DETECTION OF MODEL MIXTURE OF ORGANOPHOSPHATE AND ORGANOCARBAMATE PESTICIDES BY THIN LAYER CHROMATOGRAPHY

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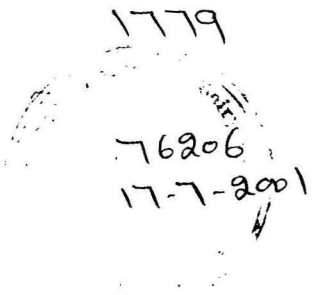
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Dedicated to
My Beloved
Achan, Amma &
Shyanchettan

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Thesis submitted to the
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MASTER OF SCIENCE
IN
DAIRYING
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(PULARI K. NAIR)

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LIST OF ABBREVIATIONS

μg	=	Microgram
μl	=	Microlitre
A	=	Acetone
AOAC	=	Association of Official Analytical Chemists
diff.	=	Different
DL	=	Detection limit
ECD	=	Electron capture detector
ElCD	=	Electrolytic conductivity detector
FAO	=	Food and Agricultural Organization
FMD	=	Fluorometric detector
FPD	=	Flame photometric detector
g	=	Gram
GC	=	Gas chromatography
GCMS	=	Gas chromatography - mass spectrometry
H	=	Hexane
HPLC	=	High performance liquid chromatography
HPTLC	=	High performance thin layer chromatography
hr	=	Hour
ITMS	=	Ion trap mass spectrometry
KCITD	=	Potassium chloride thermionic detector
LCMS	=	Liquid chromatography - mass spectrometry
max	=	Maximum
mg	=	Milligram
min	=	Minute
ml	=	Millilitre
MRL	=	Maximum residue limit
MS	=	Mass spectrometry
nm	=	Nanometer
NPD	=	Nitrogen phosphorus detector

OC	=	Organochlorine compounds
OCm	=	Organocarbamate compounds
OCmPR	=	Organocarbamate pesticide residues
OCPR	=	Organochloro pesticide residues
OP	=	Organophosphorus compounds
OPPR	=	Organophosphate pesticide residues
P	=	Phosphorus
PAM	=	Pesticide analytical manual
PFA	=	Prevention of Food Adulteration Act
Rf	=	Relative to front
RT	=	Room temperature
S	=	Sulphur
SIM	=	Single ion monitoring
TLC	=	Thin layer chromatography
TLC-Chem	=	Thin layer chromatography - chemical method
TLC-EI	=	Thin layer chromatography - enzyme inhibition technique
TS	=	Thermospray
UVD	=	UV detector
viz.	=	Namely
WHO	=	World Health Organization

CHAPTER - 1

Introduction

1. INTRODUCTION

Synthetic organic insecticides including mainly, organochloro (OC), organophosphate (OP) and organocarbamate (OCm) pesticides exhibit a high degree of persistence in the environment as compared to other classes of pesticides. Many OCs have shown undesirable effects on human beings and environment due to their persistent, bioaccumulative and lipophilic nature (Stan, 1990). These have been banned in the developed and developing countries including India and being replaced by less persistent, systemic and acute toxic OP and OCm pesticides. Because of anticholinesterase action, OP and OCm possess acute toxicity to mammalian nervous system and due to the irreversibility of their attack, risk factor is greater (Rathore *et al.*, 1995).

In India, during the period 1995-2000, the consumption pattern of pesticides have changed, OC decreased from 40 to 14.5 percent, carbamate from 15 to 4.5 percent and synthetic pyrethroids from 10 to 5 percent. There was a sharp increase in the consumption of OP from 30 to 74 percent. Out of 57 insecticides registered in agriculture, 8 OP pesticides (monocrotophos, malathion, methyl parathion, phosphamidon, phorate, quinalphos, dimethoate, chlorpyriphos), 1 OCm (carbaryl) and 1 OC (endosulfan) account for 80 percent of the total insecticides used in India (Agnihotri, 2000).

Like organochloro pesticide residues (OCPR), organophosphate pesticide residues (OPPR) and organocarbamate pesticide residues (OCmPR) have been potentially found in non-fatty as well as fatty foods including dairy products (Ivey *et al.*, 1973; Stijve and Cardinale, 1974; Baldl, 1979; IDF:9101, 1990; Alla *et al.*, 1991). In India, however, analysis of milk and milk products for OPPR and OCmPR are at its infancy.

The methods used to analyse (detect / estimate) pesticide residues range from relatively simple methods to the modern determinative techniques, like, GC, HPLC, GCMS, LCMS, etc. The high sensitivity and selectivity of modern techniques, however, is not required for all types of research on pesticides. For routine food quality control, an exact quantification will not always be necessary. The important point is to note whether the MRL has been exceeded or not. The TLC has been used as a viable alternative, since it is rapid, simple and less expensive screening method. With the availability of commercial precoated plates, the technique is adequately sensitive and especially suitable for separation of pesticide compounds. It is capable of assaying large number of samples for the presence of pesticides in relatively short time.

Further, in multiresidue isolation approach which is now preferred over individual classes of isolation (PAM1, 1999), OP and OCm are isolated together. The review of literature to-date has revealed that available TLC methods are either limited to very few OP or to the set of OP other than those in current use in India. Hence, the project has been undertaken with the following objectives:

OBJECTIVES

- Standardization of TLC methods for the separation of OP and OCm pesticides (currently in use in India).
- Determination of their detection limits so that a clear-cut proposition can be suggested for the screening of food samples including dairy products for their MRL.

Review of Literature

2. REVIEW OF LITERATURE

It is proposed to review here literature up-to-date on analytical methods used for organophosphate and organocarbamate pesticide compounds with special reference to their detection by thin layer chromatographic (TLC) technique.

2.1 ANALYTICAL METHODS

In general, the choice of analytical technique used depends upon the mode of analysis. The methods used to analyse (detect / estimate) pesticide residues range from relatively simple methods to the modern determinative techniques like, GC, HPLC, GCMS, etc. The high sensitivity and selectivity of modern techniques is required for exact quantification and is not required for all types of research on pesticides. For routine food quality control, an exact quantification will not always be necessary. Rather it will be more important to screen the food samples for their MRL. These methods have been used depending upon the nature of results required. The methods used up-to-date are illustrated below:

2.1.1 QUANTITATIVE METHODS (GLC / HPLC / GCMS / LCMS)

It is economically and physically impractical to apply large number of individual methods for all the significant chemical pesticide residues that may be present in a food commodity. Earlier evolution was towards the development of methods which could analyse a number of residues within a class, while later on this extended to development of methods which could be used to analyse pesticide residues belonging to different classes. Lower cost, shorter time of analysis, extension of scope of analysis are the major points which has given rise to multiresidue analysis concept. Table 2.1 delineates the quantitative methods involving multiresidue analysis through GLC / HPLC / GCMS / LCMS.

Table 2.1 Quantitative Methods : Multiresidue Analysis through GLC / HPLC / GCMS / LCMS.

Sample	Analytical Techniques	Detection Limit	References
Non-fatty foods (leafy vegetables and fruits) Fatty foods (milk, cheese, butter)	OCPR - **GC (ECD)** OPPR - **GC (KCITD)**	-	Wessel (1967) AOAC (1984)
Fruits and vegetables	OPPR, OCmPR - **GC (KCITD)** OCPR - **GC (ECD)**	-	Luke *et al.* (1975)
Agricultural products	OCPR (16) - **GC (EICD-X)** OCmPR (2) - **GC (EICD-N_2)** OPPR (4) - **GC (FPD)**	-	Luke *et al.* (1981) AOAC (1985)
Fruits and vegetables	19 thermally labile and non-volatile : **HPLC / TSP / MS / SIM**	0.025 - 1 μg	Liu *et al.* (1991)
Wine	OPPR + OCmPR (15) - **HPLC (UVD)**	0.006 to 0.02 μg	Cabras *et al.* (1992)
Vegetables, fruits and fatty foods (brown rice)	OCPR (7) - **GC (ECD)** OPPR (20) - **GC (FPD)** OCmPR (14) - **GC (NPD)**	0.001 μg 0.001 μg 0.01 μg	Nakamura *et al.* (1994)
Plant and animal tissues	OCPR (17) - **GC (ECD)** OPPR (43) - **GC (FPD)** OCmPR (11) - **HPLC (FMD)**	0.02 to 0.5 μg/g	Holstege *et al.* (1994)
Fruits and vegetables	OCPR (11), OPPR (21), OCmPR (3) - **GC (ITMS)**	0.0004 to 0.15 μg/g	Lehotay and Eller (1995)

Inference : OPPR - GC (FPD) - 0.001 μg DL; OCmPR - GC (NPD) - 0.01 μg DL;
OPPR + OCmPR - HPLC (UVD) - 0.006-0.02 μg DL; GCMS - 0.0004 - 0.15 μg/g DL; LCMS - 0.025 - 1 μg DL;
Hence, DL (Quantitative Methods) : 0.0004 - 1 μg

Wessel (1967) performed a collaborative study for analysis of multiple organochloro pesticide residues (OCPR) and organophosphorus pesticide residues in non-fatty and fatty foods. Gas chromatography (GC) with electron capture detector (GC-ECD) and GC-potassium chloride thermionic detector (KCITD) were used for the analysis of OCPR and OPPR, respectively. This method became AOAC (1984) multiresidue method for analysis of OCPR and OPPR in non-fatty and fatty foods.

Luke *et al.* (1975) developed a multiresidue method, for isolating OPPR and OCmPR together, which were then quantified using KCITD. OCPR were determined using GC(ECD) after clean-up of the extracts. Luke *et al.* (1981) simplified and shortened Luke *et al.* (1975) procedure by eliminating the clean-up step. They used Hall electrolytic conductivity detector (ElCD) which could be used in halogen and nitrogen modes for the determination of OC and OCm compounds, respectively. The OP compounds were determined by flame photometric detector (FPD) which provided a less complex chromatogram than that produced by KCITD. This became as AOAC (1985) method.

