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Development of analytical methods for detection of triazole pesticides based on HPLC with diode array or electrochemical detection

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LIST OF ABREVIATIONS

ACN- Acetonitrile

ad. - Dimensionless

BDDE – Boron-doped diamond electrode

CE – Capillary electrophoresis

CPZ - Cyproconazole

CV – Cyclic voltammetry

DAD – Diode-Array detector

DFZ - Difenoconazole

DPV - Differential pulse voltammetry

ECZ - Epoxiconazole

ED – Electrochemistry detector

E_{det} – Detection potential

FBZ - Fenbuconazole

FCZ - Fluconazole

F_M – Flow rate

GCE – Glassy carbon electrode

HPLC – High-performance liquid chromatography

k' – Capacity factor

LC-ESI – Liquid chromatography coupled to electrospray ionization detector

LDR – Linear dynamic range

LOD – Limit of quantification

LOQ – Limit of detection

MRL – Maximum residual level

M_w – Molecular weight

PB – Phosphate buffer

PGE - Pyrolytic graphite electrode

PNZ – Penconazole

Pot. - Potentiometry

PPZ - Propiconazole

R_t – Retention time

SD – Standard deviation

SPE – Solid phase extraction

s_α – Standard deviation of intercept

t_0 – Death time

TBZ - Tebuconazole

TTZ - Triticonazole

UPLC – Ultra performance liquid chromatography

UV/Vis – Ultraviolet/Visible

λ_{det} – Determined wavelength

ABSTRACT

Analytical methods for the determination of triazolic fungicides are needed because in human beings they can cause growth problems, alteration in sexual differentiation or even cancer. These fungicides used in agriculture are individual limited but not the combination of them being a health risk.

The similarity of the compounds made necessary a prior separation using an HPLC system operating in reversed phase and isocratic mode of elution. The mobile phase used was 57:43 (*v/v*) acetonitrile and phosphate buffer solution. Diode Array detector was chosen in the UV/vis range because of the nonbonding electrons in their structures. The separation conditions were optimized and the linearity of the responses in a wide range of concentrations was checked.

Further, the possibility to employ electrochemical detection was proven utilizing oxidation of these compounds using a boron-doped diamond electrode because of its properties. Nowadays, information of their redox activity is very limited. First, the oxidizability of the compounds was investigated and then an HPLC-ED analytical method was developed.

Métodos de separación y análisis de fungicidas triazólicos son necesarios pues pueden producir problemas de crecimiento, alteración en la diferenciación sexual e incluso cáncer en seres humanos. Estos fungicidas usados frecuentemente en la agricultura están limitados de forma individual pero no cuando se combinan siendo un riesgo para la salud.

Debido a la similitud de los compuestos se ha considerado su separación usando un equipo HPLC en fase inversa y modo de elución isocrático. La fase móvil usada fue 57:43 (*v/v*) acetonitrilo y disolución tampón fosfórica. Como detector se eligió un detector Array UV/Vis debido a los electrones no enlazantes de su estructura. Las condiciones de separación fueron optimizadas y la linealidad del método comprobada en un amplio rango.

Además, la posibilidad de detección electroquímica fue probada utilizando la oxidación de estos compuestos usando un electrodo de diamante dopado con boro. Actualmente, la información de su actividad red-ox es muy limitada. Primero se comprobó su oxidabilidad y posteriormente se desarrolló un método de análisis usando HPLC-ED.

ACKNOWLEDGEMENTS

1. INTRODUCTION

1.1 AZOLES

Azole compounds, including imidazoles and triazoles, are known for the wide range of uses in pharmaceuticals, industries and crops. They have many functions as drug and pesticides working as fungicides, bactericides, herbicides and insecticides. The action mechanism of triazole compounds as antifungals is based on the inhibition of the biosynthesis of ergosterol, which is fundamental in the biosynthesis of plasmatic membranes in fungi . It works inhibiting the activity of the CYP5A1, which catalyzes the demethylation of lanosterol producing the ergosterol [1,2].

