

Multiresidue Method for the Extraction and Detection of Organophosphate Pesticides and Their Primary and Secondary Metabolites from Beef Tissue Using HPLC[†]

Brian P. Ioerger and J. Scott Smith*

Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas 66506

A method that allows the extraction and simultaneous detection of organophosphate pesticides (OPs) and their primary and secondary metabolites from beef tissue was developed. High-performance liquid chromatography (HPLC) with UV-visible diode array detection was used. The OPs and metabolites were extracted from 10-g samples of lean (4.1% fat) and fatty (29.3% fat) beef muscle tissue. Recovery studies were performed at 1 ppm fortification levels in each sample type. Recoveries ranged from 45 to 95% in the lean muscle tissue. Coefficients of variation were between 0.9 and 24.0%, with an average of 6.8%. Recoveries ranged from 22 to 79% in the fatty muscle tissue. Coefficients of variation were between 1.1 and 16.0%, with an average of 5.9%. An experimentally established spectral library of standard UV absorbance profiles for OPs and metabolites obtained via the diode array detector aided in the confirmation of identity of the extracted compounds.

INTRODUCTION

The presence of pesticide residues in our food supply has become an important issue in recent years. In addition, there are increasing expressions of concern regarding potential toxicity of the breakdown products associated with these residues. As newer analytical instrumentation allows the detection of ever lower residue levels, the opportunity for more accurate residue determination increases proportionately. The widespread agricultural usage of the organophosphate class of pesticides (OPs) and their potential mammalian toxicity have dictated the development of several methods for the isolation and detection of the parent compounds (Brayan et al., 1988; Di Muccio et al., 1987; Ripley et al., 1974). To date, however, far less attention has been given to the development of methods that allow the concurrent detection and quantitation of the primary and secondary metabolites (oxygen analogs and hydrolysis products—aromatic alcohols) as well as the OP parent compounds.

This study investigated the potential for detection of these compounds in muscle foods, focusing on development of a method that allows the extraction and simultaneous detection of multiple OPs as well as the corresponding primary and secondary metabolites in red meat. Some work by other investigators suggested that high-performance liquid chromatography (HPLC) with UV-visible diode array detection would prove suitable for the analysis of these compound types. The OPs azinphos-methyl, diazinon, and parathion-methyl and their corresponding breakdown products obtained after basic hydrolysis were analyzed using HPLC with a UV-visible detector as well as LC-mass spectrometry (Farran and De Pablo, 1988). In another study, the utility of HPLC with UV-visible detection was demonstrated for the determination of three OPs, their oxygen analogs, and their inactive metabolites in biological tissues (Sultatos et al., 1982). Further, use of HPLC should prove advantageous by eliminating many cumbersome preparative steps common to gas chromatographic analyses (Coburn and Chau, 1974; USDA, 1987),

as well as avoiding the potential for analyte degradation of the potentially thermolabile OPs and metabolites encountered.

EXPERIMENTAL PROCEDURES

Chemicals. Ronnel, fenthion, parathion, paraoxon, chlorpyrifos, coumaphos, coumaphos oxon, famphur, famphur oxon, and chlorpyrifos-methyl reference standards were purchased from ChemService, Inc. (West Chester, PA). Ronnel oxon was supplied by Dow Elanco (Midland, MI). Fenthoxon was supplied by Mobay Corp. (Kansas City, MO). Stirofos was purchased from Supelco, Inc. (Bellefonte, PA), and 3-methyl-4-(methylthio)phenol was purchased from Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenol, 3-chloro-4-methyl-7-hydroxycoumarin, and 2,4,5-trichlorophenol were purchased from Aldrich Chemical Co. (Milwaukee, WI). High-purity OPTIMA grade ethyl acetate, methanol, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Deionized water was used from a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc., Dubuque, IA).