Liu *et al.* (1991) used high performance liquid chromatography / thermospray / mass spectrometry / selected ion monitoring (HPLC / TSP / MS / SIM) to determine nineteen thermally labile and non-volatile pesticides in fruits and vegetables and detection limits ranged from 0.025 to 1 μg. Cabras *et al.* (1992) isolated fifteen OPPR and OCmPR together from wine and separated satisfactory over HPLC with water-acetonitrile (50:50 v/v) as mobile phase and using UV-200 detector. The limit of detection ranged from 0.006 to 0.02 μg.

A method for multiresidue analysis of 48 pesticides (7 OC, 20 OP, 14 organo nitrogen and 7 pyrethroid) from samples of vegetables, fruits and fatty foods (brown rice) was developed by Nakamura *et al.* (1994). OPPR were determined directly by GC(FPD) with a limit of detection 0.001 μg. However, OCPR were quantified after florisil clean-up using GC(ECD), with

a detection limit of 0.001 μg. Organonitrogen pesticides were determined by GC(FTD) or GC-nitrogen phosphorus detector (NPD) following clean-up by silica gel and detection limits were 0.01 μg.

Holstege *et al.* (1994) developed a multiresidue method for 17 OC, 43 OP, and 11 OCm insecticides from plant and animal tissues. OCPR were analysed by GC (ECD) after florisil clean-up, while OPPR by GC (FPD) without clean-up and OCmPR by HPLC using fluorometric detector (FMD). Method detection limits ranged from 0.02 to 0.5 μg/g for 10 g sample. Lehotay and Eller (1995) developed a multiresidue method for analysis of 46 pesticides (11 OC, 21 OP, 3 OCm, 3 pyrethroid, 8 others) in fruits and vegetables by GC ion trap mass spectrometry (GC/ITMS). Limit of detection ranged from 0.0004 to 0.15 μg/g.

Thus, it could be inferred that detection limit (DL) of OPPR by GC (FPD) was 0.001 μg and DL of OCmPR by GC (NPD) was 0.01 μg. Detection limits of OPPR and OCmPR as analysed through HPLC, GCMS and LCMS were 0.006 to 0.02 μg, 0.0004 to 0.15 μg/g and 0.025 to 1.0 μg, respectively. Hence, detection limits of OPPR and OCmPR by quantitative methods ranged between 0.0004 to 1 μg.

2.2 SCREENING METHODS

2.2.1 SPECTROPHOTOMETRIC METHODS

Johnson (1963) made a collaborative study for the analysis of carbaryl (sevin) with apples and lettuce as test crops and this became AOAC (1964) method. This method was based on the alkaline hydrolysis of carbaryl and colourimetric determination of its hydrolysis product, 1-naphthol with p-nitrobenzene diazonium fluoborate as chromogenic coupling agent. It could detect 5 μg of carbaryl at λ max 475 nm.

Chopra and Chawla (1964) developed a rapid method for estimation of phosphatic pesticides from organic materials based on their total phosphorus content. Organically bound phosphorus was oxidized to orthophosphate, followed by its reduction with ascorbic acid and then

reaction with ammonium molybdate to yield a complex, phosphimolybdenum blue, having λ max at 660 nm. This method could detect total OPPR in microgram levels. Abbot *et al.* (1967) used a general screening test for the presence of OPPR in vegetable tissues. They also followed the above molybdenum blue procedures, but adopted a λ max of 820 nm and detected up to 10 μg of pesticides. Both methods suffered the interference of inorganic phosphorus.

Geitz and Watts (1964) reported a procedure based on the reaction of the organophosphorus pesticide chemicals with p-nitrobenzylpyridine in the presence of cyclohexylamine to yield chromophoric compounds having λ max 520 nm. Jain *et al.* (1974) introduced some modifications, viz., measurement at 540 nm rather than 520 nm, also doubling the quantity of p-nitrobenzyl pyridine for the reaction and thus improved the sensitivity by about 25 to 30 percent. This method detected 0.5 to 2.0 μg of OPPR from soils and various plants and plant materials.

Ramasamy (1974) described a quick and reliable method for nine carbamate residues from mud bricks and cheese clothes. The diazotizing agent, 3-nitroaniline-4-sulfonic acid, were used to obtain an orange-red colour and measured at nine different λ max corresponding to each carbamate residues. The Beer-Lambert law was obeyed by all carbamates at the range of 1 to 8 μg/ml. Analytical data included graphs and some results which indicated the wide applicability of the method.

A flow injection system for automatic determination of total P in beer was described by Fernandes *et al.* (2000). They used a 2-stage photooxidation / thermal digestion procedure, together with oxidation and hydrolysis to convert all forms of P compounds to orthophosphate. Polyphosphates were hydrolyzed by acid and heat, and organophosphates were digested by UV-catalyzed peroxo-disulphate oxidation. The orthophosphate formed was then spectrophotometrically determined by the phosphomolybdenum blue reaction, using stannous chloride as a reducing agent. Detection limit of 1 μg was achieved.

The important spectrophotometric methods and as screening for the detection of OPPR and OCmPR have been enlisted in Table 2.2. From the table, it could be inferred that spectrophotometric methods suffer from limitations, viz., interference of inorganic phosphorus and high detection limits.

2.2.2 THIN LAYER CHROMATOGRAPHY (TLC)

The TLC has been used as a viable alternative for modern quantitative methods since it is rapid, simple, sensitive and less expensive screening method. It has been used as a confirmatory method for assaying large number of samples for the presence of pesticide residues in a relatively short time. With the availability of precoated plates, which is having uniform thickness and particle size, the technique is adequately sensitive and especially suitable for the separation of pesticide compounds.

In general, the TLC methods used for analysing pesticide compounds / residues can be broadly classified into two, viz., TLC-enzyme inhibition method (TLC-EI) and TLC-Chemical method (TLC-Chem). But TLC-EI is preferred to TLC-Chem for multiresidue analysis, since TLC-EI is specific for OP and OCm compounds and hence can simultaneously screen both OP and OCm compounds. The principles involved in the TLC-EI technique can be illustrated as :

$$E + S \text{ ------- } ES \longrightarrow E + P$$

Where, E = Enzyme, S = Substrate, and P = Product.

The reaction is detected on TLC by using pH indicators or chromogenic substrates. When the enzyme is inhibited by OCm/OP compounds, the substrate will not be hydrolysed.

$$E + I \text{ ------- } EI + S \longrightarrow \text{No P}$$

Where, I = Inhibitor.

In the case of substrate such as β-naphthyl acetate pesticide locations on chromatograms appear as colourless spots on bright orange coloured backgrounds.

Table 2.2 Screening Methods : Spectrophotometric Methods

Sample	Residue	Chromogenic Agent	Coloured Complex	λ Max (nm)	Detection Limits	References
Apple and lettuce	OCmPR (Carbaryl)	P-nitrobenzene diazonium fluoborate	Purple	475	5 μg	Johnson (1963), AOAC (1964)
Vegetable tissues	Total P (inorganic + organic)	Ammonium molybdate	Molybdo-phosphate blue	820	0 to 10 μg	Abbot *et al.* (1967)
Plant and plant tissues	OPPR	P-nitrobenzyl pyridine	Coloured	540	0.5 to 2.0 μg	Jain *et al.* (1974)
Bricks, cheese cloths	9 OCmPR	3-nitroaniline-4-sulfonic acid	Orange red	Nine diff. λ max. (416-540)	1 to 8 μg	Ramasamy (1974)
Beer	Total P (inorganic + organic)	Ammonium molybdate	Molybdop hosphate blue	-	1 μg	Fernandes *et al.* (2000)

Limitations : • Interference of inorganic phosphorus; • High DL.

$$CH_3COOC_{10}H_7 + H_2O \xrightarrow{E} C_{10}H_7OH + CH_3COOH$$

β-naphthyl acetate → β-naphthol

$$C_{10}H_7OH + O_2N\text{-}C_6H_4\text{-}N^{+}\equiv N{:}B\bar{F}_4 \longrightarrow O_2N\text{-}C_6H_4\text{-}N{=}N\text{-}C_{10}H_6OH + BF_3 + HF$$

4-nitrobenzene diazonium tetrafluroborate → An azo compound (orange colour)

The TLC spots produced are very stable, hence the plates can be stored for future evaluation. In addition, this method is more sensitive than that requiring a pH indicator (e.g., bromophenol blue) (Mendoza, 1974).

2.2.2.1 TLC of OP Compounds

Table 2.3 enlists the TLC of few important OP compounds and their detection limits (DL). Braithwaite (1963) used benzene : 2-dichloroethane (DE) (1:1) for resolving 4 sulphur containing pesticides (methyl parathion, delnav, asuntol, diazinon) over lab coated silica gel G plates (pH 4.0). The compounds produced intense red spots with 0.5 percent 2,6-dibromo quinone-4-chlorimine (DBQC / Gibb's reagent) with a limit of detection 0.1-0.2 μg. Bunyan (1964) used silica gel H (250 μ) plates and benzene:acetone (9:1) for resolving 14 OP compounds. He tried bromophenol blue (BPB) reagent spray (containing 1% $AgNO_3$), followed by 5 percent acetic acid (AA) spray and UV exposure for detecting S-containing OP compounds with a detection limit 0.1 to 0.2 μg. He also used bromine exposure (30 sec) as well as bromophenol blue / bromocresol green (BPB/BCG) reagent for detecting 0.2 to 1μg of various phosphorothioates. Both the spraying agents were not specific for organocarbamates as well as non-thio-organophosphates.