The treatment of vegetables and fruits with azole compounds can put human health at risk because they do not act specifically, inhibiting other enzymes which cause growth problems, or even alteration in sexual differentiation [3]. Pesticides may also cause type 2 diabetes, asthma or even cancer [4]. For that reason they have to be regulated and, therefore, it is necessary to have analytical methods for their detection. Nowadays the single azole pesticides in food is regulated, but the problem is that there is no regulation for their mixtures. Therefore, analytical methods for determination of azole pesticides in a variety of matrixes is needed. This study represents a contribution to this problematic aiming at development of HPLC methods for determination of mixtures of azole pesticides in vegetables.
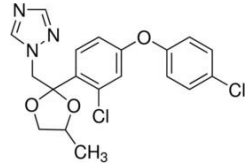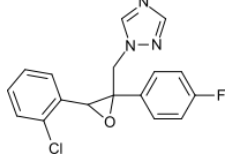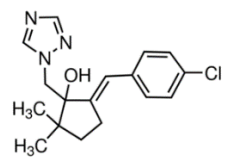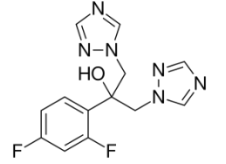
1.2 PHYSICOCHEMICAL PROPERTIES OF TRIAZOLE PESTICIDES

The triazole pesticides are a group of compounds whose physicochemical properties are not well known. They are all derivatives of 1,2,4- triazole ($C_2H_3N_3$) and the variety of compounds is wide. The triazoles pesticides studied include cyproconazole (CPZ), penconazole (PNZ), propiconazole (PPZ), tebuconazole (TBZ), fenbuconazole (FBZ), difenoconazole (DFZ), epoxiconazole (ECZ), triticonazole (TTZ) and fluconazole (FCZ). The properties established so far are displayed in Table 1:

Table 1. Physicochemical properties of the investigated triazole compounds

Name (IUPAC)	(2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol	(RS)-1-[2-(2,4-dichlorophenyl)pentyl]- 1H-1,2,4-triazole	(2RS,4RS;2RS,4SR)-1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole	(RS)-1-p-chlorophenyl- 4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol	4-(4-chlorophenyl)-2-phenyl-2-(1H-1,2,4-triazol-1-ylmethyl)butyronitrile
Common name and acronym	Cyproconazole, CPZ	Penconazole, PNZ	Propiconazole, PPZ	Tebuconazole, TBZ	Fenbuconazole, FBZ
Structure					
Molecular formula	$C_{15}H_{18}ClN_3O$	$C_{13}H_{15}Cl_2N_3$	$C_{15}H_{17}Cl_2N_3O_2$	$C_{16}H_{22}ClN_3O$	$C_{19}H_{17}ClN_4$
Molecular weight, $g \cdot mol^{-1}$	291.78	284.18	342.22	307.82	336.82
C.A.S. Number	94361-06-5	66246-88-6	60207-90-1	107534-96-3	114369-43-6
Water solubility, 20ºC, $mg \cdot L^{-1}$	93.0 [5]	73 [6]	150	36	2.47
Methanol solubility, $mg \cdot L^{-1}$	Very soluble	Not available	Miscible	Not available	Not available
Acidity constant (pk_a) at 25ºC	1.76 (CE) [7]	5.2 (Pot.) [8] 1.57 (CE) [7]	1.21 (CE) [7]	5.0 (Pot.) [8] 1.57 (CE) [7]	Not available

