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Applicability of TLC in Multiresidue Methods for the Determination of Pesticides in Wheat Grain

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The demands on the analysis of pesticides have been increasing recently, taking into account the number of compounds analysed in a single run, number of matrices handled within one method, and requirements on detection limits. Multiresidue methods (MRMs) capable of simultaneously determining more than one residue in a simple analysis have been developed (Ahmed 2001; Zrostlikova et al. 2004).

Although Gas Chromatography (GC) is still a leading separation technique in pesticide residue analysis, Thin Layer Chromatography (TLC) is an widely used technique with the some advantages over the other techniques. These advantages are simplicity, inexpensive instrumentation, and specially rapidness, which is particularly important for serial analyses. TLC was developed as a qualitative method for the seperation of various organic or inorganic compounds from complicated matrices and for the detection of impurities in synthetic products. TLC may be used when application of other methods is limited by properties of the samples, e.g., thermal lability, low volatility, or the presence of a large amount of impurities hampering the analysis (Moraes et al. 2003; Sherma 1999; Cserhati and Forgacs 1997; Rathore and Sharma 1992).

The most important requirements for TLC is to obtain reproducuble R_f value. In order to ensure the reproducibility of the R_f and the required efficiency, the conditions have to be controlled. The main parameters influencing reproducibility of HR_f (R_f x 100) are type and quality of the adsorbent, activity of the layer thickness, saturation of the chamber, humidity of the air, temperature, and elution solvent When standardized conditions are applied, reproducible Rf and RRf values can be obtained and TLC can be used for screening pesticide residues in samples of unknown origin (Lantos 1998; Ambrus et al. 1981).

The use of the marker compounds have proven to be very satisfactory for this purpose. The minimum detectable quantity (MDQ) of the marker compound is specific for a detection procedure. If the marker compounds are well detectable and their R_f values are within the expected range the analyst can be sure, and can demonstrate it at the same time, that method was applied properly. The MDQ and R_f values of marker compounds referring to the different detection methods were determined and tabulated by Ambrus et al. (1996) and Füzesi (1998).

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Our earlier publication has demonstrated that "enzyme inhibition with cow liver extract and β-naphthyl-acetate substrate (EβNA)" detection method is suitable for the qualitative and quantitative analysis of carbaryl, dichlorvos, chlorpyrifos and parathion-m within the maximum residue limit (MRL). Similarly, enzyme inhibition with horse blood serum and acethythiocholine iodide substrate (EAcI-horse) detection method is also suitable for the analysis of methomyl, and parathion-m. But fungal inhibition detection method (FAN) is only suitable for qualitative analysis of captan, fenarimol and procholoraz (Tiryaki and Aysal 2005).

The proposed paper will discuss the applicability of three TLC detection methods in combination with ethyl acetate extraction and Gel Permation Chromatography (GPC) clean-up method for screening pesticide residues in cereal grains.

MATERIALS AND METHODS

All pesticide active ingredients of atrazine, chlortoluron, cyanazine, diazinon, dioxacarb, dieldrin, dimethoate, diuron, metoxuron, oxamyl, thiabendazole, triazophos, and triforine were obtained from Dr. Ehrenstorfer Laboratories GmbH, Germany, via the International Atomic Energy Agency (IAEA). Stock solutions were prepared in acetone and composite working standard solutions were prepared by diluting the stock solutions as required. The TLC plates with silica gel 60, layer thickness of 0.25 mm, 20 X 20 cm (Merck, 1.05721) and pre-coated neutral alumina layer on aluminium sheet 20 x 20 cm, 0.2 mm thickness (aluminiumoxide F_{254} 60 Merck, 1.05550) were used. All chemicals used this study were analytical grade and solvents were reagent grade (Merck). The preparation of reagents was carried out as described by Ambrus (1996).

The GPC column (20 cm x 1 cm glass column) was filled with 8 g SX-3 gel as described in the operation manual. The KL-SX-3 GPC gel chromatograph was operated with constant nitrogen over-pressure of 0.5 bar providing a constant flow rate of approximately 1 ml/min for the ethyl acetate/cyclohexane (1:1) eluting solvent mixture (Anonymous 1998).

