

Optimized method for the determination of organophosphorus pesticides in meat and fatty matrices¹

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Received 10 June 1996; received in revised form 28 May 1997; accepted 12 June 1997

Abstract

A method was developed and optimized for determination of residues of organophosphorous pesticides (OPs) in meat and fatty matrices. The method was developed for the national Danish monitoring programme, whereby priority was given to simplify the clean-up, avoid use of toxic and harmful organic solvents and allow quantification at ppb level using GC with nitrogen–phosphorus detection (NPD). Homogenized meat was extracted using ethyl acetate, the co-extracted water being removed using anhydrous Na₂SO₄. The clean-up was done through fat precipitation by cooling the extract flowed by solid-phase extraction on C₁₈ mini-columns. Pesticide residues were determined by GC–NPD using a DB1701 capillary column. The limit of detection was 1 to 20 ppb and limit of determination was 2 to 33 ppb and the method is feasible for control of fat-soluble OPs according to the maximum residue limits set by the European Communities. The method developed covers a broad polarity range from polar OPs, such as acephate and methamidophos, through medium polar OPs to non-polar pesticides, such as prothiofos. © 1997 Elsevier Science B.V.

Keywords: Pesticides; Organophosphorus compounds; Food analysis; Sample handling

1. Introduction

Organophosphorus pesticides (OPs) are typically esters of pentavalent phosphorus acids, and are widely used within agriculture. To a large extent, these compounds have replaced the persistent organochlorine compounds, and are now the most frequently used group of insecticides [1]. Even if the insecticides of this type typically act through inhibition of the enzyme acetylcholinesterase [2,3], they display large variation in physiochemical properties such as polarity and water solubility (see Table 1).

Until recently, considering accumulation of pes-

ticides in fat and meat, the focus has been on persistent chlorinated compounds, and methods development for meat analysis has been targeted at this group of compounds [4–6]. However, the awareness that OPs may concentrate in fat and the establishment of low maximum residue limits (MRLs) for OPs in meat as set by European Communities (EC) [7,8], urges targeted development of methods suitable for surveillance programmes. The objective of this work was to develop a method suitable for determination of OPs likely to be found in meat and fatty matrices, evaluating the combination of two simple and fast clean-up techniques that are easy to operate and further leads to low solvent consumption when compared to established methodologies.

In meat, MRLs for OPs are typically set at the

¹Presented at the 1st European Workshop on Pesticide Residues, Alkmaar, 10–12 June 1996.

Table 1
Properties of pesticides included in the study

Pesticide	Solubility in water (g/l)	Partition coefficient <i>n</i> -octanol and water (log P_{ow})	log P_{ow} reference
Acephate	790 (20°C)	-0.9	[41]
Chlorpyrifos	0.014 (25°C)	5.1	[42]
Chlorpyrifos-methyl	0.004 (24°C)	4.3	[42]
Malathion	0.145 (25°C)	2.9	[42]
Methamidophos	>200 (20°C)	-0.8	[41]
Pirimiphos-methyl	0.009 (30°C)	4.2	[42]
Prothiofos	0.00007 (20°C)	5.7	[41]

Solubilities [41] and partition coefficients for (*n*-octanol–water) are shown. Pesticide fat solubility can be described by log P_{ow} . Values of log P_{ow} below 3 are indicative of non-fat soluble pesticides, whereas at log P_{ow} >4 fat solubility is anticipated [42,43].