Table 1. Continuation

Name (IUPAC)	3-chloro-4-((2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl)phenyl 4-chlorophenyl ether	1-[[3-(2-chlorophenyl)-2-(4-fluorophenyl)oxiran-2-yl]methyl]-1,2,4-triazole	(5E)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1,2,4-triazol-1-ylmethyl)cyclopentanol	2-(2,4-difluorophenyl)-1,3-bis(1,2,4-triazol-1-yl)propan-2-ol
Common name and acronym	Difenoconazole, DFZ	Epoxiconazole, ECZ	Triticonazole, TTZ	Fluconazole, FCZ
Structure				
Molecular formula	$C_{19}H_{17}Cl_2N_3O_3$	$C_{17}H_{13}ClFN_3O$	$C_{17}H_{20}ClN_3O$	$C_{13}H_{12}F_2N_6O$
Molecular weight, $g \cdot mol^{-1}$	406.26	329.8	317.8	306.27
C.A.S. Number	119446-68-3	133855-98-8	131983-72-7	86386-73-4
Water solubility at 20ºC, $mg \cdot L^{-1}$	15.0	8.4	9.3	4.4
Methanol solubility, $mg \cdot L^{-1}$	No information	Very soluble	Very soluble	Very soluble
Acidity constant (pk$_a$) at 25ºC	Not available	1.05 [7]	Not available	Not available

All these compounds have good solubility in non-polar solvents because of their hydrocarbonated structures, but they have halogen and alcohol groups and, for that reason, they are partially soluble in water. The different values of the acidity constant between the pesticides allow them to be separated as a function of their polarity. In addition, their structure with nonbonding electrons enable the use of molecular absorption detector.

1.3 ANALYTICAL METHODS

The European Union imposed a maximum residual level, MRL, for individual pesticides in tomato or general vegetables (Table 2), but it does not provide general limits for total content of azole pesticides. Analytical methods allowing simultaneous extraction, separation and determination of all present triazole pesticides with sufficient precision are nneded.

Table 2. Maximum residual level in tomatoes imposed by EU Regulation of the azole pesticides studied.

Compound	MRL in tomatoes, UE Regulation mg.kg^{-1}	Reference
CPZ	0.05	[9]
PNZ	0.1	[10]
PPZ	Not available	
TBZ	1.0	[11]
FBZ	0.5	[12]
DFZ	2.0	[13]
ECZ	0.05	[14]
TTZ	Not available	
FCZ	Not available	

Nowadays, one of the analytical methods used for the determination of mixtures of azole compounds is HPLC-MS/MS. This method has been used for the analysis of CPZ, PNZ, PPZ, TBZ and DFZ in water using a C18 column and non-isocratic gradient and reversed phase method. Using first, 20% MeOH and 80% deionized water as mobile

phase during 11 minutes, and then 12 minutes using 90% of MeOH and 10% deionized water. Limits of quantification, *LOQ*, below 1 µg·L^{-1} where achieved for the different pesticides [15].

Other method using solid phase extraction, SPE, and UPLC-MS/MS has been proposed for the determination of PPZ, PNZ, TBZ and DFZ in wine during 10 minutes, using non-isocratic, reversed phase and C18 column. Ultrapure water and acetonitrile (with 0.1% of formic acid) have been used in different proportions as mobile phase. *LOQ* of 0.066 µg·L^{-1} for PNZ and DFZ and 0.165 µg·L^{-1} for PPZ and TBZ were achieved [16].

For raw coffee samples a method using LC-ESI-MS/MS after a SPE extraction, has been suggested. It uses non-isocratic, reversed phase in C18 column and ultrapure water and ACN (with 0.1% of formic acid) in different proportions as mobile phase. A huge number of pesticides including CPZ, PNZ, TBZ, FBZ, ECZ and DFZ were successfully separated. *LOQ* values are 10 µg·kg^{-1} for CPZ, TBZ, FBZ and DFZ and 20 µg·kg^{-1} for PNZ and ECZ [17].

1.4 ELECTROCHEMISTRY STUDIES OF TRIAZOLE PESTICIDES

Not much literature exists on redox activity of the studied triazole compounds. The 1, 2, 4- triazole is neither oxidizable nor reducible and thus other parts of the molecules must be active [1].Preliminary results [18] indicate that difenoconazole is oxidizable at the potential of 1.65V using BDDE working electrode vs Ag/AgCl reference electrode in acidic media. Terconazole, a similar antifungal compound, was studied using carbon-based electrode materials including boron-doped diamond electrode, BDDE (O- and H-terminated), glassy carbon electrode, GCE, and pyrolytic graphite electrode, PGE, using differential pulse voltammetry, DPV. O- terminated BDDE had the lowest LOQ of 0.40 µmol·L^{-1}. In this diazole compound, piperazine moiety is oxidized [1].