The calibration of the GPC column was performed with diazinon and triazophos and wheat oil in triplicate. The standard mixture in 5 ml ethyl acetate/cyclohexane (1:1) containing approximately 1 µg/ml of diazinon and 0.5 µg/ml triazophos was prepared. An aliquot (250 µl) of standard mixture was injected (correspond 0.25 µg diazinon, 0.125 µg triazophos) into the stabilized GPC column. The first 7 ml was collected and discarded. Then the eluent was collected in 1 ml increments until 13 ml. The next 6 ml (fraction 14-20 ml) and the fraction of 21-30 ml were collected together. A total of 30 ml eluent was obtained. The 8 different fractions were evaporated to nearly dryness and re-dissolved in acetone to 1 ml for the GC analysis.

To get grain oil, 200 g corn or wheat flour was soaked in 200 ml water for 30 min and extracted with 400 ml ethyl acetate. The organic phase was decanted, filtered,

and evaporated to dryness and the oily material collected in a test tube. The dry extract was weighed and an ethyl acetate/cyclohexane (1:1) solution was prepared from it to obtain about 400 mg/ml solution. An aliquot (250 µl) of the solution , corresponded to 100 mg of the concentrated plant extract, was injected into GPC column. The calibrated and graduated test tubes were weighed with $\leq$ 0.0001g accuracy. The first 7 ml were discarded, then the eluent was collected in 1 ml increments until a total volume of 13 ml was eluted. The eluent was evaporated to dryness and the eluted dry material was weighed. The test was performed in three replicates.

The sample matrix studied was wheat grain. The basic extraction procedure described by Ambrus (1996) was applied in the study. Fifty ml ethyl acetate was added to the sample and reagent blank. For the recovery studies; 49 ml ethyl acetate was added to the samples. Blank samples were fortified with 1 ml standard mixture at the level of 0,1 mg/kg diazinon and 0,05 mg/kg triazophos. The final extract with about 250 µl of ethyl acetate-cyclohexane (1:1), corresponded 5 g sample equivalent, were injected GPC.

The concentrated 250 µl extract representing 5 g wheat sample was injected into the column and allowed to run at the eluent flow rate described above. The flow rate was checked during the elution. The first 9 ml eluate was discarded and the next 20 ml eluate was collected as pesticide fraction. The eluent was evaporated to nearly dryness and dissolved in acetone for application on the TLC plates and also for GC-NPD analysis to determine the recovery.

The TLC elution of pesticides were performed with Silica gel 60, 0.25 mm (Merck:1.05721): ethyl acetate system ; and aluminium oxide F_{254} 60, 0.2 mm (Merck:1.05550): ethyl acetate system.

The Rf values were determined in developing tanks kept in water bath adjusted to 23°C in order to reduce the effect of temperature variation in the laboratory. Eighty ml eluent was poured in the tank. The eluent was equilibrated with the vapour phase by inserting filter paper in the developing tank and waiting for minimum 30 minutes before the plates were placed into the tank The silica gel plates were activated at 105 °C for 30 minutes before use. Twenty µl extract and/or standard solution was applied in uniform spots of about 4-6 mm on the plate 2 cm from the bottom edge. The plate was placed into the eluting solvent at 1 cm depth in the saturated developing tank. The eluent was allowed to run up to 11 cm from the origin.

As described in the study of Ambrus (1996) and Füzesi (1998), visualization of the spots was performed with the 3 detection methods, namely, *o*-Tolidine + potassium iodide (o-TKI), photosynthesis inhibition (Hill reaction- HILL), and Aluminium oxide G incorporated with silver nitrate + UV exposition (AgUV). In the methods, the following 3 modifications and improvements were introduced in to the detection methods. Firstly the sprayer was connected with a pipe to the

