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Fluorescence polarization immunoassay for rapid screening of the pesticides thiabendazole and tetraconazole in wheat

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Keywords:	fungicides, thiabendazole, tetraconazole, fluorescence polarization immunoassay, wheat

Fluorescence polarization immunoassay for rapid screening of the pesticides thiabendazole and tetraconazole in wheat

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Abstract

Fluorescence polarization immunoassays (FPIAs) for thiabendazole and tetraconazole were first developed. Tracers for FPIAs of thiabendazole and tetraconazole were synthesized and the tracers' structures were confirmed by HPLC-MS/MS. The 4-aminomethylfluorescein-labeled tracers allowed achieving the best assay sensitivity and minimum reagent consumption in comparison with aminofluorescein-labeled and alkyldiaminefluoresceinthiocarbamyl-labeled tracers. Measurements of fluorescence polarization were performed using a portable device. The developed FPIA methods were applied for the analysis of wheat. Fast and simple sample

preparation technique earlier developed by authors for pesticides was adapted for thiabendazole and tetraconazole. The limits of detection of thiabendazole and tetraconazole in wheat were 20 and 200 μg/kg, and the lower limits of quantification were 40 and 600 μg/kg, respectively. The recovery test was performed by two methods—FPIA and HPLC-MS/MS. The results obtained by FPIA correlated well with those obtained by HPLC-MS/MS (r2 = 0.9985 for thiabendazole, r2 = 0.9952 for tetraconazole). Average recoveries of thiabendazole and tetraconazole were 74±4% and 72±3% by FPIA, and average recoveries of thiabendazole and tetraconazole were 86±2% and 74±1% by HPLC-MS/MS (n=15).

Keywords: fungicides, thiabendazole, tetraconazole, fluorescence polarization immunoassay, wheat.

Abbreviations

AF – aminofluorescein;

AMF – 4-aminomethylfluorescein;

BSA – bovine serum albumin;

$(CH_2)_4$DF – butylenediaminefluoresceinthiocarbamyl;

$(CH_2)_6$DF – hexamethylenediaminefluoresceinthiocarbamyl;

CR – cross-reactivity;

DCC – dicyclohexylcarbodiimide;

EDF – ethylenediaminefluoresceinthiocarbamyl;

ELISA – enzyme-linked immunosorbent assay;

FI – fluorescence intensity;

FITC – fluorescein isothiocyanate isomer I;

FPIA - fluorescence polarization immunoassay;

HPLC-MS/MS – high-performance liquid chromatography coupled with tandem mass spectrometry;

LOD – limit of detection;

MRL – maximum residue level;

NHS – N-hydroxysuccinimide;

SD – standard deviation;

TLC – thin-layer chromatography.

Introduction

Modern agriculture is an extensive industry where profit is affected by many factors, including plant pests and diseases. Pesticides are chemical agents targeted to be toxic to living organisms that are used to minimize yield losses. Pesticide residues in foodstuffs are controlled by legislation in many countries because of unintended pesticide toxicity for nontargeted living organisms, including humans.

Thiabendazole and tetraconazole are fungicides with protective and curative actions used against pathogens of fruits, vegetables, and cereals. Thiabendazole belongs to the benzimidazole class of pesticides, and it is applied for post-harvest treatment of food crops before dispatching for storage. Tetraconazole is a triazole class pesticide used primarily to control diseases of the vegetative organs of plants. These compounds have low toxicity to mammals [1,2]. Nevertheless, it is necessary to control their content in foodstuffs to avoid cases of chronic poisoning. For most plant products, the Maximum Residue Levels (MRLs) of thiabendazole are fixed at 0.01–0.05

mg/kg (lower limits of analytical determination) according to European regulations [3] and at 0.2–5.0 mg/kg according to Russian Hygienic standards [4]. The MRLs of tetraconazole for most plant products range from 0.02 to 0.3 mg/kg according to the European Union (EU) [5]. In the Russian Hygienic standards, the MRLs of tetraconazole are established for cereals at 0.2 mg/kg and sugar beets at 0.05 mg/kg [4].

Actually liquid and gas chromatography with different types of detectors are the main tools for pesticide analysis [6–11]. Chromatographic methods have advantages such as high sensibility and reliability, but the equipment for these methods is quite expensive, their productivity is relatively low, and such methods require laborious and time-consuming preliminary sample treatment. To make the testing of large number of samples cheaper and faster, chromatographic analysis is accomplished by preliminary screening tests. The main purpose of preliminary screening is to reduce the number of samples for confirmatory (chromatographic) analysis. This wide screening is mainly focused on the most typical contaminants for the given territory, given kind of samples, etc. For screening purposes, immunoassay methods are the most suitable because of their specificity, sensitivity, rapidity, and low cost [12, 13]. Formerly, application of enzyme-linked immunosorbent assay (ELISA) [14–16], strip-based immunoassays [17], and a surface plasmon resonance [18] method have been reported for thiabendazole analysis, and ELISA methods [19–21] have been applied for tetraconazole analysis. Besides, pseudoimmunoassay based on molecularly imprinted polymers has been reported for thiabendazole analysis [22]. No publications have been reported for fluorescence polarization immunoassay (FPIA) of these compounds.

The main advantages of FPIA as compared with other immunoassay techniques are its rapidity and simple manipulation (caused by one-stage homogeneous interaction of all analytical

reactants and immediate changes of registered fluorescence polarization after immune binding). However, due to one-stage protocol without separation of formed immune complexes from initial reaction media the FPIA results are often sensitive to interfering matrix components, and so the assays in such cases should be accomplished by preliminary sample preparation procedures.

To date, preparation techniques for various kinds of samples have been successfully adapted for determination of medicines, pesticides, mycotoxins, and other compounds using FPIA [23, 24]. Recently, we developed FPIAs for triazophos and carbaryl analysis including sample preparation technique for wheat samples [25]. This research extends the frontiers of this technique to other compounds and describes the first FPIAs methods for thiabendazole and tetraconazole.

Materials and methods

Reagents

Thiabendazole, tetraconazole, fluorescein isothiocyanate isomer I (FITC), 4-aminomethylfluorescein (AMF), aminofluorescein (AF), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), ethylenediamine dihydrochloride, 1,4-butylenediamine, and 1,6-hexamethylenediamine were purchased from Sigma Aldrich (St. Louis, MO, USA). Thin-layer chromatographic (TLC) plates (silica gel) were purchased from Merck (Darmstadt, Germany). All organic solvents and chemical reagents were of analytical reagent grade. Borate buffer (BB, 0.05 M, pH 8.6) with NaN_3 (0.01%) was used as a diluent for immunoreagents in FPIA.