level of determination (range 0.01 to 0.1 ppm) [7,8]. To be able to analyse OPs at this level in meat, removing interfering compounds is necessary. Clean-up using gel permeation chromatography (GPC) and other techniques such as liquid–liquid partitioning, column adsorption chromatography and low-temperature precipitation has been reviewed and compared [9–11]. Another review by Leoni [12] reveals that many established methods employ solvent partitioning clean-up utilizing vast quantities of methylene chloride and other organic solvents, which should be omitted due to toxicological and ecological reasons. Among the alternatives GPC is probably the most widely used technique. Separating compounds according to relative size [13] makes GPC a versatile technique and it finds extensive use analysing non-fatty matrices such as fruit and vegetables and is also used for animal products. GPC was introduced to pesticide analysis by Stalling et al. [14], extensively elaborated by Specht and Tillkes [15] and further optimized [16,17] to reduce the high solvent consumptions characteristic of applications using large columns or/and high-performance gel permeation chromatography (HPGPC) [18]. However, analysing fatty matrices, it is a drawback that the performance of the GPC is sensitive to fat [16]. Further, additional clean-up steps such as column chromatography subfractionating the pesticide in as many as seven fractions are often required following GPC [9,15,19], and GPC is quite labour intensive, if not automation is employed. Also, column chromatography on Florisil is often used to remove fat [12,20] even if it has been found to cause bad recoveries of certain OPs [12,21]. Thus, Florisil should be omitted aiming

at a general method for OPs. In this context, evaluating alternative clean-up procedures is relevant. As mentioned low-temperature fat precipitation has previously been used for a pesticide clean-up. In a very early application, low-temperature fat precipitation was used in combination with column chromatography to clean-up pesticides from plant extracts [22], and this methodology was further evaluated for clean-up of several organochlorine compounds and some OPs [23,24]. However, this was done at -78°C , requiring custom-made apparatus and chromatography using laboratory packed Florisil columns. Within the last decade manufacturing of mini-columns has reached a batch-to-batch reproducibility permitting use in routine pesticide surveillance, and mini-columns have previously been used for clean-up of OPs from various matrices [12,20,21]. On this background reevaluating the approach made by Anglin and McKinley was relevant [22]. The present work shows that gravimetric fat removal through ice cooling is sufficient for clean-up when combined with solid-phase extraction (SPE) on pre-fabricated columns and the methodology represents an alternative approach to clean-up using GPC.

2. Experimental

2.1. Chemical reagents

Pesticide standards of acephate (99.0%), chlorpyrifos (99.7%), chlorpyrifos-methyl (98.3%), malathion (96.3%), methamidophos (98.0%), para-

thion (99.7%), pirimiphos-methyl (99.0%) and prothiofos (93.2%) were purchased from Dr. Ehrentorfer (Augsburg, Germany). Acetone, ethyl acetate, cyclohexane, ethanol, methanol and acetonitrile were purchased from Rathburne (Walkerburn, UK) whereas propandiol(1,2) and diethyl ether were purchased from Merck (Darmstadt, Germany). All organic solvents were HPLC grade. Each new batch of granular anhydrous Na_2SO_4 (99.0%, BDH, Dorset, UK) was heated to 500°C for 5 h and stored in a sealed container until use. To reduce risk of residue loss due to adherence, etc., glass only was used for handling of extracts. Nitrogen (99.996%) was used for GC analysis and solvent evaporation.

2.2. Extraction

Meat was purchased from a local market, ground in a food chopper (Weisser 81K, Obersasbach, Germany) and stored at -20°C until analysis. For analysis 25 g of homogenized meat was drenched with 70 ml ethyl acetate and thoroughly mixed for 3 min using an Ultra-Thurax being submerged in an ice cooled waterbath maintaining a temperature below 10°C . Then 14 g of anhydrous Na_2SO_4 was added, and the extraction continued for 2 min whereafter the mixture was centrifuged for 2 min at 3000 g. After centrifugation the supernatant was decanted, filtered and evaporated in a stream of nitrogen (99.996%). Subsequently 1.2 ml ethyl acetate–methanol (3:7, v/v) were added and the extract transferred to a test tube. To allow fat to precipitate the tube was placed on an ice–water-bath for 5 min before SPE clean-up.

2.3. SPE clean-up

To remove fat and other matrix compounds, clean-up was done on Bond Elut 500 mg SPE- C_{18} columns using a Vac Elut SPS 24 vacuum manifold purchased from Varian (Harbor City, CA, USA). The column was conditioned using a flow of 1 ml/min and successively applying 3 ml of cyclohexane, 3 ml methanol and 3 ml water, whereafter vacuum was released and the column allowed to settle for 3 min. Subsequently, 1.00 ml of ice cool extract was sampled from the supernatant, applied to the column and allowed to warm up to room temperature sitting

in the column reservoir for 5 min. Hereafter, the vacuum was reapplied and the effluent collected. The column was then washed with 0.50 ml of acetonitrile–water (1:1, v/v). The effluent collected thus far was designated fraction 1a. Hereafter, 4 ml of methanol was applied to the column and collected in another fraction designated fraction 2a. During conditioning and elution the column was not allowed to go dry, even if this is not essential to achieve good recovery (results not shown).