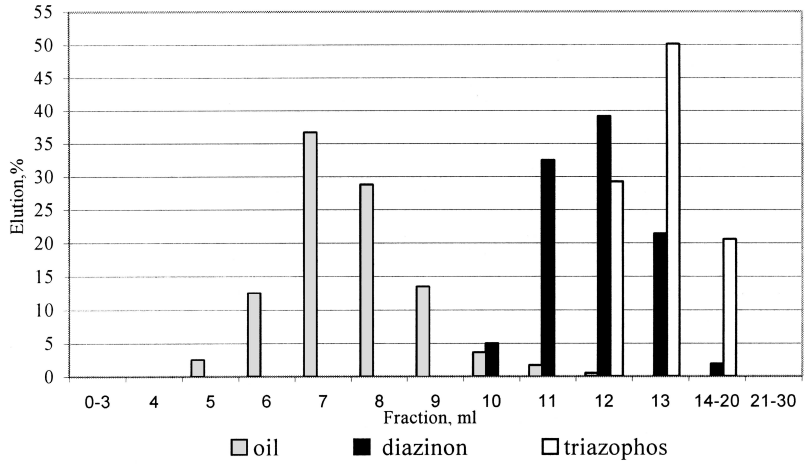

Figure 1. Distribution of wheat oil, diazinon and triazophos on Bio- Beads SX-3 gel with ethyl acetate/cyclohexane (1:1)

exhaust of the vacuum pump, so that spraying of the reagent was done effectively. Secondly,for detecting reagent of Hill detection method, wheat pressing was diluted with DCPIP reagent (224,81 mg of 2,6-dichlorophenol-indophenol Na-salt dihydrate dissolved in 500 ml borax buffer solution) at the ratio of 1:3 to 1:5 (v/v), depending on chlorophyll content of the leaves, until the colour of the mixture becomes bluish-green. The laast modification was done in the Ag-UV method. The reagent was prepared by dissolving 0.15 g $AgNO_3$ in 15 ml freshly prepared bi-distilled water. Instead of self-made plate, we used pre-coated neutral alumina layer (aluminiumoxide F_{254} 60 Merck, 1.05550) as mentioned above. The plate was sprayed with reagent solution uniformly, dried with fan and kept in an oven at 60 °C for 20 min. Pesticide solution was spotted to the plate and the plate was developed with ethyl acetate solvent. The plate was air-dried and kept in the UV chamber at 254 nm for 20-30 min. Grey coloured spots appeared on a colourless or light-grey background. The colour of the spots improved sometimes when plates were stored outside.

The GC system, HP6890 GC equipped with a NPD, was used for the analysis of GPC eluate, under the following conditions: capillary column (30.0 x 250 μm x 0.25 μm nominal film thickness, HP 19091S-433, HP-5MS 5% phenyl methyl siloxane); carrier gas N_2 1.1 ml/min; H_2 2.0 ml/min; air 60 ml/min. Operating conditions; column temperature: 140-250°C; initial time: 1 min; rise: 5°C /min; final time: 3 min; run time:26 min; detector: 280°C; injector: 270°C (splitless).

RESULTS AND DISCUSSION

The elution profiles of wheat oil and pesticide are shown in Figure 1. In the first 9 ml 94.14% of wheat oil was eluted through the column. Diazinon and triazophos were eluted in 10-20 ml and 12-20 ml fractions, respectively. The elution profiles

showed that KL-SX-3 GPC is very useful tool for cleaning-up of plant extracts. The performance of clean-up method were checked with recovery experiments by fortifying blank sample portions with diazinon and triazophos in triplicate. An aliquot of (250 µl) concentrated extract (representing 5 g wheat sample) 500 ng diazinon and 250 ng triazophos was injected to GPC. The percentage of recovery for diazinon and triazophos were 85.5% and 100.4%, respectively (Table 1).

Table 1. Recovery of pesticides from fortified wheat samples by using GC-NPD

Compound	Recovery (%)			Mean recovery (%)	CV %
	1	2	3		
Diazinon	94.2	78.56	83.8	85.53	9.39
Triazophos	102.9	89.35	108.9	100.38	9.97

Detectability of pesticide active ingredients, under the specific conditions described for the individual detection methods, were tested with three runs on different plates for each marker compound and selected compounds. The R_f, RR_f, and their CV's determined with *o*-TKI, Hill, and AgUV detection methods are given in Table 2. The R_f and RR_f values were in close agreement with those reported earlier (Füzesi 1998).

Table 2. R_f and RR_f values determined with *O*-TKI, Hill and AgUV detection methods.

