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RTI

FINAL REPORT

Study Title

Method Validation/Confirmation - Ethyl Parathion and Products in Plant Materials

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Performing Laboratory

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Laboratory Project ID

70C-4996-001

Charles M. Sparacino, Study Director Date

RESEARCH TRIANGLE INSTITUTE

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B), or (C).

Company: Chaminova Agro A/S

Company Agent: Mr. Jon Weis

Title: Manager of Patents and Registration

Date: 11/30/92

Signature: Jon Weis

GOOD LABORATORY PRACTICE STATEMENT

This study (70C-4996) meets the requirements of 40 CFR Part 160.

Charles M. Sparacino, Ph.D.
Director, Center for Applied Analytical Systems
Research Triangle Institute
Study Director

11-30-92
Date

This study was conducted in compliance with GLP regulations issued by the U.S. EPA (40 CFR, Part 160). This statement is based on the statement of the study director set forth above, the terms of the contract between the sponsor and the laboratory that conducted the study, and other assurances given by the testing facility.

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QA Statement
RTI Project 4996

Quality Assurance activities undertaken by the RTI Analytical and Chemical Sciences (ACS) Quality Assurance Office in support of this program (Method Validation) included:

- conducting periodic reviews and audits of the data measurement systems,
- meeting with project leaders on matters affecting data quality, and
- monitoring situations requiring corrective action.

The ACS QA Office conducts systems audits of current ACS studies to ascertain that data are being recorded properly, SOPs are being implemented, and that the results reported reflect the raw data of the study. Written reports of all reviews and audits are maintained by the ACS QA Officer, and results have been reported to the program management. Audits and inspections conducted for this program are listed below.

Audit	Conducted	Reported
Notebook Inspection (ACS-SOP-815-002)	10/5/92	10/7/92
Document Review (ACS-SOP-130-003)	10/7/92	10/7/92
Data Review	10/5/92	10/7/92

The ACS QA Office conducts unit-wide inspections and audits to ascertain that ACS research unit SOPs and good laboratory practices are being followed. The most recent inspections and audits and the date results were reported to management are listed below.

Inspection/Audit	Conducted	Reported
Instrument Log Notebook Inspection (ACS-SOP-815-003)	4/92	6/26/92
Notebook Inspection (ACS-SOP-815-002)	6/92	7/7/92
SOP Review (ACS-SOP-110-001)	8/92	10/28/92
Training Files Inspection (ACS-SOP-110-002)	9/92	10/5/92

To the best of my knowledge this report accurately describes the methods and procedures used for this study, and the results reported accurately reflect the raw data.

Doris Smith
ACS QA Officer

Date

SPONSOR AND TEST FACILITY

The study was sponsored by:

Cheminova Agro A/S

Lemvig, Denmark

The study was conducted at:

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SECTION 1.0

ABSTRACT

In support of a pesticide reregistration effort, analytical method validation and confirmation studies were conducted for ethyl parathion (EP) and its metabolites in four plant commodities. The parent compound and the metabolites ethyl paraoxon (EPOX) and p-nitrophenol (PNP) were assayed (using gas and liquid chromatography) in wheat straw, wheat grain, wheat flour and sunflower seed oil to validate proposed analytical methodology. Method confirmation studies (using gas chromatography/mass spectrometry) were conducted for the three compounds in wheat grain Results of the validation assays confirmed the viability of the proposed methods. EP and PNP gave acceptably high and precise recoveries. EPOX gave recoveries well in excess of 100% and with less precision than was observed for EP and PNP EPOX proved difficult to analyze due to its highly variable behavior during gas chromatographic separation. Time estimates for complete processing and analysis of sample batches were determined for each analyte

SECTION 2.0
INTRODUCTION

The Environmental Protection Agency requires that sponsors of pesticide registration efforts provide results of a validation by an independent laboratory for a proposed tolerance enforcement method (EPA PR Notice 88-5) Ethyl parathion (EP) is a pesticide applied to several cash crops, and an enforcement method for this compound and its primary metabolites, ethyl paraoxon (EPOX) and p-nitrophenol (PNP), has been proposed by the sponsors. The requisite validation and confirmation studies for these compounds were performed at the Research Triangle Institute, and are described herein. All studies were conducted in compliance with FIFRA GLPs.

SECTION 3.0

SELECTION OF SUBSTRATES FORTIFICATION LEVELS

Cheminova plans to reregister ethyl parathion (EP) on five important crops: wheat, rape, sunflower, barley, and sorghum. Major commodities for these crops are oil, straw, grain and flour. The commodities selected for method validation were sunflower oil (processed), wheat straw, wheat grain, and wheat flour (processed). Sunflower oil will also represent rape oil (canola), and wheat will represent barley and sorghum commodities. Wheat straw was selected over fresh forage because the desired analytes are more difficult to analyze in straw.

Fortifications at the low level for EP, ethyl paraoxon (EPOX), and p-nitrophenol (PNP) were 0.05 ppm each, the anticipated LOQ level with recoveries from 70% to 120%. The highest level of fortification for EP and PNP will be 2 ppm whereas the highest level for EPOX will be 0.50 ppm. Trials for sorghum resulted in residues for grain such as EP 2 ppm, EPOX, 0.2 ppm, and PNP 0.5 ppm. In hay and straw, levels of PNP can be of the same magnitude as EP. EPOX is always much lower than that for EP in crop commodities (raw and processed).

SECTION 4.0

DESCRIPTION OF METHODS

All residue analytical methods for EP, EPOX, and PNP for various plant commodities are provided in the appendices to this report. The general methodology is described in "Determination of Ethyl Parathion [EP; O,O,-diethyl-O-p-nitrophenyl phosphorothioate] and Its Metabolites (Ethyl Paraoxon [EPOX, O,O-diethyl-O-p-nitrophenyl phosphate] and p-nitrophenol [PNP; 4-nitrophenol]) in Various Matrixes," MRID #42133601. This section includes a general description of the methodology and instrumentation, and contains some representative calculations for analyte quantitation and recovery.

4.1 GENERAL METHODOLOGY

Plant homogenates were extracted by reflux with an acidified aqueous–organic solvent mixture, the extracts were purified by solvent partition. Concentrated extracts were analyzed by GC using flame photometric detection for EP and EPOX. Prior to PNP analysis by HPLC using UV detection, extracts were partially purified using a column of Florisil. For confirmatory methods, extracts were analyzed by capillary GC/MS. Data was acquired and processed using an automated digital data acquisition system.

4.1.1 Wheat commodities

A homogenized sample was spiked with known quantities of each analyte. The homogenate was extracted with acidified aqueous/organic solvent by reflux. The solvent was concentrated and the aqueous concentrate was extracted with additional solvent. The solvent was partially removed to provide a concentrate suitable for direct analysis by packed column GC/FPD for EP and EPOX. Samples were run with check standards interspersed. Quantitation was achieved by use of a calibration equation (area counts vs. concentration).

A portion of the extract was partially purified by passage through Florisil. An appropriate fraction was collected from the Florisil column and solvent-exchanged into an aqueous medium. A portion of the aqueous solution was injected onto an HPLC system for quantitation of PNP. A calibration curve was generated from standard samples. The method as given in detail in appendix [illegible] additions and modifications to the method submitted by the sponsors (appendix [illegible]) have been incorporated into this method description.

4.1.2 Sunflower seeds

Dry [illegible] was spiked with known [illegible] homogenate. The oil was extracted by reflux with acidified aqueous [illegible] portion of the extractant was removed by

evaporation, the aqueous portion was then extracted with organic solvent. After concentration, a portion of the organic concentrate was analyzed by GC/FPD for EP and EPOX. Samples were run with check standards interspersed. Quantitation was achieved by use of a calibration equation (area counts vs. concentration)

A portion of the extract was partially purified by passage through Florisil. An appropriate fraction was collected from the Florisil column and solvent exchanged into an aqueous medium. A portion of the concentrate was injected onto an HPLC system for quantitation of PNP A calibration curve was generated from standard samples. The method is given in detail in appendix II All the additions and modifications to the method submitted by the sponsors (appendix IV) have been incorporated into this method description. The major change compared to the wheat commodity method is the addition of an acetonitrile/hexane partition to remove oil from the extract

4 2 INSTRUMENTATION

4 2 1 Validation Studies

Analysis for EP and EPOX in the validation methods was conducted by packed column gas chromatography Analysis for PNP was conducted by reverse phase high performance liquid chromatography System components and run conditions are detailed below

4.2.1 1 Gas Chromatography

Model Varian 3700 with Varian Series 8000 Autoinjector

Column 10% SP 2100 on 100/120 mesh Supelcoport , 4 or 2 mm x 2 m

Carrier Gas Helium @ 40 mL/min Oven Temperature 245°C

Detector Flame Photometric in Phosphorous Mode

Detector Temperature 260°C

Injection Temperature 250°C Injection Volume 1-10 µL

Data Handling Nelson Analytical Data System

4 2.1 2 Liquid Chromatography

Solvent Delivery Waters 590 and [illegible]00 Pumps with 680 Programmer

Injector Micromeretics 728

Injection Volume 20 µL

Detector Analytical Biosystems 75[illegible] at 315 nm

Column Gas Chromosphere ODS [illegible] packing [illegible] 2 x 100 mm

Mobile Phase [illegible] Acetonitrile [illegible] Water [illegible] Acetic Acid

Flow Rate. 1.0 mL/min

Data Handling: Nelson Analytical Data System

4.2.2 Confirmation Studies

For method confirmation, wheat grain extracts were analyzed under conditions different from those used in the validation study. Instead of packed column GC and HPLC, a single analysis for all three analytes was conducted by capillary GC/MS. Instrument components and run conditions are detailed below.

4.2.2.1 Gas Chromatography

Model: Hewlett-Packard 5890 with HP 7673 Autoinjector

Column: J & W DB-1 fused silica, 30 m x 0.32 mm ID, 0 25 µ film

Carrier Gas: Helium at 1.2 mL/min

Oven Temperature 60°C to 220°C at 5°C/min, to 300°C at 12°C/min

Capillary Injector: 1 min splitless/split

Injector Temperature: 240°C Injection Volume: 1 µL

4 2.2.2 Mass Spectrometry

Model: Hewlett Packard 5988A

Ionization Mode Electron ionization, selected ion monitoring

Emission Current: 0 3 mA Electron Multiplier 1750 V

Source Temperature: 200°C

4 3 CALCULATIONS

The determination of recovery data for each analyte in each matrix at each fortification level is accomplished by use of the regression equation obtained from the analysis of calibration standards Raw chromatographic area counts from analysis of each method validation sample are substituted in the equation for the response value The sample concentration corresponding to that response is calculated. The concentration value is then divided by the nominal concentration (i e fortification level) to obtain percent recovery

To exemplify this process, the percent recovery for EP in straw at the highest fortification level (2 ppm) is determined as follows The chromatographic response from injection of an aliquot from the 2 02 ppm solution was 1,981,036 area counts The regression equation appropriate for EP in straw was $y = 1{,}204{,}155x - 15{,}393$, where y represents detector response and x represents found concentration Thus,

$$x = (y - 15{,}393) / 1{,}204{,}155$$

x = (1,981,636 + 15,393)/1,204,155

x = 1,997,029/1,204,155

x = 1.66 ppm (rounded to 2 places)

For recovery,

% recovery = (1.66/2.02)*100 = 82.2%

Mean recoveries of a specific analyte for a selected commodity (as shown in Table 7) were calculated by summation of the average recoveries for a given concentration level and dividing by the total number of concentration levels. For example, for EP in grain, the mean recovery is calculated using data from Table 1:

$$\frac{(157 + 93.2 + 99.2 + 95.0)}{4} = 111 \quad \textit{(to three significant figures)}$$

SECTION 5.0
COMMENTS

Although the two proposed methods for analysis of EP, EPOX, and PNP in wheat commodities and sunflower seed oil proved feasible, some problems were encountered. On May 29, 1992, JSC informed the Agency that RTI was having instrument problems with its mass spectrometer and was having difficulty analyzing wheat grain control samples because of interference peaks preceding the ethyl parathion peak. Control grain (organically grown) had been used for spiking with ethyl parathion and paraoxon. Control grain appeared to have been contaminated with an organophosphate and alternative wheat control grain samples had to be located. For these reasons we requested an extension for submitting these reports until November 1, 1992.

Some other problems were critical in nature, and some seemed to be inherent in the method or reflect the nature of the compounds analyzed. No major modification of the methods were required; several minor modifications were incorporated, usually for reasons of simple practicality

5.1 CRITICAL STEPS

The most important problem encountered in the analysis of the specified analytes was associated with the GC behavior of EPOX In our hands the compound gave highly variable results which necessitated extra care in developing a stable GC system for successful analysis The other analytes, EP and PNP, proved to be well-behaved in all respects, and required no precautions, other than those routinely applied, for low level pesticide analysis

5.1.1 EPOX Problems

Several factors became obvious immediately upon attempted GC analysis of EPOX. The first observation involved apparently variable response by the flame photometric detector Although there is no obvious reason why there should not be equal response for EP and EPOX the detected species is the same for both compounds), EPOX only provided approximately one fifth the response of EP This factor led to a great deal of effort in successfully obtaining the requisite sensitivity levels It was also discovered that unlike EP, the GC column required priming before analysis could be initiated The priming involved the introduction of very high levels (at least 5 times the amount associated with the highest concentration calibration standard) of EPOX onto the column It was also observed that priming with standard alone was not as effective as priming with EPOX in plant extract Matrix components were important,

particularly for GC/MS analyses, in preparing the GC system for calibration and sample analysis. It is recommended that automated run sequences include a matrix sample (blank or spike) after every ~5 analysis samples.

Column priming is considered a critical element in both proposed methods.

Although a conventional packed GC column is specified by both, it was discovered that column voids caused significant loss of EPOX signal. Again, this was in distinction to EP where, like most packed column analyses, column voids had relatively little effect on either the magnitude or shape of the GC peak. It was required that voids be completely eliminated, and that the column be filled to the top with packing material. The glass wool used to retain the column packing also required frequent change and complete silanization.

Elimination of column voids is considered a critical element in the proposed method.

5 1 2 Analyte Extraction

The initial extraction of analytes from plant materials involves either acetone (wheat grain and flour) or methanol Sample spiking was conducted by addition of the analytes as a methanol solution Following reflux, the methanol (from the extracting solvent or from the spiking solution) is removed by rotary evaporation. It was discovered that the methanol must be completely removed prior to subsequent processing. If methanol is not completely removed, emulsions resulted when the water was then partitioned with ethyl acetate or hexane. This observation was noted during trial runs, for all ensuing sample analyses, care was taken to ensure that the evaporation of methanol was complete so that emulsions were not encountered.

Complete removal of methanol from the extracting solvent mixture is considered a critical element in the proposed methods.