Haptens TN3C (3-[2-(1,3-thiazol-4-yl)-1*H*-benzimidazole-1-yl]propanoic acid), TN6C (3-[2-(1,3-thiazol-4-yl)-1H-benzimidazol-1-yl]hexanoic acid), and hapten DTPH (6-[2-(2,4-

dichlorophenyl)-3-(1*H*-1,2,4-triazole-1-yl)propoxy]hexanoic acid) were earlier obtained by A. Montoya (Universitat Politècnica de València, València, Spain). TN3C-BSA immunogen was synthesized by the active ester technique [12] and used to obtain monoclonal antibodies against thiabendazole (LIB-TN3C-13). DTPH-BSA was synthesized by the active ester technique [18] and used to obtain monoclonal antibodies against tetraconazole (LIB-DTPH-41).

Equipment

Measurements of fluorescence intensity and fluorescence polarization were performed using a portable device, Sentry 200 (Ellie, Wauwatosa, WI USA). Data were processed using Origin 8.5.1 software (OriginLab Corporation, Northampton, MA, USA). Mass-spectrometric data were obtained using a tandem mass-spectrometer, Q-Exactive, coupled to a liquid chromatograph DionexUltiMate 3000. Ionization of samples was performed using a HESI-II ion source (Thermo Scientific, Waltham, MA, USA). Possible structures of fragment ions were obtained using HighChem Mass Frontier 7.0 software from Thermo Scientific.

Synthesis of tracers

Labels purchased from Sigma Aldrich (AMF and AF) and labels synthesized by us [ethylenediaminefluoresceinthiocarbamyl (EDF), butylenediaminefluoresceinthiocarbamyl ($(CH_2)_4$DF), and hexamethylenediaminefluoresceinthiocarbamyl ($(CH_2)_6$DF)] were used for the synthesis of tracers. EDF was synthesized from FITC and ethylenediamine dihydrochloride as described previously [26]. $(CH_2)_4$DF and $(CH_2)_6$DF were synthesized following the same technique used for EDF synthesis in which 1,4-butylenediamine and 1,6-hexamethylenediamine

were used instead of ethylenediamine. Thiabendazole and tetraconazole do not contain functional groups for synthesis of tracers, so their functionalized derivatives were used (Fig. 1).

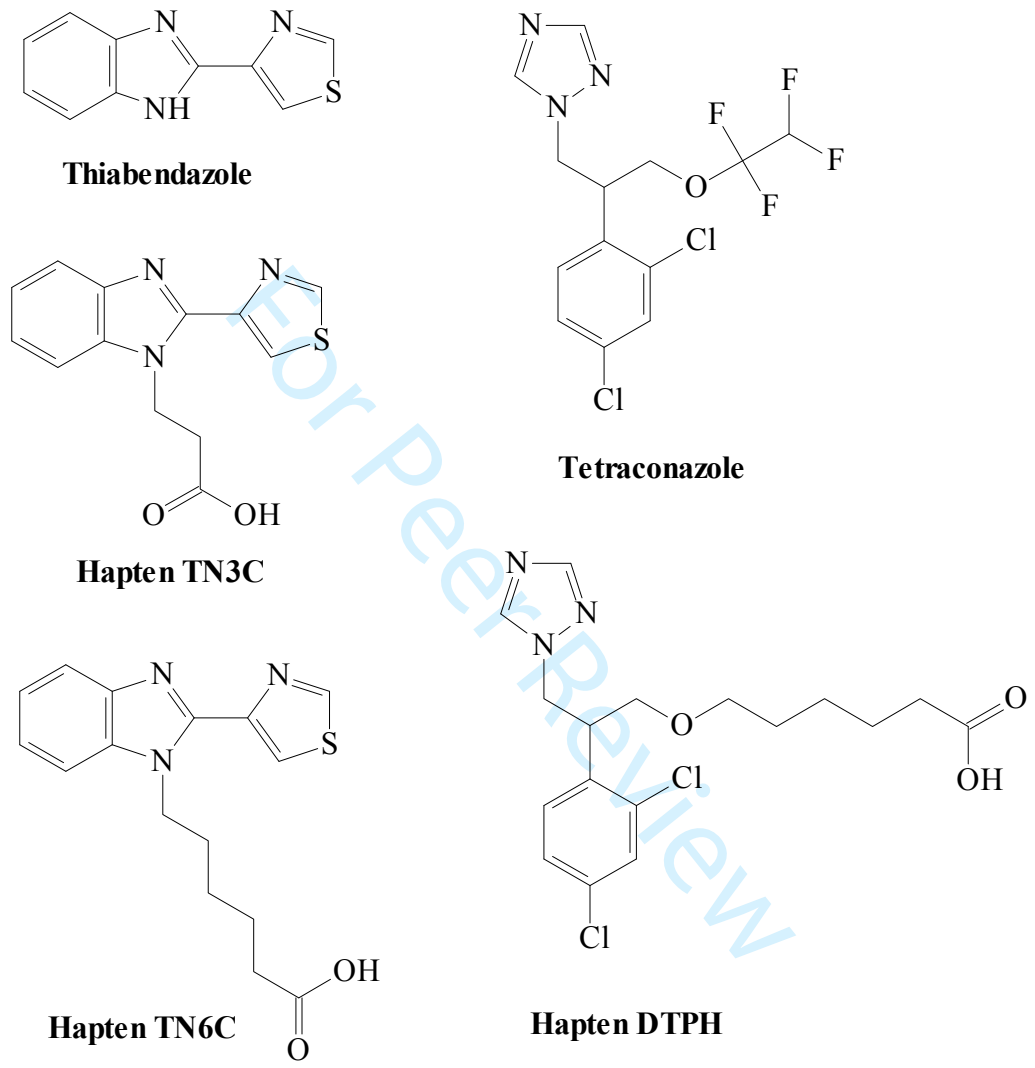


Fig. 1 Chemical structures of analytes and haptens used for the preparation of tracers

Tracers were synthesized as follows: 10 μmol of hapten were dissolved in dimethylformamide (1 mL), then 4.3 mg of DCC (20 μmol) and 2.3 mg of NHS (20 μmol) were

added to the solution. The reaction mixture was incubated for 12 h while stirring. The obtained precipitate was separated by centrifugation. Subsequently, 5 µmol of the fluorescent label were added to the supernatant, and the reaction mixture was mixed and incubated for 24 h.