Thus, the redox activity of selected triazole pesticides was tested in this study to address possibilities of their electrochemical detection. For this purpose, BDDE was chosen as working electrode. This carbon-based electrode material possess a wide potential

window (especially in the anodic region), robustness, high electrical resistivity and low signal-background ratio [19–21]. It is prepared by hot filament or microwave plasma-assisted chemical vapor deposition [20]. Nowadays it is easy to obtain this type of electrodes, as they are commercially available.

2. AIM OF THE STUDY

There is a need of fast, reliable, robust, simple and affordable analytical methods for the determination of residues of triazole pesticides in food products intended for human consumption, in order to assess that the contents of these toxicants comply with the EU regulations. Most methods developed for the simultaneous determination of mixtures of these pesticides in food matrices are based on a chromatographic separation coupled to MS detection. These methods are very sensitive, but expensive and not affordable to all laboratories. The aim of this project is to develop simpler, affordable and still accurate analytical methods for the simultaneous determination of cyproconazole, penconazole, propiconazole, tebuconazole, fenbuconazole, difenoconazole, fluconazole, epoxiconazole and triticonazole using reversed phase HPLC as separation technique, coupled to diode array detection (DAD) and electrochemical detection (ED) using BDDE as working electrode. These low cost and accurate detectors can be used as these compounds can absorb UV radiation (DAD) [18,22] and some of them are likely to be oxidable (ED) using carbon based electrodes [1].

Individual electrochemical activity of the compounds will be studied using amperometry at fixed potentials checking their oxidizability. Parameters related to the chromatographic separation and the detection steps will be optimized: flow rate and composition of the mobile phase in HPLC, wavelength in DAD or electrode potential in ED. The linearity of response and the limit of quantification will be estimated in order to ensure that the method can detect the pesticides in food products at concentrations below the MRL values established by the EU regulation.

The HPLC-DAD method will be tested using real tomatoes samples treated with single and mixtures of cyproconazole, tebuconazole and penconazole. Tomato extractions were provided by the Czech University of Life Sciences (Prague) which collaborated in this research. This study is part of a broader research project devoted to the study of interactions of triazole pesticides in the environment, their effect on the biosynthesis of steroid compounds and the development of analytical methods for their detection in various matrices.

3. MATERIALS AND METHODS

3.1 REAGENTS

The azole pesticides CPZ, PPZ, TBZ, DFZ, FCZ, ECZ, TTZ and 1,2,4-triazole were obtained from SIGMA-ALDRICH (San Luis, USA), PNZ from Honeywell Fluka^{TM} (Sleeze, Germany) and FBZ from CHEM SERVICE (Wes Cheaster, USA). All of them were kept at 4°C in the fridge for their conservation. Potassium hexacyanoferrate (II) trihydrate was bought from Lachema (Brno, CZ) and alumina from Elektrochemické Detektory (Turnov, CZ).

Acetonitrile was purchased from Honeywell Riedel-de-Haën (Sleeze, Germany), phosphoric acid 85% and potassium chloride from Lach:ner (Neratovice, CZ). The sodium hydroxide solution used was a prepared solution 8 mol.L^{-1} from Honeywell Fluka^{TM} (Sleeze, Germany). Chloroform was obtained from PENTA, CZ.

To make the aqueous solutions, deionized water from a Millipore Milli.Q.Plus system (Milipore, USA) was used.

3.2 APPARATUS AND SOFTWARE

A multichannel HPLC ELITE LaChrom MERCK HITACHI system (Fig. 1) with a Smash L-2130 gradient pump (Merck, Germany) and four available channels with a maximum flow of 5 mL·min^{-1} was used. Furthermore, it consisted of 20-µL model 7725(i) front-loading sample injector (Rheodyne, USA) and Diode Array detector L-2450 with wavelength range between 220 and 400 nm. A Kromasil Eternity-5 PhenylHexyl reversed-phase column with dimensions 4.6x150 mm, particle size of 5 µm was used. The HPLC system was operated by EZChrom Elite software (Agilent Technologies, USA).