Apparatus. A Hewlett-Packard Model 1090M Series II liquid chromatograph equipped with a DR5 (binary) solvent delivery system and a scanning UV-visible photodiode array detector was employed. Manual injection was accomplished using a Rheodyne Model 7125 injection valve with a 20- μ L loop. System control, data storage, and processing were achieved by a Hewlett-Packard ChemStation (Pascal series) using software HP79988A Rev. 5.22 and HP79997A Rev. 5.20. Separations were performed on a 250 mm \times 4.6 mm i.d. Bio-Sil (formerly RSil) C₁₈ HL 90 column (Bio-Rad Laboratories, Richmond, CA) with a particle size of 5 μ m. A guard column consisting of a 10 mm \times 4.6 mm cartridge packed with Bio-Sil C₁₈, 5 μ m, or Econosil C₁₈, 5 μ m (Alltech Associates, Inc., Deerfield, IL), was used.

Chromatographic Conditions. Organophosphate pesticides and their metabolites were separated using a step-gradient mobile phase. The initial mobile-phase composition was acetonitrile-water (55:45 v/v), which was held constant from 0.00 to 11.00 min. At 11.10 min, acetonitrile was increased to 65%. From 11.10 min, the gradient was programmed linearly to a final composition of acetonitrile-water (70:30 v/v) at 34.00 min. The flow rate was 1.0 mL/min, and injection volume was 20 μ L. Column temperature was maintained at a constant 40 °C. A post-run column flush of 100% acetonitrile was performed for 20 min after each sample run. After the post-run column flush with 100% acetonitrile, the column was re-equilibrated to initial mobile-phase composition for a period of 5.00 min. Absorption spectra of the organophosphate pesticides and metabolites were obtained by scanning wavelengths in the range 190–350 nm in 2-nm

* Author to whom correspondence should be addressed.

[†] Contribution 92-688-J from the Kansas Agricultural Experiment Station.

increments. Individual wavelengths were monitored on the basis of sensitivity and/or interference considerations. The individual wavelengths monitored were 202, 207, 230, 250, 274, and 314 nm.

Extraction Procedure. Lean (4.1% fat) and fatty (29.3% fat) beef tissues were obtained from a local supermarket and analyzed for moisture and fat content by the Soxhlet extraction procedure (AOAC, 1990). For OP residue analysis, 10-g samples of ground tissue were extracted in a Waring blender at low speed for 30 s, after addition of 2.7 g of NaCl and 20 mL of ethyl acetate-methanol (90:10 v/v) extraction solvent. The mixture was allowed to set for 10 min, briefly remixed, and filtered through Whatman No. 2 paper. The blender was washed with two aliquots of 5 mL of extraction solvent, which was added to the filter. Filtrate was collected into a 25-mL volumetric flask for 10 min and was brought to volume with the extraction solvent. The meat residue and filter paper were re-extracted in the blender for 1 min at low speed after addition of 25 mL of extraction solvent. The mixture was allowed to set for 10 min, briefly remixed, and added to another filter (Whatman No. 2). Collection of this filtrate was continued into a 10-mL volumetric flask for 10 min, and then collected filtrate was brought to volume with extraction solvent. A 10-mL aliquot of the combined filtrates (i.e., 25 mL + 10 mL) was placed in a heat block (60 °C) under a gentle stream of nitrogen until just dry. The residue was resuspended in 1.0 mL of methanol and passed through a conditioned C₁₈ solid-phase extraction cartridge (Waters Chromatography, Milford, MA). The SPE cartridge was conditioned by first passing 8.5 mL of methanol and then 8.5 mL of deionized water through the cartridge and discarding the eluted solvents. The OPs were eluted with 4 mL of acetonitrile-water (90:10 v/v), collected, dried, and resuspended in 1 mL of methanol for HPLC analysis. Before injection, samples were passed through a 0.45- μ m nylon syringe filter (Alltech Associates).