To reduce risk of residues persisting in the fat precipitated the remnant was reheated to room temperature, 1.00 ml of ethyl acetate–methanol (3:7, v/v) was added and thoroughly mixed with the fatty matrix for 30 s using a Vibro-Fix. Again the sample was cooled on ice, 1.00 ml sampled and cleaned up using another preconditioned column following the procedure described above. The respective fractions of the first and second clean-up were combined (1a+1b and 2a+2b, b indicating second clean-up), resulting in a total of two fractions (1 and 2).

To both fractions 0.1 ml keeper solution consisting of propandiol–acetone (1:9, v/v) was added to retain residues while other solvents were evaporated using N_2 , any water remaining being removed by addition of 1 to 3 ml ethyl acetate–acetone (1:1, v/v). When propandiol only remained 1.00 ml of ethyl acetate–cyclohexane (1:1, v/v) was added.

2.4. Chromatographic analysis

Organophosphorus pesticides were analysed using a HP gas chromatograph Model 5890A (Hewlett-Packard, Waldbronn, Germany) fitted with an HP 7673A automatic injector using a deactivated fused-silica capillary tubing and 2 μl splitless injection. Temperature of the injector was set to 260°C . The column was a J&W (Folsom, CA, USA) DB1701 capillary (30 m \times 0.32 mm I.D. \times 0.5 mm O.D., 0.25- μm film thickness) coated with a phenyl-cyano-propyl-methyl phase. Helium (1 ml/min on column, 99.999%) was used as carrier gas. The nitrogen–phosphorus detection (NPD) system used hydrogen (99.995%) as make up gas and a temperature set point of 250°C . The chromatographic temperature programme was: 1 min at 70°C followed successively by a $30^\circ\text{C}/\text{min}$ increase to 180°C and $4^\circ\text{C}/\text{min}$ to 260°C maintaining this final temperature for 20 min.

Total GC analysis time was 45 min. Quantification was performed using standards in ethyl acetate–cyclohexane (1:1). For normalization an internal GC standard [200 μ l of 3 μ g/ml parathion in ethyl acetate–cyclohexane (1:1, v/v)] was added.

2.5. Spiking standards

Spiking was done by adding 10 to 50 μ l of a pesticide mixture dissolved in ethyl acetate–cyclohexane (1:1, v/v) to 25 g of defrosted meat.

3. Results and discussion

Considering all OPs the polarity range covered is wide, and the feasibility of the method was checked by selecting a complete polarity range of OPs (see Table 1). Thus the polar pesticides methamidophos and acephate that are less likely to accumulate in a fatty matrix were included, even if emphasis was put on probable fat concentrating OPs. As the method was developed for routine analysis, limiting the number of clean-up steps was essential as well as achievement of a low solvent consumption and avoidance of harmful solvents.

3.1. Extraction and clean-up

OPs are often extracted from vegetables and other low-fat matrices using acetone or ethyl acetate. Developing a method that focuses on fat soluble pesticides, ethyl acetate was chosen due to its lower polarity and small carryover of water from the matrix. To reduce solvent consumption it is desirable to reduce the size of a test portion taken for analysis. Being representative of the entire sample is however essential for the subsample. The aspects of homogenisation and subsampling have been thoroughly discussed in relation to determinations of major constituents such as fat and moisture [25]. Such studies have shown that 5 g or more is required for test portions using conventional non-cryogenic homogenization [26]. An additional variation in residue distribution within the fatty matrix must be taken into account [27], and studies on pesticides in vegetables reveals a risk of higher variation in determinations using test portions below 25 g [28].