5 1 3 Solution Concentration

In two steps in the processing of wheat commodities or sunflower seed oil for PNP analysis, solvent exchange is specified The first involves complete removal of ethyl acetate prior to dissolution of the residue in hexane for mini-column clean-up In this step the ethyl acetate

must not be taken to dryness prior to the addition of hexane. The presence of small amounts (≈ 50 µL) of ethyl acetate in hexane did not affect the efficacy of the subsequent Florisil clean-up step.

Following clean-up, the final mini-column eluant is solvent-exchanged into HPLC mobile phase. The proposed method specifies complete solvent removal prior to dissolution in mobile phase. Again, the eluant must not be taken to complete dryness. Reduction of the eluant to approximately 50 µL prior to addition of mobile phase was the procedure followed in all analyses.

Prevention of complete removal of solvent is considered a critical element in the proposed method.

5 2 MINOR MODIFICATIONS

As noted earlier, several minor modifications were adopted for successful implementation of the methods. These modifications are not considered critical, but are important in preventing potential problems and in minimizing the time required for analysis Each modification is briefly discussed below.

5 2.1 Sodium Sulfate

Following extraction of the analytes from each plant material, the aqueous extract is extracted with ethyl acetate Prior to concentration of these ethyl acetate solutions by rotary evaporation they are passed through a bed of sodium sulfate The method specifies the use of 200 g of sodium sulfate This amount did not completely remove water from the organic solvent. In practice it was found that 300-400 g of sodium sulfate in the funnel were required to effect complete removal of water

5 2.2 Flask Size

Following the extraction of each plant material (using a 500 mL round bottom flask), rotary evaporation of acetonitrile or ethyl acetate is required The proposed methods specify the use of 500 mL round bottom flasks In our hands bumping during this operation caused solvent to surge into the evaporator, even when protective glassware was used between the flask and the evaporator The use of a 1000 mL round bottom flask minimized this problem significantly

5.2.3 Wheat Straw

Practical difficulties were encountered in processing straw. The proposed method specifies simple homogenization with a Polytron. It was discovered that straw cannot be homogenized unless the individual stalks are reduced in size first. This was accomplished by chopping the stalks into lengths of approximately 1 cm, then mixing the chopped straw with dry ice and grinding with a mortar and pestle. The ground mixture can then be partially homogenized.

5.2.4 Particulate Removal

In processing wheat commodities, the aqueous extract is extracted with ethyl acetate, which is then concentrated and made to 25 mL in a volumetric flask. In some cases, particularly for wheat straw, particulate matter was visible in the ethyl acetate solution. When aliquots were drawn from the volumetric for analysis of EP and EPOX, the solution was filtered through 0.45 μ Teflon filters. This filtration step is not specified in the proposed method.

5.3 GENERAL COMMENTS AND OBSERVATIONS

There are a number of observations relevant to the proposed method that did not involve method modification, but are deemed worthy of comment since they can have an effect on application of the method These observations are of variable significance.

5.3.1 GC Column Lifetime

Successful analysis of EP and EPOX required constant attention to the GC column It was observed that column lifetimes were rather short, seldom extending beyond the analysis of two complete sample sets (calibration and analysis) Some matrixes, e.g. straw, had a more pronounced deleterious effect, and produced, after some 40 injections, peaks with significant broadening

5.3.2 Mini-column Clean-up

The clean-up column (used prior to HPLC analysis for PNP) consists of a Florisil bed with a top layer of anhydrous sodium sulfate During operation it is important to prevent the level of eluting solvent from falling below the top of the sodium sulfate plug If the solvent is allowed to fall to the top of the Florisil bed, air is entrained in the column and flow is stopped Also, the addition of extract to the column must be conducted slowly or the sodium sulfate layer rises and an air space is introduced If this happens, the sodium sulfate plug can be pushed down using a small glass rod or spatula

5.3.3 Wheat commodities

Direct homogenization of wheat grain sometimes led to clogging of the Polytron head with individual grain particles. These particles were physically removed to allow for complete homogenization.

It is important that care be exercised when heating wheat flour or homogenized grain. The extracting solution must be brought to temperature slowly to prevent localized superheating and concomitant charring of the matrix.

5 3.4 Partitioning of Extracts

The proposed method specifies that separatory funnel shake-out be conducted for one minute. To prevent emulsions, the shaking operation should not be vigorous.

5.3.5 Fortification

Sample fortification is conducted, according to the proposed method, by the addition of up to 20 mL of spiking solution (at the 2 ppm level). Such an operation does not effectively introduce the analyte to the matrix, and cannot therefore provide an accurate measure of recovery efficiency.

5.3 6 Pesticide-free Plant Controls

Plant controls for use in the proposed method should be analytically screened prior to selection of a particular matrix for validation In our experience, wheat materials that were promoted as "pesticide-free" were not always so

SECTION 6.0
RESULTS AND DISCUSSION

Recovery data were tabulated for each sample and matrix. In the tables shown below the individual recoveries for each of the duplicate analyses and their averages are given. The detection limits (ppm Controls) were determined by assessing the baseline noise of control samples, and using a response value that corresponded to a value of three times the baseline noise value.

6.1 VALIDATION STUDIES

6.1.1 EP

Recovery data for EP are shown in Table 1. This analyte was well behaved as is demonstrated by the precision for duplicate recovery determinations. The mean difference in duplicate values for fourteen analyses where duplicate data was available was 6.3%. The linearity of response for EP in each of the four matrixes was very acceptable; correlation coefficients for the regression equations were all greater than 0 999 All recoveries were above the requisite level of 70% and below 120%, except for grain at the 0.05 ppm level. These recoveries were 149% and 165%.

Representative chromatograms for EP (and EPOX) are shown in Figures 1-4. The chromatograms depict analyses for samples at two fortification levels, the lowest and a medium high level Background components are present for wheat straw and grain, but not at retention values that interfered with analytes

6 1 2 EPOX

Recovery data for EPOX are shown in Table 2 The difficulties associated with the packed column analysis of EPOX have been discussed earlier, these difficulties are obvious in the precision and level of recoveries given in Table 2. The mean recovery for 16 determinations is 119%, and the average difference in duplicate determinations is 16% Linearity of response was not as high as for EP, but was nonetheless acceptable with correlation coefficients for the four regression equations greater than 0 99 The high recoveries are most likely due to the necessity of conditioning the column for EPOX The proposed methods specify that calibration curves be generated from standard solutions of analytes rather than from fortified matrix samples The possible priming effect of matrix components was noted earlier and may therefore be responsible for the high recoveries of EPOX Incorrect dilutions of stock solutions are ruled

out as a possible explanation for high recoveries since the calibration solutions contained all analytes, and recoveries for the other two analytes are not unexpectedly high.

Representative chromatograms of EPOX are shown in Figures 1-4. Low level signals were difficult to integrate owing to the broadness of the peaks, and to the variable response problem addressed earlier. There were no significant interferences for EPOX from any of the four matrixes studied.

6.1.3 PNP

Recovery data for PNP are shown in Table 3. These data more closely resemble results obtained for EP. The mean recovery value for 16 determinations is 108%, and the average difference in duplicate analyses is 9% All 32 recoveries were greater than 70% and only six were greater than 120%. Linearity of response was excellent with correlation coefficients greater than 0.999 for all four regression equations

Representative chromatograms are shown in Figures 5-8. As seen in Figure 5, straw produced many background components, some of which produced peak overlap with PNP. The control sample showed a low response component with a retention value almost identical to that of PNP. Flour and grain also showed background components, but none that interfered with the response for PNP. Sunflower seed oil produced a very clean chromatogram, as was also noted for gas chromatograms for this matrix

6 2 CONFIRMATION STUDIES

In accordance with the Acceptance Criteria for #171-4 (c and d), a confirmatory method must be developed and applied to the final extracts of the tolerance enforcement method The extracts should be analyzed using different separation/detection methods. Preliminary work in our laboratory showed that capillary GC/MS was a feasible approach for confirmatory analyses for all three analytes Consequently, extracts from wheat grain were analyzed using conditions as specified (Section 4 2 2), and with results shown in comparison with the results from the enforcement method for grain

From mass spectra generated from injections of standards (Figures 9-11), several ions were considered for monitoring purposes for each analyte A common ion for each standard was m/z 109, which corresponds to a fragment of PNP Other ions were selected for monitoring, including m/z 291 (parent ion) for EP, m/z 57 for EPOX, and m/z 139 (parent) for PNP Since m/z 109 was common to all compounds and was also a high intensity ion for all compounds, it was chosen for quantitation purposes

Although selected ion monitoring is generally a very sensitive mode of analysis for GC/MS, the variable GC behavior of EPOX led to a relatively high LOD. Initial results from introduction of EPOX into the capillary GC/MS system produced no response. Priming with high concentrations of standard solutions did little to increase system sensitivity. Priming with matrix components (controls) however resulted in responses sufficiently reproducible and intense that target LODs were reached.

6.2.1 Results

Recovery data for EP are shown in Table 4. The mean recovery for all fortification levels was 103%. The range of recoveries was small as is indicated by an estimate of precision of 11%RSD. The average difference in duplicate recovery values was approximately 13%. The regression equation was linear, with a correlation coefficient of 0.9959.

Recovery results for EPOX are shown in Table 5. The data reflect the results seen from GC/FPD analysis, with high and variable recovery values noted for the different fortification levels. Quantitation was not possible for the lowest concentration samples due to interferences for m/z 109. Similar interferences were observed for m/z 81. The average recovery for three fortification levels was 159%, and the average difference in duplicate values was approximately 24%. The calibration data were acceptable, the regression equation yielded a correlation coefficient of 0.9959.

Recovery values for PNP (Table 6) averaged 115% with 14% RSD. The average difference in duplicate determinations was approximately 20%. As with the other analytes, good linearity was observed for the regression equation (correlation coefficient of 0.9992).

Representative ion current chromatograms (total current for four ions - m/z 109, 139, 275 and 291) for all analytes are shown in Figure 2. These chromatograms were reproduced with the same scaling factor for peak intensity to allow for direct comparison for the three concentration levels. The control chromatogram of grain shows a number of peaks corresponding to background components with m/z 109 ions. None of these components interfered with the target analytes.

6.2.2 Validation/Confirmation Comparison

A comparison of recovery values for all three compounds from wheat grain as determined by analysis using the validation method (GC/FPD and LC/UV) and the confirmation method (GC/MS) is shown in Table 7. These data were taken from Tables 4-6. Considering the data as statistically paired, the differences in mean recovery for EP and PNP are not significantly

different. Given the small population sample size and the high variances associated with some of the recovery values, such a comparison is of limited meaning The recoveries for EP and PNP differ by less than 8% and 2% respectively. The EPOX values are more disparate (by more than 20%), a reflection no doubt of the previously discussed difficulties of analysis for this compound.

6.3 METHOD TIME REQUIREMENTS

Complete analysis of a set of 10 samples (8 samples plus 2 controls) required a total of seven working days. This estimate does not include the time (= 8 hours) required for the preparation of standard solutions, mobile phases, blanks, etc. Each of the seven working days was usually a "long" day, i e. in excess of 8 hours. The activities and break points associated with each time period are shown below These estimates are based on the availability of 6 reflux apparatuses, 2 Büchner filtration set-ups, and 2 rotary evaporators For certain steps, e.g the ethyl acetate partition of aqueous plant material extract, the maximum number of samples that can be processed at one time by one worker is 10

Day 1

- Set-up activities (collecting glassware, labeling, equipment set-up, etc)
- Weighing/homogenization of samples
- Extraction by reflux
- Crude extract filtration (Buchner)
- Methanol removal (rotary evaporation)

Day 2

- Ethyl acetate partition of aqueous extract
- Ethyl acetate concentration (rotary evaporation)

Day 3

- Solvent exchange to hexane
- Florisil clean-up

Day 4

- GC set-up/check initiation of EP and EPOX sample analysis
- LC set-up/check initiation of PNP sample analysis

Day 5

- Data processing - raw GC data

Day 6

- Data recording/notebook write-up/calculations
- Data processing of raw LC data

Day 7

- Data recording/notebook write-up/calculations
- Lab/equipment/glassware clean-up

SECTION 7 0
CONCLUSIONS

The proposed tolerance enforcement method for the analysis of wheat materials and sunflower seed oil for EP, EPOX and PNP consisted of basically viable methodology. The application of alternate separation and detection methodology confirmed the viability of the method Using the proposed method for the analysis of EP and PNP gave acceptably high and precise recoveries of these analytes. Analysis of ethyl paraoxon gave recoveries well in excess of 100%, and with less precision EPOX proved difficult to analyze due to its highly variable behavior during GC separation Matrix components were shown to have an important (positive) effect on this separation

Some minor modifications and precautions are considered critical for the overall success of the method. These factors are delineated in the body of this report. An important observation involved the problem of obtaining pesticide-free plant materials for use as controls or for fortification purposes A screening of controls is advised before using these two methods.