Compound Response Linearity. Pure reference standards of the pesticides and metabolites were used to make stock solutions. From these, serial dilutions were made to give concentrations in the range of interest. Calibration curves were constructed by peak area integration. Linear calibration curves were generated for concentrations in the range from 1 to 100 μ g/mL. Coefficients of determination (R^2) for the regression curves ranged from 0.993 to 1.000.

Recovery Analysis. Recovery studies were performed at the 1.0 ppm fortification level of each pesticide for three extractions in each tissue type (lean and fatty). These samples were prepared by adding 100 μ L of a 100 ppm standard of each OP and metabolite to 10 g of ground tissue before extraction. The extractions were performed as described before, and duplicate injections of 20 μ L from each extraction were made. Injections of standard at a concentration of 5 ppm containing all of the OPs and OP metabolites were made before and at the end of a day's sample runs. These peak areas were averaged and used to calculate that day's compound recoveries according to

$$\% \text{ recovery} = \frac{(5 \text{ ppm})(DF)(\text{extractd cpd area})(100)}{(5 \text{ ppm cpd area})} \quad (1)$$

where DF is the dilution factor derived as 0.35 from (35 mL of collected filtrate/10 g of tissue)/10-mL aliquot of filtrate. Compound identification by retention time and spectral absorbance profile was accomplished via a system spectral matching algorithm based on a user-generated library. This library was created from injection of reference standards described previously.

RESULTS AND DISCUSSION

Preliminary studies involved injection of the OPs and their primary (oxygen analog) and secondary (aromatic alcohols) metabolite standards onto the reversed-phase HPLC system. Relative retention times and UV absorbance profiles characteristic for each compound were established using the UV-visible diode array detector (DAD). Examples of several absorbance profiles are illustrated in Figure 1. These spectral absorbance profiles were then incorporated into a computer library for later use in identifying the extracted OPs and metabolites.

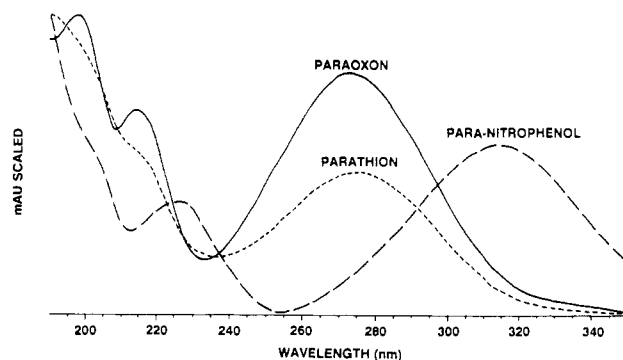


Figure 1. UV absorbance profiles of parathion, paraoxon, and *p*-nitrophenol obtained by the diode array detector.

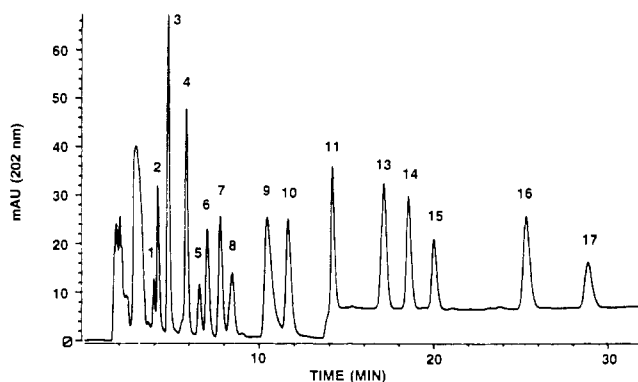


Figure 2. Chromatogram at 202 nm of organophosphate pesticide and metabolite standards demonstrating separation on reversed-phase HPLC. Compound identities are from Table I. Parathion does not show at this wavelength.