Tracers were separated from the reaction mixtures by TLC. Tracers with diamine-FITC labels were chromatographed with $CHCl_3$:CH_3OH:CH_3COOH (80:16:1, v/v) as the mobile phase. Bands of tracers with R_f = 0.8 were eluted from the TLC plate using methanol. Tracers with the AF label were chromatographed with $CHCl_3$:CH_3OH (5:1, v/v) and bands at R_f = 0.4 were eluted from the TLC plate. The separated fractions were additionally purified using TLC in the mobile phase $CHCl_3$:CH_3OH:CH_3COOH (80:16:1, v/v). After TLC separation, bands at R_f = 0.5 were eluted from the plates using methanol. For separation of the AMF-labeled tracers, the mobile phase $CHCl_3$:CH_3OH (8:1, v/v) was used; bands of tracers were at Rf = 0.5. The success of syntheses and structures of tracers were confirmed by high-resolution tandem mass-spectrometry coupled with high-performance liquid chromatography (HPLC-MS/MS).

FPIA procedure

The concentration of tracer solutions was estimated by fluorescence intensity (FI) measurement and its comparison with the FI for fluorescein. The FI of working solutions of tracers was 20 times higher than the FI of the buffer solution. The concentration of the working solutions of the tracers was approximately 5 nM.

A series of dilutions were prepared to obtain antibody dilution curves. Each solution was two times less concentrated than the previous solution. Aliquots (500 µL) of the diluted antibody solution and 500 µL of the tracer working solution were mixed in each cuvette, and fluorescence

polarization was measured. Dilution curves were produced using the results of the measurements.

The choice of optimal dilution of antibodies and concentration of tracers for the most sensitive PFIA was based on the presented experiments (Fig. 2, 3) in the accordance with common practice of FPIA protocols development [23]. Other parameters of PFIA protocols such as time of reactants incubation (2 min), pH of reaction mixture (8.5), nature and molarity of buffer (0.05 M) were chosen on the basis of previous studies of fluorescein-based FPIA as optimal for efficient immune interaction and fluorescence generation [27].

The FPIA procedure was performed as follows: 50 µL of a standard solution or a sample were mixed in a cuvette with 500 µL of the tracer working solution, and fluorescence polarization was measured. Results of the measurements of standard solutions were processed using Origin software to obtain FPIA calibration curves. The time elapsed during the measurement of the signal from a single sample was approximately 2–4 s.

For experiments in selection of immunoreagents standard solutions were prepared in 10% methanol, for experiments with wheat samples standard solutions were prepared in a mixture of extractant and BB (1:7, v/v). Concentrations of thiabendazole standard solutions were 0.1, 1, 3.5, 10, 30, 100, 1000 ng/ml, concentrations of tetraconazole standard solutions were 1, 10, 35, 100, 300, 1000, 10000 ng/ml.

Data analysis

The curves were plotted in coordinates "logarithm of concentration - mP" or "logarithm of concentration - mP/mP_0", where mP is the measured fluorescence polarization, mP_0 is the fluorescence polarization obtained for zero standard an optimized procedure of the analysis.

These curves were approximated by a 4-parameter sigmoid equation:

$$Y= (A-D)/[1+(x/C)^b]+D,$$

where A is the maximum value of the fluorescence polarization, D is the minimum value of the fluorescence polarization, b is the slope of the curve at the IC_{50} point, C (IC50) is the analyte concentration inhibiting the binding of antibodies to the tracer by 50%.

The limit of detection (LOD) was determined by performing the analysis of a blank solution (solvent without analyte) 20 times. The LOD was calculated using a calibration curve as the concentration corresponding to the difference between the average (blank) signal and three times the standard deviation. 10% methanol was used as a blank solution to calculate LOD in standard solutions and 8-fold diluted extract was used as a blank solution to calculate LOD in wheat samples.

The lower limit of quantification (IC_{20}) was calculated as the analyte concentration inhibiting binding of the tracer with antibodies by 20%; the upper limit of quantification (IC_{80}) was calculated as the analyte concentration inhibiting binding of the tracer with antibodies by 80%.

The cross-reactivity (CR) was calculated in accordance with Equation 1:

$$CR(\%) = (IC_{50}(\text{analyte}) / IC_{50}(\text{relative compound})) \times 100\% \quad (1)$$

where IC_{50} is the concentration inhibiting binding by 50%.

Obtaining of contaminated wheat grain

The initial wheat grain preparations did not contain analytes, as shown by the HPLC-MS/MS. The grain was ground in a homogenizer, then 1-gram samples of flour were contaminated. Methanolic solutions of thiabendazole (1000 ng/ml) and tetraconazole (10,000 ng/ml) were used for this purpose. The obtained preparations contained 40, 100, 200, 300, 400 µg/kg of thiabendazole and 600, 1300, 1900, 2500 , 3200 µg/kg of tetraconazole. The

contaminated samples were left for 24 hours in a fume hood at a temperature of 22 ° C and a humidity of 40%.

Sample preparation

Two milliliters of 70% methanol were added to 1 g of ground wheat. Samples were shaken thoroughly and ultrasonicated for 30 min. The methanol fraction was diluted eight times with the BB. The obtained precipitate was separated by centrifugation (5 min, 1400 *g*). The supernatant was analyzed using FPIA and HPLC-MS/MS methods.

HPLC-MS/MS-analysis

A Hypersil Gold aQ column, Thermo Scientific, Waltham, MA, USA (150 × 2.1 mm i.d., 3 µm) with a Hypersil Gold aQ pre-column (10 × 2.1 mm i.d., 3 µm) was used for chromatography. The column temperature was maintained at 30°C. The mobile phase consisted of solvent A (0.1% formic acid in a mixture of water with acetonitrile 95:5, v/v) and solvent B (0.1% formic acid in acetonitrile). The mobile phase gradient started at 5% B (0.0–2.0 min) and increased to 95% B over 15.0 min, remained constant until 18.0 min, and was followed by column equilibration to the initial conditions of 5% B (19.0–23.0 min). The flow rate of the mobile phase was 0.5 mL/min, and the injection volume was 2 µL.

Mass spectrometric detection was performed under the following conditions: sheath gas (nitrogen) flow rate, 0.4 L/min; auxiliary gas (nitrogen) flow rate, 0.1 L/min; sweep gas (nitrogen) flow rate, 0.05 L/min; capillary voltage, 4.00 kV; capillary temperature, 270°C; and auxiliary gas heater temperature, 280°C. The HESI-source was operated in the positive ion mode. MS spectra were recorded under atmospheric pressure in the range of *m/z* 100–1500 Da,

the resolution was 35,000, and the isolation window was 5 ppm. MS/MS spectra were obtained using collision-induced dissociation. Collision energy for the tracers TN3C-EDF, TN6C-EDF, TN3C-AMF, TN6C-AMF, TN3C-AF, TN6C-AF, and DTPH-AF was 35%, and for the tracers DTPH-EDF, DTPH-$(CH_2)_4$DF, DTPH-$(CH_2)_6$DF, and DTPH-AMF was 20%. Collision energy for thiabendazole and tetraconazole in confirmation analysis of wheat samples was respectively 40% and 35%. Conditions of quantitation and confirmation analysis are summarized in Table 1.