The methods required significant effort and time for complete processing of a single sample set. It was estimated that a single person required approximately seven working days (some in excess of 8 hours) for a complete analysis

Table 3 Validation Method Recovery Results - PNP

Sample	ppm Controls	ppm Fort	% Recoveries	(Average)
Wheat Straw	<0 02	0 051	162; 128	(145)
		0 510	63 1; 89 6	(76 4)
		1 28	82 8; 79 7	(81 2)
		2 04	77 9; 80 9	(79 4)
Wheat Grain	0 02	0 051	104, 96.9	(100)
		0 510	101; 118	(110)
		1 28	119; 117	(118)
		2 04	123; 125	(124)
Wheat Flour	<0 02	0 051	102; 90 2[a]	(96 0)
		0 510	92.5, 106	(99 2)
		1 28	103; 101	(102)
		2 04	94.1, 112	(103)
Sunflower Seed Oil	<0 02	0 051	159; 159	(159)
		0 510	115, 111	(113)
		1 28	113; 112	(112)
		2 04	107 106	(106)

Curves

Wheat Straw	y = 81,389x - 266 0,	r = 0 9999
Wheat Grain	y = 105,819x - 806 0,	r = 0 9999
Wheat Flour	y = 82,710x + 142.5,	r = 0 9998
Sunflower Seed Oil	y = 84,873x - 1,058;	r = 0 9996

[a]Repeat sample analysis required for this sample; analyzed and quantitated with sunflower seed oil samples

Table 2 Validation Method Recovery Results - EPOX

Sample	ppm Controls	ppm Fort	% Recoveries	(Average)
Wheat Straw	<0 05	0 0500	105; 130	(118)
		0.200	124, 129	(126)
		0.350	116; 114	(115)
		0.500	113; 117	(115)
Wheat Grain	0 05	0 0500	122; 105	(114)
		0.200	109; 125	(114)
		0.350	123, 143	(133)
		0.500	134; 118	(126)
Wheat Flour	<0 05	0 0500	150, 101[a]	(126)
		0 200	104[a], 150	(127)
		0 350	152, 151	(152)
		0 500	144, 185	(164)
Sunflower Seed Oil	<0 05	0 0500	78.8, 78 0	(78 4)
		0 200	98 6, 94 7	(96 6)
		0 350	100; 96 9	(98 4)
		0 500	90 6, 101	(95 8)

Curves

Wheat Straw	y = 280,412x + 218 o.	r = 0 9980
Wheat Grain	y = 514,289x + 974 9.	r = 0 9989
Wheat Flour	y = 340,876x - 2,863.	r = 0 9989
Sunflower Seed Oil	y = 476,960x + 6,868.	r = 0 9998

[a]Integration may be unreliable due to negative baseline

Table 1. Validation Method Recovery Results - EP

Sample	ppm Controls	ppm Fort	% Recoveries	(Average)
Wheat Straw	<0 04	0 0504	104, 100	(102)
		0 504	83 1, 80 0	(81 6)
		1 26	77 9, 80 2	(79 0)
		2 02	75 2; 82.2	(78 7)
Wheat Grain	<0 04	0 0504	165; 149	(157)
		0 504	87 9; 98 4	(93 2)
		1 26	98 4; 100	(99 2)
		2 02	97 0, 93 1	(95 0)
Wheat Flour	<0 04	0 0504	102, Lost	(102)
		0 504	Lost; 82 7	(82 7)
		1 26	93 7, 89 7	(91 7)
		2 02	87 6, 105	(96 3)
Sunflower Seed Oil	<0 04	0 0504	86 5, 86 7	(86 6)
		0 504	92 1, 90 3	(91 2)
		1 26	102, 89 7	(95 8)
		2 02	88 6, 86 1	(87 4)

Curves

Wheat Straw	y = 1,204,155x - 15,393,	r = 0 9999
Wheat Grain	y = 1,751,462x - 42,876,	r = 0 9995
Wheat Flour	y = 1,650,220x - 16,179,	r = 0 9999
Sunflower Seed Oil	y = 1,272,215x - 1,998,	r = 0 9999

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TABLES

Table 4 Confirmation Method Recovery Results - EP

Sample	ppm Controls	ppm Fort.	% Recoveries	(Average)
Wheat Grain	<0 04	0 0504	96 1, 77 2	(86.6)
		0 504	104, 105	(104)
		1 26	107, 113	(110)
		2.02	124, 99 6	(112)

Curve: y = 0 03755x - 6 391; r = 0 9959
where x = area counts
y = found concentration

Table 5 Confirmation Method Recovery Results - EPOX

Sample	ppm Controls	ppm Fort	% Recoveries	(Average)
Wheat Grain	<0 05	0 050	NQ; NQ	(NQ)
		0 200	174, 153	(164)
		0 350	157, 162	(160)
		0 500	176, 129	(152)

NQ Not quantifiable due to interferences
Curve y = 0 1105x - 31 69, r = 0 9959
where x = area counts
y = found concentration

Table 6 Confirmation Method Recovery Results - PNP

Sample	ppm Controls	ppm Fort	% Recoveries	(Average)
Wheat Grain	<0 02	0 051	118, 150	(134)
		0 510	107, 132	(120)
		1 28	107, 113	(110)
		2 04	104, 88 3	(96 3)

Curve y = 0 03306x - 1 927 r = 0 9992
where x = area counts
y = found concentration

Table 7 Comparison of Recovery Values from Method Validation and Confirmation Studies (Wheat Grain)

Analyte	Validation (GC/FPD or LC/UV)	Confirmation (GC/MS
EP	111	103
EPOX	122	159
PNP	113	115

SECTION 9.0
FIGURES

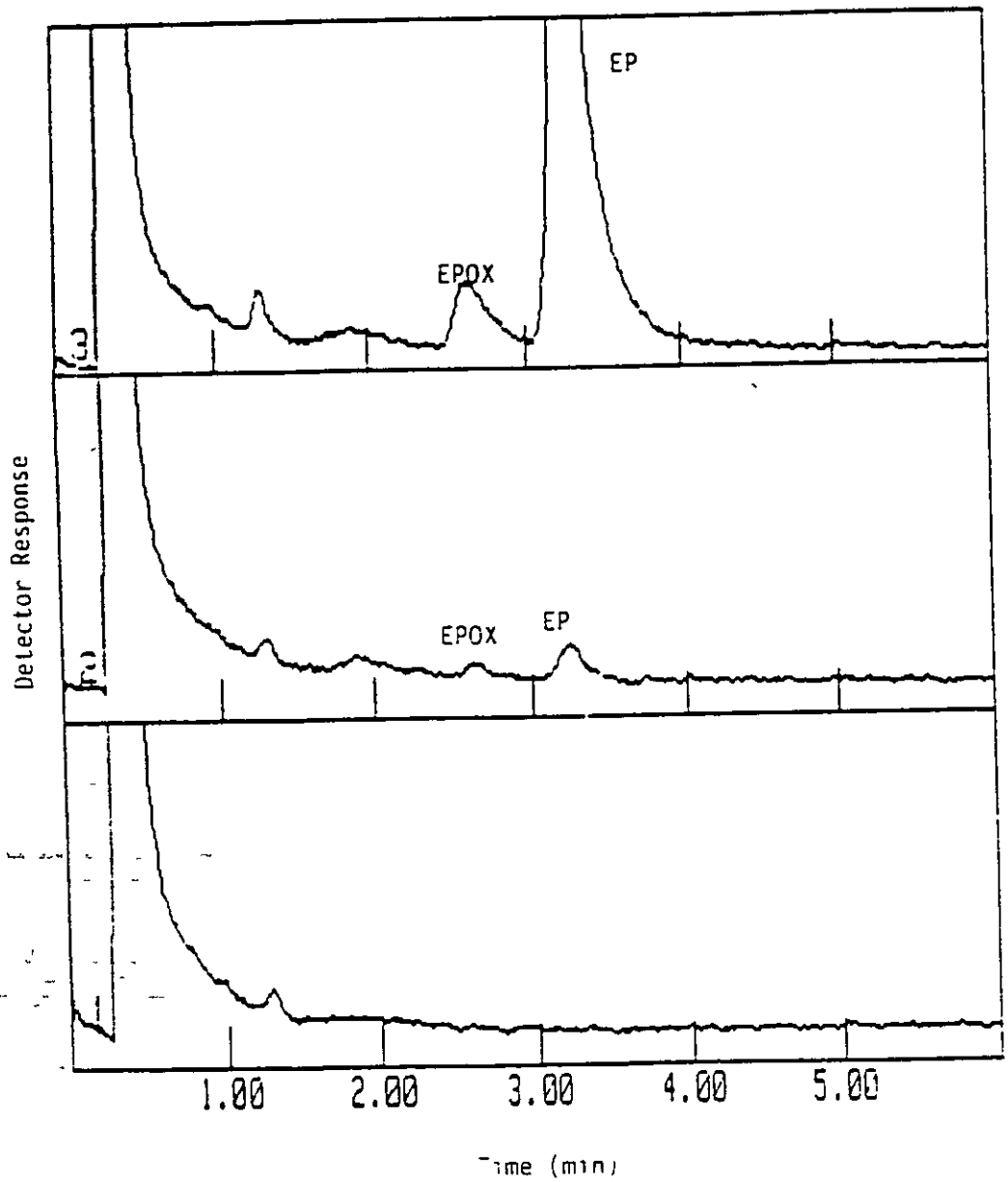


Figure . Gas Chromatograms - Wheat Straw
1) Control
2) 0.05 ppm EPOX, 0.05 ppm EP
3) 0.05 ppm EPOX, 0.26 ppm EP

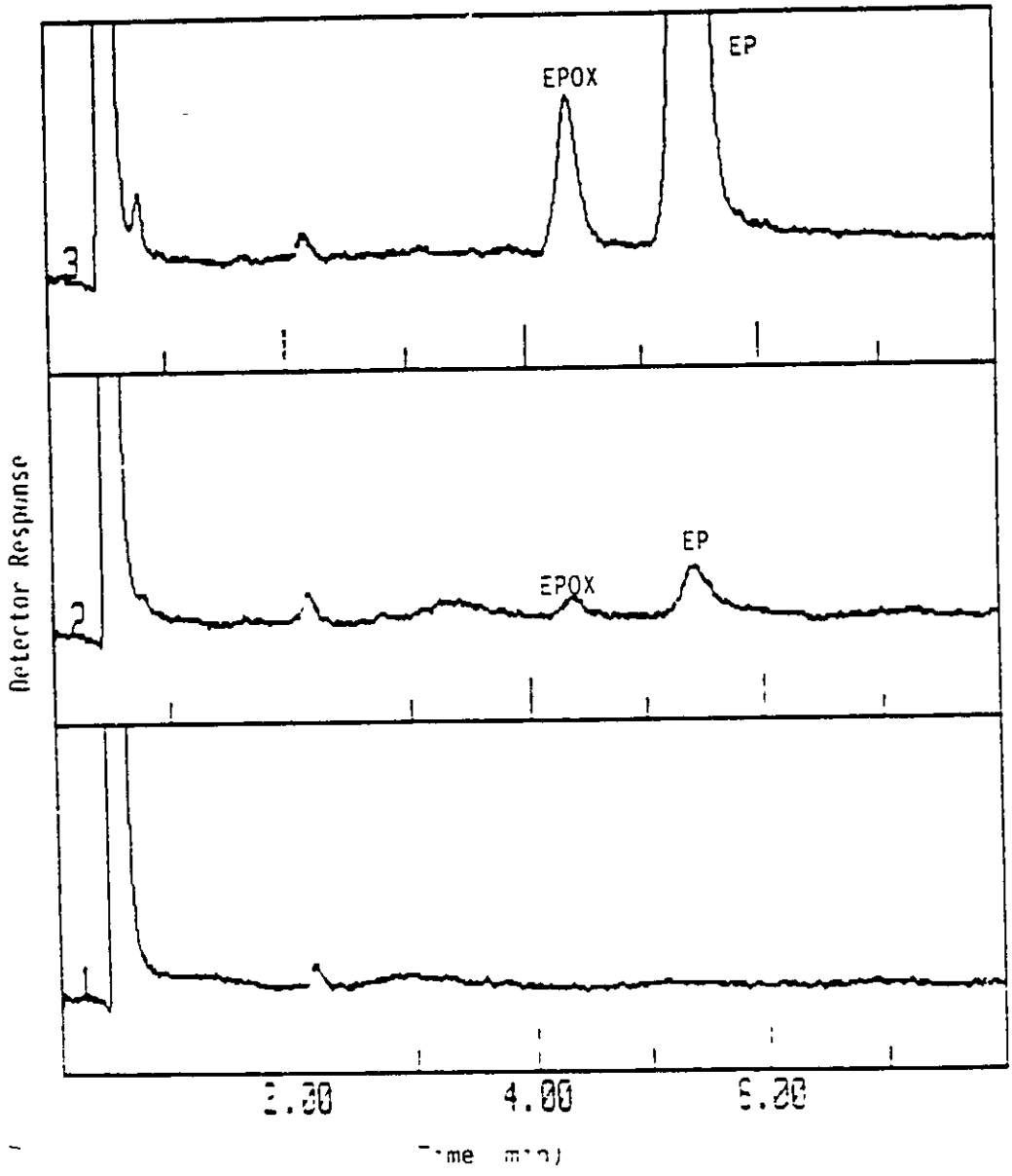


Figure 1 Gas Chromatograms - Wheat Grain
1 Control
2 0.05 ppm EPOX, 0.05 ppm EP
3 0.05 ppm EPOX, 0.06 ppm EP

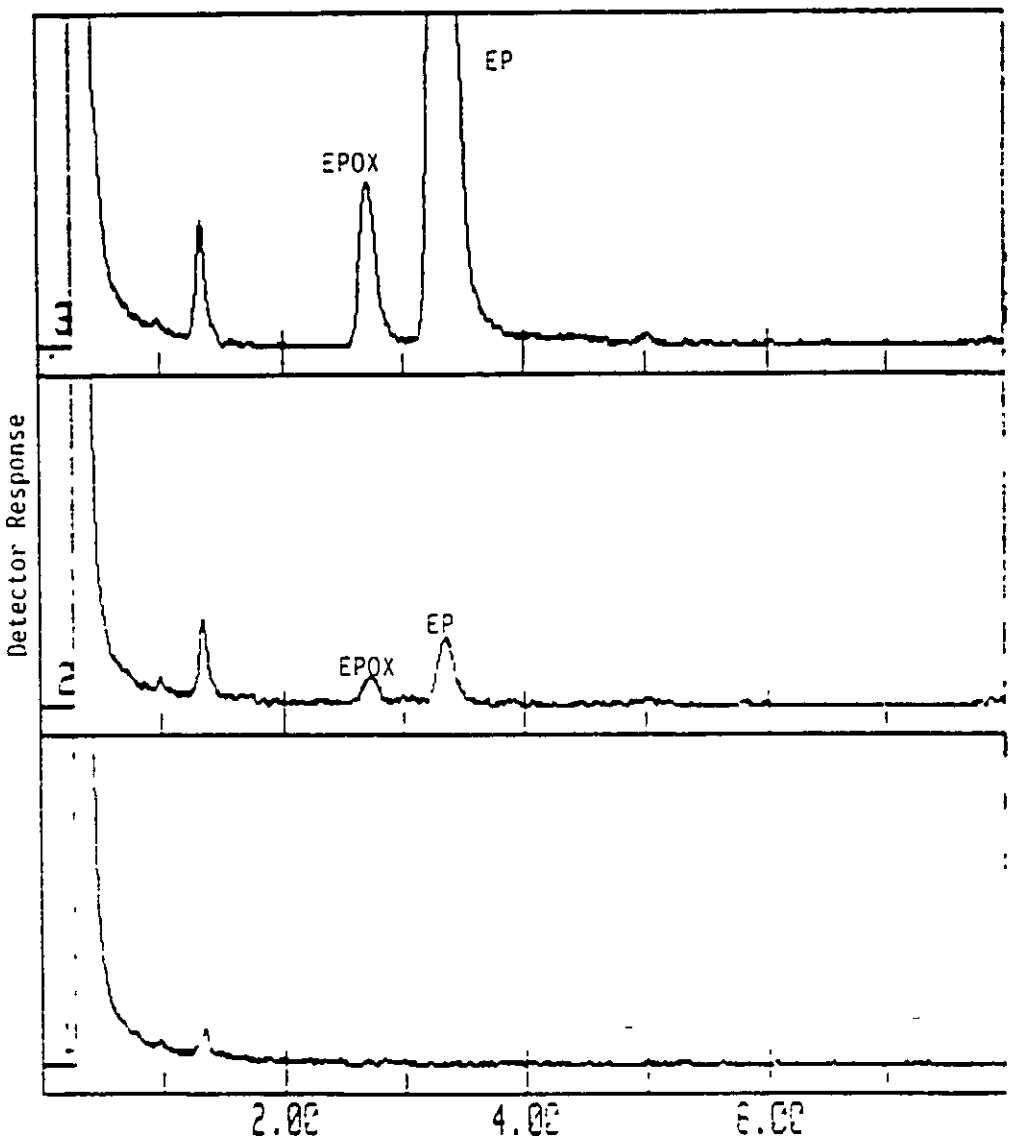


Figure 2 Gas Chromatograms - Wheat Flour
1) Control
2) 0.05 ppm EPOX, 0.05 ppm EP
3) 0.05 ppm EPOX, 0.10 ppm EP