Due to such homogeneity considerations and in consistency with several other works [18,29,30] a test portion of 25 g was chosen. Accordingly, the total solvent consumption used for extraction and clean-up was 92 ml. This is comparable to the volume used in a downsized GPC methodology [16], but relatively small compared with the American Food and Drug Administration (FDA) small scale method [31] using a total of 370 ml and suggested CEN methods [18] using 100 to 950 ml organic solvent for extraction, and additionally 70 to 1000 ml for extract clean-up.

Removing fat gravimetrically through cooling is a simple procedure that can easily be combined with SPE for clean-up of OP residues. Using this approach, the composition of the application solvent mixture had to be optimized in several aspects to allow good recovery of pesticides without fat contamination. Thus the solvent must effectively extract residues from the fat, it must solubilize fat at room temperature and yet permit solvated fat to precipitate on cooling with ice. Finally the solvent must be compatible with the active sites of the SPE column to accomplish clean-up.

Several organic solvents were evaluated: methanol, isopropanol, acetone, ethyl acetate, diethyl ether, ethanol and hexane. From initial experiments, methanol, diethyl-ether and ethyl acetate were selected for further optimization through a ternary simplex design [32]. Solvent composition was evaluated for the ability to both solvate fat at room temperature and promote fat precipitation when placed on ice (see Table 2). At room temperature 200 mg fat was weighed into a dried test tube, extracted with 1.20 ml solvent mixture and thoroughly mixed for 30 s using a Vibro-Fix. Fat was precipitated on ice for 5 min whereafter 1.00 ml was transferred into another tube. Contents of both test tubes were evaporated using N_2 , dried in an oven for two hours at 70°C, whereafter fat remaining in both tubes was determined gravimetrically. Low fat transfer was observed when sampling from methanol and a mixture of methanol–ethyl acetate (1:1, v/v) (experiments 1 and 5 in Table 2). A final optimization step was thus done to evaluate clean-up for fat using various mixtures of methanol and ethyl acetate. A general optimum was found using ethyl acetate–methanol (3:7, v/v), which was selected for the present method.

Table 2
Optimizing solvent composition for fat precipitation by cooling

Experiment	Solvent composition (volume part is indicated)			<i>T</i> Fat transferred/fat remaining
	Methanol	Diethyl ether	Ethyl acetate	
1	1	0	0	0.02
2	0	1	0	12.92
3	0	0	1	0.84
4	0.5	0.5	0	0.24
5	0.5	0	0.5	0.09
6	0	0.5	0.5	17.48
7	0.33	0.33	0.33	1.61

A ternary simplex design was used [32] in which the composition of the solvent mixture was varied in seven experiments. The ratio *T* describes the relative part of fat transferred when sampling 1.00 ml from the cooled supernatant (*T*=fat transferred/fat remaining, see text for details).

In principle, the two SPE fractions could be combined before further analysis, but screening for OPs in the range 10–50 ppb it is desirable to have a primary fraction, one that is as free of matrix compounds as feasible. This is particularly true using NPD for detection, whereas requirement for clean-up may be less, using mass spectrometry for detection (GC–MS or GC–MS–MS). Also, the majority of the residues are contained within fraction one (Fig. 1) and this alone may thus be used when screening is done. However, to fully quantify the residues both fractions should be analysed. It should be noticed, that using the vacuum manifold labour needed for SPE conditioning and clean-up is considerably diminished, whereby two extractions and the use of two SPE columns per clean-up can be justified. To assure acceptable recovery of pesticide and avoid breakthrough of the SPE column, overloading with fat must be avoided. Using meat with high fat content, the sample amount thus needs to be adjusted to a total of 4.5 g fat before precipitation. This is a high capacity when compared with clean-up using GPC, where a maximum of 100 mg fat can be loaded onto a 450×10 mm column [16]. Thus, analysing meat samples with a high fat content using GPC would require reduction in sample size or an increase in column dimensions leading to larger solvent consumption (up to 200 ml for GPC alone [15]). Also, overloading a GPC column, the following samples are affected, and repacking of the column may be required. However, the experiences from these studies may also be employed working with

GPC and matrixes with high fat content. Thus, gravimetric removal of fat may be applied before GPC through cooling of the extracts or alternatively through cooled centrifugation.