Figure 1. Multichannel HPLC ELITE LaChrom MERCK HITACHI system

The electrochemical detection was made using µAUTOLAB III from Metrohm (Herisau, Switzerland) using Nova 1.7 software (Fig. 2a). An overflow home-made wall-jet electrochemical cell [23] was employed (Fig. 2b) housing three electrode detection system: BDD working electrode diameter 3 mm, BioLogic SAS (Seyssinet-Pariset, France), a Ag/AgCl 3 mol·L^{-1} KCl as reference electrode and auxiliary platinum electrode both from Elektrochemické Detektory (Turnov, CZ). The capillary outlet (diameter 0.15 mm) and electrode surface distance was kept at 0.5 mm.

a)   b)

Figure 2. a) µAUTOLAB III   b) overflow home-made wall-jet electrochemical cell

One-way Agilent 8453 spectrophotometer (Santa Clara, USA) was used for measurement of absorption spectra. The spectra were measured in quartz cuvettes (width 0.5 cm) in wavelength range 200 nm – 700 nm.

Eko-Tribo polarograph from Polaro-Sensors (Prague, CZ) was used for voltammetric measurements. A three electrode system was used with the same working, auxiliary and reference electrodes used in HPLC-ED in the wall-jet detection cell.

For preparation of solutions, an Ultrasonic compact cleaner PS02000A with a capacity of 1.25 L from Pájecí Technika (Dolní Roveň, CZ) was employed. A Jenway 33510 pH meter (Staffordshire, UK) calibrated using three solutions of pH 4.0, 7.0 and 10.0 was used to control the pH of the prepared buffers.

Microliter Syringes from Hamilton Company (Bonaduz, The Switzerland) of 100 µL and 10 µL were used to prepare standards by dilution of stock solutions and to measure the volume of the sample in the standard additions method.

The weighting was made using a Sartorius 2204 analytical balance (Groettinger, Germany).

3.3 ANALYTICAL PROCEDURES

3.3.1 Solutions

Stock solutions of triazole pesticides were prepared in acetonitrile dissolving the exact mass of each azole pesticide (Table 3) in 50 mL volumetric flask to get a final concentration of $1 \cdot 10^{-3}$ mol·L^{-1}. The compounds were weighted using an analytical balance and the prepared stock solutions were stored at 4°C for their conservation.

Table 3. Molecular weight of the compounds and masses needed for the preparation of stock solutions

Compound	Mw / g·mol^{-1}	Mass used / mg
CPZ	291.78	14.6
PNZ	284.18	14.2
PPZ	342.44	17.1
TBZ	307.82	15.4
FBZ	336.8	16.8
DFZ	406.26	20.3
FCZ	306.27	15.3
ECZ	329.76	16.5
TTZ	317.81	15.9
1,2,4-triazole	69.07	3.45

Solutions of lower concentrations were made diluting stock solutions with acetonitrile and likewise the mixtures of various compounds.

3.3.2 HPLC coupled to diode array detector

The HPLC equipment allows us to separate compounds as a function of their different chemical properties. In this case, separation in reversed phase was used. The stationary phase is apolar (PhenylHexyl column) and a moderately polar mobile phase composed by acetonitrile and phosphate buffer in 57:43 (*v/v*) ratio was used. The detection was made with DAD and ED. Four replicates of each measurement were made to check for repeatability.

The DAD is based on the Beer-Lambert law which relates the concentration to the absorption of the compound. The absorbance of triazole pesticides in UV range is because of presence of aromatic rings, eventually double bonds (as in the case of TTZ) in the structure.

To optimize the separation optimization, two different solutions of phosphate buffer were used: 0.05 mol·L^{-1} phosphate at pH 6.8 and 0.1 mol·L^{-1} phosphate at pH 2.0. Phosphate buffers were prepared diluting phosphoric acid 85% in deionized water and adjusting the pH using 0.2 mol·L^{-1} NaOH solution.

