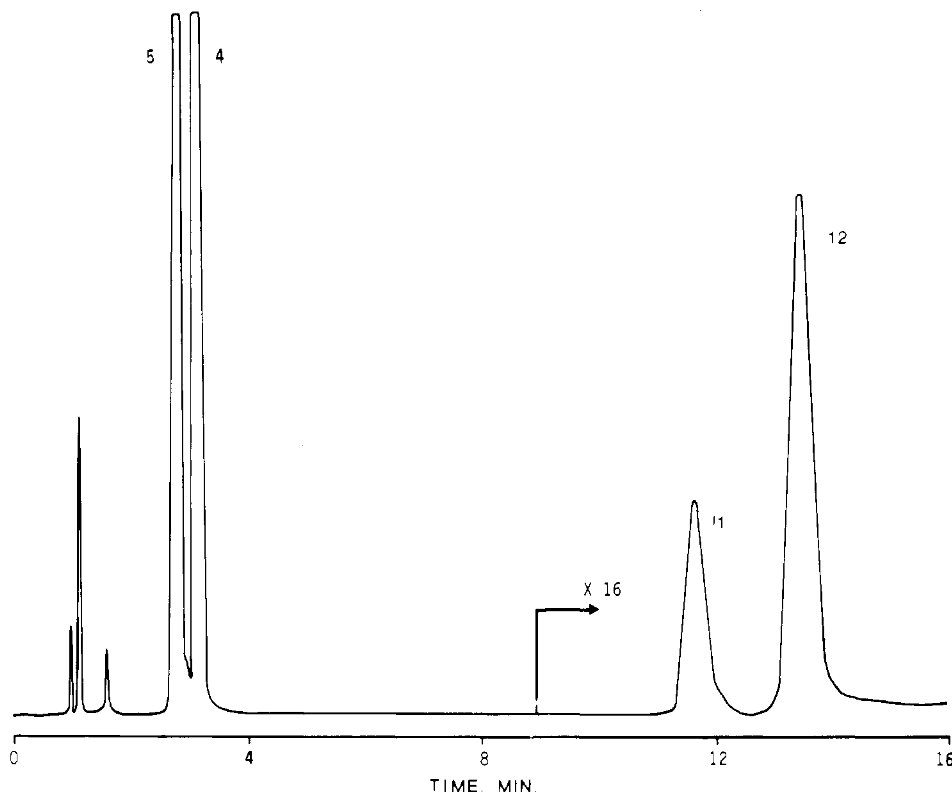

Figure 4. Chromatogram of phorate, phorate oxygen analogue, phorate sulfoxide, and phorate sulfone obtained with a 2-m column consisting of 66% OV-17 and 33% OV-225; injector and column temperatures, 220 and 205 °C, respectively. The nitrogen flow rate was 40 cm^3/min; the initial attention, 1 × 128.

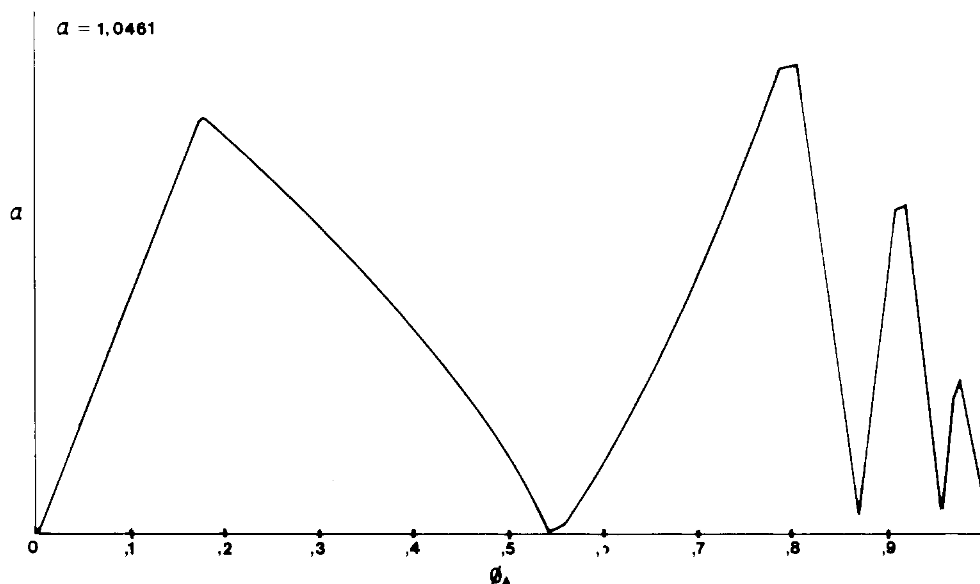

Figure 5. "α plot" for phorate and its five metabolites.

