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Method validation for the analysis of pesticide residues in grain by thin-layer chromatography

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Abstract Method validation is an important requirement in the practice of pesticide residue analysis and is the process of verifying that a method is fit for its purpose. To make a correct decision on the validity of the method, the following method performance parameters have to be taken into consideration: scope, specificity, limit of detection, limit of quantification, linear range, accuracy, precision, repeatability, reproducibility, recovery, ruggedness

and robustness. The goal of this study was to validate previously adapted thin-layer chromatographic methods for the pesticide residue analysis in grain. Confirmation of validation parameters for some compounds was also performed by gas chromatographic analysis.

Keywords Method validation · TLC detection method · Precision · Calibration

Introduction

The implementation of a quality system in analytical laboratories, based on 17025, is now a reality. The requirements of this standard deeply modified the organization of the laboratories, whereas it also improved the quality of the analytical results [1].

One of the quality control/quality assurance (QA/QC) requirements in residue analysis is method validation. The International Standardisation Organisation (ISO) definition of validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled [2]. The EURACHEM Guide defines method validation as the process of establishing the performance characteristics and limitations of a method and the identification of the influences which may change these characteristics and to what extent [3].

The process of method validation has a direct impact on the quality of residue analytical data. Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones [4]. Parallel to this concept, another definition of method validation is the process of verifying that a method is fit for purpose, i.e., for use for solving a particular analytical problems [3].

Validation studies for quantitative analytical methods typically determine some or all of the following parameters: accuracy, scope, specificity, sensitivity, precision (repeatability and reproducibility), bias, linearity, detection limit, robustness, ruggedness and selectivity. Some information on all characteristics should be established prior to the analysis of samples [5, 6].

There are several levels of validation. The lowest one is when a single analyst within a single laboratory fortifies his or her own samples to gather recovery data. But the validity of methods has often been established on the basis of inter-laboratory studies of performance. Therefore, the highest level is the collaborative study, which includes many collaborating laboratories and samples containing the analytes in unknown concentration [7].

Thin-layer chromatography (TLC) is used in many analytical laboratories for pesticide residue analysis. Although gas chromatography (GC) is still a leading separation technique in residue analysis, qualitative determination of pesticide residue by TLC is more precise without any interference, since the visualization of the spot is performed by using a chemical reagent and bioassay techniques. The acceptance and importance of TLC is mainly due to its simplicity, speed, and low cost. When standardized conditions are applied, TLC can be used for screening pesticide residues in sample of unknown origin [8, 9].

The minimum detectable quantity (MDQ) of a pesticide is defined as the minimum mass of analytical standard, expressed in nanograms, spotted on the TLC plate, which gives a clear visible spot after elution under average chromatographic conditions [10].

To ensure the reproducibility of the retention factor (R_f) values and required efficiency, the conditions have to be controlled. The use of marker compounds has proven to be very satisfactory for this purpose. The marker compounds are specific for a TLC detection procedure. The MDQ of the marker compounds should be spotted on each plate on which qualitative and quantitative determinations are carried out. The MDQs of these result in well-defined spots only with optimum conditions. 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 R_f values of the marker compounds can also be used as a reference for the relative R_f (RR_f) values, which greatly facilitates the identification of the spots detected on the plates. The marker compounds selected should be relatively stable in standard solutions, sensitive for the detection conditions (not appearing on the plate if the conditions are not optimal) and have reproducible R_f [10, 11].

The definition of limit of detection (LOD) is defined in the EU Document No. SANCO 10476 as the minimum concentration or mass of the analyte that can be detected with acceptable certainty, though not quantifiable with acceptable precision. Various definitions are used but, for convenience, it is often the quantity of analyte that generates a response three times greater than the noise level of the detection system. Definitions based on standard deviation of blank values can be difficult to apply in chromatographic analysis [6]. According to IUPAC definitions, limit of detection is the lowest concentration of a pesticide residue in a defined matrix where positive identification can be achieved using a specified method. The limit of detection, expressed as the concentration or the quantity, is derived from the smallest measure that can be detected with reasonable certainty for a given analytical procedure and calculated based on the standard deviation of blank values [12, 13]. In TLC basis study, it can be defined as the lowest spotted amount of the analyte that can be detected on the plate in the presence of the coextractives from a sample matrix. Therefore, the LOD is calculated based on MDQ and sample equivalent applied (M), according to Eq. (1) [13]. The term of sample equivalent is the sample mass in the spotted extract volume.