Table I. Order of Elution (in Order of Compound No.) and Wavelengths Used for Quantifying Organophosphate Pesticides and Metabolites

compd ID no.	compd	detection wavelength, nm
1	famphur oxon	230
2	<i>p</i> -nitrophenol	314
3	3-chloro-4-methyl-7-hydroxycoumarin	314
4	3-methyl-4-(methylthio)phenol	250
5	paraoxon	274
6	fenthoxon	250
7	coumaphos oxon	314
8	famphur	230
9	2,4,5-trichlorophenol	202
10	ronnel oxon	202
11	stirofos	207
12	parathion	274
13	fenthion	250
14	coumaphos	314
15	chlorpyrifos-methyl	202
16	ronnel	202
17	chlorpyrifos	202

Absorbance wavelength shifts were noted in the comparison of parent compounds and their respective metabolites (Figure 1). For example, the hydrolysis product of parathion, *p*-nitrophenol, exhibits intense absorbance at 314 nm. Attachment of the substituted phosphate functional group (chromophore) to the hydrolysis product results in a hypsochromic shift (blue shift). The phosphate-substituted phenols (parent and oxon) showed absorbance shifts from 314 nm for the hydrolysis product to 274 nm for parathion and paraoxon.

The order of elution of the OPs and metabolites from the analytical column as well as the detection wavelength used for each is provided in Figure 2 and Table I. The relative interaction of these compounds with the reversed-phase column packing material, apparent from the elution

order, exhibited some notable trends (see Figure 2). Nine of the first 10 compounds to elute from the column were either primary or secondary metabolites of the OPs being studied. This result implies a more polar character for the metabolites, an attribute that has been noted previously (Bowman, 1981). This finding is logical when considered in terms of toxicant biotransformation. The resultant metabolite is usually more hydrophilic (i.e., polar) than the parent molecule from which it was derived (Sipes and Gandolfi, 1986). The elution order pattern for a given OP "family" (i.e., parent, oxon, and hydrolysis product) was the same in every instance. The hydrolysis product (alcohol) eluted first followed by the oxygen analog, with the parent compound being the last to elute. Similar behavior on nonpolar GC columns has been reported for some of the OP parent compounds and corresponding oxons (Bowman and Beroza, 1967).

The sensitivity of the UV-visible diode array detector for the OPs and metabolites was determined by injection of mixed OP and metabolite standards. The mixed standards were serially diluted to the 0.1 ppm level. Of the 17 compounds used in the study, chlorpyrifos, chlorpyrifos-methyl, 3-chloro-4-methyl-7-hydroxycoumarin, coumaphos, famphur, famphur oxon, fenthion, fenthoxon, 3-methyl-4-(methylthio)phenol, paraoxon, and ronnel oxon were detectable at the 0.25 ppm level (5 ng). Coumaphos oxon, *p*-nitrophenol, and ronnel had limits of detection of 0.5 ppm (10 ng), whereas parathion, stirofos, and 2,4,5-trichlorophenol were detectable to 1.0 ppm (20 ng). A compound was considered detectable if a response of at least 3 times baseline was noted and the library compound match was equal to or greater than 900 on the 1000-point scale. At the 0.1 ppm concentration level, several peaks on the chromatogram suspected of being OP or metabolite compounds exhibited 3 times baseline peaks. However, none of the library compound matches were equal to or greater than 900 for these peaks.

Various extraction solvent systems were tried on spiked tissue samples in developing the current method. These included methanol, acetonitrile, chloroform-methanol (90:10 v/v), ethyl acetate-methanol (80:20 v/v), and ethyl acetate-methanol (60:40 v/v). Ethyl acetate-methanol (90:10 v/v) provided the greatest overall recovery of the OP compounds investigated. The presence of 10% methanol in an ethyl acetate extraction solvent has been found to be beneficial for the extraction of compounds including very polar metabolites, such as sulfoxides and phenols (Chau et al., 1982). Extraction efficiency was further enhanced by addition of sodium chloride before solvent extraction of the tissue sample. Sodium chloride was added to saturate the water in the meat system, assuming 75% moisture according to the solubility limit of sodium chloride in water of 1 g/2.8 mL of H₂O (Windholz et al., 1989). This "salting out" effect has been noted in the literature (Chau et al., 1982).