Detection and	R_f values					RR_f values		Reported[a]	
Pesticide	1	2	3	Mean	CV%	Mean	CV%	Rf	RRf
O-TKI									
Atrazine	0.61	0.62	0.60	0.61	1.98	1	0	0.62	1
Diuron	0.36	0.36	0.35	0.36	1.87	0.587	1.06	0.368	0.594
Oxamyl	0.15	0.16	0.14	0.15	5.82	0.247	4.29	0.189	0.305
Dimethoate	0.27	0.26	0.30	0.276	6.48	0.440	3.43	0.275	0.443
Dioxacarb	0.42	0.42	0.46	0.44	5.44	0.695	8.10	0.454	0.732
HIIL									
Atrazine	0.62	0.66	0.68	0.65	4.55	1	0	0.62	1
Chlortoluron	0.43	0.47	0.45	0.45	3.87	0.691	3.49	0.398	0.642
Metoxuron	0.34	0.37	0.33	0.35	5.43	0.533	6.19	0.303	0.489
Cyanazin	0.58	0.59	0.59	0.59	1.38	0.979	0.47	0.602	0.971
Thiabendazole	0.39	0.36	0.36	0.37	4.60	0.618	5.46	0.335	0.540
AgUV									
Dieldrin	0.89	0.86	0.87	0.87	1.72	1	0	0.834	1
Triforine	0.54	0.56	0.47	0.52	9.15	0.600	9.08	0.788	0.729

[a] Reported Rf by Füzesi (1998)

The MDQ values determined with the same detection methods are given in Table 3. To compare our data Füzesi et al. (1998)'s findings also included in the table.

The MDQ values were also verified in the presence of co-extractives being in the cleaned-up extracts. If the co-extractives substantially affect the detectability of the compounds then the sample equivalent spotted onto the TLC plates should be

Table 3. MDQ and LOD of the pesticides in the wheat extracts.

Method	Compound		MDQ (ng)		LOD[b]	MRL
			Reported[a]	Found		(EU)[c]
O-TKI	Marker	Atrazine	25	12.5	1.25	-
		Diuron	30	15	1.5	-
		Oxamyl	100	50	5	-
	Selected	Dimethoate	100	50	5	0.02
		Dioxacarb	25	12.5	1.25	-
HILL	Marker	Atrazine	1	0.5	0.05	-
		Chlortoluron	1	0.5	0.05	-
		Metoxuron	5	2.5	0.25	-
	Selected	Cyanazin	1	0.5	0.05	
		Thiabendazole	100	50	5	0.05
AgUV	Marker	Dieldrin	12.5	12.5	2.5	0.01
		Triforine	50	50	10	0.05

[a] Reported by Füzesi (1998)
[b] ng/mg or mg/kg
[c] EU limit, mg/kg ; http://europa.eu.int/comm/food/plant/protection/resources /mrl_commodity.pdf

reduced. The tests were carried out in three replicates with the blank extracts of wheat after the GPC clean-up. The amounts of extracts that could be spotted on to the TLC plate was determined experimentally. Ten mg sample equivalent applied in 20 µl did not affect the detectability of the compounds with the detection procedures except in case of detection with AgUV method, where only 5 mg sample equivalent in 20 µl could be spotted.

The limit of detection (LOD), based on MDQ and sample equivalent applied, was calculated as follows:

$$LOD=MDQ(ng) / M(mg)$$

where M is the sample equivalent applied on the layer.

The calculated LOD values are summarized in Table 3. The LOD levels of any detection methods should not be higher than MRL in the pesticide residue analysis. As it is shown in table, generally LOD's of tested compounds is higher than European Union MRL. According to our findings, these three TLC detection methods is only suitable for qualitative analysis.

Although determined MDQ values in the solvent are lower than Füzesi et al. (1998)'s findings, LOD values is very high in the sample matrix. The reason may be that it is used very small sample equivalent (10 mg) for loading the plates to verifiy determined MDQ. To increase the loadability to the TLC plate, additional cleanup procedure was required, besides GPC clean-up (Lantos 1998)

Qualitative determination of pesticide residue by TLC is more precise without any interference, since the visualisation of spot is performed by using chemical reagent (O-TKI, AgUV and HILL-reaction) and bioassay technique (FAN, EßNA, and

EAcI-horse). Similar findings have been reported earlier indicating that TLC has lower sensitivity, i.e, higher LOD, compared to other chromatographic techniques, the methodology is suitable for semi-quantitative and qualitative determination of various thermally unstable materials and for confirmation purposes (Moraes et al. 2003).

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