Partition thin-layer chromatography technique was developed by El-Refai and Hopkins (1965) for separating 7 sulphur containing OP over cellulose layers coated with different concentrations of mineral oil in ethyl ether (EE) and development with immiscible mobile phase using different concentrations of acetonitrile in water. They made a comparison between

Table 2.3 Screening methods : TLC of OP compounds.

MM / Isolate	Plate and Solvent System	Chromogenic Agent / Coloured Spots	Compounds Resolved and DL in μg	Remarks	References
MM	P : Silica gel G SS : benzene : DE (1:1)	Gibb's reagent - DBQC - Red	Methyl parathion, Delnav Diazion, Asuntol 0.1 - 0.2	Less sensitive	Braithwaite (1963)
MM	P : Silica gel H (250μ) SS : benzene - acetone (9+1)	M-I : BPB, AA and UV - Blue / mauve M-II : Br_2, BPB / BCG - Yellow on blue	M-I M-II Malathion 0.1 1.0 Methyl parathion 0.1 1.0 Phosphamidon 0.2 -	M-I specific for S-OPPR M-I, M-II - NS for OCm	Bunyan (1964)
MM	P : Cellulose coated with mineral oil in EE SS : CH_3CN in water	TLC-Chem. $AgNO_3$ - BPB, Citric acid - Blue spots TLC-EI : Br_2, Serum (horse / human), Ac. choline Br, BPB - Blue on yellow	Chem. EI Gluthion 0.1 0.000001 Malathion 0.2 0.002 Methyl parathion - 0.0005	Sensitive to thiano, thiol and dithio compds. S-containing OC Interferes TLC-EI : more sensitive	El-Refai and Hopkins (1965)

Contd....

Contd....(Table 2.3)

MM / Isolate	Plate and Solvent System	Chromogenic Agent / Coloured Spots	Compounds Resolved and DL in μg	Remarks	References
MM	P : Silica gel SS : EA : n-hexane (1:3)	Br_2,$FeCl_3$,Benzoxazole and UV - Fluor. blue	Methyl parathion Dimethoate Malathion - 0.2-5.0	Phosphamidon - ND	Ragab (1967)
Cauli-flower, paddy straw and grain - OPPR	P : Silica gel G (450μ) SS : acetone - hexane (1+4)	Br_2, Rat liver homogenate, NA, PNBDF - White on orange	Parathion 0.0001	Comparable with GLC	Kumar *et al.* (1976)
Paddy grain - OPPR	P : Silica gel G (450μ) SS : acetone - hexane (1+4)	Br_2, Pig liver acetone powder, Liver homogenate (sheep / rat), NA, PNBDF - White on orange	Methyl parathion 0.0001	Comparable with GLC	Bhaskar and Kumar (1981)

MM = Model mixture; DL = Detection limit; P = Plate; SS = Solvent system; DE = 2-dichloroethane; DBQC = 2,6-dibromoquinone 4-chlorimine; BPB = Bromophenol blue; AA = Acetic acid; BCG = Bromocresol green; NS = Not specific; ND = Not detected; EE = Ethyl ether; Ac.choline.Br = Acetyl choline bromide; EA = Ethyl acetate; NA = β-naphthyl acetate; PNBDF = 4-nitrobenzene diazonium tetrafluroborate.

TLC-EI, involving bromine vapour exposure, human / horse serum as enzyme source, acetyl choline bromide (Ac.choline Br.) as substrate and bromophenol blue as pH indicator solution, and TLC-Chem involving silver nitrate-bromophenol blue reagent spray which contains 1.0 percent silver nitrate and 0.4 percent bromophenol blue, followed by 0.01 percent citric acid spray. TLC-Chem is sensitive towards thiono, thiol and dithio compounds and interference of sulphur containing OCPR may not be neglected. However, TLC-EI was more sensitive than TLC-Chem.

Ragab (1967) developed a simple, rapid, convenient and widely applicable method for the direct fluorescent detection of organothiophosphorus pesticides. The compounds were spotted on lab made silica gel plates, developed in ethyl acetate (EA) : n-hexane (1:3) and made visible by exposure to bromine vapour followed by spraying with ferric chloride and 2-(o-hydroxyphenyl) benzoxazole and viewed under UV. The sensitivity of this method was in the range of 0.2 to 5 μg, depending on the specific compound, but failed to detect phosphamidon.

Kumar *et al.* (1976) developed a simple, sensitive and rapid method for the quantitative estimation of nanogram amounts of parathion from cauliflower, paddy straw and grain as paraoxon on silica gel G plates (450 μ), developed in acetone : hexane (20:80). Paraoxon was detected by inhibition using rat liver homogenate as the enzyme source, 2-naphthyl acetate (NA) as substrate and 4-nitrobenzene diazonium tetra fluroborate (PNBDF) as the chromogenic agent. The detection limit was 0.0001 μg and could be used as a viable alternative to gas chromatographic analysis. Bhaskar and Kumar (1981) used the method of Kumar *et al.* (1976) and detected methyl parathion from paddy grain as methyl paraoxon by pig liver acetone powder / sheep or rat liver homogenate as the enzyme source, and 1-naphthyl acetate as substrate. Commercial pig liver acetone powder was advantageous than raw liver sources because it is readily available and can be preserved indefinitely. Method detection limit was 0.0001 μg.

From the review, it can be concluded that specificity of TLC-EI is for cholinesterase inhibitors (OP compounds) while that of TLC-Chem is towards

sulphur containing OP compounds only, and there may be interference of sulphur containing OC compounds. Further, the sensitivity of TLC-El is higher than TLC-Chem.

2.2.2.2 TLC of OCm Compounds

Table 2.4 delineates the TLC of few important OCm compounds. Finocchiaro and Benson (1967) separated eight N-methyl carbamate pesticides on alumina-G plates (250 μ) with solvent system ethyl ether : toluene (1:3) and were made visible with 1 N alcoholic KOH spray, followed by 4-nitrobenzene diazonium tetrafluroborate spray (Chiba and Morley, 1964). Detection limit was 0.1 μg.

Mendoza and Shields (1970) separated 12 carbamate pesticides on silica gel G-HR (450 μ) with acetone : cyclohexane (20:80) and detected by bromine exposure followed by spray of pig / beef liver extract and 5-bromo indoxyl acetate (BIA) reagent. A comparison of pig and beef liver extracts for detection of 12 carbamates residues (DL : 0.1 - 0.00005 μg) including carbaryl at 0.0001 μg level was discussed. In general, pig liver extracts and frozen extracts were more sensitive than the steer liver extracts and freeze dried extracts, respectively. Bromine / UV exposure was found to destroy the inhibitory property of the pesticides. Padalikar *et al.* (1988) used a specific spray reagent for the detection of carbaryl, viz., 1 percent $CuCl_2$, followed by 0.1 percent ammonium metavandate (Amm. MV) and 0.5 percent potassium hexacyanoferrate in 0.5 percent NaOH. The pesticide was spotted over silica gel G plates (250 μm) and developed using hexane : acetone (4:1) and quantified carbaryl from water. The DL of the method was 1 μg. This reagent is not specific for other OP components such as malathion, parathion, dimethoate, etc.

Rathore and Sexena (1990) analysed river as well as deep well water for 5 organocarbamates with TLC, developed using benzene and detected using 0.1 percent dibromo quinone chlorimine in benzene. The limit of detection was 0.1 μg.

Table 2.4 Screening Methods : TLC of OCm Compounds.

MM / Isolate	Plate & Solvent Systems	Chromogenic Agent / Coloured Spots	Compounds Resolved and DL in μg	Remarks	References
MM	P : Al_2O_3 G (250 μ) SS : ether : toluene (1:3)	Alco. KOH, PNBDF - Diff. colours on yellow	Eight N-methyl carbamates including carbaryl - 0.1	OP - ND	Finacchiaro and Benson (1967)
MM	P : Silica gel G-HR (450 μ) SS : acetone : cyclohexane (20:80)	Br_2, Liver extract (pig / beef), BIA	*12 OCm(0.1-0.00005)* Carbaryl - 0.001	-	Mendoza and Shields (1970)
MM and water - OCmPR	P : Silica gel G (250μ) SS : hexane : acetone (1+4)	$CuCl_2$, Amm. MV, K-hexacyano ferrate - Violet	Carbaryl - 1	Malathion Parathion dimethoate OCPR - ND	Padalikar *et al.* (1988)
River and Deep well water - OCmPR	P : Silica gel G SS : benzene	DBQC in benzene	Carbaryl - 0.1	-	Rathore and Sexena (1990)

BIA = 5-bromoindoxyl acetate; Amm.MV = Ammonium metavandate.

From Table 2.4, it can be concluded that TLC-Chem for OCm compounds is not specific for OP compounds and DL for carbaryl is in the order of 0.1 to 1 μg. Also, the sensitivity of TLC-EI for the detection of OCm compounds is higher than that of TLC-Chem.

2.2.2.3 TLC of OC, OP and OCm Compounds / Multiresidues

Table 2.5 describes the TLC of model mixture of few important OC, OP and OCm compounds / multiresidues and their detection limits. Walker and Beroza (1963) developed a single, simple procedure for the analysis of 62 pesticides over silica gel G plates (275 μ) and developed using benzene : acetone (9:1). Spots were made visible by bromine vapour exposure, fluorescein solution spray and by a final spray of Mitchell's reagent (added solution of 1.7 g silver nitrate in 5 ml water to 10 ml 2-phenoxyethanol and diluted to 200 ml with acetone) and obtained a detection limit of 1 to 10 μg. In general, the sensitivity of the method was very less and was not specific towards phosphamidon, diazinon and OC compounds.

