# Solid-Phase Extraction and Cleanup of Organophosphorus Pesticide Residues in Bovine Muscle with Gas Chromatographic Detection

**Jorma Kuivinen** and **Staffan Bengtsson**
National Food Administration, Chemistry Division 2, Box 622, SE-751 26 Uppsala, Sweden

## Abstract

**The purpose of this investigation is to develop a rapid and simple method for the assessment of organophosphorus pesticides in bovine muscle by using solid-phase extraction (SPE). After extraction with ethyl acetate (EtOAc) the homogenate is centrifuged and filtered through sodium sulfate. The fat is precipitated in methanol by cooling and the extract is diluted with water and passed through a SPE column (Isolute ENV+). After elution with EtOAc, evaporation, and redissolution, the sample is injected into a gas chromatographic (GC) capillary column DB-1701 and detected by a flame photometric detector. Recoveries from bovine muscle fortified with 12 pesticides between 4 and 65 µg/kg include three levels ranging between 59% and 109% for ten of them. The two most polar pesticides (metamidophos and acephate) are not successful. The relative standard deviations are between 1% and 10% for the ten pesticides. A simplex method is used to optimize the GC conditions.**

## Introduction

Organophosphorus pesticides (OPs) are one of the most common classes involved in poisonings because of the inhibition of acetyl-cholinesterase (1,2). OPs may also be concentrated in fat. The degradation of the OPs and the formation of primary and secondary metabolites are induced by exposure to high temperature; light; enzymatic systems; microorganisms; and acidic, neutral, and alkaline pHs. However, in beef muscle some OPs and their metabolites could not be completely eliminated after cooking (3).

The European Communities have established maximum residue limits (MRLs) in meat (4,5). The conventional methods for the analysis of pesticides in meat are rather time-consuming, labor-intensive, and require relatively large volumes of solvents (6–8).

Another problem is that the MRLs for OPs are near the level of determination, which make them difficult to analyze because of interfering compounds in fat. However, Juhler (9) has shown that fat removal by ice cooling in combination with solid-phase extraction (SPE) is sufficient for cleanup.

A rather new technique, matrix solid-phase dispersion has also been reported for food analysis (10). Another technique using liquid chromatographic preparation and gas chromatography (GC)–mass spectrometry (MS) detection (11) has also recently been published.

In this study, the use of SPE with Isolute ENV+ columns reduced analysis time without affecting the quality of residue detection and measurement. There have also been other recently published papers in which ENV+ columns were used for cleanup in the pesticide analysis of vegetables (12) and honey (13).

The aim of this investigation was to develop a more rapid method procedure for the assessment of pesticides in a meat matrix and optimize the GC conditions with a simplex method (14).

## Experimental

### Apparatus

An Ultra-Turrax homogenizer TP 18-10 (Janke & Kunkel, IKA Werk, GmbH & Co., Staufen, Germany) was used. An SPE vacuum manifold 24-port model was obtained from Supelco (Sigma-Aldrich Co., Milwaukee, WI).

The GC system used was a Hewlett-Packard (Wilmington, DE) 5890 GC Series II with an electronic pressure control and equipped with a flame photometric detector (FPD). A split/splitless injector with a one-end tapered liner and an HP 6890 autoinjector was used. A Teflon-faced underside injector septum was obtained from Varian (Walnut Creek, CA). The capillary column that was used was a DB-1701 with a 30-m × 0.32-mm i.d. and a 0.25-µm film thickness (J&W Scientific, Folsom, CA).

The operating conditions were as follows: the carrier gas was helium at 1.5 mL/min, the diluted gas was nitrogen at 30 mL/min, the FPD gases were hydrogen at 75 mL/min and air at 100 mL/min, and the injection was splitless at 2 µL.

The injector temperature was 250°C, and the FPD temperature was 260°C. The temperature program was 63°C for 0 min and then to 270°C at 35°C/min for 10 min (total time 16 min).

**Material and reagents**

*Pesticide standards*

In general, the pesticide standards were ≥ 95% purity (Dr. Ehrenstorfer GmbH, Augsburg, Germany).