$$LOD = MDQ/M \quad (1)$$

Similarly, the limit of quantification (quantitation), also known as limit of determination, (LOQ) is the minimum concentration or mass of the analyte that can be quantified with acceptable accuracy and precision. It is defined in different ways but must be a value greater than the limit of detection, and is often calculated as the analyte concentration that gives a signal-to-noise ratio of 10 [4, 6]. In the IUPAC definition, it is the lowest concentration of a

pesticide residue in a defined matrix where positive identification quantitative measurement can be achieved using a specified method. LOQ has been defined as three times the LOD [12]. In the TLC method, the estimated limit of quantification (LOQ_e) was calculated from the LOD verified in the presence of sample matrix and the recovery, Q . It was used as an estimated recovery of 70%. LOQ_e was determined from Eq. (2) [14].

$$LOQ_e = \frac{LOD}{Q} \quad (2)$$

A traditional method for testing the linearity of calibration functions after linear regression is to compute the correlation coefficient, r (or the similar coefficient of determination, r^2). But further comments have been made on the misuse of r for testing the linearity. The value of r correctly describes a correlation between two files; it describes the quality of the fit only poorly and its linearity not at all. Since equal results can have different meanings, depending on the number of the degrees of freedom, the use of r is not reliable measure of linearity [15, 16]. The virtue of TLC and GC calibration can be very well characterized by the standard deviation ($S_{\Delta y/\hat{y}, n-2}$) of the relative residuals (residuals/predicted $\Delta y_i = y_i - \hat{y}$; $Y_i = \Delta y_i/\hat{y}$), which is calculated with $n - 2$ degrees of freedom by applying Eq. (3) [7, 17].

$$S_{\Delta y/\hat{y}} = \sqrt{\frac{\sum (Y_i - \bar{Y})^2}{n - 2}} \quad (3)$$

where y_i is the response obtained from spotting/injecting analytical standard. \hat{y}_i is the point corresponding with analytical standard on the regression line. \bar{Y} is the mean value of Y_i . n is the total number of standard spots/injections e.g., when the calibration is made at three level with duplicate injections, then n is equal to 6.

A good calibration curve has a standard deviation of relative residuals less than 0.1. Since the standard deviation of the relative residuals is not constant but generally proportional to the injected analyte, standard deviation of the relative residuals reflect the average variability of the calibration points even if unweighted regression equation is used for the estimation of the calibration relationship [17].

The standard deviation of the relative residuals clearly better to interpret than r , because of their linear response to the random errors of the signals combined with possible systematic errors produced by non-linearity of the real calibration function. By using this concept, problems due to different numbers of degrees of freedom between calibration and analytical data could be avoided [15].

According to EC Directive, the analytical calibration should extend over a range appropriate to the lowest and highest nominal concentration of the analyte in relevant analytical solutions, at least $\pm 20\%$. Details of the linear range should be given, e.g., $\mu\text{g mL}^{-1}$ [18]. From the point of view both qualitative and quantitative determination, the

significance of the knowledge on the loadability to TLC, the linear calibration range has to be emphasized. Beyond its upper limit it can alter the R_f values and make the quantitative determination impossible or false [11].

As for accuracy, it is the closeness of the determined value to the true value. It refers to the method's ability to measure the "true" value, while precision refers to the closeness of repeated measurements. A measure of accuracy is the difference between the mean value and its true value, expressed as a percentage of the true value. For example, if the true value and measured mean value are 0.05 and 0.045 mg/kg, respectively, then the accuracy is 10% [19]. But, there is different approach related to accuracy. Accuracy is a qualitative indication (but mostly and erroneously) used as a quantitative deviation from the true value. The term of 'true value' may not be a practical concept since one never knows the 'truth' [20].