Table II indicates the recoveries obtained by the application of the current extraction method to the spiked lean and fatty muscle tissue samples, as well as the library compound matches based on the software spectral matching algorithm. The lean and fatty tissue samples were chosen to represent the potential variation in beef cuts commonly available to the consumer. In addition, the method allowed a comparison of differential recovery of the analytes from very lean versus higher fat-containing cuts.

Recoveries from the lean beef muscle tissue samples (4.1% fat) ranged from 45 to 95% at the 1 ppm level. Coefficients of variation (CV) were between 0.9 and 24.0%,

Table II. Recoveries of Organophosphate Pesticides and Metabolites from Lean (4.1% Fat) and Fatty (29.3% Fat) Beef Tissue Containing 1 ppm; Library Compound Match Scale 0 to 1000 (1000 = Perfect Match; See Text)

compd	recovery, %		library match
	lean ^a	fatty ^b	
chlorpyrifos	59 (2.0) ^c	22 (13.0)	940
chlorpyrifos-methyl	73 (15.1)	36 (5.6)	967
3-chloro-4-methyl-7-hydroxycoumarin	71 (1.8)	54 (1.0)	992
coumaphos	62 (2.1)	40 (3.8)	958
coumaphos oxon	68 (0.9)	53 (1.9)	992
famphur	95 (3.7)	62 (2.8)	990
famphur oxon	85 (16.3)	31	971
fenthion	45 (24.0)	33 (5.2)	962
fenthoxon	69 (7.7)	53 (2.9)	994
3-methyl-4-(methylthio)phenol	ND ^d	ND	
parathion	56 (4.5)	39 (4.4)	914
paraoxon	71 (3.5)	56 (3.1)	978
<i>p</i> -nitrophenol	72 (2.9)	56 (1.8)	940
ronnel	74	25 (16.0)	962
ronnel oxon	74 (3.4)	52 (3.8)	997
stirofos	55 (8.7)	79 (11.3)	924
2,4,5-trichlorophenol	52 (5.2)	29 (12.0)	988

^a Average of four extractions. ^b Average of three extractions. ^c Number in parentheses is the coefficient of variation. ^d ND, not detected.

with an average of 6.8%. The highest recovery from the lean muscle tissue was 95%, obtained for famphur. In the reversed-phase system used in this study, famphur was eighth among 17 OPs and metabolites to elute, exhibiting a retention time of approximately 8.5 min. This would indicate that famphur possesses an intermediate polarity relative to the other OP compounds examined. The high recovery of this intermediate polarity compound illustrates the compromise inherent in a multicomponent extraction method required to recover compounds like the OPs and metabolites that exhibit a range of polarity. CV values for some of the recovered OP compounds appeared to be high. However, results from other pesticide recovery studies indicate that these values are not unreasonable.

Recoveries from the fatty beef muscle tissue samples (29.3% fat) ranged from 22 to 79% at the 1 ppm level. Coefficients of variation were between 1.1 and 16.0%, with an average of 5.9%. With one exception, recoveries from fatty muscle tissue were lower than the recoveries of the corresponding OPs and metabolites from the lean tissue.

These lower percentage recoveries from the fatty muscle tissue samples are logical in relation to the overall polarity of the OP and metabolite compounds studied. Many of the OPs and metabolites used in the study can be considered to be relatively nonpolar, a characteristic of phosphate esters containing aromatic moieties. These OPs and metabolites are likely to partition into the lipid portion of the tissue sample matrix (Ivey et al., 1969). As the percentage of tissue lipid in the sample matrix increases, more of the available OP and/or metabolite will partition into that lipid component. The low-polarity analyte is then less available for extraction from the sample matrix, resulting in the observed lower recovery. Lowering the polarity of the extraction solvent to increase analyte to solvent affinity is an option. However, an increase in coextracted lipid is likely to occur, making isolation of the compounds of interest more difficult.