The SPE column used was 200 mg Isolute ENV+ with a 6-mL reservoir volume (International Sorbent Technology, Hengoed, U.K.). ENV+ is a polystyrene–divinylbenzene sorbent copolymer.

*Solvents*

The methanol (MeOH) used was gradient grade (LiChrosolv, Merck, Darmstadt, Germany). Pestiscan-grade acetone, cyclohexane (Cy), and ethyl acetate (EtOAc) were obtained from Lab-Scan (Dublin, Ireland). Milli-Q water was used. 1,2-Propanediol (99.8% reagent) was obtained from Sigma-Aldrich (Chemie GmbH, Munich, Germany). Sodium sulfate anhydrous granular ($Na_2SO_4$) was also obtained from Merck. Each new batch was dried at 500°C for 5 h and stored in an desiccator with bluegel.

**Standard solutions**

From standard stock solutions of each phosphorus pesticide, an intermediate solution mix was prepared and diluted in acetone (approximately 10 µg/mL of each (except for phorate, which was approximately 15 µg/mL) including all 12 pesticides). Four working standard mix solutions were prepared between approximately 0.02 and 1 (1.5) µg/mL by diluting with EtOAc–Cy (1:1). The stability at 4°C was between 3 and 6 months except for phorate, which was not stable more than approximately 1 month.

Parathion was used as the internal standard (IS) and prepared by dilution with EtOAc–Cy (1:1) to approximately 3 µg/mL from the stock solution in acetone.

Preparing the final working standard matrix solutions directly in GC vials with a matrix background was performed from a matrix blank (25 g bovine muscle) that was taken through the analysis procedure and redissolved at the end with 1.0 mL EtOAc–Cy (1:1). From this solution, 250 µL was transferred to four GC vials and evaporated at 40°C to dryness with nitrogen. To the respective vials, 0.5 mL of each working standard mix solution was transferred plus 0.1 mL of the IS parathion (3 µg/mL). The vials were sealed and mixed before injection. These matrix standards had a longer stability compared with pure standards and were used for preparing the standard calibration curves.

**Extraction**

The extraction was carried out according to Juhler (9). Homogenized meat (25 g) was extracted with 70 mL of EtOAc by an Ultra-Turrax for 3 min submerged in an ice water bath. $Na_2SO_4$ was added (14 g) and the extraction continued for a further 2 min. After centrifugation for 5 min at approximately 1300 g, the extract was filtered through $Na_2SO_4$ (11 g). Normally, 50 mL of the extract was then evaporated with a rotary evaporator at 30°C. The residue was transferred into a 13-mL test tube with small volumes of EtOAc–Cy (1:1). In order to remove the remaining water residues, EtOAc–acetone (1:1) was added then evaporated with nitrogen at 40°C in a thermal block.

In this method, the sample residue was redissolved in 3 mL MeOH and placed in a freezer for at least 5 min to precipitate the fat. The tubes were centrifuged for 5 min at 2000 g.

From the cold extract an aliquot of 2.7 mL was transferred to the Isolute ENV+ column. The column was preconditioned with 5 mL of EtOAc, 5 mL MeOH, and 5 mL MeOH–$H_2O$ (1:1); a flow of approximately 1 drop/s Milli-Q water (2.7 mL) was added and mixed with the extract in the reservoir; and the solution was discarded. Afterwards, the column was vacuum dried for 10 min at 0.1 bar (10 kPa). The column was eluted with 3 mL of EtOAc, and the eluate was collected in a test tube. A keeper (compound that retained the analytes) of 0.1 mL 1,2-propanediol–acetone (1:9) was added to the eluate and evaporated to dryness with nitrogen at 40°C.

The residue was immediately redissolved with 1.0 mL of EtOAc–Cy (1:1), and 0.2 mL of the parathion solution as the IS (3 µg/mL) was added. After the tube was mixed, the solution was transferred to a GC vial for injection.

A flow chart of this procedure is shown in Figure 1.