Wessel (1967) performed a collaborative study which became AOAC (1984) method for detecting 7 OPPR from leafy vegetables and fruits using gas chromatographic method and were confirmed by TLC. Partition thin layer chromatography was performed over lab made alumina G plates (250 μ) with immobile phase 15 / 20 percent N, N´ dimethyl formamide (DMF) in ether and methyl cyclohexane as mobile phase. Ronnel, ethion, trithion were not resolved by this method. The sulphur containing OPPR (methyl parathion, parathion, diazinon, malathion, ronnel, ethion, trithion) present in 6, 15 and 50 percent eluate were detected using 0.2 percent tetrabromophenophthalein ethyl ester spray, followed by 0.5 percent silver nitrate spray and by a final spray with 5 percent citric acid. However, there is no mention of detection limit of these sulphur containing compounds. Non-thiophosphates were not detected by this method. OCPR present in 6 and 15 percent eluate were detected by Mitchell's reagent as violet black spots.

Table 2.5 Screening Methods : TLC of OC, OP and OCm compounds / Multiresidues.

MM / Isolate	Plate and Solvent Systems	Chromogenic Agent / Coloured Spots	Compounds Resolved and DL in μg	Remarks	Reference s
MM - OC, OP, OCm	P : Silica gel G (275μ) SS : benzene : acetone (9+1)	Br_2 + fluorescein + Mitchell's reagent - Greenish yellow spots on pink	Dimethoate - 1 Malathion - 5 Methyl parathion - 2 Carbaryl - 10	Sensitivity less. Diazinon, phosphamidon, OC - ND	Walker and Beroza (1963)
100 g non-fatty and fatty foods - 6% eluate (OCPR + few OPPR) - 15% eluate (few OCPR + OPPR) - 50% eluate (OPPR - malathion)	P : Al_2O_3-G (250 μ) OCPR : SS : n-heptane for 6% SS : n-heptane : acetone(98:2) for 15% OPPR : Immo : 15/20% DMF in ether mo : methyl cyclohexane	OCPR : Mitchell's reagent - violet black spots OPPR : TBP, $AgNO_3$, Citric acid - blue / purple on yellow	Methyl parathion Malathion Parathion Diazinon	Unresolved : Ronnel Ethion Trithion	Wessel (1967) AOAC (1984)
MM - OP and OCm	P : Silica gel G-HR (450 μ) SS : 15% acetone in hexane	Br_2, Steer liver homogenate, IA / Sub. IA - White on indigo	Malathion - 0.005 Methyl parathion - 0.005 Carbaryl - 0.005	Sensitivity much less than that of NA and PNBDF	Mendoza *et al.* (1968a)
Carrots and peas - 50-100g OPPR,OCmPR	P : Silica gel G (450-500 μ) SS : 20% acetone in hexane	Br_2, pig / beef liver esterase, BIA - White on blue	Carbaryl - 0.0005 Malathion - 0.0025	DL comparable with GLC	Mendoza and Shields (1971)

Contd....

Contd....(Table 2.5)

MM / Isolate	Plate and Solvent Systems	Chromogenic Agent / Coloured Spots	Compounds Resolved and DL in μg			Remarks	Reference s
MM - OP and OCm	P : Alumina - G (250 μ)	M-I : TBP, $AgNO_3$ - Blue or purple on yellow M-II : i) Br_2 vapour ii) Enzymatic detection	Dimethoate Methyl parathion Malathion Phorate	EI 0.2 0.014 0.8 0.6	Chem 1.3 0.5 0.5 0.1	TLC-EI is more sensitive	Leoni and Puccetti (1971)
Fatty foods - OCPR, OPPR	P : Precoated silica gel - G (250 μ) SS : PE : MC : EE (8:2:2)	Br_2, Lyo. human serum, NA, Fast blue - White on red	6 OPPR 0.005			Sensitivity less compared to PNBDF	Stijve and Cardinale (1974)
Fruits & Vegetables OPPR, OCmPR	P : HPTLC silica gel 60 SS : hexane / acetone (8:2); MC:EA (7:3); methanol : water (7:3)	Cholinesterase inhibition	Methyl carbamates and OPPR 1 μg/kg			Densitometry	Gardyan and Thier (1991)

Immo = Immobile; mo = Mobile; DMF = N,N'dimethyl formamide; TBP = Tetrabromophenophthalein ethyl ester; IA/Sub.IA = Indoxyl acetate / substituted indoxyl acetate; PE = Light petroleum; MC = Methylene chloride; Lyo = Lyophilised; HPTLC = High performance TLC.

Mendoza *et al.* (1968a) described a reproducible thin layer chromatography-enzyme inhibition method for detecting 0.005 μg amounts of ten OP pesticides and carbaryl with 450 μ thick silica gel G layer, steer liver homogenate as source of esterase and indoxyl or substituted indoxyl acetate (IA / sub. IA) as chromogenic substrate. The esterase and substrate spray solutions being used at a pH of about 8. However, sensitivity of this chromogenic agent is much less than that of 2-naphthyl acetate and 4-nitrobenzene diazonium tetra fluroborate (Kumar *et al.*, 1976), Mendoza and Shields (1971) used the above method and detected 5 OPPR and 5 OCmPR from 50 to 100 g of carrots and peas using pig / beef liver homogenate as source of esterase and 5-bromo indoxyl acetate (BIA) as chromogenic substrate. Detection limit of carbaryl was 0.0005 μg and that of malathion was 0.0025 μg which were comparable to that of GLC.

Leoni and Puccetti (1971) compared TLC-EI and TLC-Chem, viz., tetra bromophenophthalein ethyl ester, silver nitrate and citric acid spray, over alumina-G plates (250 μ). TLC-EI was found to be more sensitive than TLC-Chem. Stijve and Cardinale (1974) carried out a confirmatory analysis of GC for 6 OPPR (fenchlorophos, iodofenphos, bromophos, ethion, dursban, phenkapton) from fatty foods by thin layer chromatogrphy using precoated silica gel 60 (250 μg) and developed using light petroleum : methylene chloride : ethyl ether (DE:MC:EE) (8:2:2) pesticides were made visible using lyophilised human serum as source of esterase, 2-naphthyl acetate as substrate and fast blue as chromogenic coupling agent. The detection limit of the method is 0.005 μg. The sensitivity of fast blue reagent is much less compared to 4-nitrobenzene diazonium tetra fluroborate (Kumar *et al.*, 1976).

Gardyan and Thier (1991) used high performance TLC (HPTLC) with densitometric evaluation for detecting OPPR and methyl carbamate residues over silica gel 60 HPTLC layer. This was developed using hexane : acetone (8:2); methylenechloride : ethyl acetate (MC:EA) (7:3); and methanol : water (7:3) and detected using EI technique with a limit of detection 1.0 μg/kg.

Table 2.6 Advantages of TLC-EI over TLC-Chem.

Characteristics	TLC-EI	TLC-Chem.
Sensitivity	High	Low
Detection limit	Low	High
Interference	Nil	Some S-containing OC compounds may interfere
Specificity	OP and OCm components	S-containing OP and OCm compounds
Screening for MRL	Highly suitable	Suitable

From this review, it can be concluded that TLC-EI is specific towards cholinesterase inhibitors, i.e., OP and OCm compounds, while TLC-Chem is specific towards sulphur containing pesticides. TLC-Chem suffered the interference of sulphur containing OC compounds. Detection limit of TLC-EI is comparable with GLC. Sensitivity of TLC-EI is higher than TLC-Chem. Thus, TLC-EI is more suitable for screening of food items for their MRL (Table 2.6).

The review of literature to-date has revealed that available TLC methods are either limited to very few OP / OCm or to the set of OP and OCm other than those in current use in India. Hence, the project has been undertaken with a view to standardise a method for separating the OP and OCm compounds that are currently in use in India and to determine their detection limits so that it can be extended for screening of food samples for their MRL (0.002 - 5 μg/g) as recommended by Codex Alimentarius / PFA..

Materials and Methods

3. MATERIALS AND METHODS

3.1 SOLVENTS

Acetone extra pure	:	55.5 - 56.5°C (LR, SD fine)
Hexane	:	65 - 70°C (LR, BDH)
Diethyl ether	:	34 - 36°C (LR, SD fine)
Ethyl acetate	:	76.5 - 77.5°C (AR, BDH)
Benzene	:	79 - 81°C (LR, SD fine)
Methanol	:	64.5 - 65.5°C (AR, BDH)
Chloroform	:	60 - 62°C (AR, Qualigens)
Ethyl alcohol	:	99.9% (AR, P.R. China)
Acetic acid glacial	:	(AR, BDH)

3.2 REAGENTS

i) Bromine (AR, SD fine)

ii) Isotonic phosphate buffer (pH 7.4, 0.01 M)

a) Stock solution : Prepared a stock solution containing 90 g NaCl, 13.65 g Na_2HPO_4 and 2.43 g $NaH_2PO_4.H_2O$ made up to 1 litre with distilled water.

b) Working buffer solution : Diluted solution 'a' to 9:100 to obtain required buffer.

iii) 0.5 Percent β-naphthyl acetate reagent : Dissolved 20 mg β-naphthyl acetate (Sigma) in 4 ml acetone and stored the solution in the refrigerator.

iv) 4-Nitrobenzene diazonium tetrafluroborate reagent :

a) For TLC-EI : Prepared 0.4 percent 4-Nitrobenzene diazonium tetrafluroborate (Merck) in acetone and stored the solution in the refrigerator.

b) For TLC-Chem : Prepared 0.01 percent 4-Nitrobenzene diazonium tetrafluroborate (Merck) in diethyl ether : methanol (1:1).

v) 1.5 N methanolic NaOH : Prepared 1.5 N methanolic NaOH by dissolving 6 g of NaOH (AR, BDH) in 100 ml methanol.

vi) 3′, 3″, 5′, 5″ tetrabromophenophthalein ethyl ester reagent :

a) Stock solution : Dissolved 1 g tetrabromophenophthalein ethyl ester (Sigma) in 100 ml acetone.

b) Working dye solution : Diluted 10 ml stock dye solution to 50 ml with acetone.

vii) 0.5 Percent silver nitrate solution : Dissolved 0.5 g $AgNO_3$ (AR, SD fine) in 25 ml H_2O and diluted to 100 ml with acetone. Kept under dark.