at approximately 80% of the first phase ($\phi_A = 0.8$). To facilitate the construction of these "α plots", a computor program (in Basic) was prepared in our laboratory. This facility also permits peak identity to be maintained and retention times to be predicted for proposed mixed phases.

EXPERIMENTAL SECTION

For the effective determination of phorate and its metabolites, proper packing, conditioning, and deactivation of the column was found to be essential. Columns required conditioning for at least 72 h prior to use. The temperature at which the column is conditioned should not cause the phase ratio to alter through selective bleeding. Additional deactivation of the column was achieved by passing three successive 10-μL "plugs" of a 50 ppm solution of phorate and its metabolites through the column.

The procedure for preparing the mixed-phase columns was as follows: the support material was coated separately with the two liquid phases in the usual manner, after which it was dried and thoroughly mixed in the required ratio prior to packing the column.

The support material used for all columns was Chromosorb W-HP (AW-DMCS), 100–120 mesh. Chromatograms were obtained with a Pye-Philips GCV instrument, fitted with a flame photometric phosphorus detector.

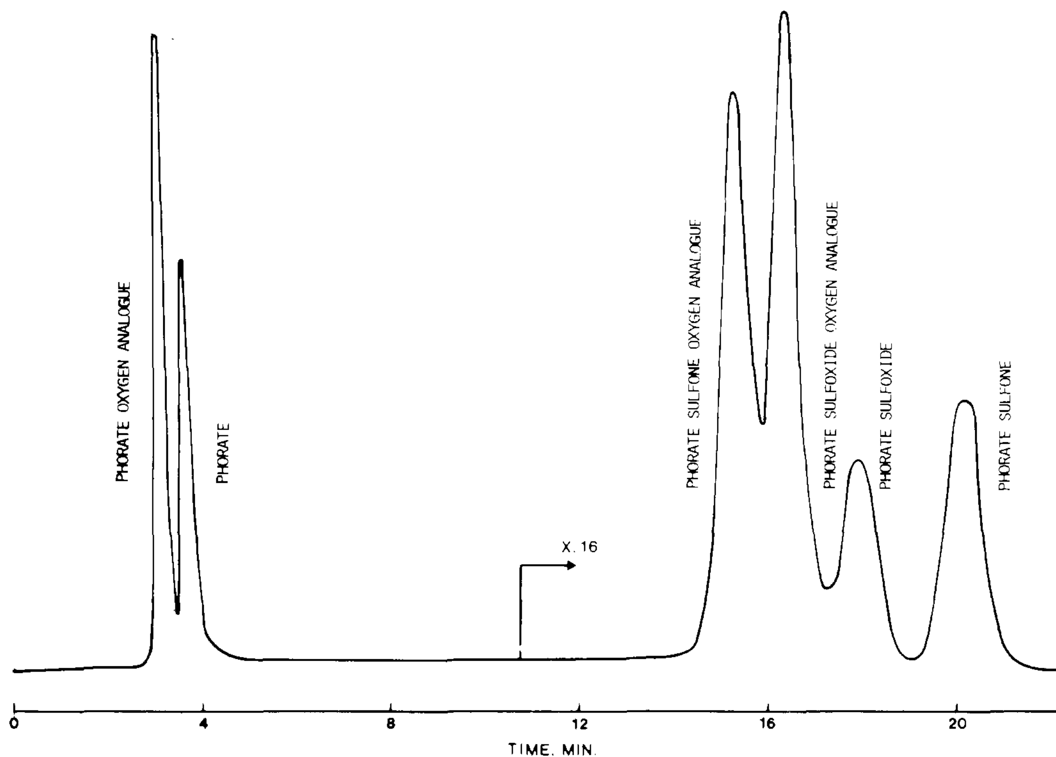

Figure 6. Chromatogram of phorate and its five metabolites, obtained with a 2.5-m column consisting of 83% OV-17 plus 17% OV-225. The column temperature was 205 °C, the nitrogen carrier gas flow rate 37 cm^3/min.