**Preparation of spiked samples**

The homogenized sample was weighed (25 g bovine muscle) in the centrifuge tube, and 1.0 mL of a working standard mix

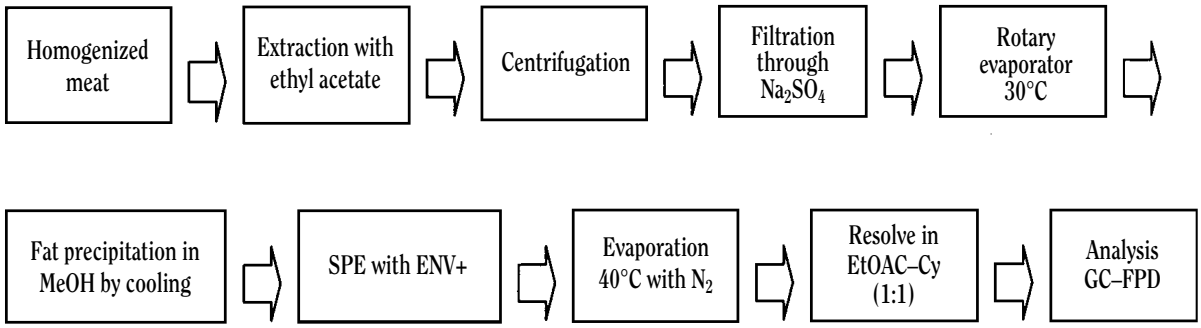


**Figure 1.** Flow chart of the analytical procedure.

solution in EtOAc–Cy (1:1) was added, followed by 69 mL EtOAc. Then, the extraction procedure as described previously was continued.

### Simplex optimization

The MultiSimplex software (Grabitech Solutions AB, Timrå, Sweden) uses optimization algorithms from two simplex methods (15,16) and a fuzzy set membership function (17) for handling multiple responses.

For the optimization of GC conditions in this study we used four different control variables: initial temperature, initial time, temperature gradient, and maximum oven temperature.

The initial control parameters were chosen

**Table I. LOD and Recovery at Three Different Spiking Levels**

| Pesticide | LOD µg/kg | %Recovery* 4–6 µg/kg | 15–26 µg/kg | 36–65 µg/kg | $K_{ow}$† log P |
|---|---|---|---|---|---|
| Dichlorvos | 0.1 | 85 ± 4 | 65 ± 9 | 59 ± 2 | 1.9 |
| Phorate | 0.5 | 107 ± 1 | 73 ± 3 | 68 ± 3 | 3.9 |
| Chlorpyrifos-methyl | 0.2 | 82 ± 9 | 86 ± 3 | 83 ± 1 | 4.2 |
| Pirimiphos-methyl | 0.6 | 101 ± 4 | 91 ± 4 | 83 ± 3 | 4.2 |
| Chlorpyrifos | 0.1 | 97 ± 5 | 83 ± 3 | 74 ± 2 | 4.7 |
| Malathion | 0.3 | 109 ± 3 | 93 ± 4 | 83 ± 1 | 2.8 |
| Chlorfenvinphos | 0.1 | 97 ± 10 | 99 ± 8 | 89 ± 2 | 3.9 |
| Protiophos | 0.1 | 91 ± 3 | 78 ± 3 | 69 ± 2 | 5.7 |
| Methidathion | 0.2 | 106 ± 5 | 89 ± 3 | 83 ± 2 | 2.2 |
| Triazophos | 4.3 | 97 ± 3 | 90 ± 4 | 81 ± 2 | 3.3 |

* Recovery and RSD percentages are given in the table ($n$ = 4 for LOD and each spiked level).
† Found in reference 18. $K_{ow}$ log P is the partition coefficient of $n$-octanol and water.