In this work, method validation criteria above mentioned for the analysis pesticide residues in grain were used for the validation of the previously adapted four different TLC detection methods, namely, *o*-tolidine + potassium iodide (*O*-TKI), photosynthesis inhibition (HILL-reaction), enzyme inhibition with cow liver extract and β -naphthyl-acetate substrate (E β NA), and aluminum oxide G incorporated with silver nitrate + UV exposition (AgUV) [10, 21]. For confirmation, the residues of some pesticides were determined with gas chromatography-nitrogen-phosphorous detector (GC-NPD).

Materials and methods

Pesticides

The following pesticide active ingredients (with the indicated purity in parenthesis and obtained from Dr. Ehrenstorfer Laboratories GmbH, Germany via IAEA) were used in this study: Atrazine (98.4%), carbaryl (99.5%), chlorpyrifos (99.5%), chlortoluron (97.5%), cyanazine (98.5%), dichlorvos (97.0%), dioxacarb (97.0%), dieldrin (96%), dimethoate (98.5%), diuron (97.5%), metoxuron (99.5%), oxamyl (98.6%), parathion-methyl (97.0%), thiabendazole (99.9%), and triforine (99.5%).

Chemicals

All chemicals used were of analytical grade and solvents were reagent grade (Merck).

Reagents

The reagents *o*-tolidine + potassium iodide, DCPIP solution (224.81 mg of 2,6-dichlorophenol-indophenol Na-salt dihydrate dissolved in a 500-mL borax buffer solution), enzyme solution and substrate solution (mixture of 10 mL β -naphthyl-acetate solution and 16 mL Echtblau-salt solu-

tion), and AgNO₃ solution are used for the *O*-TKI, HILL, E β NA, and AgUV detection, respectively.

Equipment

The following equipment was used to perform the analysis: Ultra Turrax (T25 basic IKA-WERKE), rotary evaporator (Heidolph OB 2200), TLC plate (Silica gel 60, 0.25 mm, Merck:1.05721 and aluminum oxide F₂₅₄ 60, 0.2 mm Merck:1.05550), TLC basic set (e.g., Camag or Desaga including spreader, application guide, atomizer, developing tanks, etc.) semi-automatic gel permeation chromatography (GPC) system operated with constant nitrogen pressure, Bio Beads SX3 gel, GC (Hewlett Packard -HP6890 Agilent) and other basic glassware and equipment such as measuring cylinders and Hamilton micro syringe.

Extraction and cleanup

For the method validation study, durum wheat (Kiziltan variety, which is grown in the Central Anatolia Region) was used. A 10-g analytical portion of ground wheat was transferred into an extraction vessel. Ten mL of distilled water was added, mixed vigorously, and soaked for 5 min. Then 50 mL ethylacetate (EtAc) – for recovery samples 49 mL EtAc and 1 mL standard fortification mixture – 5 g sodium hydrogen carbonate and 35 g anhydrous sodium sulphate were added. The vessel was placed on a water bath and the sample solvent mixture was allowed to reach a temperature of about 28 °C. The mixture was homogenized with Ultra Turax at about 25 °C for 30 s. A 25-mL extract was decanted and filtered through a cotton-wool plug in a filter funnel. The extract was evaporated to near dryness in a rotary evaporator. The residue was transferred to a calibrated conical test tube with 1 mL EtAc and the solvent was evaporated to nearly dryness. The residue was dissolved in 250 μ L of EtAc/cyclohexane 1/1 v/v, representing a 5-g sample. After the calibration of GPC, the extracts were cleaned-up on a SX-3 GPC column with elution solvent of EtAc/cyclohexane 1/1 v/v.

Elution of pesticides and evaluation

The cleaned-up sample extracts were analyzed by TLC detection methods, namely, *O*-TKI, HILL, E β NA, and AgUV [10]. Found R_f and MDQ of compound in the previous work were used to carry out this study [21]. The TLC elution of pesticides was performed with Silica gel 60: EtAc system for *O*-TKI, HILL and E β NA detection; and aluminum oxide F₂₅₄ 60: EtAc system for AgUV detection. The quantitation was performed immediately after appearance of the spots as they disappear within a few minutes. The evaluation of the marked spots was carried out by measuring the diameter of the circular spot and the vertical and horizontal diameter are averaged.