Table 1 HPLC-MS/MS conditions for thiabendazole and tetraconazole analysis

Analyte	ESI mode	Precursor ion $[M+H]^+$, Da	RT, min	Quantitation product ion (m/z)	Confirmation product ion (m/z)
Thiabendazole	positive	202.0439	3.9	175.0330	131.0608
Tetraconazole	positive	372.0294	10.1	158.9768	70.0405

Results and Discussion

FPIA development

The FPIA method is based on the competition between an antigen and a fluorescently-labeled antigen–tracer for a limited number of antibody binding sites. Immunoreagents should be selected so that the tracer can easily form the bond with the antibody and be displaced by the analyte. During the assay development, analytical characteristics of FPIA methods involving different tracers were compared. Structures of tracers were varied in two ways: by selecting different fluorescent labels for synthesis (EDF, AMF, AF) and by varying the fragment

connecting the antigen with the fluorescent label (the length of carbon bridge in TN3C and TN6C, the length of carbon bridge in EDF, $(CH_2)_4$DF, $(CH_2)_6$DF).

Confirmation of the structures of tracers

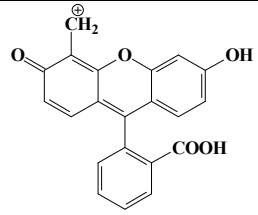
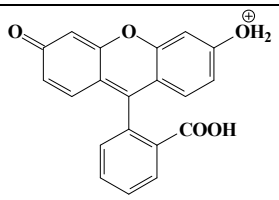
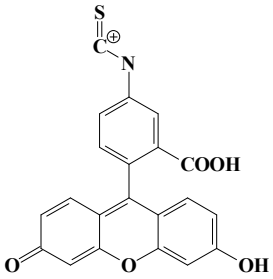
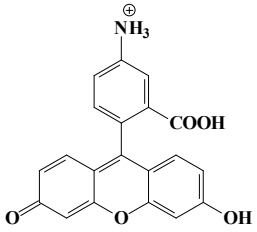
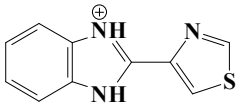
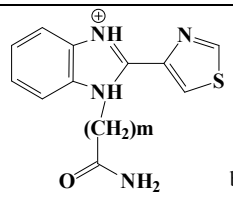
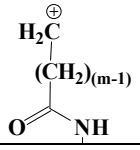
All the tracers used in this work were synthesized for the first time, so it was essential to confirm the success of syntheses and isolation of tracers from the reaction mixtures. HPLC coupled with tandem high-resolution mass spectrometry was used to identify the synthesized compounds.

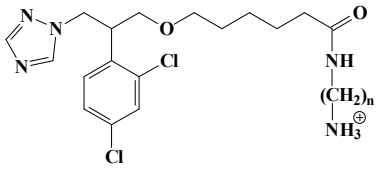
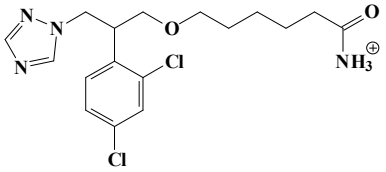
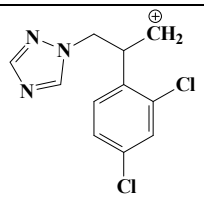
Firstly, full scan mass spectra of tracers were obtained. Signals corresponding to singly and doubly protonated tracer molecules ($[M+H]^+$ and $[M+2H]^{2+}$) were present in positive ion mode spectra. Singly charged ions were used as precursor ions to obtain MS/MS spectra.

Peaks that were characteristic for fluorescent fragments of tracers were observed in MS/MS spectra of all the tracers. Peaks at m/z 345.0763 and 333.0763 were observed in spectra of tracers with the AMF label, peaks at m/z 390.0436 and 348.0872 were observed in spectra of tracers with diamine-FITC labels, and a peak at m/z 348.0872 was observed in spectra of tracers with the AF label. Molecular formulas and potential chemical structures are shown in Table 2.

Table 2 Accurate masses, molecular formulas and potential chemical structures of the product ions of tracers

Tracer molecule or tracer molecule section	m/z of product ion, Da	Molecular formulas of product ions	Potential structure of product ion

AMF-	345.0763	$C_{21}H_{13}O_5$	
	333.0763	$C_{20}H_{13}O_5$	
$(CH_2)_nDF$-[a]	390.0436	$C_{21}H_{12}O_5NS$	
$(CH_2)_nDF$-, AF-	348.0872	$C_{20}H_{14}O_5N$	
TN3C-, TN6C-	202.0433	$C_{10}H_8N_3S$	
TN3C-AMF	273.0810	$C_{13}H_{13}N_4OS$	 b
TN6C-AMF	315.1280	$C_{16}H_{19}N_4OS$	
TN3C- EDF	115.0866	$C_5H_{11}N_2O$	

TN6C- EDF	157.1341	$C_8H_{17}N_2O$	
DTPH-EDF	428.1620	$C_{19}H_{28}Cl_2N_5O_2$	
DTPH-$(CH_2)_4$DF	456.1933	$C_{21}H_{32}Cl_2N_5O_2$	
DTPH-$(CH_2)_6$DF	484.2246	$C_{23}H_{36}Cl_2N_5O_2$	
DTPH-AMF	385.1198	$C_{17}H_{23}Cl_2N_4O_2$	
DTPH-AF	254.0252	$C_{11}H_{10}Cl_2N_3$	

[a]n is the number of methylene groups; n=2, 4, 6 for tracers with EDF, $(CH_2)_4$DF, and $(CH_2)_6$DF labels, respectively;

[b]m is the number of methylene groups; n=2 and 5 for tracers with TN3C and TN6C haptens, respectively

In the MS/MS spectra of tracers synthesized from the haptens TN3C and TN6C, an intense peak of a product ion corresponding to the antigen fragment of the tracer molecules (m/z 202.0433) was observed. Also, peaks corresponding to a carbon bridge between the antigen and the fluorophore of the tracer molecules (m/z 115.0866 and 157.1341) and peaks corresponding to a carbon bridge connected with the antigen section of the tracer (m/z 273.0810 and 315.1280) were obtained.