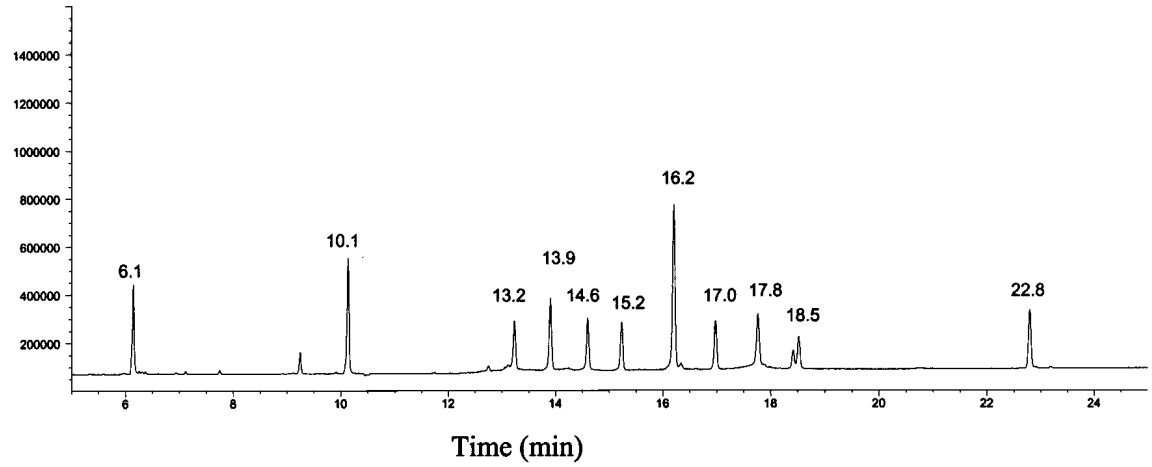


**Figure 2.** Before optimization. The temperature program began at 70°C, increased to 180°C at 30°C/min, increased to 260°C at 4°C/min, and then remained for 20 min (similar to Juhler (9)). The carrier gas was helium at 1.5 mL/min. The sample used for this test was bovine muscle spiked with the middle range standard of 15 to 26 µg/kg.

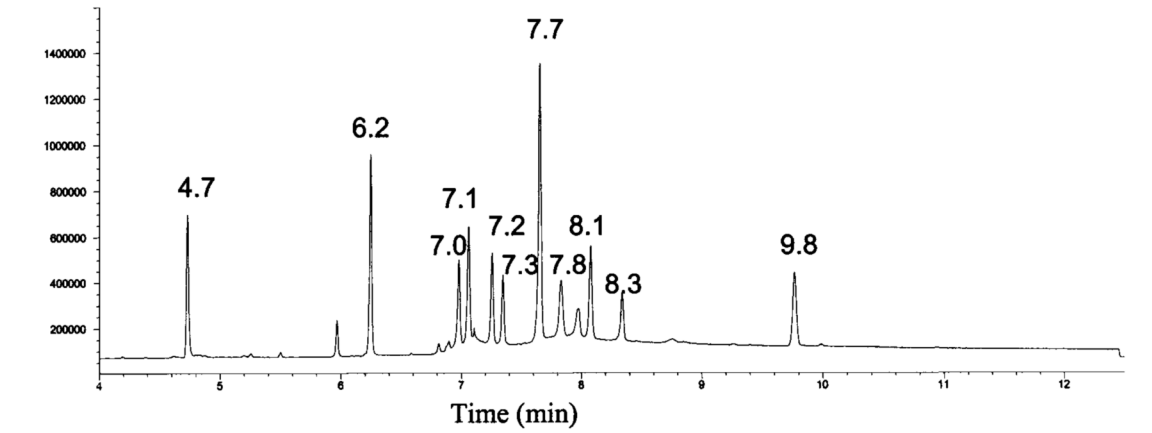


**Figure 3.** After optimization. With MultiSimplex optimization the GC retention time for triazophos was shortened from 22.8 to 9.8 min. The temperature program began at 63°C, increased to 270°C at 35°C/min, and then remained for 10 min. The carrier gas was helium at 1.5 mL/min. The sample used for this test was bovine muscle spiked with the middle range standard of 15 to 26 µg/kg.

from Juhler's (9) temperature program and varied with appropriate steps in the MultiSimplex optimization. The sample used for this test was a bovine muscle spiked with the middle range standard (15–26 µg/kg).

As response variables, we used chromatographic resolution. The first (R1) was between two peaks at the beginning of the chromatogram and the second (R2) was between two peaks at the later part of the chromatogram. The criteria for the R values were selected between 2 and 5. As a third response variable, the retention time for triazophos was used. The influence of weight for each response variable was also chosen in the MultiSimplex program (more details about MultiSimplex can be found at www.multisimplex.com).