3.2. GC analysis

The GC temperature programme agrees with a previously optimized method by Andersson and Ohlin [33], only the initial temperature was lowered from 90 to 70°C to promote reconcentration through a possible solvent effect [34]. A typical chromatogram is shown in Fig. 1. Two polar OPs, acephate and methamidophos were included in the development of the method. These pesticides have low t_R at the chromatographic conditions used and have been in some works where they have displayed low recoveries partly due to instability on injection [35–38]. Rather low recoveries were also observed for these pesticides at low levels in the present work (Table 3). With some matrices, impurities emerge within the first 10 min of the GC analysis, the main effect being on acephate eluting as a shoulder on a matrix peak (m2 in Fig. 1). These major matrix peaks are not observed extracting pure fat (results not shown). Also, quantifying acephate and methamidophos is possible even in the presence of matrix peaks (Table 3). For verification purposes, confirmation on pesticide identity using retention characteristics can be supplemented using a J&W DB01 column.

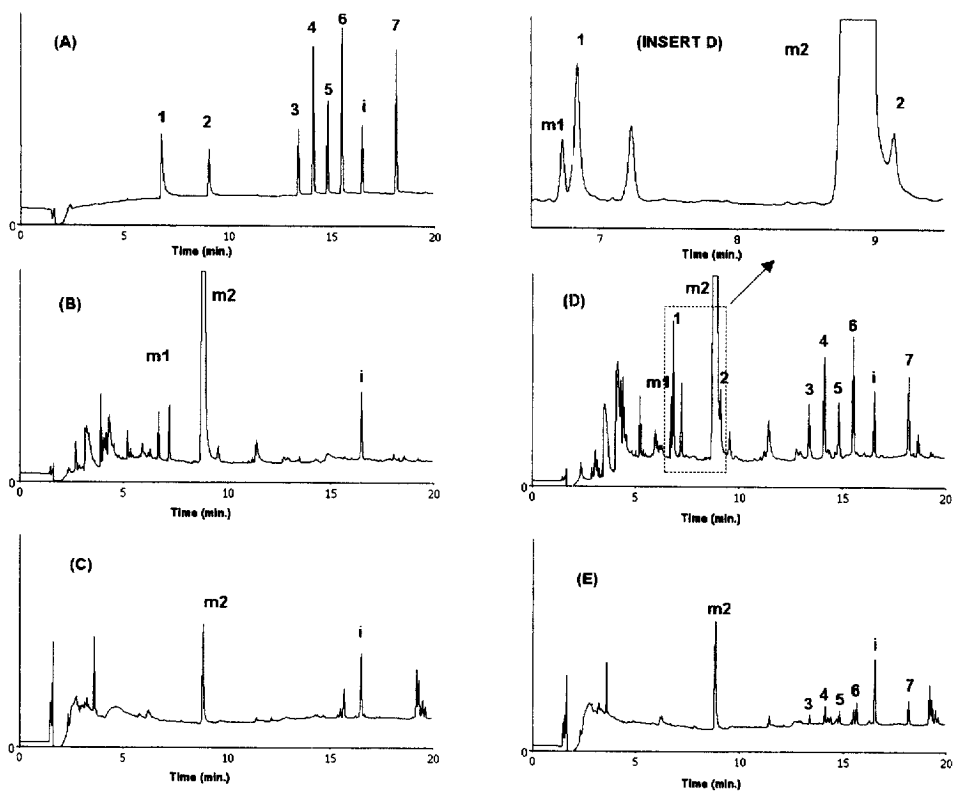


Fig. 1. Representative gas chromatograms of meat extract using NPD and OPs. Chromatograms are normalized to parathion standard. (A) Pure standard set at level of 1 ppm. Blank extracts fraction 1 (B) and fraction 2 (C). Meat spiked at level 120 ppb fraction 1 (D) and fraction 2 (E). Insert D is an enlargement of the boxed section of (D). The (insert D) shows the separation of methamidophos (1) from a matrix peak (m1) and acephate (2) as a shoulder on matrix peak m2. Peaks: 1=methamidophos, 2=acephate, 3=chlorpyrifos-methyl, 4=pirimiphos-methyl, 5=chlorpyrifos, 6=malathion, 7=prothiofos, i=parathion standard.