In the MS/MS spectra of the tracers synthesized from the DTPH hapten and the diamine-FITC labels, peaks corresponding to a carbon bridge connected with the antigen section of the

molecule (m/z 428.1620, 456.1933, 484.2246) were observed. In the MS/MS spectrum of DTPH-AMF, a peak corresponding to the hapten (m/z 385.1198) was observed. In the MS/MS spectrum of DTPH-AF, a peak corresponding to the antigen section of the tracer molecule (m/z 254.0252) was observed.

Selection of immunoreagents

Tracers for thiabendazole analysis Monoclonal antibodies obtained against the immunogen TN3C-BSA by means of active ester method [14] were used to develop the FPIA of thiabendazole. TN3C is a thiabendazole derivative containing propanoic acid with a terminal carboxyl group as a spacer arm. TN3C and its homolog TN6C containing hexanoic acid with a terminal carboxyl group were used for the synthesis of tracers. AF, AMF, and EDF were used for the synthesis of tracers as fluorescent labels. Firstly, antibody dilution curves were obtained (Fig. 2, A). Working concentrations of antibodies were chosen from the linear ranges of antibody dilution curves. Concentrations of antibodies were chosen for each tracer to compare them under the same conditions such that the difference between the maximum and minimum mP values on the calibration curve would be the same (70–80 mP). In subsequent experiments, antibodies were used in these concentrations. For illustrative purposes, data are shown at the coordinates of the plot of the mP/mP_0 versus analyte concentration, where mP is the measured fluorescence polarization, and mP_0 is the fluorescence polarization of the blank solution analyzed by the FPIA method (Fig. 2, B). Experiments were made in 3 or 4 replicates and errors varied from 1 to 5%.

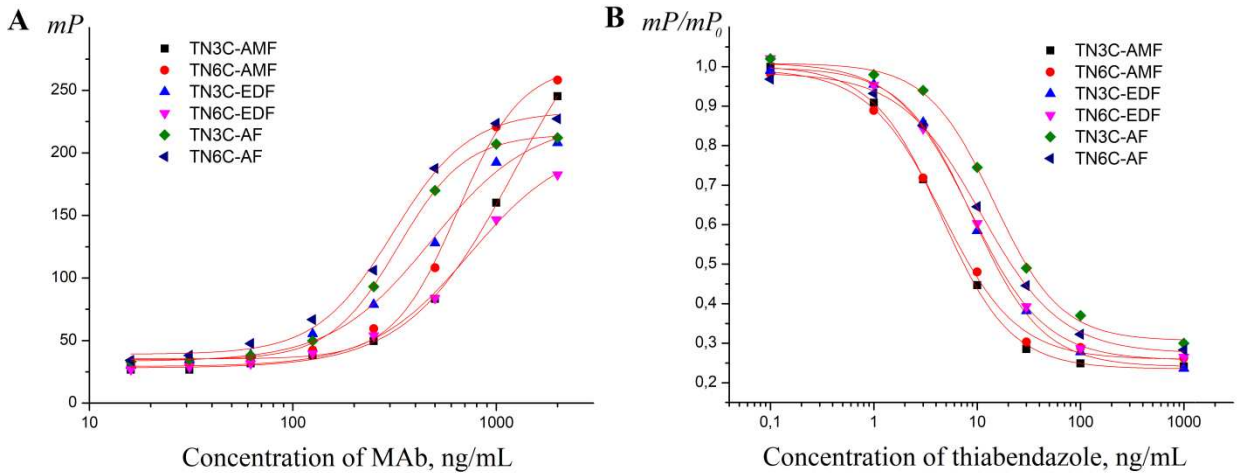


Fig. 2 (A) Antibody dilution curves and (B) calibration curves for thiabendazole determination using different tracers

When using tracers with the same fluorescent label and haptens having spacer arms of different lengths, the sensitivity of the FPIA method remained the same. Also, linear ranges of all the calibration curves accord to close concentrations; the minimal thiabendazole concentrations for these ranges with different tracers vary from 1.5 to 5.0 ng/mL, and the maximal thiabendazole concentrations – from 13 to 41 ng/mL (Table 3).

The choice of antibody concentrations for analysis was based on the need to ensure sufficient changes of analytic signal at the lowest antibody concentration and, by this way, to reach the best sensitivity. The standard deviations for the mP values in our experiment were from 0.8 to 2.5 mP. In comparison with the values of the analytical signal of 70-80 mP the deviations are less than 5%.

Using tracers with the AMF label yielded a slight advantage in sensitivity, whereas using TN6C-AMF reduced the amounts, and therefore the costs, of the antibodies required for the analysis. Consequently, TN6C-AMF was chosen for the subsequent experiments.

Table 3 Characteristics of thiabendazole determination using different tracers

Tracer	Working concentration of antibodies, ng/mL	Linear range, ng/mL
TN3C-AMF	670	1.5–13
TN6C- AMF	500	1.5–15
TN3C-EDF	400	3.0–27
TN6C-EDF	670	2.8–27
TN3C-AF	280	5.0–41
TN6C-AF	250	3.2–38

Tracers for tetraconazole analysis

Antibodies used in the development of the FPIA for tetraconazole were obtained against the synthesized by active ester method immunogen DTPH-BSA [20]. The hapten DTPH is a tetraconazole derivative containing a terminal carboxyl group. In this study, the hapten DTPH and the fluorescent labels AMF, EDF, $(CH_2)_4$DF, $(CH_2)_6$DF, and AF were used for the synthesis of tracers. Antibody dilution curves and calibration curves were obtained using the synthesized tracers (Fig. 3).

The difference between the maximum and minimum possible values of fluorescence polarization was calculated using the antibody dilution curves for each tracer. For DTPH-AMF, DTPH-EDF, DTPH-$(CH_2)_4$DF, DTPH-$(CH_2)_6$DF, and DTPH-AF this difference was 130, 100, 70, 90, and 60 mP, respectively. The calibration curve for DTPH-AMF was obtained using a working solution of antibodies with a concentration of 1000 ng/mL to adjust the difference between the maximum and minimum mP values to be equal to 70–80 mP. Calibration curves

generated for other tracers were obtained using the same concentration of antibodies as used for DTPH-AMF. Other tracers bind antibodies significantly worse, and so the same range of fluorescence polarization values cannot be reached for them. Therefore, the conditions were standardized by selecting the same antibody concentration for all, namely 1000 ng/mL.