3.3 PRECOATED TLC PLATES

TLC aluminium sheets (20x20 cm) Merck.

a) Aluminium oxide 60 F_{254} neutral (200 μ) Type E

b) Silica gel 60 (200 μ)

c) Silica gel 60 F_{254} (200 μ)

3.4 REFERENCE STANDARDS

Methyl parathion	99 % (Supelco, Pesticides Kit 52)
Dimethoate	99 % (Supelco, Pesticides Kit 52)
Malathion	98 % (Supelco, Pesticides Kit 52)
Phorate	99 % (Supelco, Pesticides Kit 52)
Monocrotophos	99 % (Supelco)
Phosphamidon	98 % (Supelco)
Quinalphos	99 % (Supelco)
Chlorpyriphos	99 % (Supelco)
Carbaryl	100 % (Atul Limited)

3.5 EQUIPMENTS

i) Anamed electronic balance

ii) Mettler AT-200 electronic balance

iii) Remi glass homogeniser

iv) Remi centrifuge

v) TLC chamber and spray bottles

vi) Spotting syringe - 10 μl syringe (Hamilton)

3.6 PREPARATION OF LIVER HOMOGENATE

Fresh liver lobes (goat) were collected from the slaughter house of local market and stored in isotonic sodium phosphate buffer (pH 7.4, 0.01 M) at -20°C immediately. Chopped the liver well before preparing a 10 percent homogenate in ice cold pH 7.4 isotonic sodium phosphate buffer (0.01 M) in a glass homogenizer at 0°C. The homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was decanted and the precipitate was discarded. The supernatant can be stored at 4°C for about 1 week without loosing its activity.

3.7 STANDARDIZATION OF A TLC METHOD FOR THE SEPARATION OF MODEL MIXTURE OF OP AND OCm COMPOUNDS

The TLC aluminium sheet (20x20 cm) of aluminium oxide 60 F_{254} neutral Type E (200 μ) was loaded with 10 μl of each pesticide standard solutions (1 $\mu g/\mu l$) as well as their mixtures. It was developed for about 45 min in a well saturated TLC chamber (which has been lined with Whatman No.1 filter paper) with the solvent system acetone : hexane (30:70) at 25°C.

3.8 DETECTION OF MODEL MIXTURE OF OP AND OCm COMPOUNDS BY TLC-EI

The model mixture of organophosphate and organocarbamate pesticide compounds were detected essentially by Kumar *et al.* (1976) procedure with some changes as described below:

The plate developed in Section 3.7 was dried at room temperature and exposed for 30 sec to evenly distributed bromine vapour for oxidation of pesticide compounds by keeping plate in a well closed TLC chamber containing about 0.5 ml liquid bromine. The plate was removed and allowed the bromine to evaporate completely in air for about 1 hr. The plate was then sprayed heavily and uniformly with goat liver homogenate solution as prepared under Section 3.6, thoroughly wetting the plate. About 6 to 8 ml liver homogenate solution was required for 20x20 aluminium oxide plate of 200 μ thickness. Kept the plate at room temperature (30°C) for 5 min, followed by spraying heavily with 0.5 percent β-naphthyl acetate substrate solution in acetone. Allowed the plate to stay undisturbed for 1 min at room temperature. Then sprayed the plate lightly and uniformly with 0.4 percent 4-nitrobenzene diazonium tetrafluroborate in acetone. Orange colour was developed immediately and the pesticides inhibition of enzyme appeared as clear white spots on orange background.

For determining the limit of detection 10, 7.5, 5, 2.5, 1.0, 0.75, 0.5, 0.25, 0.1 μl of each pesticide standard solutions (1 μg/μl) and 0.75, 0.50 μl of each pesticide standard solutions (0.1 μg/μl) were spotted as mixtures, developed as described under Section 3.7 and detected by the TLC procedures described above.

3.9 DETECTION OF S-CONTAINING OP COMPOUNDS (MALATHION, METHYL PARATHION, PHORATE, CHLORPYRIPHOS, DIMETHOATE AND QUINALPHOS) BY TLC-CHEM

The plate was developed as per Section 3.7 and sulphur containing organophosphate pesticides were detected by AOAC (1984) method. The method in brief follows as :

The plate was dried at room temperature and immediately sprayed moderately heavily and uniformly with working solution of tetrabromophenophthalein ethyl ester. Plate was vivid blue after spraying.

Then oversprayed the plate lightly and uniformly with 0.5 percent $AgNO_3$ solution. At this point, plate was bluish purple and spots were discernible.

After 2 min, oversprayed plate with citric acid solution. After spraying, S-containing pesticides immediately appeared as vivid blue or purple spots against yellow background. Colour of spots reached maximum intensity within 5-10 min after citric acid spraying.

For determining the limit of detection of S-containing pesticides, 10, 5, 1, 0.75, 0.5, 0.25, 0.1 μl of each pesticide standard solutions (1 μg/μl) and 0.75, 0.50, 0.25, 0.1 μl of each pesticide standard solutions (0.1 μg/μl) were spotted as mixtures, developed as described under Section 3.7 and detected S-containing organophosphate compounds by TLC-Chem procedure as described above..

3.10 DETECTION OF CARBARYL BY TLC-CHEM

The plate was developed by TLC procedure described under Section 3.7. It was air dired and carbaryl was detected by Chiba and Morley (1964) method. This method in brief follows as :

The plate after development was sprayed with methanolic NaOH (1.5 N). While the plate was still wet, this was further sprayed by 4-nitro benzene diazonium tetrafluroborate reagent. Carbaryl gave a brilliant blue spot which gradually changed to purple colour after 10 min.

For determining the limit of detection of carbaryl 10, 5, 1, 0.75, 0.5, 0.25, 0.1 μl of pesticide standard solutions (1 μg/μl) and 0.75, 0.50, 0.25, 0.1 μl of pesticide standard solutions (0.1 μg/μl) were spotted as mixtures, developed as described under Section 3.7 and detected carbaryl by TLC-Chem procedure as described above.

Results and Discussion

4. RESULTS AND DISCUSSION

4.1 STANDARDIZATION OF A TLC METHOD FOR THE SEPARATION OF MODEL MIXTURE OF OP AND OCm COMPOUNDS

The major insecticides used in agriculture, currently in use in India, include 8 OP pesticides (monocrotophos, phosphamidon, phorate, malathion, methyl parathion, quinalphos, dimethoate, chlorpyriphos), 1 OCm (carbaryl) and 1 OC (endosulfan) (Agnihotri, 2000). This was confirmed by local market survey also. Accordingly, an attempt has been made to standardize a method for the separation of above mentioned OP and OCm compounds.

For the resolution of OP and OCm pesticides, various solvent system combinations, viz., acetone : hexane (10:90, 20:80, 30:70, 40:60); acetone : benzene (30:70); ethyl acetate : hexane (30:70); acetic acid : chloroform (10:90) were tried. Table 4.1 showed the Rf of OP and OCm compounds in various ratios of acetone and hexane solvent systems during winter (room temp. 15-20°C). With acetone : hexane (10:90), methyl parathion - malathion - quinalphos (Rf 0.32, 0.33 and 0.35, respectively) and similarly phorate - chlorpyriphos (Rf 0.63 and 0.64, respectively) appeared as mixed spots. In the solvent system acetone : hexane (20:80), the same problem of mixing together of pesticides, viz., methyl parathion - malathion - quinalphos (Rf 0.36, 0.38 and 0.40, respectively) and phorate - chlorpyriphos (Rf 0.68 and 0.69, respectively) was observed. However, methyl parathion (Rf 0.56), malathion (Rf 0.59) and quinalphos (Rf 0.62) were well separated, when polarity of the solvent system was increased as acetone : hexane (30:70). On further increasing, the polarity as acetone : hexane (40:60), Rf of almost all pesticide compounds were increased with simultaneous overlapping of methyl parathion - malathion (Rf 0.68 and 0.70, respectively) and quinalphos - phorate - chlorpyriphos (Rf 0.76, 0.76 and

Table 4.1 Rf of OP and OCm compounds* in acetone - hexane (A:H) solvent systems during winter (room temperature 15-20°C).

Pesticide Compounds	A:H (10:90)	A:H (20:80)	A:H (30:70)	A:H (40:60)
Monocrotophos	0.03	0.05	0.09	0.11
Phosphamidon	0.14	0.17	0.19	0.41
Dimethoate	0.20	0.22	0.24	0.36
Carbaryl	0.27	0.31	0.39	0.59
Methyl parathion	0.32	0.36	0.56	0.68
Malathion	0.33	0.38	0.59	0.70
Quinalphos	0.35	0.40	0.62	0.80
Phorate	0.63	0.68	0.75	0.81
Chlorpyriphos	0.64	0.69	0.77	0.82

* Average of three trials.

0.77, respectively). Thus, the best possible separation was obtained with the solvent system acetone : hexane (30:70).

It was observed during summer season at RT 35°C, the solvent system acetone : hexane (30:70) standardized above did not work smoothly and run time increased very much. This might be due to the improper saturation of TLC chamber at high temperature (35°C). Accordingly, attempts were made to run the solvent system acetone : hexane (30:70) at relatively low temperature (25°C) maintained through air-conditioner in a small closed cabin. Table 4.2 showed the Rf of OP and OCm compounds in solvent system acetone : hexane (30:70) along with two other solvent systems, such as, ethyl acetate : hexane (30:70) and acetone : benzene (30:70) at 25°C. In general, Rf of all pesticide compounds in acetone : hexane (30:70) increased at 25°C (Fig. 4.1), viz., maximum Rf 0.92 at 25°C compared to 0.77 at 15 to 20°C.