3.3. Calibration plots, LD and LOD

The method performance and OP calibration is characterized in Table 3. The detection limit (LD) and the limit of determination (LOD) has been established using spiked samples according to the recommendations from the Association of Official Analytical Chemist (AOAC) [39]. Thus, LD was determined as the mean value of the blank matrix reading plus 3 standard deviations and LOD as the mean of the measured content of a blank sample plus 6 times standard deviation. The LODs of the non-polar OPs are in accordance with the EC MRLs ([7,8] and Table 3) and Fig. 2 shows a chromatogram addressing this level for non-polar OPs. Polar pesticides can only be screened due to LOD above the EC MRL. However, in Fig. 3 it is demonstrated that

the chromatographically most problematic pesticide acephate can be screened down to its EC LOD at 1 ppb and in practice the limit of quantitation is in agreement with the MRLs for polar OPs. Developing methods to measure pesticide residues at very low levels at the LOD, Codex Alimentarius operates with a "lower practical limit to be determined (LPL)", and when the MRL is set at the LOD, the LPL will also be at this level [40]. However, in Table 3 data for recovery below the EC LOD is provided for non-polar OPs. From Table 3 it can also be seen, that all OPs except acephate are within the acceptable 40–120% range at 5 ppb as set by AOAC [39].

Considering quantification in a daily routine, using pure GC standards is simpler and thus more desirable than employing standard solutions in a residue free matrix extract. Though, using neat standards to

Table 3
Detection limit (LD) and limit of determination (LOD)

Pesticide	LD in meat ($\mu\text{g}/\text{kg}$) ppb	LOD in meat ($\mu\text{g}/\text{kg}$) ppb	EC MRL ($\mu\text{g}/\text{kg}$) ppb	Recovery				r^2 of calibration equations
				2 ppb	5 ppb	100 ppb	400 ppb	
Acephate	10 ^a	25 ^a	20 ^{*b}	35 \pm 13 ^a	32 \pm 3 ^a	69 \pm 9	82 \pm 16	0.996
Chlorpyrifos	3	5	50 ^{*c}	100 \pm 11	99 \pm 10	116 \pm 6	102 \pm 10	0.997
Chlorpyrifos-methyl	15	25	50 ^{*c}	103 \pm 5	89 \pm 6	121 \pm 10	108 \pm 4	0.995
Malathion	4	7	—	132 \pm 14	107 \pm 6	99 \pm 18	91 \pm 4	0.997
Methamidophos	10 ^a	30 ^a	10 ^{*b}	60 \pm 4 ^a	48 \pm 6 ^a	109 \pm 8	88 \pm 19	0.996
Pririmiphos-methyl	1	2	50 ^{*c}	90 \pm 2	91 \pm 5	101 \pm 10	91 \pm 8	0.994
Prothiophos	20	34	—	97 \pm 13	102 \pm 6	98 \pm 9	87 \pm 8	0.992

^a The polar OPs acephate and methamidophos are included for screening primarily and both are eluting within a group of matrix peaks (see Fig. 1 insert D). Where established, EC MRL for meat ^(b) or fat ^(c) is given.

* Indicates EC LOD.

LDs and LODs were established using meat extracts containing no residues (see text for details). Further, the EC MRL is shown where established [7,8]. Characterization of detector response was performed on three different days towards neat standards at 0.13, 0.32, 0.25, 0.65, 1.13 mg/ml and the square of the correlation coefficient of linear regression is shown (r^2). Also, recovery is given for levels 2, 5, 100 and 400 ppb and the standard deviation indicated. Recovery data are based upon 5 individual determinations on spiked meat samples and data given are the sum of residues in fraction 1 and 2.