Calibration curves were prepared in the coordinates of plots of mP versus tetraconazole concentration for ease of comparison (Fig. 3, B). Experiments were made in 3 replicates and errors varied from 2 to 5%. The tracers DTPH-AMF, DTPH-EDF, DTPH-$(CH_2)_4$DF, and DTPH-$(CH_2)_6$DF yielded the same FPIA sensitivity (Table 4). However, using antibodies at a concentration of 1000 ng/mL with DTPH-AMF allowed operation in the range approximately from 30 to 100 mP (ΔmP = 70), whereas using of any of the other tracers with antibodies in the same concentration reduced the ΔmP value to 40–50. Therefore, DTPH-AMF was chosen for FPIA development in wheat samples.

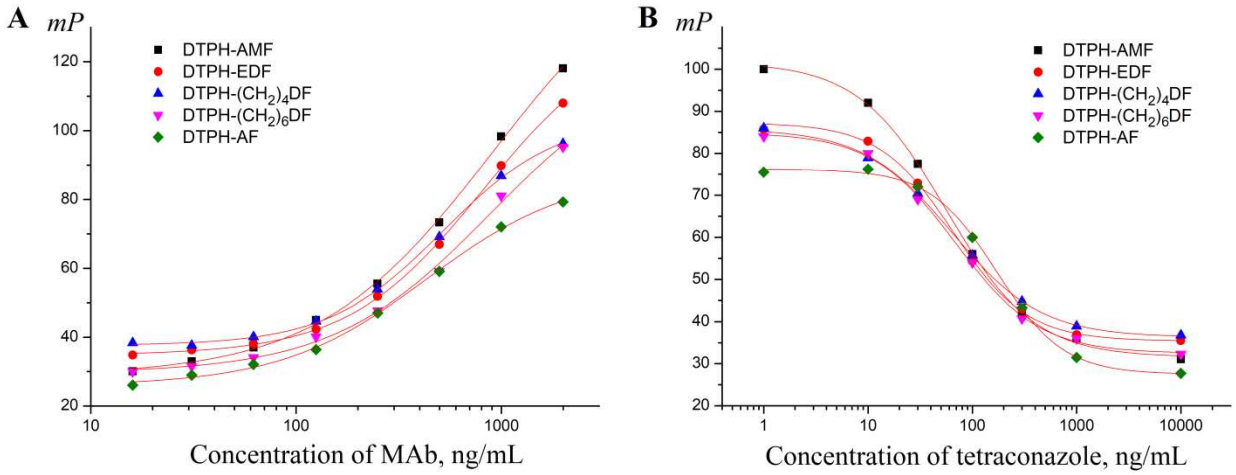


Fig. 3 (A) Antibody dilution curves and (B) calibration curves with different tracers for tetraconazole determination

Table 4 Characteristics of tetraconazole determination using different tracers

Tracer	Working concentration of antibodies, ng/mL	Linear range, ng/mL
DTPH-AMF	1000	16–210
DTPH-EDF	1000	21–180
DTPH-$(CH_2)_4$DF	1000	20–250
DTPH-$(CH_2)_6$DF	1000	20–250
DTPH-AF	1000	62–480

Characteristics of optimized FPIAs

Calibration curves were prepared using the results of the analyses of thiabendazole and tetraconazole standard solutions (Fig. 4). The linear range of determination for thiabendazole was from 1.5 to 16 ng/mL, and the LOD was 1 ng/mL. The linear range of determination for tetraconazole was from 16 to 210 ng/mL, and the LOD was 10 ng/ml. The specificity of the developed methods was determined by comparison of the cross-reactivity with structurally related compounds. Tetraconazole does not influence the interaction between anti-thiabendazole antibodies and thiabendazole tracer (i.e. does not cause changes in the registered fluorescence polarisation); thiabendazole does not influence the interaction between anti-tetraconazole antibodies and tetraconazole tracer. The specificity of antibodies against thiabendazole was investigated using the most similar compounds—benomyl and carbendazim. The cross-reactivity for both compounds was less than 0.1%. The specificity of antibodies against tetraconazole LIB-DTPH-41 was investigated using pesticides from the triazole class (Table 5). A high cross-reactivity was observed for penconazole (35%) and cyproconazole (23%). Other tested triazoles demonstrated negligible cross-reactivity. These results agree well with results obtained in

previous reports [20] using the ELISA method. When normalized to a tetraconazole cross-reactivity of 100%, penconazole and cyproconazole demonstrated cross-reactivity at the levels of 44% and 33%, respectively. Thus, the developed methods allowed a highly specific determination of thiabendazole and a less specific assay of tetraconazole because LIB-DTPH-41 antibodies exhibited cross-reaction with other chemicals applied in agriculture.

Table 5 Cross-reactivity of anti-tetraconazole antibodies LIB-DTPH-41

Compound	Cross-reactivity, %
Tetraconazole	100
Penconazole	35
Cyproconazole	23
Triadimefon	0.5
Propiconazole	0.3
Difenoconazole	<0.1
Tebuconazole	<0.1
Triadimenol	<0.1
Triticonazole	<0.1

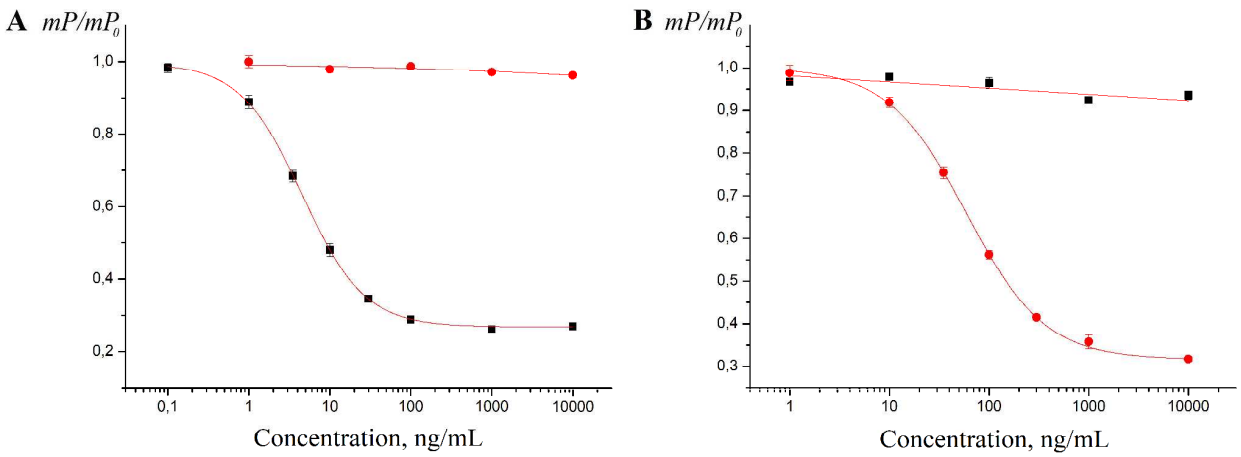


Fig. 4 (A) FPIA standard curve for thiabendazole (■) and cross-reactivity with tetraconazole (●); (B) FPIA standard curve for tetraconazole (●) and cross-reactivity with thiabendazole (■) (n=3)

Analysis of wheat samples

Thiabendazole and tetraconazole can occur in foodstuffs including cereals. The developed FPIA methods for thiabendazole and tetraconazole were applied for the analysis of wheat grain. The FPIA-adopted sample preparation technique for grain was published earlier [25].