Validation of the method

A recovery test was done for marker and selected compounds with the corresponding TLC detection method in wheat. Fortifications were carried out based on determined LOQ levels (Table 1), and maximum residue level (MRL) of the tested compounds. Marker compounds were included in the tests as representative compounds regardless their MRLs. Five analytical portions were spiked at 1 LOQ, 2 LOQ (or 1 MRL) and 5 LOQ (or 3 MRL) and recovered amounts of analytes were determined applying of three-point calibration with the corresponding TLC detection method. The fortification, extraction, and recovery studies were performed in different days including all fortification levels in the sets on each day.

The individual recoveries, their average, accuracy, standard deviation (SD) and relative standard deviation (RSD) were calculated.

GC-NPD determination

For confirmation and comparison, the residues of some pesticides (parathion-m, dichlorvos, atrazine, diuron, and chlorpyrifos) in the spiked and blank sample extracts were determined with GC-NPD analysis, simultaneously applying five-point calibration. For this aim, HP GC with

equipped NPD were used at the following conditions: capillary column (30.0 m length \times 250 μ m inside diameter \times 0.25 μ m nominal film thickness, HP 19091S-433, HP-5MS 5% phenyl methyl siloxane); carrier gas nitrogen 1.3 mL min⁻¹, hydrogen 2.0 mL min⁻¹, air 60.0 mL min⁻¹· μ g/mL. Operating conditions; column temperature: 140–250 °C; initial time: 1 min; rise: 5 °C/min; final time: 3 min; run time: 26 min; Detector T: 280 °C; Injector T: 270 °C (splitless).

The results of method validation including the calibration functions were reported in this study.

Results and discussion

LOD and LOQ

The MDQ, LOD and LOQ_e values of marker and selected compounds were calculated basis on each detection method are given in Table 1. The MRLs of the compound for wheat commodity are also given in the last column of the table.

As is indicated in Table 1, LOQ_e values are higher than MRLs for the most of the tested compounds, except in case of atrazine with HILL detection, and parathion-m and chlorpyrifos with E β NA detection. Although our determined MDQ values in the solvent are lower than the

Table 1 The MDQ, LOD and LOQ_e values of the marker and selected compounds with the corresponding TLC detection method in the wheat extracts and their MRLs

TLC detection method	Active ingredient	MDQ values, ng		LOD ^b , mg kg ⁻¹	MRL ^c in grain, mg kg ⁻¹
		Found ^a	Reported ^b		
O-TKI					
Marker	Atrazine	12.5	25	1.250	0.200 (a)
	Diuron	15	30	1.500	
	Oxamyl	50	100	5.000	
Selected	Dimethoate	50	100	5.000	0.020 (b)
	Dioxacarb	12.5	25	1.250	
HILL					
Marker	Atrazine	0.5	1	0.50	0.200 (a)
	Chlortoluron	0.5	1	0.50	
	Metoxuron	2.5	5	0.250	
Selected	Thiabendazole	50	100	5.000	0.50 (b)
	Cyanazine	0.5	1	0.50	
EβNA					
Marker	Parathion-M	0.5	1	0.50	0.200 (a)
	Dichlorvos	10	20	1.000	
	Oxamyl	10	10	1.000	
Selected	Carbaryl	5	10	0.500	0.500 (a,b)
AgUV					
Marker	Chlorpyrifos	0.25	0.5	0.25	0.50 (a,b)
	Dieldrin	12.5	12.5	2.500	
	Triforine	50	50	10.000	0.50 (b)

^aValues from the previous part of this study [21] LOQ_e is calculated by dividing of LOD to Q, estimated recovery of 0.70

^bReported values by Ambrus et al. [22]

^cMRL regulations in: (a) Türk Gıda Kodeksi. <http://www.kkgm.gov.tr/Genel/index.asp?Prm=/Mevzuat/Kodeks.asp?Adres=KodeksList.htm> (b) EU limits http://europa.eu.int/comm/food/plant/protection/resources/mrl_commodity.pdf

Table 2 Summary of calibration parameters for some compounds for TLC detection methods and GC-NPD detection with five-level calibration