Calibration lines were plotted using ORIGIN software and, for the calculation of *LOD* (limit of detection) and *LOQ* (limit of quantification), the standard deviation of the y-intercepts was used using the following expressions:

$$LOD = \frac{3\ S_\alpha}{b}$$

$$LOQ = \frac{10\ S_\alpha}{b}$$

Where *b* is the slope of the calibration line and the S_α is the standard deviation of the intercepts.

3.3.3 Treatment of tomato samples

Different samples of tomatoes treated with CPZ, TBZ and PNZ, individually or in combination, were prepared by the Czech University of Life Sciences (Prague), and analyzed using the developed HPLC-DAD method. Tomatoes were grown in a greenhouse with nonregulated temperature and light, the plants were irrigated with demineralized water. Each seedling of tomato (Solanum lycopersicum L. var. 'Sweet Cherry') was embedded in 4 L pot with ca 2 kg of a prefabricated mix "Horticultural Substrate with Active Humus" containing mineral nutrients (AGRO CS a.s., CZ). The fertilization was performed twice, after 4 and 7 weeks of maturing, with liquid NPK mixture (LOVOFLOR NPK 4-2.5-3, Lovochemie, CZ) in optional dose.

The application of triazole pesticides was conducted with commercially available individual pesticides, with exception of cyproconazole which could be only purchased in mixture with other non-azolic fungicides.

For application, pesticides were sprayed on 15 cm tomato plant with a given triazole or with the mixture of various triazoles, administer every week during 7 weeks. Dose was always based on the ranges in application sheets given by the manufacturer for spraying tomatoes to have the total triazole amount of $3.52 \cdot 10^{-6}$ mol per plant (calculated from their declared content).

The total amount of each applied pesticide per plant is depicted in Table 4. Five tomato plants were treated with the same number of pesticides and the same conditions.

Table 4. Amount of each azole applied in the treated tomatoes plants. The total concentration of azole is always 3.52 µmol per plant.

Sample n°	Triazole(s) applied in a mixture	Amount of each azole (µmol/plant)
1	CPZ	3.52
2	CPZ + TBZ	1.76
3	TBZ + PNZ	1.76
4	CPZ + TBZ + PNZ	1.17

For the measurement of the residual level of pesticides in the tomato samples, the azole pesticides were extracted from the peel seven days after the last fungicide application. The tomatoes were harvested the same day and frozen at -20°C to preserve them. Peel was cut and weighted into conical centrifugal tubes (approximately between 2 and 3 grams) following the addition of 4 mL of chloroform acidified with 0.1% of acetic acid. After mixing for two minutes and centrifuged for 20 min at 5G, 2 mL of the extracts were transferred and cleaned trough a SPE cartridge, following the rinsing with chloroform having a final volume of 5 mL. Two additions of 2.5 mL of chloroform were used for the conditioning of the SPE C18 (Sigma-Aldrich, Prague, CZ) and after that the prepared

samples were stored at 4 °C. All this process was made by the Czech University of Life Sciences (Prague).

The chloroform of the tomatoes extract is not miscible in the mobile phase because of the high percent of aqueous solution. This behavior was tested adding a drop of chloroform to our mobile phase, obtaining a two phases system. For that reason, is required another extraction of the triazole pesticides: Taking a volume of the sample (above 450 µL) in a chromatography vial, the chloroform was evaporated using nitrogen gas. After that, the same volume of acetonitrile was added. After mixing the vial for 2 minutes by sonication, sample is prepared for the injection.

3.2.4 Electrochemical detection of triazole pesticides

A wall-jet flow-through electrochemical cell was connected to the HPLC. The three-electrode cell consisting of BDDE as working electrode, Ag/AgCl 3 mol·L^{-1} KCl reference electrode and platinum auxiliary electrode, was used to investigate if the target pesticides are electrochemically active using amperometry at constant potential and plotting hydrodynamic voltammograms. Once the optimum potential was selected, concentration dependence was tested. Four replicates of each measurement were made.

Prior to measurement, BDDE surface was polished for five minutes on a polishing pad with a suspension containing alumina, both obtained from Elektrochemické detektory (Turnov, CZ).