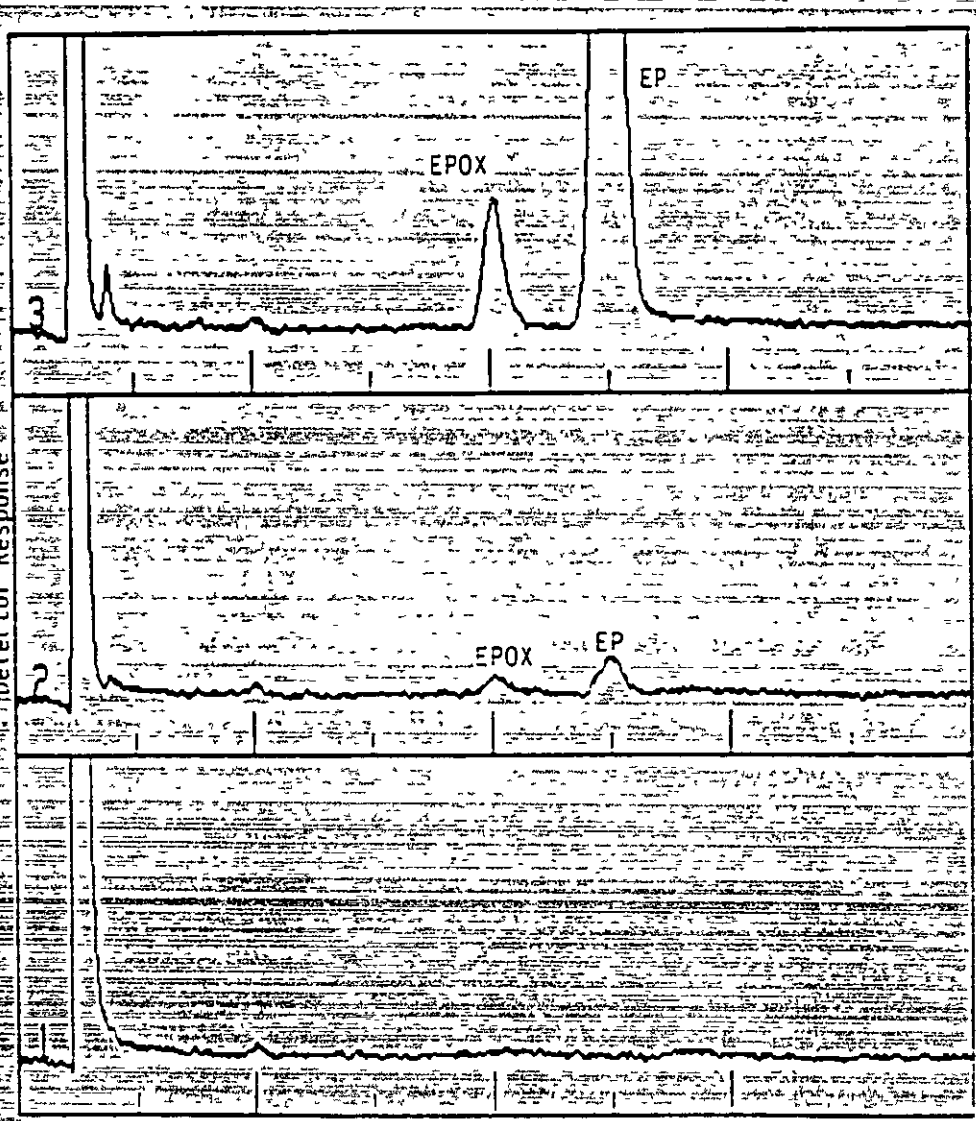


Figure 1. Gas Chromatograms of Sunflower Seed Oil:
(1) Control
(2) 0.05 ppm EPOX, 0.05 ppm EP
(3) 0.05 ppm EPOX, 1.26 ppm EP

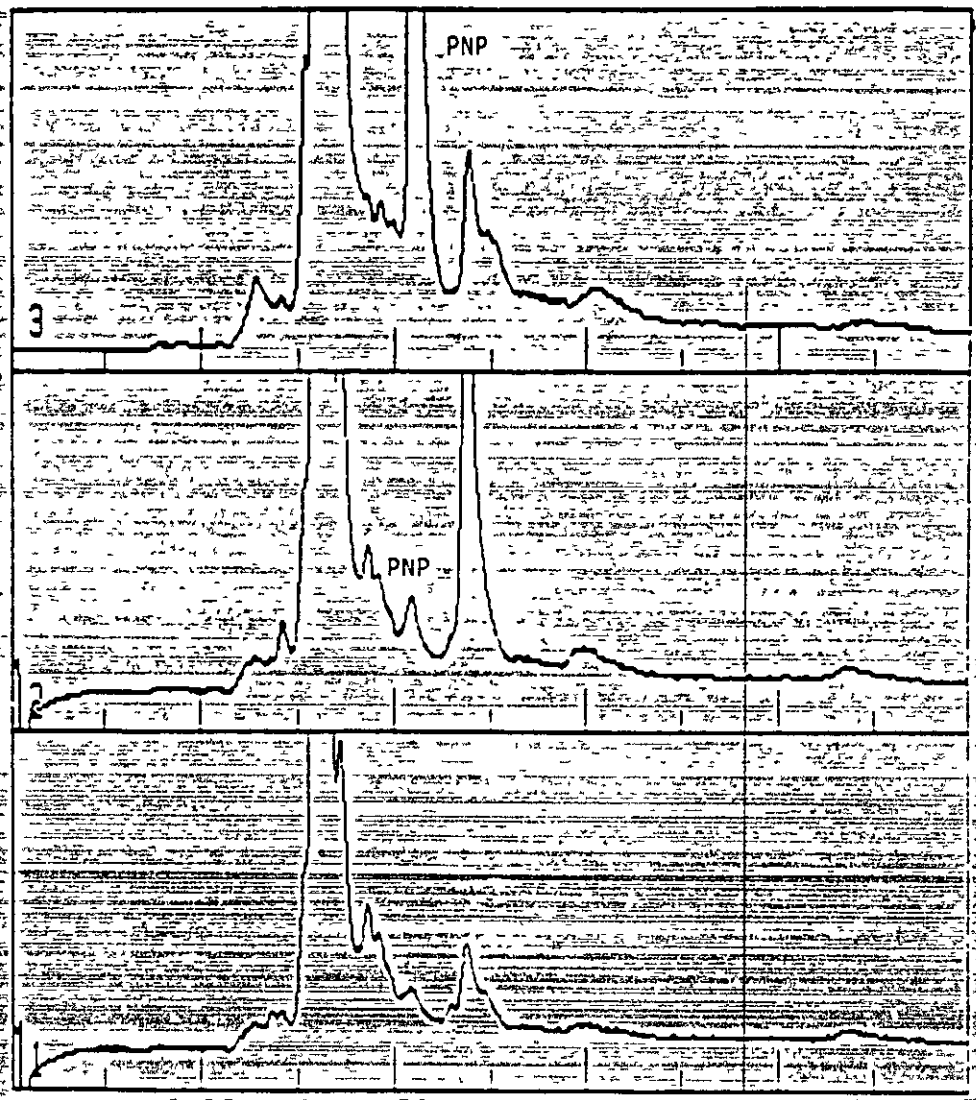
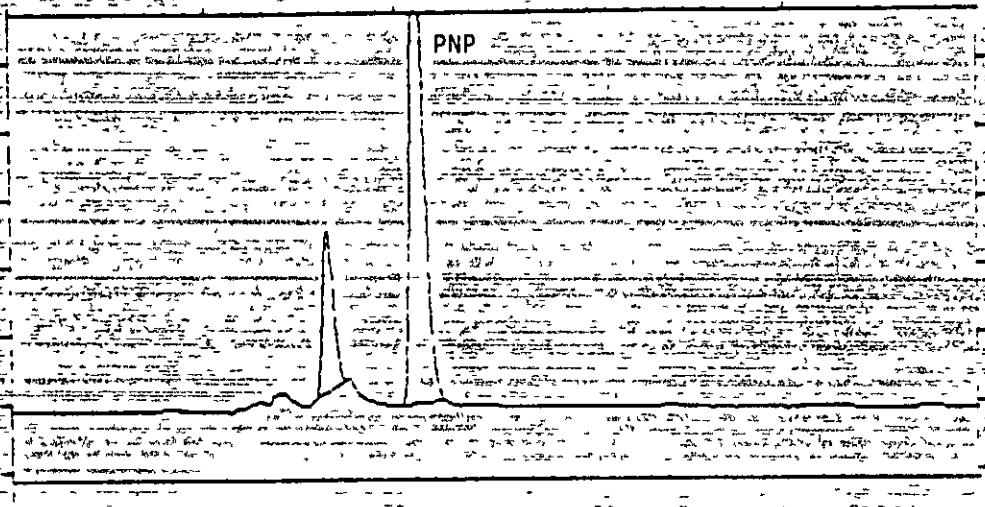
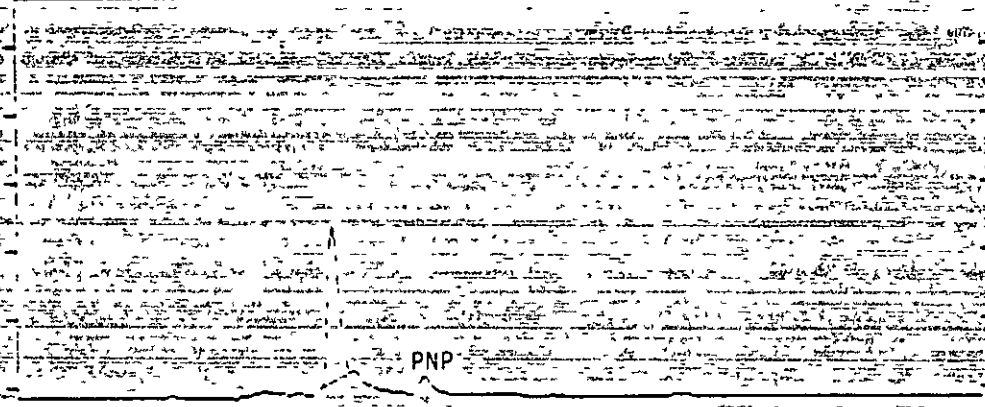


Figure 5. Liquid Chromatograms - Wheat Straw
(1) Control
(2) 0.051 ppm PNP
(3) [illegible]8 ppm PNP





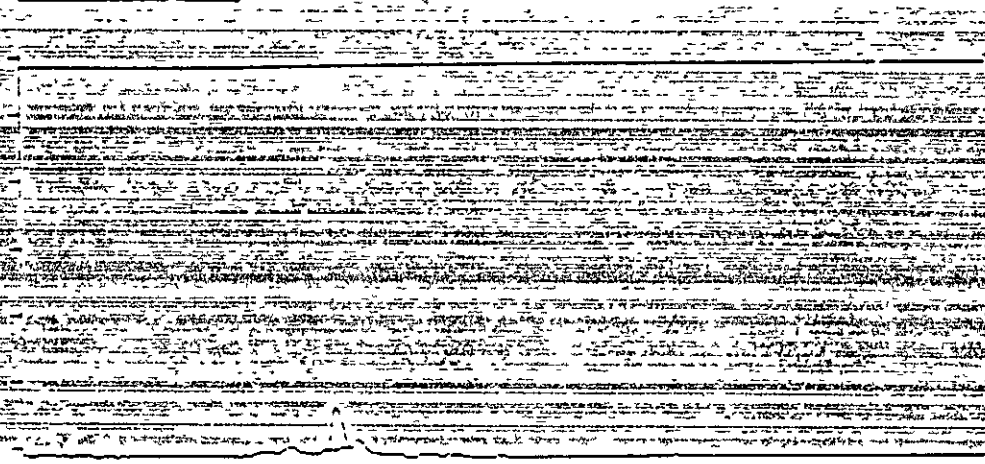


Figure 5 Liquid Chromatograms of Wheat Grain
(1) Control
(2) 0.051 ppm - PNP
(3) 0.28 ppm - PNP