## Results and Discussion

The purpose of this investigation was to reduce the analysis time for the SPE and optimize the GC conditions to achieve a shorter run time.

The OPs display a large variation in physiochemical properties (such as polarity) and partition coefficients between octanol and water. If the $K_{ow}$ log P is greater than 4, the substances are assumed to be fat-soluble (see Table I). The solvent composition used for the extraction of OPs in meat was taken from Juhler's method (9).

Many of the OPs in Table I have limit values in Sweden at 10 µg/kg meat except phorate, which has a limit of 50 µg/kg.

The recovery and repeatability of the method were investigated by analyzing bovine muscle samples spiked at three levels (Table I) (see also the Preparation of spiked samples section). A matrix peak interfered with the triazophos response, resulting in a higher limit of detection (LOD). Ten of the twelve OPs resulted in good recoveries and acceptable relative standard deviations (RSDs). The most polar pesticides (metamidophos and acephate) did not work well with this method and resulted in poor recoveries. A few high recoveries were obtained at the lowest spiked level, which probably depends on the difficulties encountered by the integration of the peaks at such a low level.

Pesticide-free bovine muscle was used for the LOD determinations ($n = 4$). The calculation of LOD was performed with the following equation:

$$\text{LOD} = \text{average of matrix blanks} + (3 \times \text{SD}) \qquad \text{Eq. 1}$$

The recovery tests were based on four real spiked determinations at each level. The recoveries were corrected for matrix blanks.

In order to reduce the GC running time, the temperature program was optimized with the MultiSimplex software to a run time of only 9.8 min for triazophos compared with 22.8 min before the optimization (Figures 2 and 3). This is very important because these substances are unstable in room temperature. The retention times before and after optimization are compared in Table II.

The benefit gained by using a MultiSimplex design for the optimization of the chromatographic conditions is the possibility to use several response variables (in this case the resolution and retention time for triazophos).

The improved method has several advantages compared with the earlier method (9). Only one SPE column (ENV+) was needed instead of two and only one fraction was collected, thus saving not only extraction time but also time for the GC analysis.

The evaporation step (at 40°C with a nitrogen stream) after the SPE column took only 30–45 min compared with approximately 2.5–6 h with the earlier method, because of the elimination of water residues.

A matrix blank was added to each working standard solution in order to get a more equal amount of the matrix background for both samples and standards (see the Standard solutions section). The specific reason for this was that the two latest peaks (methidathion and triazophos) had considerably lower responses without a matrix background. The other phosphorus pesticides did not need any matrix background for response compensation, but some of them (e.g., phorate) showed better stability with matrix.

All of the substances showed very good linearity with correlation coefficients between 0.995 and 1.000.

Different matrices may lead to problems with various small interference peaks, which makes it necessary to investigate the chromatograms from the matrix blanks. In the optimization step with MultiSimplex it is important to take this into consideration as well as for the matrix background to the standard solutions.

The method was evaluated using spiked samples as low as 4–6 µg/kg, which yielded good recoveries thus making it suitable for sample quantitation under the limit value of 10 µg/kg.

In order to achieve good peak resolution with as short a run time as possible, the simplex optimization method (MultiSimplex) improved the performance of the GC method. The SPE procedure with Isolute ENV+ columns is a fast and efficient method for extracting most of the pesticides from bovine muscle samples.

**Table II. Retention Times (min) for Phosphorus Pesticides Before and After Optimization**

| Pesticide | Figure 2 (before) | Figure 3 (after) |
|---|---|---|
| Dichlorvos | 6.1 | 4.7 |
| Phorate | 10.1 | 6.2 |
| Chlorpyrifos-methyl | 13.2 | 7.0 |
| Pirimiphos-methyl | 13.9 | 7.1 |
| Chlorpyrifos | 14.6 | 7.2 |
| Malathion | 15.2 | 7.3 |
| Parathion (IS) | 16.2 | 7.7 |
| Chlorfenvinphos | 17.0 | 7.8 |
| Protiophos | 17.8 | 8.1 |
| Methidathion | 18.5 | 8.3 |
| Triazophos | 22.8 | 9.8 |

With this method it should be possible to analyze other matrices such as pig, chicken, or lamb muscle as with the method by Juhler (9).