4. RESULTS AND DISCUSSIONS

4.1 UV/Vis absorption spectra of triazole pesticides

Using Agilent 8453 UV-visible spectrometer, the spectra of $1 \cdot 10^{-4}$ mol$\cdot$L^{-1} dissolutions of pure triazol pesticides were scanned between 200 and 700 nm. Measured solutions were prepared diluting 1.0 mL of stock solution of each pesticide in acetonitrile in 9.0 mL of the mobile phase solution of composition 57:43 (*v/v*) acetonitrile and 0.1 mol$\cdot$L^{-1} phosphate buffer of pH 2.0, having a final concentration 61:39 (*v:v*) . Blank solutions were made diluting 1.0 mL of ACN in 9.0 mL of the mobile phase solution. The spectra registered for DFZ, FCZ, TTC and 1,2,4-triazole are shown in Fig. 3. No signals were registered after 400 nm and therefore the absorbance above 400 nm is not shown.
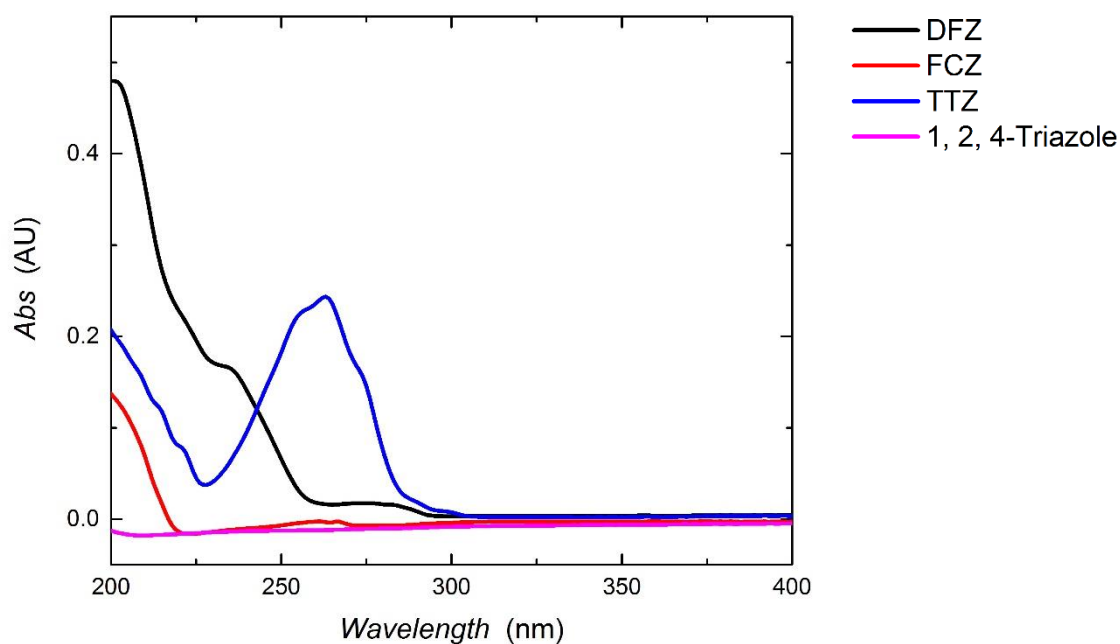
Figure 3. Absorbance spectra of $1 \cdot 10^{-4}$ mol$\cdot$L^{-1} solutions of DFZ, FCZ, TTZ and 1,2,4-triazole in 61:39 (v:v) acetonitrile and 0.1 mol$\cdot$L^{-1} phosphate buffer at pH 2.0 in the range between 200 and 400 nm.

All studied compounds except TTZ, FCZ and 1,2,4-triazole had similar spectra. Therefore, only DFZ was included in Fig. 3. All of them exhibited continuous increase of absorbance from ca 300 nm to 200 nm without any clear maxima, but higher value at lower absorbance wavelength, 200 nm. Different patterns were recognized for the other compounds.