With acetone : hexane (10:90), Walker and Beroza (1963) tried to resolve 47 pesticides (OC, OP and OCm), but most of the compounds were mixing together or near to origin. Walker and Beroza (1963) resolved 43 pesticides (OC, OP and OCm) using acetone : hexane (20:80), while Mendoza and Shields (1971) resolved 10 OP and OCm compounds in this solvent system. The resolution pattern in this solvent system was comparatively poor compared to that of acetone : hexane (30:70) in the present study. However, there is no comparative report in the literature for the separation of set of OP and OCm compounds (used in this study) in the solvent system acetone : hexane (30:70).

The eluting solvent, acetone was replaced by less polar ethyl acetate, while still retaining hexane, as the background solvent. Here, mixing up of methyl parathion - malathion - quinalphos (Rf 0.63, 0.66 and 0.62, respectively) and similarly phorate - chlorpyriphos (Rf 0.77 and 0.78, respectively) were resulted. Simultaneous decrease in Rf value of all pesticide compounds were noted (maximum Rf obtained was 0.78). On replacing the background solvent hexane with benzene, while retaining acetone as the eluting solvent, the Rf of pesticide pairs, viz., malathion -

Table 4.2 Rf of OP and OCm compounds* in various solvent systems at 25°C.

Pesticide Compounds	Acetone : Hexane (30:70)	Ethyl Acetate : Hexane (30:70)	Acetone : Benzene (30:70)
Monocrotophos	0.11	0.02	0.35
Phosphamidon	0.29	0.07	0.42
Dimethoate	0.32	0.11	0.37
Carbaryl	0.54	0.43	0.64
Methyl parathion	0.69	0.63	0.77
Malathion	0.72	0.66	0.77
Quinalphos	0.84	0.62	0.79
Phorate	0.90	0.77	0.80
Chlorpyriphos	0.92	0.78	0.80

* Average of three trials.

Fig. 4.1 Resolution pattern of OP and OCm compounds in the solvent system acetone : hexane (30:70) at 25°C as detected by TLC-EI.

1. Quinalphos
2. Carbaryl
3. Dimethoate
4. Chlorpyriphos
5. Phorate
6. Malathion
7. Methyl parathion
8. Phosphamidon
9. Monocrotophos

methyl parathion (Rf 0.77 and 0.77, respectively), and similarly for chlorpyriphos - phorate (Rf 0.80 and 0.80, respectively) were same. Thus, the solvent systems ethyl acetate : hexane (30:70) and acetone : benzene (30:70) did not help in giving better separation of OP and OCm compounds (especially of phorate and chlorpyriphos) when compared with that achieved through the solvent system acetone : hexane (30:70).

The solvent system chloroform : acetic acid (90:10) was also tried with a view to achieve better separation of pesticides, but it did not work. Developing time of the system was nearly trippled when chloroform : acetic acid (90:10) solvent system was tried. Here spots of only two pesticide compounds, i.e., monocrotophos (Rf 0.93) and malathion (Rf 0.74) were obtained. All other pesticide compounds were moved along with the solvent front. Similar observations were made by Walker and Beroza (1963).

Eventhough acetone : hexane (30:70) was selected as the best solvent for resolving the pesticide compounds, at higher concentrations (10 μg) phorate and chlorpyriphos (0.90 and 0.92, respectively) were just overlapping. Attempts were made to change the polarity of the solvent system by adding acetic acid at 2.5 percent and at 5 percent levels. This also failed to separate phorate and chlorpyriphos. However, it had been noted that, at lower concentrations (0.1 μg), these compounds were reasonably separated.

Also, attempts were made for achieving better resolution of phorate and chlorpyriphos by silica gel 60 and silica gel 60 F_{254} plates. Both silica gel 60 and silica gel 60 F_{254} yielded same Rf for the compounds studied. In general, the Rf of all compounds showed a decreasing trend. Further, a well scattered distribution was not obtained over silica gel 60 / silica gel 60 F_{254}.

4.2 DETECTION OF MODEL MIXTURE OF OP AND OCm COMPOUNDS BY TLC-EI

The pesticide compounds resolved were detected essentially by Kumar *et al.* (1976) procedure. In the present study, however, we have to make some changes as illustrated below :

4.2.1 OPTIMUM STABILITY OF THE ENZYME

During the initial course of study, stability of enzyme (acetyl cholineesterase) was not satisfactory. Enzyme showed poor stability in sodium phosphate buffer (0.01 M, pH 7.0) as suggested by Kumar *et al.* (1976) as well as in distilled water as reported by Bhaskar and Kumar (1981)

Attempts were made to obtain optimum stability for the enzyme present in goat liver homogenate. For this isotonic solution of sodium phosphate buffer (0.01 M, pH 7.4) as reported by Dacie (1954) was tried, which is usually used as a medium for preparing the enzyme solutions for testing its activity in analytical biochemistry purposes (Cohen *et al.*, 1970). Reproducible results were obtained by using above isotonic solution. Isotonic solution of rat liver homogenate was also tried as suggested by Kumar *et al.* (1976), but no added advantage was obtained in terms of sensitivity.

Other causes for failure in the development of the background colours and its remedial measures were :

i) **Insufficient amount of the enzyme on the plate :** This can be rectified by re-spraying with the homogenate solution. About 6-8 ml liver homogenate solution was required for 20x20 Alumina plate of 200 μ thickness.

ii) **Insufficient amount of the substrate on the plate :** This can be rectified by re-spraying the same substrate. About 5-6 ml of substrate solution was required for 20x20 Alumina plate of 200 μ thickness.

iii) **Inadequate evaporation of excess bromine vapour :** This cannot be corrected. Hence, sufficient time (usually 1 hr) was given for the complete evaporation of bromine from the plate, so that the plate was completely free off the smell. The presence of residual bromine vapours could inhibit the enzyme itself and hence, there will be no orange background colour on the plate.

4.2.2 INCUBATION PERIOD

After development, plates were dried and was then sprayed with the isotonic liver homogenate solution. It was incubated at RT (30-35°C) for 5 min instead of moist conditions at 37°C for 20 min as in the original procedure. Both these conditions yielded same background colour. β-naphthyl acetate spray was followed immediately after the incubations. The plates were again incubated at RT (30-35°C) for 1 min instead of moist conditions at 37°C for 2 min in the original procedure. Since the turn over rate of acetyl cholinesterase for the substrate is very high, the later step can be reduced to 1 min. The sensitivity of the method under both the conditions were same. It was then followed by the chromogenic agent spray and the background colour developed immediately.

4.3 DETERMINATION OF DETECTION LIMITS OF OP AND OCm COMPOUNDS BY TLC-EI

Table 4.3 showed the detection limits of 8 OP and 1 OCm compounds (as separated under Section 4.1) enzymatically detected on a 200 μ thick Alumina 60 F_{254} (neutral type) plate, with 10 percent goat liver homogenate in isotonic phosphate buffer (0.01 M, pH 7.4). After a short incubation period, the substrate solution, viz., 0.5 percent β-naphthyl acetate was sprayed, followed by spraying of 0.4 percent 4-nitrobenzene diazonium tetrafluroborate reagent. Orange colour developed immediately and the pesticides inhibition of enzyme appeared as clear white spots on orange background.

The detection limit for most of the pesticides, viz., quinalphos, carbaryl, methyl parathion, malathion, phorate and chlorpyriphos was found to be 0.1 μg. However, for dimethoate, detection limit was 2.5 μg and were exceptionally high (7.5 μg) for monocrotophos and phosphamidon.

El-Refai and Hopkins (1965) used human / horse serum as the enzyme source and obtained a detection limit of 0.002 μg for malathion and 0.0005 μg for methyl parathion. The detection limit for malathion, methyl

Table 4.3 Detection limits of OP and OCm compounds* using TLC-EI.

Pesticide Compounds	Amount Spotted (μg)										
	10	7.5	5.0	2.5	1	0.75	0.5	0.25	0.10	0.075	0.050
Monocrotophos	+	+	-	-	-	-	-	-	-	-	-
Phosphamidon	+	+	-	-	-	-	-	-	-	-	-
Dimethoate	+	+	+	+	-	-	-	-	-	-	-
Carbaryl	+	+	+	+	+	+	+	+	+	-	-
Methyl parathion	+	+	+	+	+	+	+	+	+	-	-
Malathion	+	+	+	+	+	+	+	+	+	-	-
Quinalphos	+	+	+	+	+	+	+	+	+	-	-
Phorate	+	+	+	+	+	+	+	+	+	-	-
Chlorpyriphos	+	+	+	+	+	+	+	+	+	-	-

* Verified three times

parathion and carbaryl was 0.005 μg when Mendoza *et al.* (1968a) used indoxyl acetate or substituted indoxyl acetate as the substrate. Later, Leoni and Puccetti (1971) obtained detection limit of 0.014 and 0.8 μg for methyl parathion and malathion, respectively. Mendoza and Shields (1971) obtained a much lesser detection limit for carbaryl (0.0005 μg) as compared to 0.005 μg (Mendoza *et al.*, 1968a) and 0.0001 μg (Mendoza and Shields, 1970) under identical conditions. Bhaskar and Kumar (1981), used TLC-EI technique of Kumar *et al.* (1976) and obtained high sensitivity of 0.0001 μg for methyl parathion using sheep liver homogenate as enzyme source, β-naphthyl acetate as substrate and 4-nitrobenzene diazonium tetrafluroborate as chromogenic agent; which gave very stable and sensitive inhibition zones as compared to other chromogenic agents cited. In PL-480 report (1983), OPPR were determined using Kumar *et al.* (1976) procedures and obtained a detection limit of 0.3 μg for carbaryl, 0.2 μg for quinalphos as compared with 0.1 μg obtained in the present study. No literature was available about the detection limits of phosphamidon and monocrotophos.