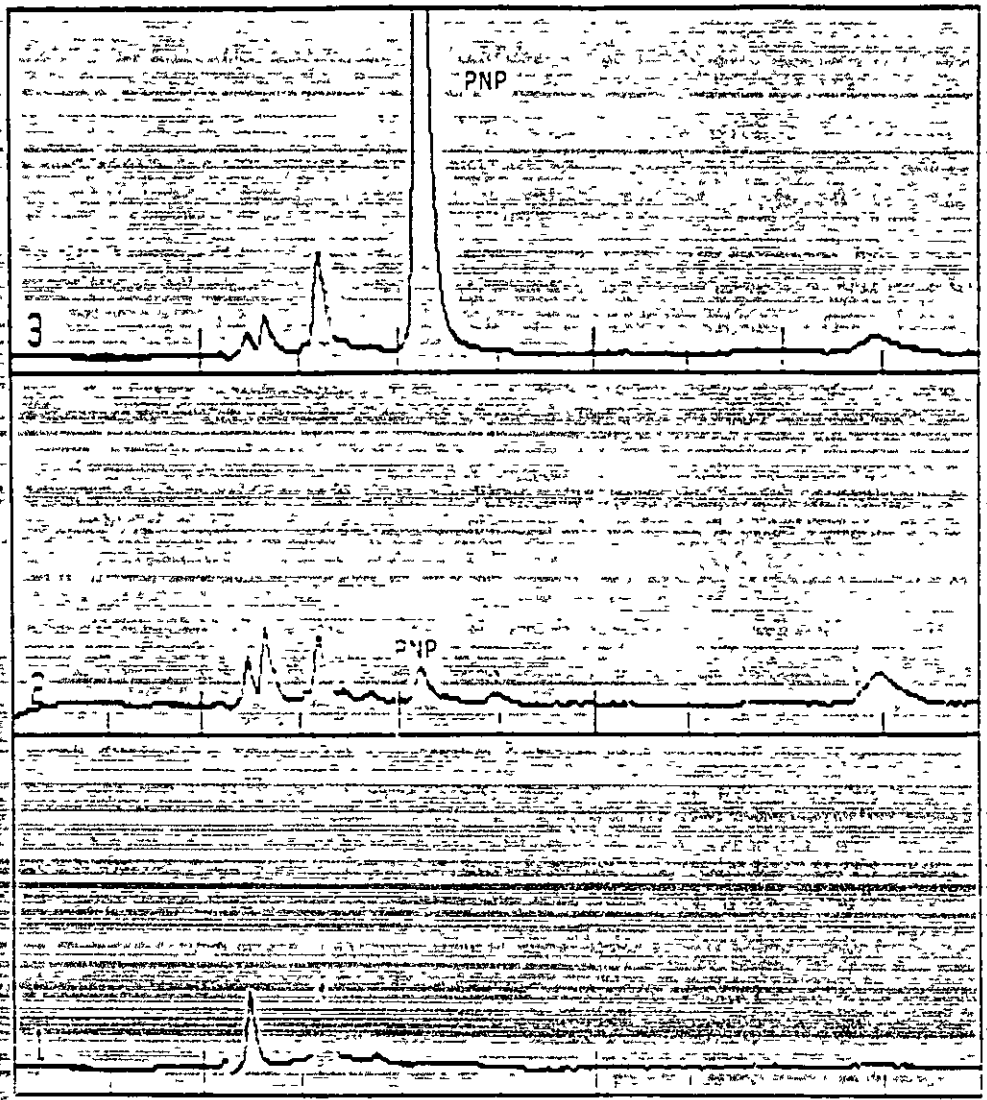


Figure [illegible] Liquid Chromatograms Wheat Flour

[illegible] PNP

[illegible] PNP

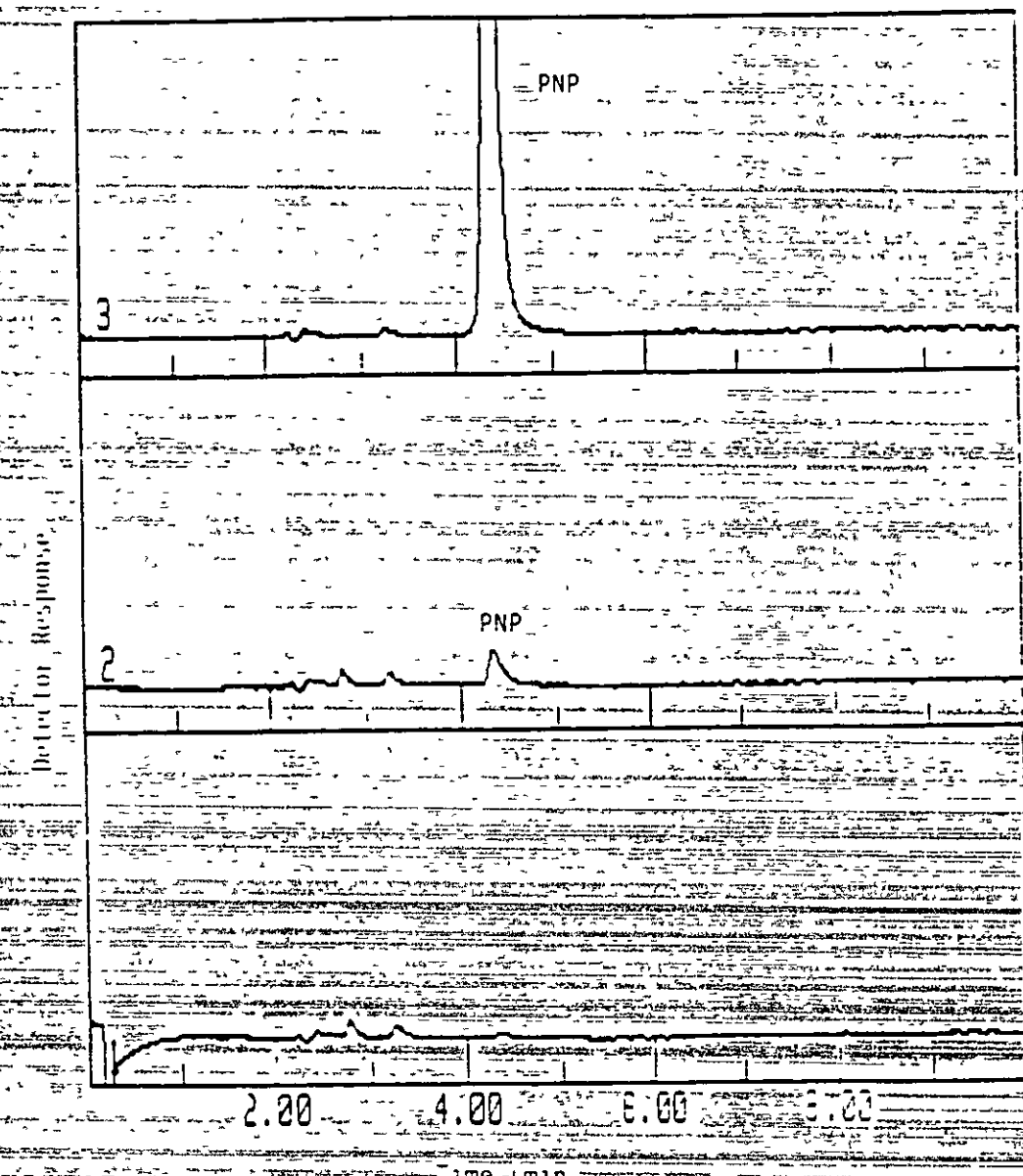


Figure [illegible] Chromatograms - Sunflower Seed [illegible]
[illegible] Control
[illegible] ppm PNP
[illegible] ppm PNP