Two observations suggest that lipid material was being extracted from the beef tissue matrix by the ethyl acetate-methanol extraction solvent. First, extract filtrate that was stored at refrigerator or freezer temperatures (5 to -13°C) exhibited a sizable amount of whitish precipitate.

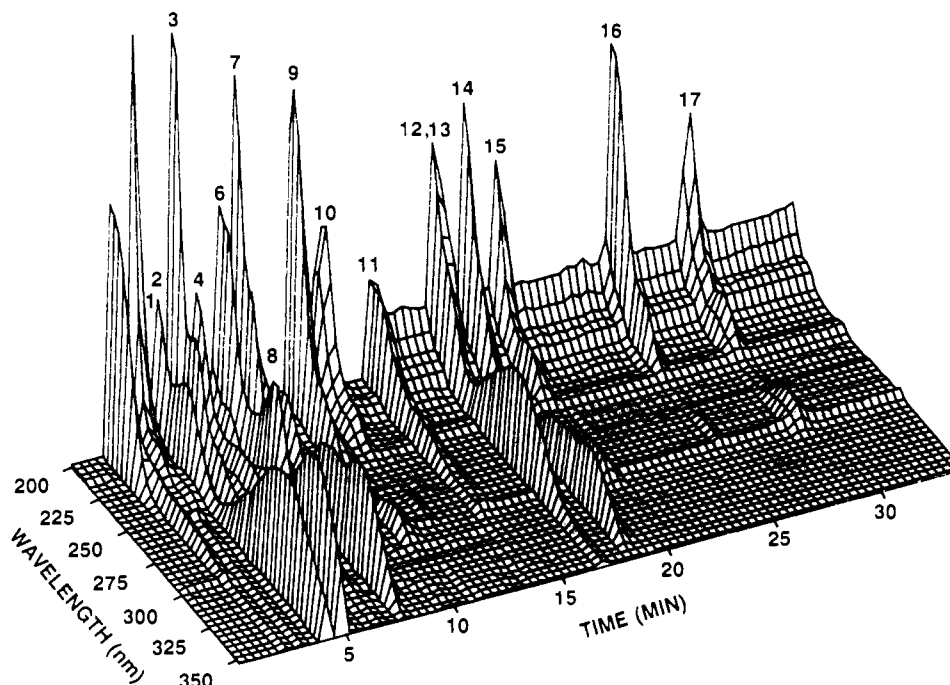


Figure 3. Three-dimensional chromatogram of organophosphate pesticide and metabolite standards. Famphur oxon (1) does not show and parathion (12) and fenthion (13) are not resolved in this graph.

This precipitate had a greasy tactile character and dissolved back into the filtrate solution upon reattaining ambient temperature. The second indication is illustrated by the need for a post-run column flush with 100% acetonitrile following the tissue extract injections on the HPLC. This flush resulted in the elution of several UV-absorbing peaks that would otherwise interfere with later HPLC analyses of extracted tissues. We believe that these tenaciously retained compounds represented lipid material that had been coextracted from the tissue sample with the compounds of interest. The incorporation of a C_{18} solid-phase extraction (SPE) cleanup step into the method was an attempt to alleviate this problem. The strong retention of these interfering compounds on the C_{18} analytical column and the requirement of 100% acetonitrile to elute them as a group suggested a possible cleanup solution. To exploit the tenacious affinity of the interfering compounds for the reversed-phase packing material, a C_{18} SPE cartridge was used to mimic the analytical column. By selecting an eluting solvent slightly weaker than 100% acetonitrile, we reasoned that the OPs and metabolites would elute from the SPE cartridge while the lipid material would remain and, therefore, be unavailable to interfere in the analytical system. This approach was only partially successful, however. A post-run flush was still necessary for complete removal of these strongly retained compounds. However, the chromatograms of the extracted OPs and metabolites were somewhat improved in quality, particularly for the compound library match. Therefore, we decided to incorporate the C_{18} SPE cleanup step into the method. Attempts at alternate SPE cleanup techniques such as Florisil resulted in unacceptably high analyte losses from the OP-fortified tissue.