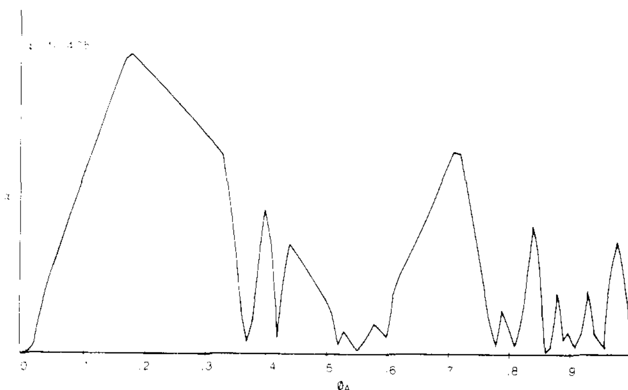

Figure 7. "α plot" for phorate, its five metabolites, and nine other organophosphorus pesticides listed in Table I.

RETENTION DATA

Four liquid phase materials were considered: OV-101, OV-17, OV-225, and Apolar-5CP. These were chosen because of their high stability, wide polarity range, and general acceptance in pesticide analysis. Retention data were obtained (Table I) for phorate and its metabolites as well as for a number of other commonly used organophosphorus insecticides. The nonretained peak for each column was also obtained.

In considering the data in Table I it was found that certain phase combinations could be eliminated. For example, the possibility of combining OV-225 and Apolar-5CP would be unsuccessful since phorate and phorate oxygen analogues are not separated on either phase and would therefore not be separated by a combination of the two. Phases OV-17 and OV-225, however, appear to complement each other in this respect, as do OV-17 and Apolar-5CP. For some reason OV-101 produced poor peak shapes with most of the phorate metabolites and was dropped from further consideration.

SEPARATION OF PHORATE AND ITS THREE MAJOR METABOLITES

A mixed-phase column for the optimum separation of phorate, phorate oxygen analogue, phorate sulfoxide, and phorate sulfone, prepared from OV-17 and OV-225, was considered first.

From the following computer printout it can be seen that the necessary "input" data consists of the unretained peak value and the retention times of each of the four compounds, on each of the two liquid phases selected:

```
* Enter "Specicolumn"
* Run TØ(1) ? 7, T Ø(2) ? 6, Number of peaks ? 4
Retention times on first phase
? 172 ? 2Ø ? 472 ? 474
Retention times on second phase
? 147 ? 147 ? 923 ? 1184
Largest alpha of 1.14339 occurs at PHI = .66 of first
phase
1          156.5
2          178.94
3          618.34
4          708.4
```

From the above data and the "α plot" (Figure 3), the phase ratio (ϕ_A) for optimal separation is indicated as being 0.66 or 66% (volume percent) of the first phase (OV-17) and 33% of the second (OV-225). Also included in the data are the predicted retention times based on the optimum phase ratio.

A 2 m × 3 mm i.d. glass column was packed with material consisting of 66% OV-17 and 33% OV-225. The phase loading for each was 3%. The resultant chromatogram (Figure 4) was obtained from approximately 25 ng of each of the four compounds.

SEPARATION OF PHORATE AND ITS FIVE METABOLITES

Separation of phorate and its five metabolites was more difficult than for the previous analysis and a relatively high