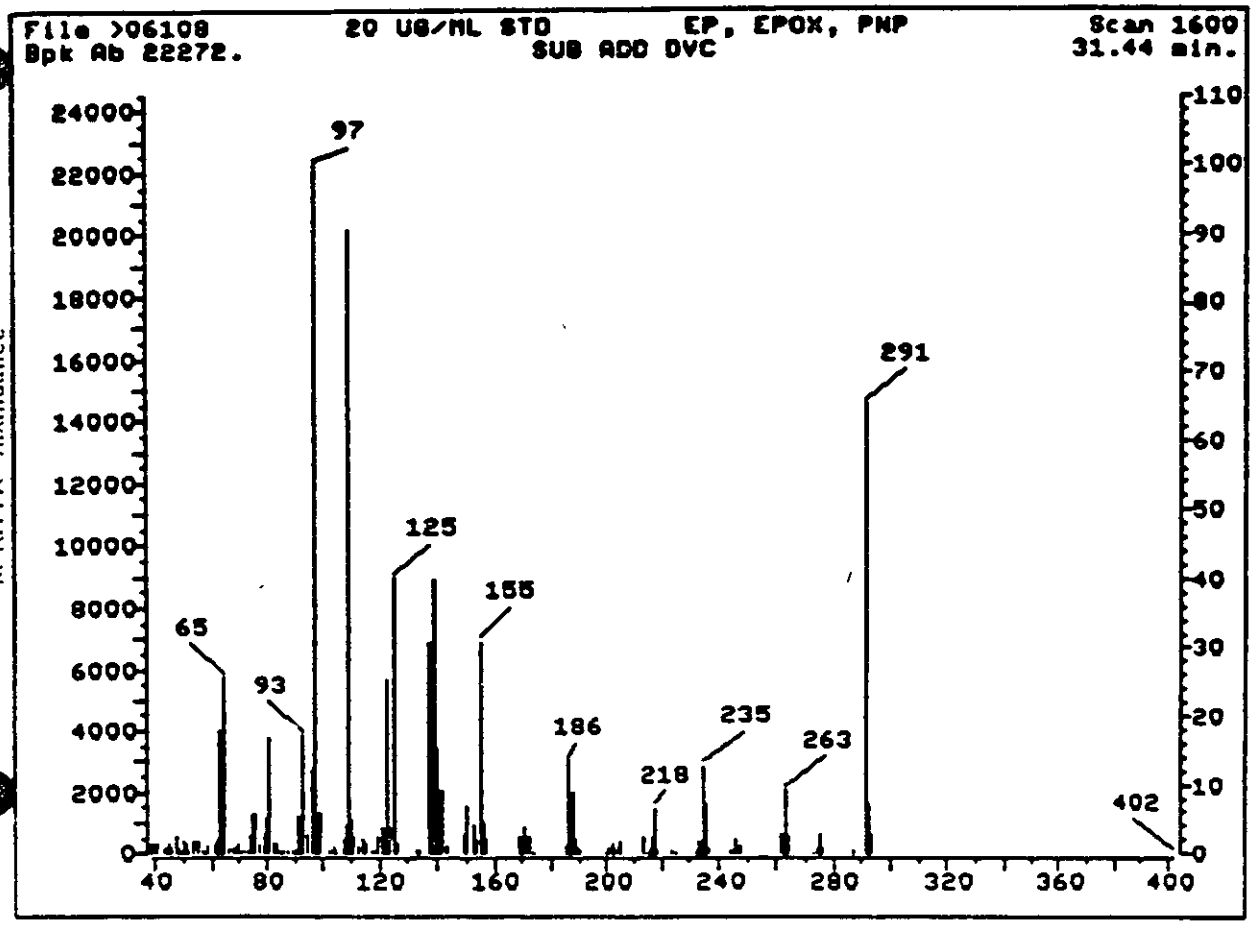


Figure 9. Mass Spectrum - Parathion

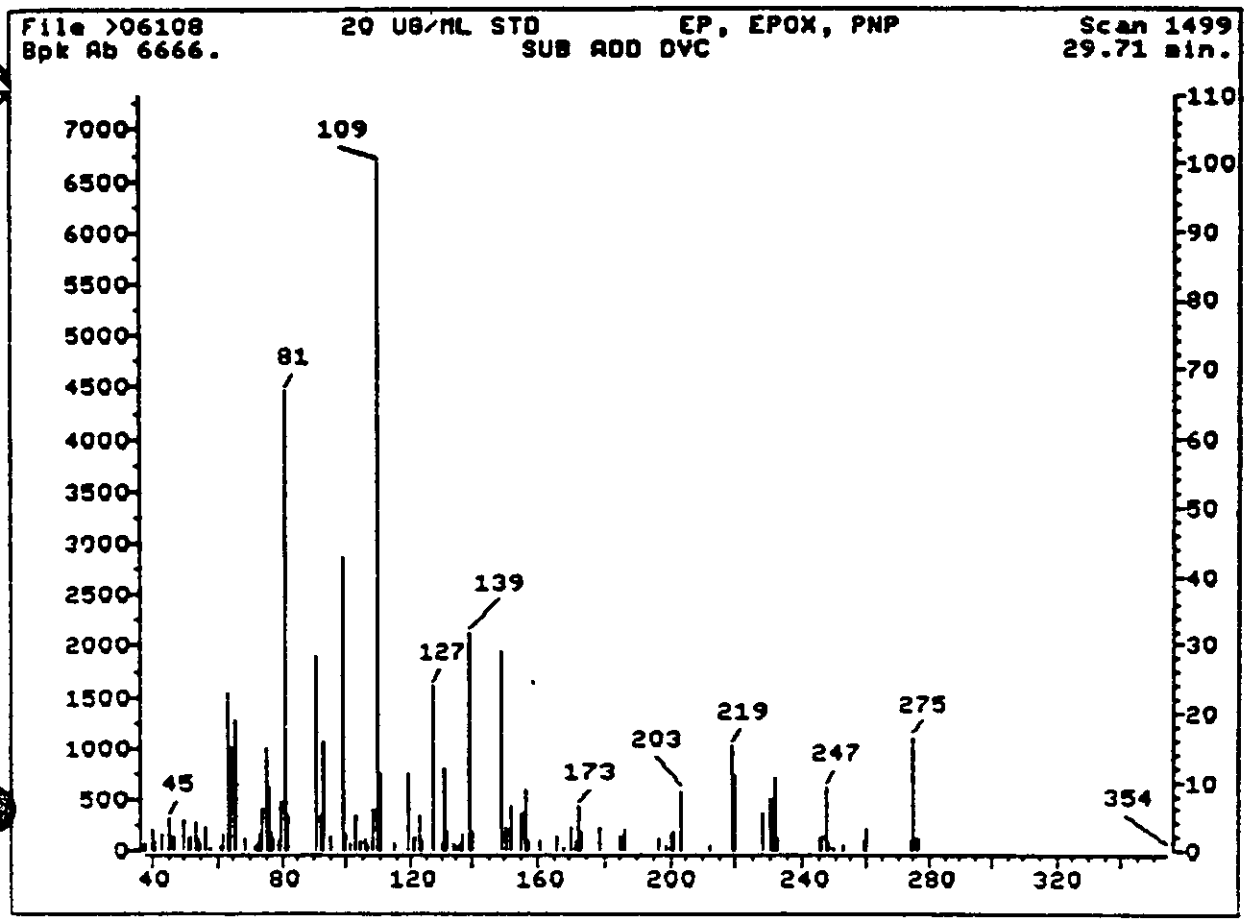


Figure 11 Mass Spectrum - Paraoxon

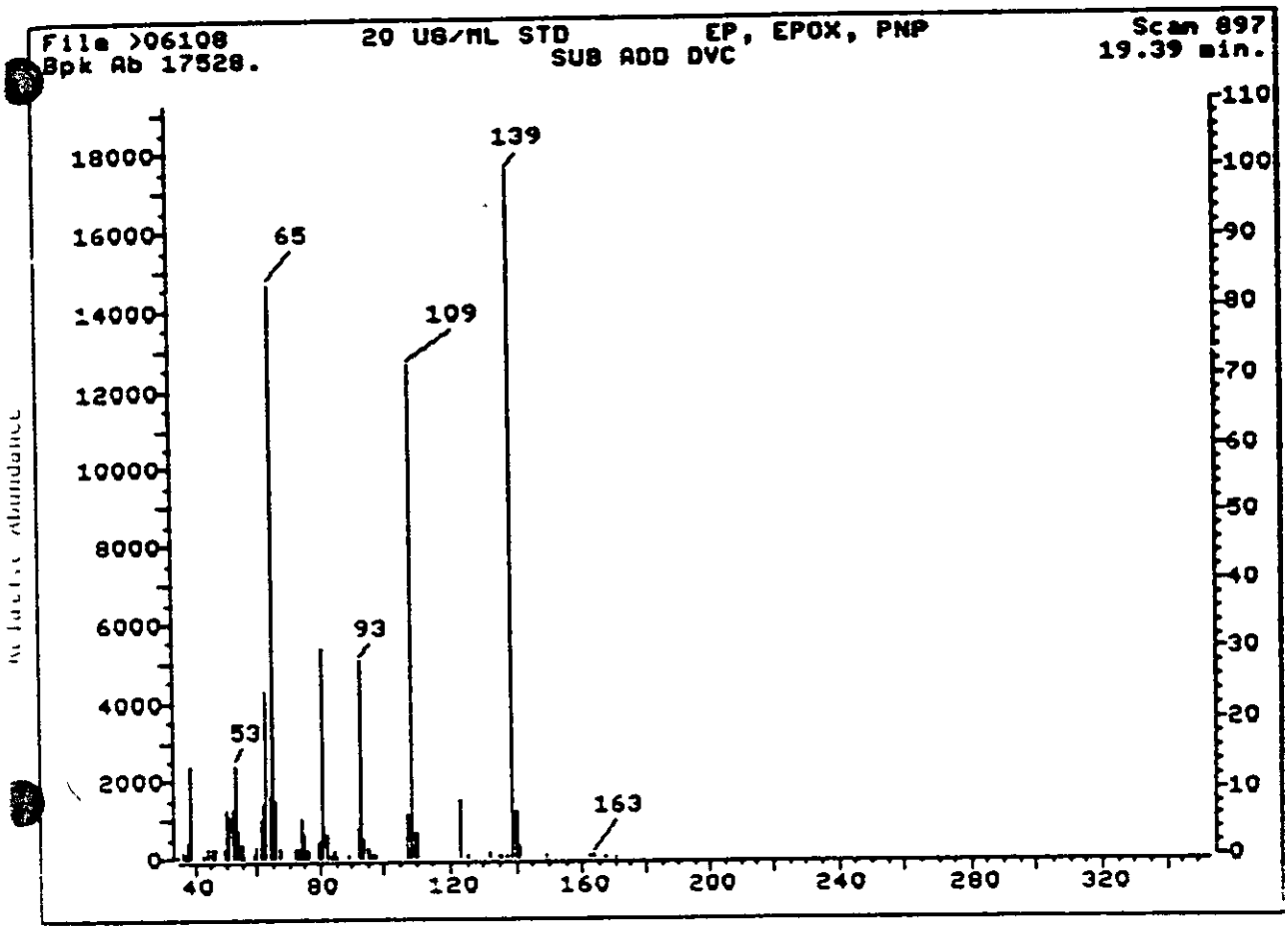


Figure 11 Mass Spectrum - p-Nitrophenol

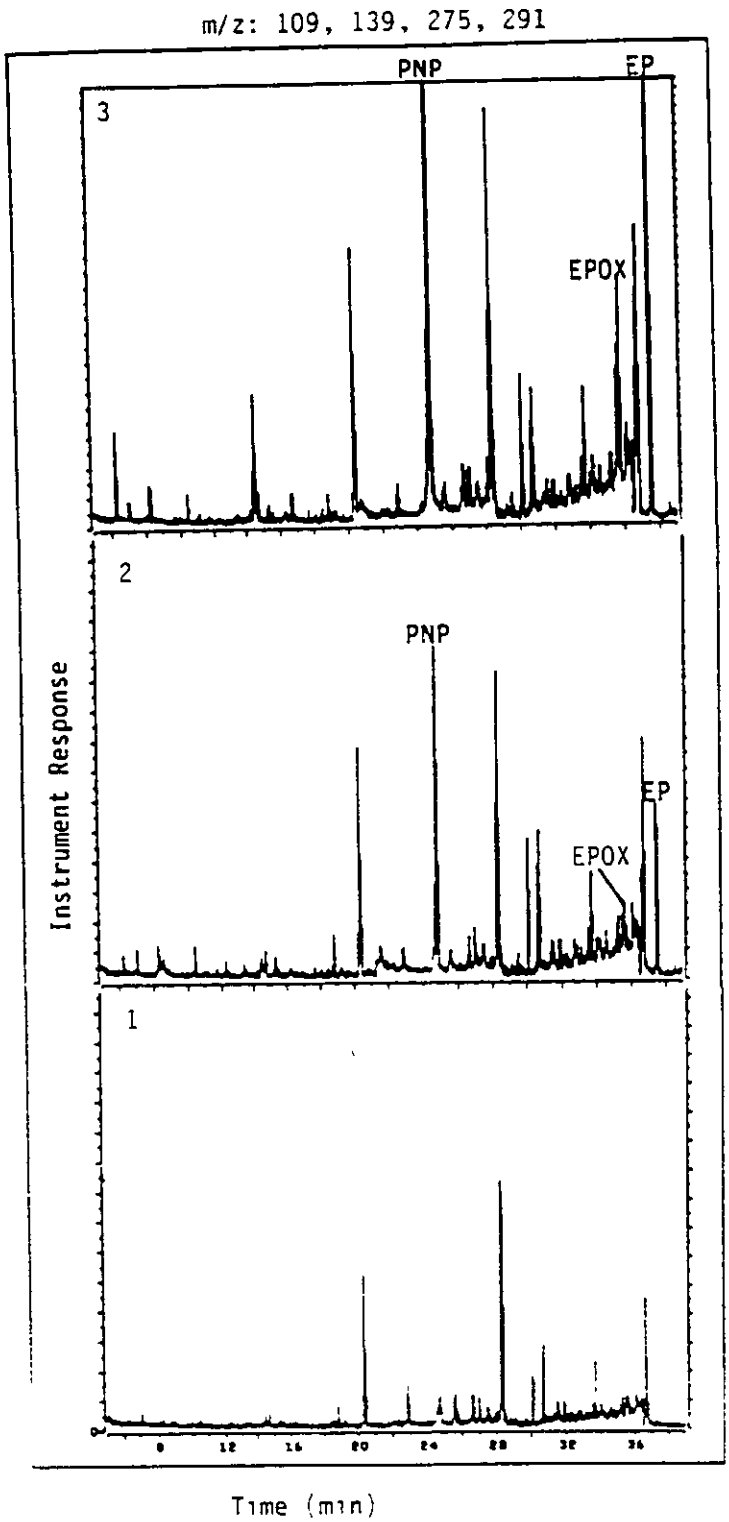


Figure 12. Total Ion Current Chromatograms - Wheat Grain
(1) Control
(2) 0.61 ppm PNP, 0.20 ppm - EPOX, 0.60 ppm EP
(3) 2.0 ppm PNP, 0.50 ppm - EPOX, 2.0 ppm EP

SECTION 10 0

APPENDICES

Appendix I

Proposed Method 1 Analysis of Wheat Commodities for Ethyl Parathion, Ethyl Paraoxon and p-Nitrophenol

1.0 GENERAL

This assay is designed to determine levels of ethyl parathion (EP), ethyl paraoxon (EPOX), and p-nitrophenol (PNP) in wheat straw, wheat grain, and wheat flour. For EP and EPOX, the plant materials are subjected to an extraction sequence, and portions of the final extract are analyzed by gas chromatography using phosphorous-specific detection (GC/FPD). Another portion of the final extract is partially purified by open column chromatography, then analyzed by high performance liquid chromatography (HPLC) using ultraviolet absorption detection. The procedure is designed so that EP and EPOX sample preparation and extraction is performed in two working days, with analysis of those samples overnight prior to the third working day. On the third day, the PNP sample processing is conducted, with those samples analyzed overnight. Data reduction is performed on day four The entire assay spans approximately 5 working days. This Analytical Protocol provides the procedural details of the assay.

2.0 INSTRUMENTATION

2.1 Homogenizer

Model Brinkmann PT 10/35 with PTA 2DTS rotor, or equivalent

2.2 Gas Chromatographic System

Model Varian 3700 with Varian Series 8000 Autoinjector, or equivalent

Column 10% SP 2100 on 100/120 mesh Supelcoport , 4 or 2 mm x 1.8 m

Carrier Gas Helium @ 40 mL/min (measured at ambient temp)

Oven Temperature 235°C - 245°C (isothermal)

Detector Flame Photometric in Phosphorous Mode

Detector Temperature 260°C

Injection Temperature 250°C Injection Volume 1-5 µL

Data Handling Nelson Analytical Data System

2.3 High Performance Liquid Chromatographic System

Solvent Delivery Waters 590 and 6000 Pumps with 680 Programmer, or equivalent

Injector Micromeretics [illegible] utoinjector, or equivalent

Injection Volume 20 µL

Detector Analytical Bio-[illegible] or equivalent, at 315 nm

Column: Customsphere ODS (3 micron packing), 6.2 x 100 mm

Mobile Phase: 40% Acetonitrile, 60% Water, 0.1% Acetic Acid

Flow Rate: 1.0 mL/min

Data Handling: Nelson Analytical Data System

3.0 ASSAY PROCEDURE

3.1 Preparation of Standards

A reference standard for each of the three analytes (EP, EPOX, PNP) is obtained from Ultra Scientific (North Kingstown, RI). EPA Certified standards should be obtained if available. Approximately 10-20 mg of each substance is accurately weighed (nearest 0.1 mg) into a 25 mL volumetric flask. The sample is dissolved in HPLC-grade methanol to the mark. An appropriate aliquot (= 5 mL) of the primary stock solution is then accurately transferred to a 25 mL volumetric flask and diluted to the mark with methanol. The aliquot volume is chosen so that each final stock solution contains each standard at a concentration of 100.0 µg/mL.

Aliquots (5.0 mL) of each of the EP and PNP individual stock solutions are quantitatively transferred to a single 200 mL volumetric flask. The samples are diluted to volume with methanol. Twenty mL of this solution is quantitatively transferred to another 200 mL volumetric flask and diluted to volume with methanol. These two spiking solutions contain 2.5 µg and 0.25 µg EP and PNP/mL. For EPOX 2.5 mL of the 100 µg/mL solution is diluted to 200 mL. Forty mL of this solution is quantitatively transferred to another 200 mL volumetric flask and diluted to volume with methanol. These two solutions contain 1.25 µg and 0.25 µg/mL.

3.1.1 Solutions for Gas Chromatographic Calibration

Standard solutions containing EP and EPOX are prepared by serially diluting the primary stock solution as follows. An accurate aliquot of each EP and EPOX primary stock is transferred to a single 200 mL volumetric flask which is diluted to volume with ethyl acetate. The actual volume of primary stock aliquot is chosen so that a final solution concentration of 10 µg EP/mL and 5 µg EPOX/mL is obtained.

An aliquot of the combination stock solution is transferred to a series of volumetric flasks, each being diluted to the mark with ethyl acetate. An aliquotting/dilution sequence guide is shown below:

EP

Initial Conc. (µg/mL)	Aliquot (mL)	Final Volume (mL)	Final Conc. (µg/mL)
10.0	5 5	25.0	2.2
10.0	4 0	25.0	1.6
10.0	5 0	50 0	1.0
10.0	6 0	100 0	0.60
10.0	2 0	100 0	0.20
10.0	1 0	100.0	0.10
10.0	0 8	200.0	0.04

EPOX

Initial Conc (µg/mL)	Aliquot (mL)	Final Volume (mL)	Final Conc. (µg/mL)
5.0	5 5	25.0	1.1
5.0	4 0	25.0	0 80
5 0	5 0	50.0	0.50
5 0	6 0	100 0	0 30
5 0	2 0	100 0	0 10
5.0	1 0	100 0	0 05
5 0	0 8	200.0	0 02

3 : 2 Solutions for HPLC Calibration

Standard solutions containing PNP are prepared by serially diluting the primary stock solution as follows An accurate aliquot of PNP primary stock is transferred to a single 200 mL volumetric flask which is diluted to volume with HPLC mobile phase (methanol/water/acetic acid) The actual volume of primary stock aliquot is chosen so that a final solution concentration of 10 µg PNP/mL is obtained

An aliquot of the stock solution is transferred to a series of volumetric flasks, each being diluted to the mark with HPLC mobile phase An aliquotting/dilution sequence guide is shown below

Initial Conc. (µg/mL)	Aliquot (mL)	Final Volume (mL)	Final Conc. (µg/mL)
10.0	5.5	25.0	2.2
10.0	4.0	25.0	1.6
10.0	5.0	50.0	1.0
10.0	6.0	100.0	0.60
10.0	2.0	100.0	0.20
10.0	0.8	200.0	0.04

3.2 Sample Preparation and Extraction

For method validation the method samples are fortified in duplicate at four levels: 0.05, 0.5, 1.25 and 2.0 ppm for EP and PNP, and 0.05, 0.20, 0.35 and 0.50 ppm for EPOX. Wheat grain or wheat flour (25 g) is weighed into an 8 oz glass jar. For EP and PNP, five mL of the 0.25 µg/mL spiking solution, and 5, 12.5 and 20 mL of the 2.5 µg/mL spiking solution are added to fortify the method samples. For EPOX, 5 mL of the 0.25 µg/mL spiking solution and 4, 7 and 10 mL of the 1.25 µg/mL spiking solution are added to fortify the method samples. A solvent mixture (100 mL) of 80:20 acetone/0.1 N HCl is added and the mixture is blended at highest speed for 1 minute using a Polytron homogenizer. The blended mixture is transferred to a 500 mL round bottom flask. Additional extraction solvent (50 mL) is added to the 8 oz jar and the contents are blended at the highest speed for 30 seconds. This mixture is added to the round bottom flask. The blending shaft is washed with 2 x 25 mL of the extraction solvent, each of which is also transferred to the round bottom flask. Boiling chips are added and the mixture is refluxed for one hour.

For the extraction of wheat straw, 10 g of straw (individual stalks cut into ≈ 2 cm pieces) are weighed into an 8 oz glass jar. An appropriate amount of spiking solution is added to obtain samples fortified at 0.05, 0.5, 1.25 and 2.0 ppm. A solvent mixture (100 mL) of 80:20 methanol/0.1 N HCl is added and the mixture is blended at highest speed for 1 minute using a Polytron homogenizer. The blended mixture is transferred to a 500 mL round bottom flask. Additional extraction solvent (50 mL) is added to the 8 oz jar and the contents are blended at the highest speed for 30 seconds. This mixture is added to the round bottom flask. The blending shaft is washed with 2 x 25 mL of the extraction solvent, each of which is also transferred to the round bottom flask. Boiling chips are added and the mixture is refluxed for one hour.

At the end of the reflux period the sample is allowed to cool to room temperature. The flask contents are filtered through a Buchner funnel fitted with Whatman filter paper (#1 or #4) and topped with ≈ 1 cm of Celite. The reflux flask is then rinsed with 2 x 20 mL acetone or methanol (allowing the marc to dry between rinses), each rinse is added to the funnel. The filtrate is collected in a 1000 mL round bottom flask. The filtrate is concentrated by rotary evaporation (30°-50°C) until all acetone and methanol are removed. The aqueous residue is transferred to a 500 mL separatory funnel. The round bottom flask is rinsed with 3 x 20 mL

portions of water (Milli-Q or equivalent) and the rinses are added to the separatory funnel.

The aqueous phase is extracted with 3 x 125 mL portions of ethyl acetate. After shaking for one minute for each portion, the aqueous phase is removed to a second separatory funnel. The ethyl acetate phase is filtered through a sodium sulfate filled funnel plugged with glass wool into a clean 1000 mL round bottom flask.

NOTE It may be useful to run portions of fresh ethyl acetate through the prepared funnel prior to the filtration of extract to ensure removal of potentially interfering materials from the sodium sulfate and glass wool. Discard such "pre-conditioning" ethyl acetate

After all three portions have been collected, rinse the sodium sulfate with 10-15 mL portions of ethyl acetate and collect this rinse with other portions. The combined portions and rinses are concentrated to approximately 10 mL by rotary evaporation (40°-45°C). This volume is transferred quantitatively to a 25 mL volumetric flask, and diluted to the mark with ethyl acetate. Aliquots of this solution are used for analysis of EP and EPOX by direct injection onto the GC system (Section 3.3.1)

The ethyl acetate solution is further processed for analysis of PNP. An aliquot (1.0 mL) is pipetted into a conical tube (e.g., centrifuge tube) and concentrated to near dryness (approximately 50 µL) by nitrogen blow-down.