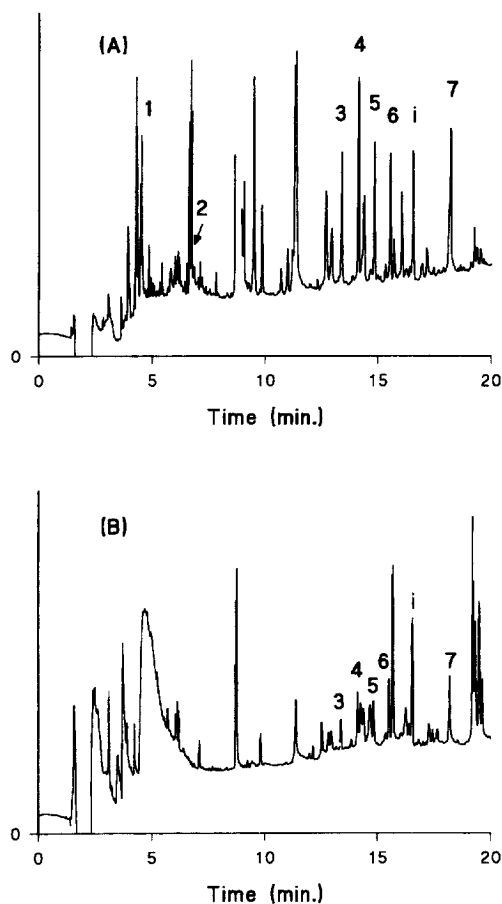


Fig. 2. Normalized GC-NPD chromatogram at the EC MRL level for non-polar OPs in fraction 1 (A) and 2 (B). Blank meat samples were spiked at level 5 ppb. Legends as in Fig. 1.

quantify acephate and methamidophos attention must be given to recovery, as some previous works have shown too high recoveries of OPs with P=O bonds. Higher response is thought to result from a matrix mediated protection against degradation and adsorption in the GC system [38]. However, in this study such effects were not observed (see Table 3).

Screening for pesticides, GC analysis can be done without internal normalization standard. Though, quantifying residue contents, including internal GC-standards is desirable. In this study parathion was used for the following reasons, i.e. elutes within relevant OPs, it is quite stable, it has no EC MRL in

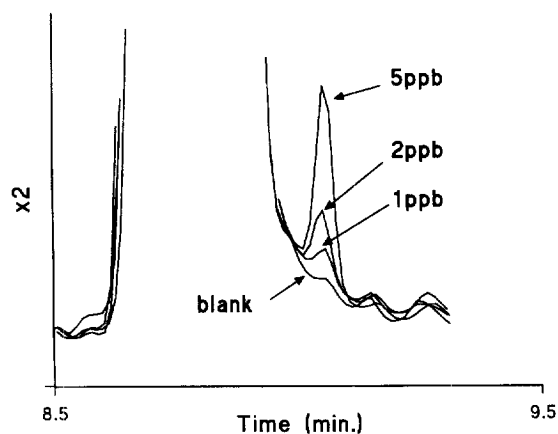


Fig. 3. Normalized GC-NPD chromatograms showing the possible screening for the polar OP acephate using fraction 1 extracts. Acephate is found as a shoulder on a major matrix peak (see Fig. 1). Blank meat samples were spiked at levels 1 to 5 ppb as indicated, and also the response of a blank sample is shown. The response is multiplied by two relative to the axis used in Fig. 2.

meat or fat, and further parathion gives satisfactory response on the NPD. Whenever a parathion residue is detected in samples, dithalimphos may be used.

4. Conclusion

The method presented has proven to work at the ppb level required for control of MRLs of OPs likely to be found in fatty matrices. Extraction and clean-up is performed without any liquid-liquid partitioning or comparable methodologies leading to extended use of undesirable solvents, and it supplements the extensively used GPC methodology. The novelty of the approach presented lies in the clean-up achieved through the combined use of gravimetric fat removal through cooling and SPE clean-up on pre-fabricated mini-columns. Considering the broad polarity range covered by the method, the prospects for inclusion of several OPs are promising, and work is currently being done to increase the number of pesticides covered by the method. Further, the method may perform well on other similar pesticide groups, whereby an attempt is currently being made to incorporate pyrethroids.

Acknowledgments

The author acknowledges the skilled technical assistance of Mrs. I. Schröder.

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