The OP and metabolite compounds added to the beef tissue at the 1 ppm level were readily apparent on the HPLC chromatogram. The compounds of interest began eluting at about 4 min, immediately after a group of large background peaks. These early eluting background peaks were characteristic of all the meat extracts. The last OP compound to elute was chlorpyrifos, at about 30 min.

The hydrolysis product of fenthion, 3-methyl-4-(methylthio)phenol (MTP), was not recovered in amounts

great enough to allow positive identification. Preliminary work done in the study indicated that MTP was recovered at approximately 75% levels, but recoveries were intermittent and unpredictable. Why such inconsistency in the recovery of this compound occurs is unknown at this time, though oxidation and interaction of the thio group with other components of the beef tissue, forming sulfur bridges, is a possibility. This interaction might then render the compound unrecoverable using the current method. MTP was readily detected when injected as a pure standard.

An important aspect of using the diode array detector is the capability to scan the spectral absorbance of given compounds over a specified range of wavelengths (Figure 3). This allows generation of absorbance profiles unique for those compounds. Two major advantages were derived directly as a result of this potential. First, wavelengths could be chosen to optimize the detector sensitivity for a given compound by choosing the primary or secondary absorbance maxima of that compound. Most of the compounds exhibited relatively high UV absorbance at or below a wavelength of 205 nm. However, it was sometimes preferable to monitor a compound's secondary absorbance maxima at a higher wavelength to compensate for coextracted UV-absorbing material. An example of this was with the oxygen analog of famphur. At 202 nm, UV absorbance was very high for this compound. The famphur oxon was the first OP to elute and appeared immediately after a group of unretained interference peaks that absorbed at the lower UV wavelengths. At a wavelength of 230 nm, however, the coextracted interference had diminished sufficiently to allow resolution of the famphur oxon. Also, compounds such as parathion and fenthion that had nearly identical retention times (approximately 17.1 and 17.2 min, respectively) could be resolved because of slightly different UV absorbance maxima (274 nm for parathion and 250 nm for fenthion). The various wavelengths used to monitor each of the respective OPs and metabolites are included in Table I.

The second advantage of diode array detection was the ability to create a library of UV absorbance profiles of standards. Retention times of unknowns relative to

standards were used for preliminary identification of the recovered OPs and metabolites. This preliminary identification then could be confirmed by computer matching of the compound with the stored UV absorbance profiles of the standards. The Hewlett-Packard software uses a match scale of 0–1000 based on the spectral library. Numbers over 990 indicate a probable match. Numbers between 900 and 990 indicate the possibility of a match or similar structures, and numbers below 900 indicate that a match is unlikely. All library compound matches of recovered OPs and metabolites in this study were within software parameters for positive identification with standard retention times.

The objective of this study was the development of a rapid, accurate, and reproducible method utilizing HPLC for the separation and determination of OPs and their metabolites in beef muscle tissue. The results indicated clearly that these compounds can be separated on a reversed-phase HPLC system (see Figure 2). Further, utilization of the diode array detector allowed the detection of several of the separated compounds at or below regulatory tolerance levels that have been established (CFR, 1991). The recoveries obtained in the study would allow quantitative determination of this class of pesticides and metabolites in beef tissue. The range of compounds determined using this method supports its use as a true multiresidue detection technique. The method's primary utility would appear to be as a screening technique to determine the presence of suspected OPs and/or associated metabolites. No method currently exists for extraction and determination of OP metabolites as a group in beef tissue. The present method, utilizing a straightforward extraction procedure and the improved optics of diode array detection, verifies the applicability of HPLC for analysis of OP metabolite residues.

ACKNOWLEDGMENT

This material is based upon work supported by The Cooperative State Research Services, U.S. Department of Agriculture, under Agreement 89-34187-4511.

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Received for review August 11, 1992. Accepted November 30, 1992.