NOTE The solution must <u>not</u> be taken to complete dryness. It may be helpful to premark the conical tube at a level corresponding to 50 µL of ethyl acetate

The concentrated solution is dissolved in 2.0 mL of hexane and sonicated for approximately 10 seconds to ensure complete dissolution

A minicolumn is prepared from a Pasteur pipet by adding activated Florisil (Activity Grade I) to a clean pipet (insert a small amount of glass wool to retain the Florisil). Florisil is added to the level of the indentation of the pipet. Add anhydrous sodium sulfate (approximately 0.5 cm) to the top of the Florisil. The column is conditioned by washing with 3.0 mL of hexane. The eluate is discarded.

The sample (2.0 mL hexane solution) is added to the column, discarding the eluate. To the sample tube, add 2.0 mL of column eluant (49.5% ethyl ether, 49.4% hexane, 1% methanol, and 0.1% acetic acid) and sonicate for approximately 10 seconds. This rinse is transferred to the minicolumn, the eluate is discarded. The sample tube is rinsed again with 6.0 mL of eluant, and the eluant is added to the minicolumn. The eluate from this addition is collected in a clean 15 mL conical centrifuge tube. This solution is concentrated to near dryness (approximately 50 µL) by nitrogen blow-down.

NOTE The solution must <u>not</u> be taken to complete dryness. It may be helpful to premark the conical tube at a level corresponding to 50 µL of eluant

To the concentrated eluant is added 1.0 mL of the solvent mixture of acetonitrile/water (45:55) containing 0.1% acetic acid. This solution is sonicated for approximately 10 seconds. The

solution is then filtered through a 0.45 micron filter into an autosampler vial. The sample is analyzed by HPLC as described below in Section 3.3.2.

3.3 Analysis

3.3.1 Ethyl Parathion/Ethyl Paraoxon

3.3.1.1 Calibration Curve

The GC is first equilibrated with several injections of conditioning samples (analyte standards and matrix blank) before the actual quantitative run begins. The system is considered equilibrated when successive injections produce peaks that differ no more than 15% in peak area or height for the same amount injected. Calibration curve samples and method validation samples are injected during the same run. The run sequence is shown below in Section 3.3.1.2.

A calibration curve is derived by linear regression treatment of GC detector response results for the calibration samples analyzed at each of the 7 concentration levels in the approximate range of 0.04 - 2.20 ppm. The regression equation is of the form

$$y = ax + b$$

where

y = GC detector response (as peak area counts)

x = concentration of the calibration standard (µg/mL)

a = slope of the calculated calibration line

b = intercept of the calculated calibration line

Any acceptable calibration curve for sample analysis must have $\geq$ 96% of the total variance (equivalent to r (correlation coefficient) $\geq$ 0.98) explained by the regression line over the specified calibration range. The overall assay linearity is determined by

$$\% \text{ Total Variance Explained} = r^2 * 100$$

3.3.1.2 Sample Analysis

To validate the methodology described above, a series of samples of varying concentrations within the range 0.05 - 2.0 ppm is prepared. For each plant material four method samples are prepared in duplicate at the following approximate concentrations: 0.05, 0.5, 1.2 and 2.0 ppm for EP and 0.05, 0.20, 0.35 and 0.5 ppm for EPOX. Samples are analyzed via autoinjection onto the GC system. Included in the set of method samples and blanks are two calibration curve check samples, two method controls (unspiked plant homogenates) and a solvent blank. The calibration curve check samples consist of any two mid-range samples used to prepare the calibration curve. The entire sample set of 20 samples is analyzed in duplicate in the following order:

Sample	Order
Solvent Blank	1
Cal Curve Sample	2
Cal Curve Sample	3
Cal Curve Sample	4
Cal. Curve Sample	5
Cal Curve Sample	6
Cal Curve Sample	7
Cal Curve Sample	8
Method Control	9
Method Sample	10
Method Sample	11
Method Sample	12
Curve Check Sample	12
Method Sample	14
Method Sample	15
Method Sample	16
Curve Check Sample	17
Method Sample	18
Method Sample	19
Method Control	20

The method samples in the above sequence are randomized

3.3.2 p-Nitrophenol

3.3.2.1 Calibration Curve

The HPLC is first equilibrated with several injections of conditioning samples (analyte standards and matrix blank) before the actual quantitative run begins. The system is considered equilibrated when successive injections produce peaks that differ no more than 5% in peak height or area for the same amount injected. Calibration curve samples and method validation samples are injected during the same run. The run sequence is shown below in Section 3.3.2.

Calibration curves are determined by linear regression treatment of HPLC detector response results for the calibration samples analyzed at each of the concentration levels in the approximate range [illegible] ppm. The regression equation for the form

$$y = ax - b$$

where

y = HPLC detector response (as peak area counts)
x = concentration of the calibration standard (µg/mL)
a = slope of the calculated calibration line
b = intercept of the calculated calibration line

The required criterion for linearity (% Total Variance Explained) is as described in Section 3.3.1.2.

3.3.2.2 Sample Analysis

To validate the methodology described above, a series of samples of varying concentrations within the range 0.05 - 20 ppm is prepared. For each plant material, four method samples are prepared in duplicate at the following approximate concentrations: 0.05, 0.5, 1.2 and 20 ppm. Samples are analyzed via autoinjection onto the HPLC system. Included in the set of method samples and blanks are two calibration curve check samples and a solvent blank. The calibration curve check samples consist of any two mid-range samples used to prepare the calibration curve. The entire sample set of 19 samples is analyzed in the following order:

Sample	Order
Solvent blank	1
Cal. Curve Sample	2
Cal. Curve Sample	3
Cal. Curve Sample	4
Cal. Curve Sample	5
Cal. Curve Sample	6
Cal. Curve Sample	7
[illegible]	8
[illegible]	9
[illegible]	10
[illegible]	11
[illegible]	12
[illegible]	13
[illegible]	14
[illegible]	15

Curve Check Sample	16
Method Sample	17
Method Sample	18
Method Control	19

The method samples in the above sequence are randomized.

3 4 Results Reported

Sample results are reported as the found concentration of each test sample as determined from the regression equations for EP/EPOX and PNP The mean found concentrations for each concentration point, and the precision (as %RSD) will be reported, as will the % Recovery (found/nominal) Found concentrations are not corrected for recovery with found blank concentrations

Appendix II

Proposed Method 2. Analysis of Sunflower Seed Oil for Ethyl Parathion, Ethyl Paraoxon and p-Nitrophenol

1.0 GENERAL

This assay is designed to determine levels of ethyl parathion (EP - CAS No. 56-38-2), ethyl paraoxon (EPOX - CAS No 311-45-5), and p-nitrophenol (PNP - CAS No. 100-02-7) in sunflower seed oil. For EP and EPOX, the oil is subjected to a solvent extraction and partition sequence, and portions of the final partition solution are analyzed by gas chromatography using phosphorous-specific detection (GC/FPD). Another portion of the final partition solution is partially purified by open column chromatography, then analyzed by high performance liquid chromatography using ultraviolet absorption detection (HPLC/UV). The procedure is designed so that EP and EPOX sample preparation and extraction is performed in two working days, with analysis of those samples overnight prior to the third working day. On the third day, the PNP sample processing is conducted, with those samples analyzed overnight. Data reduction is performed on day four The entire assay spans approximately 5 working days. This Analytical Protocol provides the procedural details of the assay.

2.0 INSTRUMENTATION

2.1 Gas Chromatographic System

Model Varian 3700 with Varian Series 8000 Autoinjector, or equivalent

Column 10% SP 2100 on 100/120 mesh Supelcoport, 4 or 2 mm x 1.8 m

Carrier Gas: Helium @ 40 mL/min

Oven Temperature 235°C

Detector Flame Photometric in Phosphorous Mode

Detector Temperature 260°C

Injection Temperature 250°C Injection Volume 1-10 µL

Data Handling Nelson Analytical Data System

2.2 High Performance Liquid Chromatographic System

Solvent Delivery Waters 590 and 6000 Pumps with 680 Programmer, or equivalent

Injector Micromeretics 728 or equivalent

Injection Volume 20 µL

Detector Analytical Biosystems 75[illegible] or equivalent, at 315 nm

Column Customsphere [illegible] packing, 6.2 x 100 mm

Mobile Phase: 40% Acetonitrile, 60% Water, 0.1% Acetic Acid

Flow Rate: 1.0 mL/min

Data Handling: Nelson Analytical Data System

3.0 ASSAY PROCEDURE

3.1 Preparation of Standards

A reference standard for each of the three analytes (EP, EPOX, PNP) is obtained from Ultra Scientific (North Kingstown, RI). EPA Certified standards should be obtained if available. Approximately 10-20 mg of each substance is accurately weighed (nearest 0.1 mg) into a 25 mL volumetric flask. The sample is dissolved in HPLC-grade methanol to the mark. An appropriate aliquot (≈ 5 mL) of this stock solution is then accurately transferred to a 25 mL volumetric flask and diluted to the mark with methanol. The aliquot volume is chosen so that each primary stock solution contains each standard at a concentration of 100.0 µg/mL.

Aliquots (5.0 mL) of each of the EP and PNP individual stock solutions are quantitatively transferred to a single 200 mL volumetric flask. The samples are diluted to volume with methanol. Twenty mL of this solution is quantitatively transferred to another 200 mL volumetric flask and diluted to volume with methanol. These two spiking solutions contain 2.5 µg and 0.25 µg EP and PNP/mL. For EPOX 2.5 mL of the 100 µg/mL solution is diluted to 200 mL. Forty mL of this solution is quantitatively transferred to another 200 mL volumetric flask and diluted to volume with methanol. These two solutions contain 1.25 µg and 0.25 µg/mL.

3.1.1 Solutions for Gas Chromatographic Calibration

Standard solutions containing EP and EPOX are prepared by diluting the primary stock solution as follows. An accurate aliquot of each EP and EPOX primary stock (≈ 1 mg/mL) is transferred to a single 200 mL volumetric flask which is diluted to volume with ethyl acetate. The actual volume of primary stock aliquot is chosen so that a final solution concentration of 10 µg EP/mL and 5 µg EPOX/mL is obtained.

An aliquot of the combination stock solution is transferred to a series of volumetric flasks, each being diluted to the mark with ethyl acetate. An aliquotting/dilution sequence guide is shown below:

EP

Initial Conc (µg/mL)	Aliquot (mL)	Final Volume (mL)	Final Conc. (µg/mL)
10 0	5 5	25 0	2 2
10 0	4 0	25 0	1 6
10 0	5 0	50 0	1 0
10 0	6 0	100 0	0 60
10 0	2 0	100 0	0 20
10 0	1 0	100 0	0 10
10 0	0 8	200 0	0 04

EPOX

Initial Conc (µg/mL)	Aliquot (mL)	Final Volume (mL)	Final Conc (µg/mL)
5 0	5 5	25 0	1.1
5 0	4 0	25 0	0 80
5 0	5 0	50 0	0.50
5 0	6 0	100 0	0 30
5 0	2 0	100 0	0 10
5 0	1 0	100 0	0 05
5 0	0 8	200 0	0 02

3 1 2 Solutions for HPLC Calibration

Standard solutions containing PNP are prepared by diluting the primary stock solution as follows An accurate aliquot of PNP primary stock is transferred to a single 200 mL volumetric flask which is diluted to volume with HPLC mobile phase (methanol/water/acetic acid) The actual volume of primary stock aliquot is chosen so that a final solution concentration of 10 µg PNP/mL is obtained

An aliquot of the stock solution is transferred to a series of volumetric flasks, each being diluted to the mark with HPLC mobile phase. An aliquotting/dilution sequence guide is shown below:

Initial Conc (µg/mL)	Aliquot (mL)	Final Volume (mL)	Final Conc (µg/mL)
100	5 5	25 0	2 2
100	4 0	25 0	1 6
100	5 0	50 0	1 0
100	6 0	100 0	0 60
100	2 0	100 0	0 20
100	0 8	200 0	0.04

3.2 Sample Preparation and Extraction

For method validation, the method samples are fortified in duplicate at four levels: 0 05, 0 5, 1.25 and 2.0 ppm for EP and PNP, and 0 05, 0.20, 0 35 and 0 50 ppm for EPOX. Sunflower seed oil (25 g) is weighed into an 8 oz. glass jar. For EP and PNP, five mL of the 0.25 µg/mL spiking solution, and 5, 12.5 and 20 mL of the 2.5 µg/mL spiking solution are added to fortify the method samples. For EPOX, 5 mL of the 0 25 µg/mL spiking solution and 4, 7 and 10 mL of the 1.25 µg/mL spiking solution are added to fortify the method samples. After addition of the spiking solutions, the mixture is shaken vigorously by hand for 1 minute. A solvent mixture (100 mL) of 80 20 methanol/0.1 N HCl is added and the mixture is transferred to a 500 mL round bottom flask. Additional extraction solvent (50 mL) is added to the 8 oz. jar and the contents are added to the round bottom flask. Boiling chips are added and the mixture is refluxed for one hour.

At the end of the reflux period the sample is allowed to cool to room temperature The flask contents are filtered through a Buchner funnel fitted with Whatman filter paper (#1 or #4) and topped with ≈ 1 cm of Celite

NOTE Vacuum should be applied before adding Celite to the funnel. The Celite bed should be washed with 50 mL of extraction solvent; the wash solvent is discarded.

The reflux flask is then rinsed with 2 x 25 mL methanol. Each rinse is added to the funnel. All filtrate is collected in a 1000 mL round bottom flask The filtrate is concentrated by rotary evaporation (45°-50°C) until all methanol is removed

NOTE: It is very important that all methanol be removed to prevent emulsions in the partition steps.

The aqueous residue (≈ 30-40 mL) is transferred to a 500 mL separatory funnel. The round bottom flask is rinsed with 200 mL of acetonitrile and the rinses are added to the separatory funnel. The funnel is shaken for 30 seconds.

The round bottom flask is rinsed with 100 mL of hexane, and the rinse is added to the separatory funnel. The funnel is shaken for 30 seconds. The lower layer (acetonitrile) is drained into a second separatory funnel, the hexane layer is discarded. The round bottom flask is rinsed with an additional 50 mL of hexane and the rinse is added to the second separatory funnel. The funnel is shaken for 30 seconds. The lower layer (acetonitrile) is drained through a bed of sodium sulfate held in a narrow necked funnel by a plug of glass wool. The filtrate is collected in a clean 500 mL round bottom flask.