Analyte _{detection method}	Linear range, ng μL^{-1}	Regression equation $y = a + bx$	Correlation coefficient, r^2	Relative residual standard deviation, $S_{\Delta y/\bar{y}}$
Atrazine _{O-TKI}	0.625–3.125	$y = 4.152 + 0.037x$	0.980	0.24
Atrazine _{HILL}	0.25–0.2	$y = 5.66 + 0.657x$	0.969	0.43
Carbaryl _{EβNA}	0.25–1	$y = 4.618 + 0.1x$	1.000	0.02
Chlorpyrifos _{EβNA}	0.125–0.05	$y = 4.888 + 1.679x$	0.998	0.05
Chlortoluron _{HILL}	0.025–0.1	$y = 5.121 + 1.512x$	0.999	0.06
Cyanazine _{HILL}	25–100	$y = 3.821 + 0.7214x$	0.996	0.06
Dichlorvos _{EβNA}	0.5–2	$y = 3.325 + 0.056x$	0.996	0.12
Dieldrin _{AgUV}	0.625–3.125	$y = 4.935 + 0.0175x$	0.993	0.06
Dimethoate _{O-TKI}	2.5–7.5	$y = 3.904 + 0.0067x$	0.999	0.04
Dioxacarb _{O-TKI}	0.625–3.125	$y = 3.7 + 0.018x$	0.995	0.07
Diuron _{O-TKI}	0.75–3.75	$y = 3.821 + 0.018x$	0.999	0.02
Metoxuron _{HILL}	0.125–0.5	$y = 5.624 + 0.283x$	0.997	0.11
Oxamyl _{O-TKI}	2.5–12.5	$y = 4.294 + 0.01x$	0.996	0.11
Oxamyl _{EβNA}	0.5–2	$y = 3.138 + 0.04x$	0.999	0.02
Parathion-M _{EβNA}	0.05–0.2	$y = 5.792 + 0.75x$	0.998	0.07
Triforine _{AgUV}	2.5–12.5	$y = 4.771 + 0.0083x$	0.999	0.03
Atrazine _{GC-NPD}	0.1–0.5	$y = 0.189 + 0.0106x$	0.994	0.99
Chlorpyrifos _{GC-NPD}	0.055–0.5	$y = -0.443 + 0.0156x$	0.999	0.93
Dichlorvos _{GC-NPD}	0.1–0.6	$y = 0.008 + 0.003x$	0.997	0.93
Diuron _{GC-NPD}	0.6–1	$y = 0.084 + 0.0014x$	0.998	0.12
Parathion-M _{GC-NPD}	0.135–0.5	$y = -0.149 + 0.0124x$	0.999	0.29

Table 3 Method validation parameters of O-TKI-TLC detection and HILL-TLC detection method for the marker and selected compounds

Analyte _{detection method}	Fortification mg kg^{-1}	Found, mg kg^{-1}	Recovery, Q, %	SD	RSD %	Accuracy %
Atrazine _{O-TKI}	1.786	1.657	92.78	8.13	8.70	7.22
	3.571	3.445	96.47	13.41	13.90	3.53
	8.928	7.990	89.49	7.52	8.41	10.50
Diuron _{O-TKI}	2.142	2.145	100.10	12.67	12.65	-0.10
	4.286	3.871	90.32	7.34	8.13	9.68
	10.714	10.110	94.36	11.59	12.28	5.64
Oxamyl _{O-TKI}	7.143	7.110	99.54	11.94	12.00	0.46
	14.286	13.240	92.68	13.55	14.62	7.32
	35.714	32.160	90.05	11.77	13.07	9.95
Dimethoate _{O-TKI}	7.143	7.370	103.20	8.16	7.91	-3.20
	14.280	14.681	102.80	7.22	7.02	-2.80
	35.700	34.295	96.06	8.73	9.09	3.94
Dioxacarb _{O-TKI}	1.780	1.643	92.30	17.51	18.97	7.70
	3.570	3.329	93.25	4.49	4.81	6.75
	8.930	8.784	98.37	13.03	13.25	1.63
Atrazine _{HILL}	0.107	0.114	106.50	5.53	5.35	-6.50
	0.250	0.247	98.80	7.79	8.11	1.20
	0.750	0.666	88.80	10.48	11.74	11.20
Chlortoluron _{HILL}	0.071	0.062	87.32	11.11	12.71	12.70
	0.143	0.129	90.21	8.88	9.99	9.79
	0.357	0.335	93.84	5.56	5.95	6.16
Metoxuron _{HILL}	0.357	0.373	104.50	8.98	8.59	-4.50
	0.714	0.667	93.42	3.69	3.96	6.58
	1.786	1.656	92.72	7.02	7.57	7.28
Cyanazine _{HILL}	0.107	0.107	100.00	9.61	9.59	0.00
	0.143	0.143	100.00	9.43	9.42	0.00
	0.357	0.327	91.60	9.16	9.99	8.40