Grain is a complex matrix containing proteins, lipids, carbohydrates—starch, hemicelluloses, mucilages, and simple sugars—and mineral compounds. During sample preparation of grain, pesticides are usually extracted with organic solvents. The extracts obtained are then subjected to purification and concentration. Such sample preparation is not suitable for screening methods because it is time-consuming, whereas the main advantage of screening methods is their rapidity.

The sample preparation technique for FPIA in this research included ultrasonic extraction with 70% methanol, dilution of the extract with a buffer solution, and centrifugation. Scheme of sample preparation represented on Fig. 5. The choice of extractant was described in a recent

manuscript [25] devoted to the FPIA of triazophos and carbaryl in wheat. Methanol was determined not to have a significant effect on the sensitivity of thiabendazole and tetraconazole assay.

The extract was diluted to eliminate matrix influence. The optimum extract dilution was determined in the following manner. An extract of uncontaminated wheat was prepared, and it was diluted 2-, 4-, 6-, and 8-fold. The diluted extracts were used to prepare standard solutions of analyte. The obtained standard solutions were analyzed via FPIA, and calibration curves were plotted using the results of this analysis. The calibration curves were compared with those obtained using the results of analysis of standard solutions in extractant diluted 2-, 4-, 6-, and 8-fold. Dilution of the extract was considered sufficient if the results analysis of standard solutions in diluted extracts and the corresponding diluted extractant nearly coincided, and the IC_{20}, IC_{50}, IC_{80}, and mP_0 values differed insignificantly. The 8-fold dilution was concluded to be sufficient for the FPIA of thiabendazole and tetraconazole (Fig. 6, Table 6). Thiabendazole could be determined in wheat in the range from 40 to 500 µg/kg with a LOD value of 20 µg/kg using the developed FPIA method. Tetraconazole could be determined in the range from 600 to 3200 µg/kg with a LOD value of 200 µg/kg.

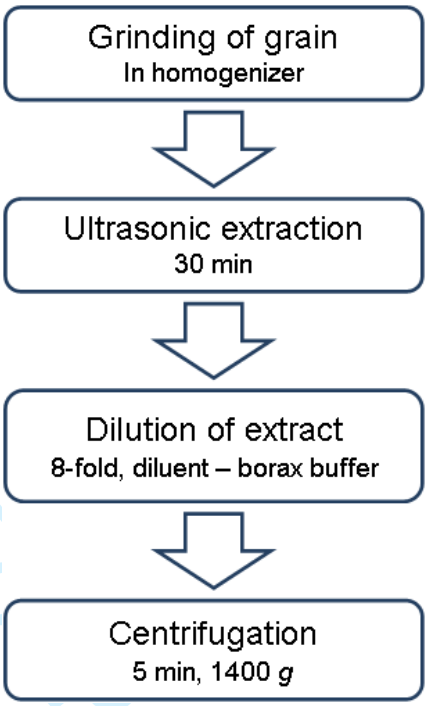


Fig. 5 Scheme of sample preparation

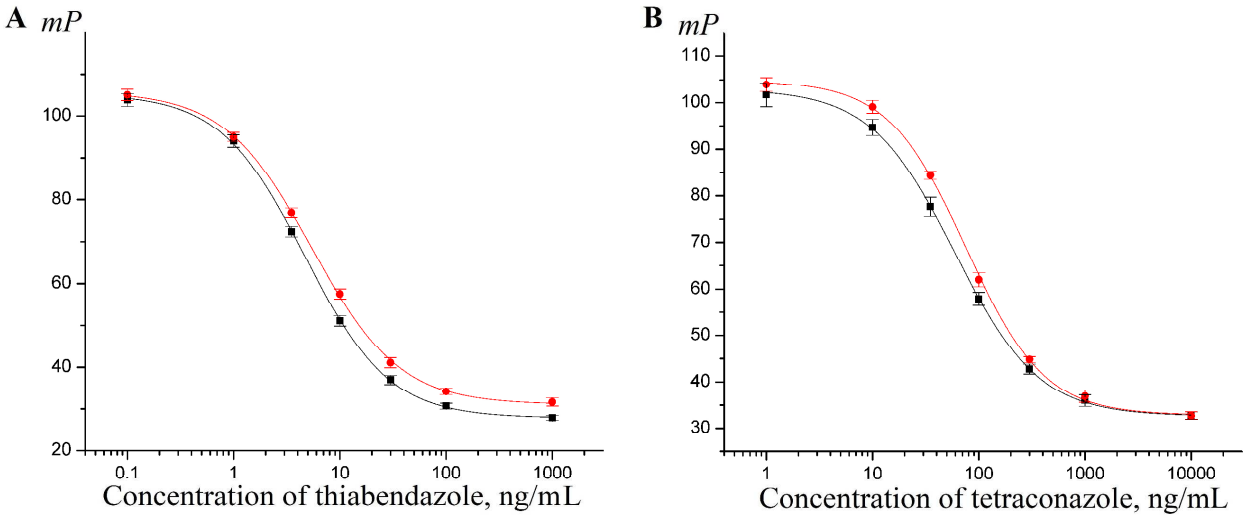


Fig. 6 FPIA standard curves for thiabendazole (A) and for tetraconazole (B) (■ - standards in diluted extractant, ● – standards in diluted extract, n=3)

Table 6 Parameters of sigmoidal fitting for calibration curves

	TBZ	TBZ	TBZ	TBZ

	(extractant)	(extract)	(extractant)	(extract)
IC20	1.3	1.5	17	24
IC50	4.7	5.4	59	76
IC80	17	20	210	245
R^2	0.999	0.999	0.999	0.999
ΔmP	77	75	70	72

Thus, the sample preparation technique developed earlier for the FPIA of triazophos and carbaryl [25] was adapted for thiabendazole and tetraconazole. The dilution ratio of the extract was determined for each compound. For triazophos, thiabendazole, and tetraconazole analysis the minimum dilution ratio was 8, and for carbaryl analysis, it was 4. However, the sensitivity of the carbaryl analysis was adequate for its determination below the existing MRLs even if an 8-fold dilution was used, so the sample preparation technique for the FPIA of these four compounds is identical.