## Acknowledgments

The authors wish to thank Dr. Samuel Atuma, Dr. Marie Aune, and chemist Sören Wretling for their valuable comments.

## References

1. C.D. Klaassen. "Nonmetallic Environmental Toxicants: Air Pollutants, Solvents and Pesticides". In *Goodman and Gilman's, The Pharmacological Basis of Therapeutics, International Edition.* J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon, and A. Goodman-Gilman, Eds. McGraw-Hill, New York, NY, 1996, pp. 1673–96.
2. L.G. Sultatos. Mammalian toxicology of organophosphorus pesticides. *J. Toxicol. Environ. Health* **43:** 271–89 (1994).
3. K. Coulibaly and J.S. Smith. Effect of pH and cooking temperature on the stability of organophosphate pesticides in beef muscle. *J. Agric. Food Chem.* **42:** 2035–39 (1994).
4. Council Directive 93/57/EEC. *Off. J. Eur. Commun.* **36:** L211/1 (1993).
5. Council Directive 95/39/EEC. *Off. J. Eur. Commun.* **38:** L197/29 (1995).
6. V. Leoni, A.M. Caricchia, and S. Chiavarini. Multiresidue method for quantification of organophosphorus pesticides in vegetable and animal foods. *J. Assoc. Off. Anal. Chem. Int.* **75:** 511–18 (1992).
7. D.M. Holstege, D.L. Scharberg, E.R. Tor, L.C. Hart, and F.D. Galey. A rapid multiresidue screen for organophosphorus, organochlorine, and *N*-methyl carbamate insecticides in plant and animal tissues. *J. Assoc. Off. Anal. Chem. Int.* **77:** 1263–74 (1994).
8. F. Hernández, R. Serrano, J. Beltran, and F.J. López. Comparison of cleanup techniques for simple method for analysis of selected organophosphorus pesticide residues in molluscs. *J. Assoc. Off. Anal. Chem. Int.* **79:** 123–31 (1996).
9. R.K. Juhler. Optimized method for the determination of organophosphorus pesticides in meat and fatty matrices. *J. Chromatogr. A* **786:** 145–53 (1997).
10. S.A. Baker. Applications of matrix solid-phase dispersion in food analysis. *J. Chromatogr. A* **880:** 63–68 (2000).
11. R. Serrano, F.J. López, and F. Hernández. Multiresidue determination of persistent organochlorine and organophosphorus compounds in whale tissues using automated liquid chromatographic clean up and gas chromatographic–mass spectrometric detection. *J. Chromatogr. A* **855:** 633–43 (1999).
12. T. Philström and B.-G. Österdahl. Analysis of pesticide residues in fruit and vegetables after cleanup with solid-phase extraction using ENV+ (polystyrene–divinylbenzene) cartridges. *J. Agric. Food Chem.* **47:** 2549–52 (1999).
13. C. Jansson. Multiresidue method for the gas chromatographic determination of pesticides in honey after solid-phase extraction cleanup. *J. Assoc. Off. Anal. Chem. Int.* **83:** 714–19 (2000).
14. O. Zuloaga, N. Etxebarria, L.A. Fernandez, and J.M. Madariaga. MultiSimplex optimisation and comparision of different purge-and-trap extractions of volatile organic compounds in soil samples. *Anal. Chim. Acta.* **416:** 43–53 (2000).
15. W. Spendley, G.R. Hext, and F.R. Himsworth. Sequential application of simplex designs in optimisation and evolutionary operation. *Technometrics* **4:** 441–61 (1962).
16. D. Betteridge, A.P. Wade, and A.G. Howard. Reflections on the modified simplex methods. *Anal. Chim. Acta.* **144:** 39–53 (1985).
17. L.A. Zadeh. Fuzzy sets. *Info. Control* **8:** 338–63 (1965).
18. C.D.S. Tomlin. *The Pesticide Manual*, 12th ed. The British Crop Protection Council, Surrey, U.K., 2000.

Manuscript accepted May 1, 2002.