Table 4 Method validation parameters of E β NA-TLC detection and AgUV-TLC detection method for the marker and selected compounds

Analyte _{detection method}	Fortification mg kg ⁻¹	Found, mg kg ⁻¹	Recovery, Q, %	SD	RSD %	Accuracy %
Parathion-M _{EβNA}	0.107	0.95	88.79	12.92	14.48	11.20
	0.200	0.175	87.5	3.91	4.47	12.50
	0.600	0.541	90.17	4.00	4.41	9.83
Dichlorvos _{EβNA}	1.428	1.356	94.96	9.29	9.78	5.4
	2.857	2.675	93.63	16.85	17.99	6.37
	7.143	6.208	86.91	1.58	1.82	13.10
Oxamyl _{EβNA}	1.428	1.295	90.69	13.04	14.38	9.31
	2.857	2.490	87.15	8.88	10.19	12.80
	7.143	6.115	85.61	3.75	4.39	14.40
Carbaryl _{EβNA}	0.714	0.682	95.52	15.33	16.4	4.48
	1.428	1.249	87.46	14.6	16.08	12.50
	3.571	3.185	89.19	10.28	11.52	10.80
Chlorpyrifos _{EβNA}	0.036	0.033	91.67	12.85	13.67	8.33
	0.050	0.42	84.00	8.75	10.28	16.00
	0.150	0.131	87.33	10.12	11.63	12.70
Triforin _{AgUV}	14.280	13.950	97.69	10.8	11.5	2.31
	28.570	25.340	88.69	10.89	12.28	11.30
	71.430	64.950	90.93	6.47	7.12	9.7
Dieldrin _{AgUV}	3.570	3.10	84.31	4.6	4.81	15.70
	7.140	6.920	96.92	14.76	15.23	3.8
	17.86	16.580	92.83	12.23	13.18	7.17

findings of Ambrus et al. [22], LOD values are very high in the sample matrix. The reason may be that it is used as a very small sample equivalent for loading the plates to in order to verify determined MDQ. In our case, it was 10 mg sample mass in 20 μ L spotted extract volume. By carrying out only GPC cleanup, the 10 mg sample equivalent did not affect the detectability of the compounds with the detection procedures except in the case of detection with the AgUV method, where only a 5-mg sample equivalent could be spotted. To increase the loadability to the TLC plate, an additional cleanup procedure was required. This in turn corresponded with the decrease at the LOQ_e values. These comments are parallel with Füzési and Su-

san's [14] findings that the loadability of the TLC plate increased with the combination of GPC and silica cartridge cleanup.

Calibration and linear range

Standard concentrations of calibration mixtures embraced all fortification levels and were spotted on the TLC plates in duplicate. Linear calibration curve between the spot diameter and the analyte concentration is used in this study. This can be expressed as $y = a + bx$, where y is the spot diameter (mm) and x is the concentration analyte. The spot diameter