The thickness of alumina plates (200 μ) might be the reason for obtaining a higher detection limit in the present study as compared to those reported in the literature. For labcoated plate, an average thickness of 450 μ was recommended by Mendoza *et al.* (1968a,b), Mendoza and Shields (1971), Kumar *et al.* (1976), Bhaskar and Kumar (1981). For the present project, plates of thickness 200 μ were procured since, 450 μ thick precoated plates were not available commercially.

A few attempts with isotonic solution of rat liver homogenate as reported by Kumar *et al.* (1976) were also done. But these trials did not improve the sensitivity of the method.

4.4 SUGGESTED PROPOSITION FOR SCREENING OF FOOD PRODUCTS FOR THEIR MRL

From the above obtained detection limits, a proposition can be suggested for screening food samples including dairy products for their maximum residue limit (MRL). MRL is the maximum concentration for a

pesticide residue recommended by Codex Alimentarius Commission to be legally permitted or recognised as acceptable in or on a food, agricultural commodity or animal feed. The concentration is expressed in mg of pesticide residue/kg of the commodity or μg of pesticide residue/g of the commodity (IDF, 9101, 1990). In general, if detection limits of a pesticide is lower than its MRL, volume/g equivalent of the sample can give information for screening purpose. However, while dealing with variety of pesticides with different detection limits and different MRL in various food commodities, it becomes necessary to derive a general formula, which can be applied for screening purposes.

If w(g) is the weight of milk / milk product / food product used for extracting pesticide residues, V (μl) is the total volume made up after extracting pesticide residues, DL (μg) is the detection limit and MRL (μg/g) is the maximum residue limit in μg and per g of milk or milk product or any food product, then

Volume (μl/g equivalent) to be spotted for screening a pesticide residue with a particular MRL (μg) :

$$= \frac{V\ (\mu l)}{W\ (g)}$$

Volume (μl/g equivalent) to be spotted for screening a pesticide residue with a particular DL (μg) :

$$= \frac{V\ (\mu l) \times DL\ (\mu g)}{W\ (g) \times MRL\ (\mu g)}$$

Table 4.4 showed the amount to be spotted for screening milk / milk products / food products for MRL of OPPR and OCmPR. This is on the assumption that weight (W) of food product is 100 g, volume (V) made up is 100 μl and recovery of pesticides is 100 percent. To be more precise, percent recovery can be included in the formula as follows:

$$\text{Vol } (\mu l) \text{ to be spotted} = \frac{V\ (\mu l)}{W\ (g)} \quad \frac{DL\ (\mu g)}{MRL\ (\mu g/g)} \times \frac{100}{\%\ \text{recovery}}$$

Table 4.4 Volume to be spotted for screening milk / milk products / food products for MRL of OPPR and OCmPR*.

Pesticide Residues	DL (μg)	Milk or Milk Products		Food Grain		Spices		Fruits and Vegetables	
		MRL (μg)	Vol. (μl) to be Spotted	MRL*** (μg)	Vol. (μl) to be Spotted	MRL*** (μg)	Vol. (μl) to be Spotted	MRL*** (μg)	Vol. (μl) to be Spotted
Monocrotophos	7.5	0.002**	3750	0.025	300	0.1	75	1.0	7.5
Phosphamidon	7.5	-	-	0.05	150	-	-	0.02	375
Dimethoate	2.5	-	-	-	-	-	-	5.0	0.5
Carbaryl	0.1	0.1**	1	0.05	2.0	-	-	5.0	0.02
Methyl parathion	0.1	-	-	-	-	1.0	0.1	-	-
Malathion	0.1	0.1***	1	4.0	0.025	-	-	4.0 (fruit) 3.0 (veg.)	0.025 0.033
Quinalphos	0.1	-	-	-	-	0.2	0.5	-	-
Phorate	0.1	0.05**	2	-	-	-	-	-	-
Chlorpyriphos	0.1	0.01**	10	0.5	0.2	-	-	0.5	0.2

* w (μg) = 100, v (μl) = 100; ** MRL = As set by WHO / FAO; *** MRL = As set by PFA.

Volume (μl) to be spotted for screening milk / milk products / food products for MRL of OPPR and OCmPR is practically feasible for almost all OPPR and OCmPR studied except for monocrotophos and phosphamidon. If volume (μl) to be spotted is less than 1 μl, either use microsyringes of 1 μl capacity or make suitable dilutions so that it can be conveniently spotted. By this method, it is practically impossible to screen food commodities for the presence of monocrotophos and phosphamidon. This can be overcome, provided extraction for pesticide residues is done from large quantities of milk / milk products / food commodities. However, this may not be economically feasible.

Thus, a semi-quantitative analysis could be demonstrated by spotting a fraction of the entire aliquot. Based on the lowest limit of detection of individual pesticides by this procedure, a fraction of an aliquot may be selected to screen that pesticide for its presence below or above MRL.

4.5 DETECTION AND DETERMINATION OF DETECTION LIMITS BY TLC-CHEM

Here, sulphur containing OP compounds (dimethoate, malathion, methyl parathion, quinalphos, phorate and chlorpyriphos) were detected using AOAC (1984) method, which involved spraying of tetrabromophenophthalein ethyl ester solution, followed by 0.5 percent $AgNO_3$ solution spray. Clarity of the spots were obtained after a final spray with 5 percent citric acid solution. In general, the S-containing OP compounds appeared as grey to blue spots (Fig. 4.2). Carbaryl was detected using Chiba and Morley procedure (1964) which involved spraying of 1.5 N methanolic NaOH solution followed by a final spray with 0.01 percent 4-nitrobenzene diazonium tetrafluroborate reagent. Carbaryl gave a brilliant blue spot and was stable for 10 min (Fig. 4.3).

Table 4.5 showed the detection limits of S-containing OP compounds and carbaryl as detected by TLC-Chem. The detection limit for most of the S-containing pesticides, viz., malathion, methyl parathion, quinalphos, phorate, chlorpyriphos was found to be 0.05 μg. However, detection limit

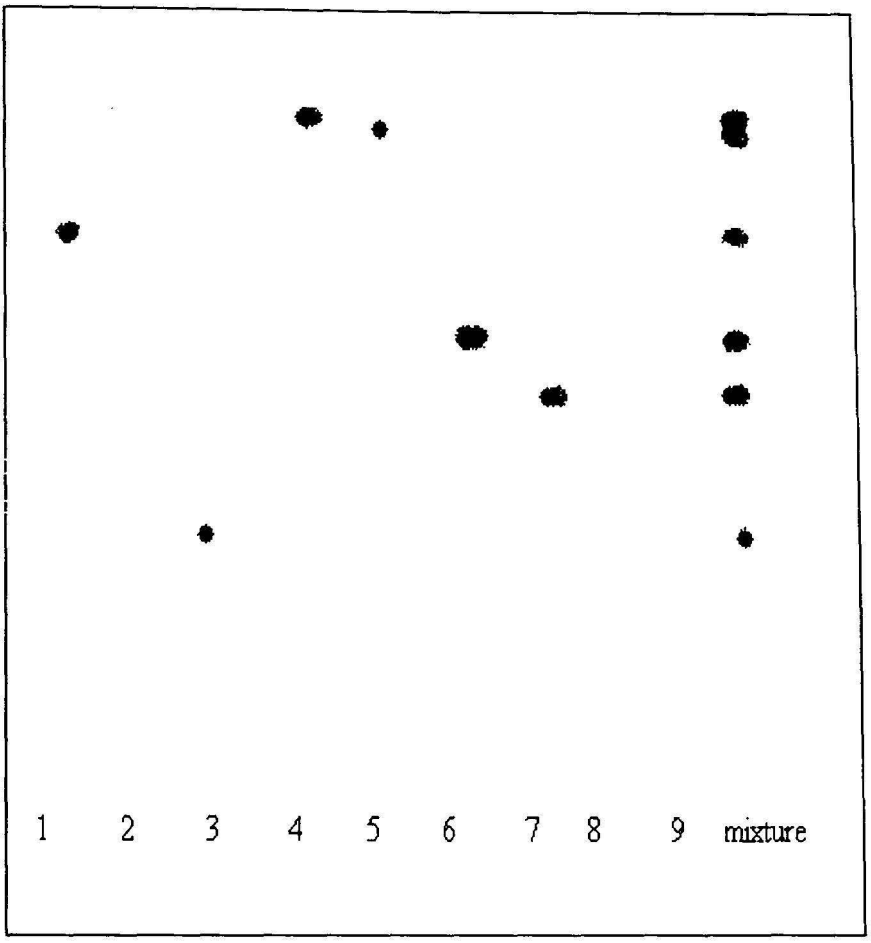

Fig. 4.2. **Detection of S-containing OP compounds by TLC-Chem (AOAC, 1984 Method)**

1. **Quinalphos**
2. **Carbaryl**
3. **Dimethoate**
4. **Chlorpyriphos**
5. **Phorate**
6. **Malathion**
7. **Methyl parathion**
8. **Phosphamidon**
9. **Monocrotophos**