Recovery test

Wheat grain used for the recovery test was not contaminated with pesticides. Wheat samples were grinded; subsequently, they were spiked with thiabendazole and tetraconazole at several concentrations, and the solvent was evaporated for 24 h. Preparation of spiked wheat samples was conducted using the optimized conditions. The obtained diluted extracts were analyzed in parallel by two methods—FPIA and HPLC-MS/MS.

The results of the recovery tests are presented in Table 6. The recoveries of thiabendazole ranged from 71 to 86% by FPIA and from 83 to 89% by HPLC-MS/MS. The coefficient of

variation was less than 10% for the FPIA and less than 4% for the HPLC-MS/MS method (n=3). The recoveries of tetraconazole were from 60 to 77% by the FPIA and from 72 to 75% by the HPLC-MS/MS method; the coefficient of variation was less than 6% for the FPIA and less than 2% for the HPLC-MS/MS method (n=3). The linear correlation between the results obtained by the two methods was observed. The regression equation for thiabendazole was $y = 0.819 x + 0.411$, with an R^2 value of 0.9985, and for tetraconazole it was $y = 0.989 x - 0.467$, with an R^2 value of 0.9952. In general, the results obtained by the FPIA agreed with the results obtained by the HPLC-MS/MS method. Therefore, the developed methods of analysis using a fast and simple sample preparation technique were appropriate for the determination of thiabendazole and tetraconazole.

Table 7 Analytical results and recoveries of thiabendazole and tetraconazole in wheat by the FPIA and HPLC-MS/MS methods.

Spiking level, µg/kg	FPIA		HPLC-MS/MS	
	Detected concentration ± SD, mg/kg	Recovery ± SD, %	Detected concentration ± SD, mg/kg	Recovery ± SD, %
Thiabendazole				
0	N. d.[a]	–	N. d.	–
40	34±3.4	86±8	36±1.1	89±3
100	75±6.8	75±7	88±0.7	88±1
200	144±4.5	72±2	171±6.8	86±3
300	213±6.8	71±2	248±2.8	83±1

400	272±4.6	68±1	340±7.9	85±2
Tetraconazole				
0	N. d.	–	N. d.	–
600	358±23	60±4	439±10	73±2
1300	900±31	69±2	936±9.1	72±1
1900	1470±57	77±3	1430±9.1	75±1
2500	1900±68	76±3	1850±12	74±1
3200	2450±68	77±2	2400±23	75±1

[a]N. d. – not detected.

Conclusions

FPIAs of thiabendazole and tetraconazole were developed for the first time. Tracers for FPIAs of these compounds were synthesized, and their structures were confirmed by HPLC-MS/MS. The influence of the structures of tracers on assay sensitivity was estimated. The sensitivity of the developed FPIAs depended on the structures of the fluorophores and antigen fragments of the tracer molecules and did not depend on the length of the bridge between them. FPIAs of thiabendazole and tetraconazole were applied for analysis of wheat using a sample preparation technique developed earlier that required less than an hour. This sample preparation technique has now been adapted for analysis of the four pesticides triazophos, carbaryl, thiabendazole, and tetraconazole. The LODs of thiabendazole and tetraconazole in wheat were 20 and 200 µg/kg, respectively. The linear range of thiabendazole determination was from 40 to 500 µg/kg, and the linear range of tetraconazole determination was from 600 to 3200 µg/kg. The results obtained by the proposed FPIA method exhibited a good correlation with the results obtained by the HPLC-

MS/MS method. The developed methods are rapid, sensitive, and selective. Therefore, they are appropriate for high-throughput screening of thiabendazole and tetraconazole in wheat.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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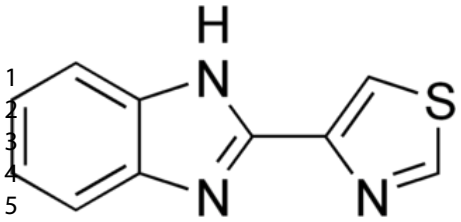
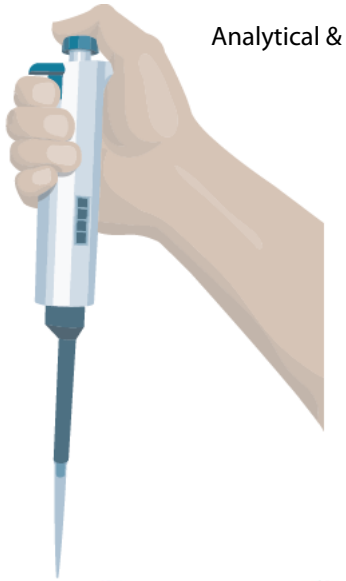
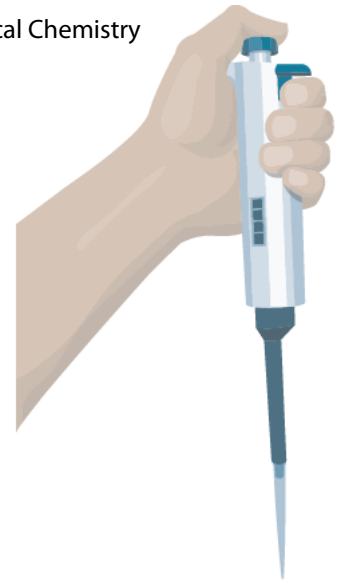
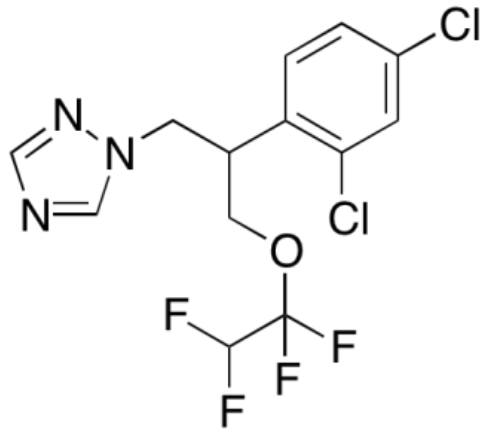


Thiabendazole







Tetraconazole

Two analytes – one procedure

- *Unified sample preparation*
- *Unified immune stage*
- *Unified measurements of fluorescence polarization*