Table 5 Method validation parameters of GC-NPD detection for some compounds

Analyte	Fortification mg kg ⁻¹	Found, mg kg ⁻¹	Recovery, Q, %	SD	RSD %	Accuracy %
Atrazine	1.786	1.814	101.60	4.83	4.76	-1.60
	3.571	3.869	108.30	8.18	7.54	-8.30
	8.928	9.132	102.30	4.99	4.88	-2.30
Diuron	2.142	2.125	99.210	4.15	4.18	0.79
	4.286	4.473	104.40	2.74	2.63	-4.40
	10.714	11.196	104.50	9.23	8.83	-4.50
Parathion-M	0.107	0.097	90.65	12.47	13.75	9.35
	0.200	0.170	85.00	10.97	12.91	15.00
	0.600	0.569	94.83	7.259	7.65	5.17
Dichlorvos	1.428	1.535	107.50	11.19	10.41	-7.50
	2.857	2.925	102.40	18.54	18.11	-2.40
	7.143	8.463	118.50	12.04	10.16	-18.00
Chlorpyrifos	0.036	0.041	113.90	10.74	9.59	-14.00
	0.050	0.050	100.00	6.59	6.20	0.00
	0.150	0.135	90.00	6.90	6.75	10.00

(y) and the concentration (x) compound is entered into an Excel spreadsheet to calculate intercept (a), slope (b) and correlation coefficient, r^2 [22]. To check the linearity, regression equations, corresponding regression coefficients, and relative residual standard deviations ($S_{\Delta y/y}$) of three-level calibrations with the corresponding TLC detection method are presented in Table 2. $S_{\Delta y/y}$ were between 0.002 and 0.043, which was lower than the acceptable limit of 0.1.

The linear range for each compound extends from the MDQ value to about 4–8 times the MDQ, which allows spotted pesticide in the fortified sample to be within the calibration range, is also given in Table 2. Since 20 μL of extract and/or standard solutions were applied in uniform spots of about 4–6 mm on the plate, calculation of the linear range was performed by dividing by 20. For example, for Atrazine_{OTKI}, the linear range of 12.5–62.5 ng, which equals one and five times the found MDQ (Table 1), respectively, is seen in Table 2 as 0.625–3.125 ng μL^{-1} .

Recovery

The recoveries of the pesticides determined on 2 days with four detection methods are given in Tables 3 and 4. A recovery test was performed at three fortification levels, in five analytical portions from each level and duplicate spotting of the sample extracts on each plate. The recovery is obtained by dividing the found value by the fortification level of the analyte. The recoveries ranged between 84.00 and 106.50%. They are within the limits based on the analyte content in the sample [6, 7].

Precision

Precision refers to the closeness of repeated measurements. The within-laboratory reproducibility of the measurements, characterized by the RSD value of the recoveries was between 1.82 and 18.97% (Tables 3 and 4). Most of them fit the expected intralaboratory repeatability RSDs based on the fortification concentration [6, 7].

Accuracy of method

Accuracy is the closeness of the agreement between the result of measurement and a true value of the measurand [24]. The calculated accuracies are given in the last columns of each table (Tables 3 and 4).

Confirmation with GC-NPD analysis

The same sample extracts, which were applied on TLC plates, were checked with GC analyses. The recovery results of GC analyses in five analytical portions from each fortification level and duplicate injecting to GC are presented in Table 5. Recoveries were between 85.00 and 118.50% with RSDs of 2.63–18.11%. The recovered values varied based on the analyte content in the sample. Most fit the required intralaboratory repeatability, based on fortification concentration [6, 7].

Calculations of standard deviations of relative residuals, that is, the measure of relative deviations of the system, and coefficient of correlations that show how well the experimental points fit to a straight line, are presented in Table 2. Standard deviations of relative residuals with five-level calibration were between 0.012 and 0.099. The linear ranges for a compound, which allows injected pesticide in fortified sample, is within the calibration range, analyzed GC-NPD, are also included in the table.

Conclusion

All methods used for pesticide residue determinations must be validated prior to implementation in the analysis of laboratory samples. The analyst can face many problems during the performance of any method. The best way to minimize method problems is to perform adequate validation experiment.

The four TLC detection methods validated based on single-laboratory validation criteria are in this work. Some parameters of method validation, such as recovery, precision, accuracy, calibration and function were found within the required range. These parameters were also confirmed by GC-NPD analysis for some compounds. According to our findings, a 10-g sample matrix is not enough to achieve the required LOQ for most of the compounds applying TLC detection. Therefore, to get low LOQ, the loadability of the TLC plate should be increased by combining GPC with another cleanup system, such as silica cartridge.

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