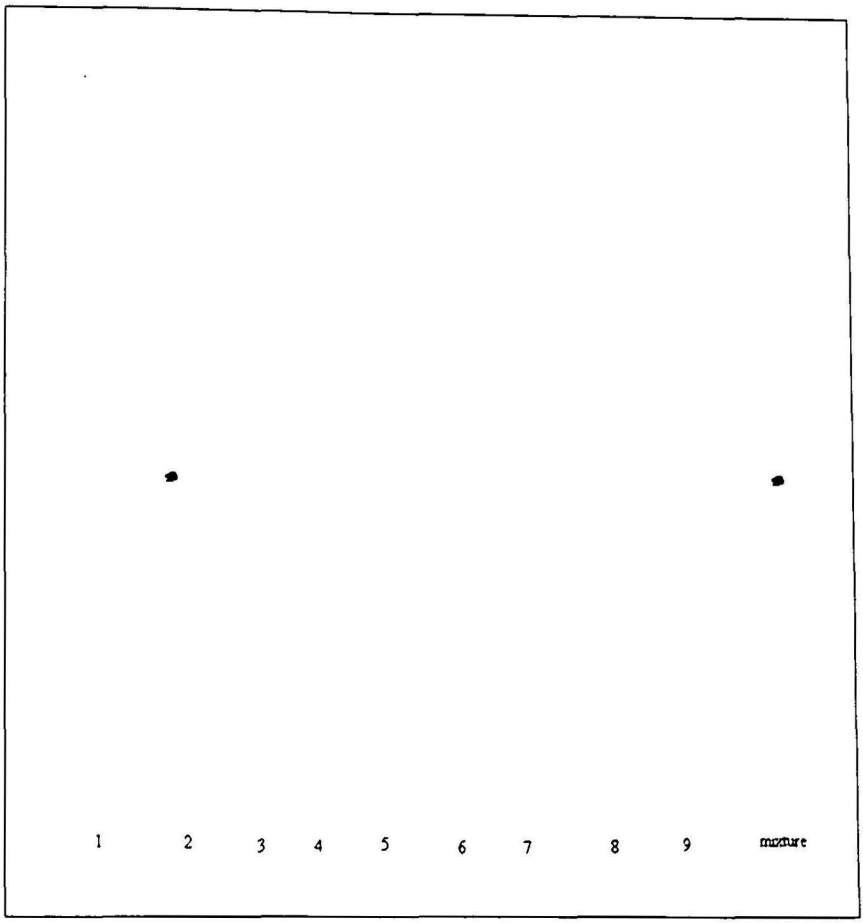

Fig. 4.3 Detection of Carbaryl by TLC-Chem as detected by Chiba and Morley (1964) Method.

1. Quinalphos
2. Carbaryl
3. Dimethoate
4. Chlorpyriphos
5. Phorate
6. Malathion
7. Methyl parathion
8. Phosphamidon
9. Monocrotophos

Table 4.5 Detection limits of S-containing OP compounds and carbaryl using TLC-Chem***.

Pesticide Compounds	Colour of Spots	Amount Spotted (μg)										
		10	5	1	0.75	0.5	0.25	0.1	0.075	0.05	0.025	0.010
Dimethoate*	Grey	+	+	+	+	+	+	-	-	-	-	-
Carbaryl**	Brilliant blue	+	+	+	+	+	+	+	-	-	-	-
Methyl parathion*	Greyish blue	+	+	+	+	+	+	+	+	+	-	-
Malathion*	Blue	+	+	+	+	+	+	+	+	+	-	-
Quinalphos*	Greyish blue	+	+	+	+	+	+	+	+	+	-	-
Phorate*	Blue	+	+	+	+	+	+	+	+	+	-	-
Chlorpyriphos*	Vivid blue	+	+	+	+	+	+	+	+	+	-	-

* S-containing OP compounds detected by AOAC (1984) method.

** Detected by Chiba and Morley (1964) method.

*** Verified three times.

Table 4.6 Comparative assessment of detection limits of OP and OCm compounds by TLC-EI and TLC-Chem.

Pesticide Compounds	Detection Limit (μg)	
	TLC-EI	TLC-Chem
Monocrotophos	7.5	-
Phosphamidon	7.5	-
Dimethoate	2.5	0.25
Carbaryl	0.1	0.1
Malathion	0.1	0.05
Methyl parathion	0.1	0.05
Quinalphos	0.1	0.05
Phorate	0.1	0.05
Chlorpyriphos	0.1	0.05

for dimethoate was 0.25 μg and that of carbaryl was 0.1 μg. The detection limit of carbaryl (0.1 μg) obtained in the present study was in accordance with that obtained by Chiba and Morley (1964). However, no information is available in the literature for the detection limits of S-containing compounds (used in the present investigation) by TLC-Chem method of AOAC (1984).

4.6 COMPARATIVE ASSESSMENT OF TLC-EI AND TLC-CHEM

A comparison of the detection limits of OP and OCm compounds by TLC-EI and TLC-Chem were given in Table 4.6. A comparatively low detection limit of 0.05 μg was obtained for almost all S-containing OP compounds in comparison to 0.1 μg in the case of TLC-EI technique. Both TLC-Chem and TLC-EI yielded same detection limit (0.1 μg) for carbaryl. While in the case of dimethoate TLC-Chem yielded a detection limit of 0.25 μg in comparison to 2.5 μg in the case of TLC-EI. Based on a comparative study between TLC-EI and TLC-Chem, El-Refai and Hopkins (1965) and Leoni and Puccetti (1971) concluded that TLC-EI was more sensitive than TLC-Chem. However, the TLC-Chem procedures followed by the above workers were different from AOAC (1984) methods adopted in the present study for the detection of S-containing compounds.

Eventhough a high sensitivity was obtained by TLC-Chem, TLC-EI should be preferred for multiresidue analysis, since TLC-EI is specific for OP and OCm compounds and hence can simultaneously screen both OP and OCm compounds. Some S-containing organochlorine pesticide compounds (e.g., endosulfan) may interfere in the case of TLC-Chem (Wise, 1967), while no such interference was reported for TLC-EI (Mendoza, 1974) as TLC-EI is specific for OP and OCm compounds. Further, the stability of the spots were observed to be very less by TLC-Chem (10 min) as compared to those obtained by TLC-EI (indefinite).

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

The present study was undertaken with a view to standardize a TLC method for the separation of model mixture of 8 organophosphorus OP (malathion, methyl parathion, phorate, chlorpyriphos, quinalphos, phosphamidon, monocrotophos, dimethoate) and 1 organocarbamate OCm (carbaryl) pesticide compounds, which are currently in use in India and to determine their detection limits, so that a clear cut proposition can be suggested for screening of food samples including dairy products for their MRL.

5.1 STANDARDIZATION OF A TLC METHOD FOR THE SEPARATION OF MODEL MIXTURE OF OP AND OCm COMPOUNDS

A TLC method has been standardized for the separation of OP and OCm compounds on a precoated TLC plate of aluminium oxide 60 F_{254} neutral Type E (200 μ) using the solvent system acetone : hexane (30:70) at 25°C.

5.2 DETECTION BY TLC-EI

The OP and OCm compounds separated by TLC as above were detected by TLC-enzyme inhibition (TLC-EI) method by spraying 10 percent goat liver homogenate in isotonic sodium phosphate buffer (0.01 M, pH 7.4) as a source of acetyl cholinesterase, followed by spraying of 0.5 percent β-naphthyl acetate in acetone as substrate and the final spray of 0.4 percent 4-nitrobenzene diazonium tetrafluroborate in acetone as chromogenic agent. The locations of pesticide inhibition of enzyme on the chromatogram appeared as clear white spots on orange background.

5.3 DETERMINATION OF DETECTION LIMITS BY TLC-EI

The detection limits by TLC-EI for most of the pesticides, viz., quinalphos, carbaryl, methyl parathion, malathion, phorate and chlorpyriphos were found to be 0.1 μg. However, detection limit for dimethoate was 2.5 μg and were exceptionally high (7.5 μg) for monocrotophos and phosphamidon.

5.4 SUGGESTED PROPOSITION FOR SCREENING OF FOOD PRODUCTS FOR THEIR MRL

The following formula can be applied, which will be giving volume (μl/g equivalent) to be spotted for screening a pesticide residue with a particular MRL:

$$= \frac{V\ (\mu l) \times DL\ (\mu g)}{W\ (g) \times MRL\ (\mu g/g)}$$

Where,

W (g) = Weight of milk / milk product / food product used for extracting pesticide residues,

V (μl) = Total volume made up after extracting pesticide residues,

DL = Detection limit in μg, and

MRL (μg/g) = Maximum residue limit in μg per g of milk or milk product or any food product.

Volume (μl/g equivalent) to be spotted for screening milk / milk products / food products for MRL of OPPR and OCmPR is practically feasible for almost all OPPR and OCmPR studied except for monocrotophos and phosphamidon. Thus, a semi-quantitative analysis could be demonstrated by spotting a fraction of the entire aliquot. Based on the lowest limit of detection of individual pesticides by this procedure, a fraction of an aliquot may be selected to screen that pesticide for its presence below or above MRL.

5.5 DETECTION AND DETERMINATION OF DETECTION LIMITS BY TLC-CHEM

Sulphur containing OP compounds (dimethoate, malathion, methyl parathion, quinalphos, phorate and chlorpyriphos) were detected using AOAC (1984) method, while carbaryl was detected using Chiba and Morley (1964) method. The detection limits for S-containing pesticides was found to be 0.05 μg except for dimethoate (0.25 μg) and that of carbaryl was 0.1 μg.

5.6 COMPARATIVE ASSESSMENT OF TLC-EI AND TLC-CHEM

A comparatively low detection limit of 0.05 μg was obtained for almost all S-containing OP compounds by TLC-Chem in comparison to 0.1 μg in the case of TLC-EI. Both TLC-Chem and TLC-EI yielded same detection limit (0.1 μg) for carbaryl. Eventhough, a high sensitivity was obtained by TLC-Chem, TLC-EI should be preferred for multiresidue analysis, since TLC-EI is specific for OP and OCm compounds, and hence, can simultaneously screen both OP and OCm compounds.

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