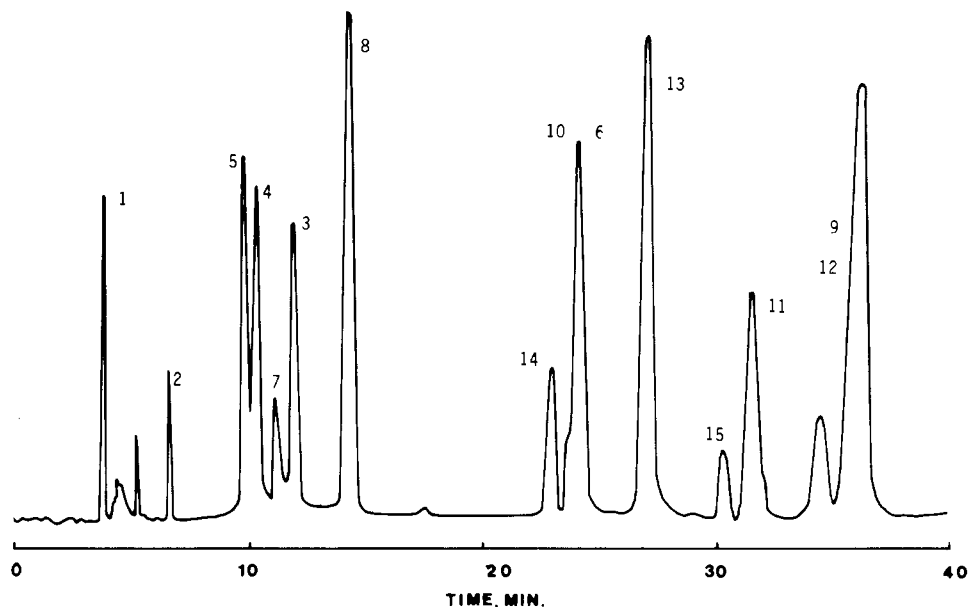

Figure 8. Chromatogram of phorate, its metabolites, and nine other organophosphorus pesticides listed in Table I, obtained on a 4-m column consisting of 25% OV-17 and 75% OV-225. The column temperature was 205 °C; the nitrogen carrier gas flow rate was 37 cm^3/min.

efficiency was required of the column. With reference to Figure 5, the best phase ratios are indicated as being: 17% OV-17 and 83% OV-225 or alternatively 80% OV-17 and 20% OV-225. The latter phase ratio provided the higher α valve ($\alpha = 1.046$), and since this value indicates the need for a relatively high theoretical plate number, it was chosen for the separation. The chromatogram in Figure 6 was therefore obtained using a 2.5 m × 3 mm i.d. glass column consisting of 83% OV-17 plus 17% OV-225. Ten nanograms of each compound was injected, except for the oxygen analogue of the sulfoxide and the sulfone in which case approximately 50 ng was injected. The initial attenuation was 1 × 64. The 3% discrepency between the theoretical and the de facto optimum α value arises in the measurement of gas hold-up time while determining retention data for the pure phases.

SEPARATION OF PHORATE AND ITS FIVE METABOLITES IN THE PRESENCE OF OTHER ORGANOPHOSPHORUS INSECTICIDES

The separation of phorate and its five metabolites in the presence of nine organophosphorus insecticides which are commonly used on vegetable crops was also investigated. The combination of OV-17 with Apolar-5CP resulted in a low α value (1.025) as well as narrow "windows", both of which detract from this choice. The data obtained from OV-17 and OV-225, however, showed much better results. The best phase ratio as supplied by the "α plot" (Figure 7) is: 18–30% OV-17 and 70–82% OV-225. The resultant chromatogram (Figure 8) was obtained from a 4-m glass column consisting of 25% OV-17 and 75% OV-225, the phase loading for each being 5%. The relative amount of phorate to other organophosphorus pesticides was approximately 3:1.

CONCLUSION

The rapid determination of phorate and its five metabolites, even in the presence of many other organophosphorus insecticides was achieved with the aid of recent developments in mixed-phase gas chromatography. This approach should facilitate the treatment of similar problems and lead to the existence of more suitable phases for multiresidue analysis of insecticides.

LITERATURE CITED

Blinn, R. C., *J. Assoc. Off. Anal. Chem.* **47**(4), 641–645 (1964).
Brown, M. J., *J. Agric. Food Chem.* **23**(2), 334–335 (1975).
Higgins, I. J., Burns R. G., "The Chemistry and Microbiology of Pollution", Academic Press, New York, 1975, pp 17, 39.
Laub, R. J., Purnell, J. H., *J. Chromatogr.* **112**, 71–79 (1975).
Laub, R. J., Purnell, J. H., Williams, P. S., *J. Chromatogr.* **134**, 249–261 (1977).
Saunders, J. L., Getzin, L. W., *J. Econ. Entomol.* **66**(2), 530–531 (1973).

Received for review December 7, 1977. Accepted October 27, 1978.