NOTE. It may be useful to run portions of fresh acetonitrile through the prepared funnel prior to the filtration of extract to ensure removal of potentially interfering materials from the sodium sulfate and glass wool. Discard such "pre-conditioning" acetonitrile

The solution is concentrated by rotary evaporation (35-40°C) to approximately 10 mL.

CAUTION. Do not take to dryness!

To the concentrate is added ≈ 100 mL of ethyl acetate. The contents are mixed by swirling and then poured over a bed of sodium sulfate into a 1000 mL round bottom flask. The 500 mL flask is rinsed by addition of an additional 100 mL of ethyl acetate, swirling, then pouring this rinse through the sodium sulfate bed into the second round bottom flask.

The ethyl acetate solution is concentrated to less than 5 mL by rotary evaporation (35-40°C).

CAUTION Do not take to dryness!

The residue is quantitatively transferred to a 25 mL volumetric flask with ethyl acetate. The flask is made to volume with ethyl acetate. This solution, after filtration through a 0.45 micron filter (Teflon), is ready for analysis of EP and EPOX by GC.

The ethyl acetate solution is further processed for analysis of PNP. An aliquot (1.0 mL) is pipetted into a conical tube (e.g., centrifuge tube) and concentrated to near dryness (approximately 50 µL) by nitrogen blow-down.

NOTE The solution must not be taken to complete dryness. It may be helpful to premark the conical tube at a level corresponding to 50 µL of ethyl acetate.

The concentrated solution is dissolved in 2.0 mL of hexane and sonicated for approximately 10 seconds to ensure complete dissolution.

A minicolumn is prepared from a Pasteur pipet by adding activated Florisil (Activity Grade I) to a clean pipet (insert a small amount of glass wool to retain the Florisil). Florisil is added to the level of the indentation of the pipet. Add anhydrous sodium sulfate (approximately 0.5 cm) to the top of the Florisil. The column is conditioned by washing with 8.0 mL of hexane. The eluate is discarded.

The sample (2.0 mL hexane solution) is added to the column, discarding the eluate. To the sample tube, add 2.0 mL of column eluant (49.5% ethyl ether, 49.4% hexane, 1% methanol, and 0.1% acetic acid) and sonicate for approximately 10 seconds. This rinse is transferred to the minicolumn, the eluate is discarded. The sample tube is rinsed again with 6.0 mL of eluant, and the eluant is added to the minicolumn. The eluate from this addition is collected in a clean 15 mL conical centrifuge tube. This solution is concentrated to near dryness (approximately 50 µL) by nitrogen blow-down.

NOTE: The solution must not be taken to complete dryness. It may be helpful to premark the conical tube at a level corresponding to 50 µL of eluant.

To the concentrated eluant is added 1.0 mL of a solvent mixture of acetonitrile/water (45/55) containing 0.1% acetic acid. This solution is sonicated for approximately 10 seconds. The solution is then filtered through a 0.45 micron filter into an autosampler vial. The sample is analyzed by HPLC as described below in Section 3.3.2.

3.3 Analysis

3.3.1 Ethyl Parathion/Ethyl Paraoxon

3.3.1.1 Calibration Curve

The GC is first equilibrated with several injections of conditioning samples (analyte standards and matrix blank) before the actual quantitative run begins. The system is considered equilibrated when successive injections produce peaks that differ no more than 15% in peak height or area (for the same amount injected). Calibration curve samples and method validation samples are injected during the same run sequence. The run sequence is shown below in Section 3.3.1.2.

A calibration curve is derived by linear regression treatment of GC detector response results for the calibration samples analyzed at each of the 6 concentration levels in the approximate range of 0.04 - 2.20 (ppm). The regression equation is of the form

$$y = ax + b$$

where

y = GC detector response (as peak area counts)

x = concentration of the calibration standard (µg/mL)

a = slope of the calculated calibration line

b = intercept of the calculated calibration line

Any acceptable calibration curve for sample analysis must have $\geq$ 96% of the total variance (equivalent to r (correlation coefficient) $\geq$ 0.98) explained by the regression line over the specified calibration range. The overall assay linearity is determined by:

$$\% \text{ Total Variance Explained} = r^2 * 100$$

3.3.1.2 Sample Analysis

To validate the methodology described above, a series of samples of varying concentrations within the range 0.05 - 2.0 ppm is prepared. Four "method" samples are prepared in duplicate at the following approximate concentrations 0.05, 0.5, 1.2 and 2.0 ppm for EP and 0.05, 0.2, 0.35 and 0.5 for EPOX. Samples are analyzed via autoinjection onto the GC system. Included in the set of method samples and blanks are two calibration curve check samples and a solvent blank. The calibration curve check samples consist of any two mid-range samples used to prepare the calibration curve The entire sample set of 19 samples is analyzed in the following order

Sample	Order
Solvent Blank	1
Cal. Curve Sample	2
Cal. Curve Sample	3
Cal. Curve Sample	4
Cal. Curve Sample	5
Cal. Curve Sample	6
Cal. Curve Sample	7
Method Control	8
Method Sample	9
Method Sample	10
Method Sample	11
Curve Check Sample	12
Method Sample	13
Method Sample	14
Method Sample	15
Curve Check Sample	16
Method Sample	17
Method Sample	18
Method Control	19

The method samples in the above sequence are analyzed in no specified order

3.3.2 p-Nitrophenol

3.3.2.1 Calibration Curve

The HPLC is first equilibrated with several injections of conditioning samples (analyte standard and matrix blank) before the actual quantitative run begins. The system is considered equilibrated when successive injections produce peaks that differ no more than 15% in peak height or area (for the same amount injected). Calibration curve samples and method validation samples are injected during the same run. The run sequence is shown below in Section 3.3.1.2.

A calibration curve is derived by linear regression treatment of HPLC detector response results for the calibration samples analyzed at each of the 5 concentration levels in the approximate range of 0.04 - 2.20 (ppm). The regression equation is of the form

$$y = ax + b$$

where

y = HPLC detector response (as peak area counts)

x = concentration of the calibration standard (µg/mL)

a = slope of the calculated calibration line

b = intercept of the calculated calibration line

The criteria for accuracy (% Error) and linearity (% Total Variance Explained) as described in Section 3 3 1 2 are required.

3 3 2 2 Sample Analysis

To validate the methodology described above, a series of samples of varying concentrations within the range 0 05 - 2.0 ppm is prepared. Four "method" samples are prepared in duplicate at the following approximate concentrations 0.05, 0 5, 1 2 and 2.0 ppm. Samples are analyzed via autoinjection onto the HPLC system. Included in the set of method samples and blanks are two calibration curve check samples and a solvent blank. The calibration curve check samples consist of any two mid-range samples used to prepare the calibration curve The entire sample set of 19 samples is analyzed in the following order:

Sample	Order
Solvent Blank	1
Cal Curve Sample	2
Cal. Curve Sample	3
Cal. Curve Sample	4
Cal Curve Sample	5
Cal Curve Sample	6
Cal. Curve Sample	7
Method Control	8
Method Sample	9
Method Sample	10
Method Sample	11
Curve Check Sample	12
Method Sample	13

Method Sample	14
Method Sample	15
Curve Check Sample	16
Method Sample	17
Method Sample	18
Method Control	19

The method samples in the above sequence are randomized.

3.4 Results Reported

Sample results are reported as the found concentration of each test sample as determined from the regression equations for EP/EPOX and PNP. The mean found concentrations for each concentration point, and the precision (as %RSD) will be reported, as will the % Recovery (found/nominal). Found concentrations are not corrected for recovery with found blank concentrations.

MODIFIED CRAVEN METHOD

ANALYTICAL DETAILS - ETHYL PARATHION AND ETHYL PARAOXON ASSAY

1. Weigh 25.00 ± 0.05 grams of homogenized substrate into a 8 oz. glass jar. Add 100 mL of extraction solvent (80:20 MeOH:0.1 N HCl in Milli-Q H_2O), blend using a PowrPulse Homogenizer at high speed for 60 seconds

2. Transfer the entire sample (substrate plus extraction solvent) to a 1 liter round bottom flask with the aid of a glass funnel. Rinse the blending shaft and 8 oz. jar with 2 x 25 mL of the extraction solvent

3. Transfer this to the same 1 liter round bottom flask as before, add boiling chips and gently reflux for one hour

4. At the end of the reflux period, allow the sample to cool to room temperature; vacuum filter the sample through a Buchner funnel fitted with Whatman #4 filter paper and topped with 0.25 inches of Celite (use a filter adapter), collecting in a 500 mL round bottom flask. Rinse the 1 liter round bottom flask with 2 x 20 mL MeCH, allowing the marc to dry between rinses

5. Dry down (roto-evaporate 45-50°C, water bath) until all methanol is removed, then transfer aqueous solution to a 500 mL separatory funnel. Rinse the flask with 3 x 30 mL portions of water and add rinses to the separatory funnel

6. Extract the aqueous phase with 3 x 1.0 mL portions of ethyl acetate, shake for one minute each, drain the lower aqueous layer into the second 500 mL separatory funnel and filter the top layer of ethyl acetate through a sodium sulfate filled funnel plugged with glass wool into a clean 500 mL round bottom flask. (It may be useful to run portions of fresh ethyl acetate through the prepared funnel prior to the filtration extract to ensure removal of the potentially interfering materials from the sodium sulfate and glass wool. Discard such 'pre-conditioning' ethyl acetate.) After all three portions have been collected, rinse the sodium sulfate with 10-25 mL of ethyl acetate and collect this rinse with other portions

7. Dry down the ethyl acetate (roto-evaporate 40-45°C) to 10 mL, transfer quantitatively into 40 mL vials with small portions of ethyl acetate and adjust final volume to 25.0 mL

8. The ethyl parathion and ethyl paraoxon are ready to inject on the GLC

ANALYTICAL DETAILS - p-NITROPHENOL ASSAY

1. Transfer 5 mL of the ethyl acetate substrate extract into a 15 mL flat bottom test tube and evaporate to dryness and re-dissolve in 2.0 mL of 1 N NaOH. Sonicate to assure complete dissolution

10 Prepare a minicolumn by filling it up to the indentation with 0% deactivated Florisil. Top this with sodium sulfate to fill the column. Condition the column by flushing with 8 0 mL hexane. Discard the eluate

11 Transfer the sample to the minicolumn and discard the eluate. To the sample tube, add 2.0 mL eluant (49.5% ethyl ether, 49 45% hexane, 1 0% methanol, and 0 1% acetic acid) and sonicate Transfer this rinse to the minicolumn and discard the eluate

12. Rinse the sample tube again with 6 0 mL eluant and add the rinse to the minicolumn. Collect this eluate in a clean, 15 mL flat bottom tube. Evaporate the eluate to dryness under a nitrogen stream. Add 1.0 mL 45 55 acetonitrile:water with 0.1% acetic acid and then sonicate the sample tube

13 Filter the sample through a 0 2 microfilter into an autosampler vial The sample is ready for HPLC analysis

Modification Number 1

For olives, pecans and wheat grain use 80 20 acetone 0 1 N HCl to replace 80 20 Methanol 0 10 N HCl

Modification Number 2

For wheat forage, wheat straw, and alfalfa use a 10 0 g sample instead of 25 0 g Adjust final volumes to 10 0 mL instead of 25 0 mL

Modification Number 3

For onions, when doing the PNP extraction, elute the sample with 8 0 mL eluant instead of 6 0 mL

Modification Number 4

For all potato products add about six drops Dow Antifoam B before refluxing

Appendix IV

Revised Soybean Refined Oil Method for Ethyl Parathion, Ethyl Paraoxon and p-Nitrophenol

Analytical Development Corporation

ADC REFINED OIL METHOD

I A.1 EXTRACTION

I.A 1.1 Weigh 25 0 g of soybean refined oil into an 8 oz French square bottle

I A 1 2. Extract sample with 100 mL of 80% MeOH/20% 0.1N HCl extraction solution

I A 1 3 Blend sample mixture for one minute with a polytron at high speed.

I.A.1.4. Pour sample into a 500 mL flat bottom flask (#1)

I A 1 5 Rinse sample jar and polytron blade by blending 50 mL of the extraction solvent with the polytron at high speed

I A 1 6 Pour rinse into the 500 mL flask (#1)

I A 1 7 Reflux with medium heat for one hour, allow to cool briefly

I A.1 8 Vacuum filter the sample through a Buchner funnel fitted with Whatman #1 filter paper and topped with approximately 0 25 inches of Celite 545 (Turn on vacuum before adding Celite to filter paper Wash the Celite bed with 50 mL of the extraction solvent and discard solvent.)

I A 1 9 Rinse the sample flask (#1) 7 x 25 mL portion of MeOH and add rinses to funnel

I A 1 10 Quantitatively transfer the filtrate into a 1000 mL flat bottom flask (#2 with 2 x 10 mL portions of MeOH

I A 1 11 Evaporate MeOH with a rotary evaporator with a water bath temperature maintained at 35C-40°C

[Using a distillation trap between the flask and the vapor tube is helpful Bumping is common in the early states of evaporation There is usually 30-40 mL solution left in the flask when all of the methanol has evaporated Other signs of complete methanol evaporation are the condensation of water in the distillation trap, slow rate of bubbling in the sample flask. It is imperative that all methanol is removed Even trace amounts of methanol can lead to severe emulsions in the partition steps that follow]

I A 2 PARTITION AND CONCENTRATION

I A 2.1 Pour sample into a 500 mL separatory funnel (#1) Rinse flask #2 with 2 mL of ACN and pour rinse into funnel Shake sep funnel 30 seconds

I A 2 2 Rinse flask (#2 with 100 mL of hexane and pour rinse into the sep funnel #1) Shake sep funnel 30 seconds Drain lower ACN layer into a second 500 mL sep funnel #2) discard hexane

I A 2 3 Rinse flask #2 with 50 mL of hexane and pour rinse into sep funnel #2 Shake sep funnel 30 seconds

I A 2.4 Drain lower, ACN, layer through a 200 g pad of anhydrous Na_2SO_4 held in a narrow-necked funnel by a plug of glass wool Collect the ACN in a 500 mL flat bottom flask (#3)

I A 2 5 Add two drops of mineral oil, swirl Evaporate sample to approximately 10 mL with a roto-vap with a water bath temperature maintained at 35°C to 40°C

I A 2 6 Measure 200 mL of ethyl acetate Add approximately half of it to the flask (#3), swirl and pour over a 200 g pad of Na_2SO_4 into a second 500 mL flat bottom flask (#4) (as in I A 2 4) Add the rest of the EtOAc to the original flask (#3), swirl and pour over the Na_2SO_4 pad into the second flask (#4)

I A 2 7 Evaporate sample to less than 5 mL with a roto-vap with a water temperature maintained at 35°C to 40°C

I A 2 8 Quantitatively transfer sample to a 25 mL volumetric flask with EtOAc and q s to the line with the EtOAc

I A 2 9 Filter sample with a 0 45 µm Gelman Acrodisc, PTFE, filter

I A 2 10 Inject sample on GC