TTZ showed the maximum of absorbance at λ = 263 nm. It possesses a double bond in its structure, not present in the structure of the other pesticides. Thus, energy levels are less separated, meaning less energy for excitation of π electrons, resulting in a higher maximum absorption wavelength.

FCZ spectrum had a pattern similar to DFZ but with a signal about three times lower and the plateau appears before, being absorbance null at 220 nm.

On the other hand, 1,2,4-triazole had negligible absorbance in the whole range studied, not presenting any kind of interaction with UV/Vis radiation.

Because the diode-array detector used in HPLC had a minimum wavelength of 220 nm, a decrease in sensitivity had to be assumed. FCZ did not present absorption at 220 nm and for that reason, it had been determined as UV/Vis inactive in this setup. Other compounds wavelengths selected for detection in HPLC-DAD of the target pesticides are shown in Table 5.

Table 5. Wavelength chosen for the determination of each compound by HPLC-DAD

Analyte	λ_{det} / nm
TTZ	263
CPZ	223
ECZ	220
TBZ	222
PNZ	223
FBZ	220
FCZ	-
PPZ	223
DFZ	221

4.2 HPLC with diode array detection

4.2.1 Optimization of the separation conditions

In a previous study of HPLC separation [18] of five triazole pesticides, CPZ, FBZ, TBZ, PNZ and PPZ, the following optimum conditions were found: isocratic elution with mobile phase composed of 57% ACN and 43% 0.05 mol·L^{-1} phosphate buffer at pH 6.8 and flow rate of 0.5 ml·min^{-1}. In the present study, the number of pesticides was increased to nine and the composition of the mobile phase was modified, so that it enables electrochemical detection (ED) of the compound. According to preliminary results published in [18], DFZ is oxidizable in acidic media and thus, the pH of the aqueous part of the mobile phase was lowered to pH 2.0 to meet the conditions presumably enabling ED. Firstly, a mixture of all nine azole pesticides in concentration of $1 \cdot 10^{-4}$ mol·L^{-1} was measured using the conditions used in [18], i.e, 57% ACN and 43% 0.05 mol·L^{-1} phosphate buffer at pH 6.8. Five different peaks were obtained in a total separation time of 16 min (Fig. 4a). Thus, the pesticides were injected separately to identify the peaks. Insufficient separation was observed for CPZ-TBZ, ECZ-TBZ and PNZ-FBZ.

Using the mobile phase containing 0.1 mol·L^{-1} phosphate buffer at pH 2.0, better separation was achieved. Six peaks can be seen in the chromatogram shown in Fig. 4b, because separation of PNZ and FBZ, which were co-eluting using previous conditions. Total separation time was 18 min, retention times and factors are given in Table 6.

Figure 4. Chromatograms of nine triazole compounds using HPLC-DAD, 0.5 mL·min^{-1} flow and mobile phase 57:43 (v:v) acetonitrile and two different phosphate buffers: a) 0.05 mol·L^{-1} pH 6.8 b) 0.1 mol·L^{-1} pH 2.0

Table 6. Retention times of the studied pesticides using HPLC-DAD in optimum conditions of phosphate buffer pH in different flows.

pH of the PB	2.0			
F_M / mL·min^{-1}	0.5		1	
	R_t / min	k' / ad.	R_t / min	k' / ad.
TTZ	8.2	2.7	2.8	1.8
CPZ	8.3	2.8	2.8	1.8
ECZ	10.3	3.7	3.5	2.5
TBZ	10.3	3.7	3.2	2.2
PNZ	11.7	4.3	3.7	2.7
FBZ	12.3	4.6	3.4	2.4
FCZ	-	-	-	-
PPZ	13.0	4.9	4.0	3.0
DFZ	16.7	6.6	4.8	3.7
	t_0 = 2.2		t_0 = 1.1	

The other co-eluting compounds (CPZ-TTZ and ECZ-TBZ) could not be separated using the more acidic mobile phase, thus a selection of only five pesticides was made: CPZ, TBZ, PNZ, PPZ and DFZ. For that reason, the optimization of the chromatographic separation of these compounds continued, focusing on improvement of separation and
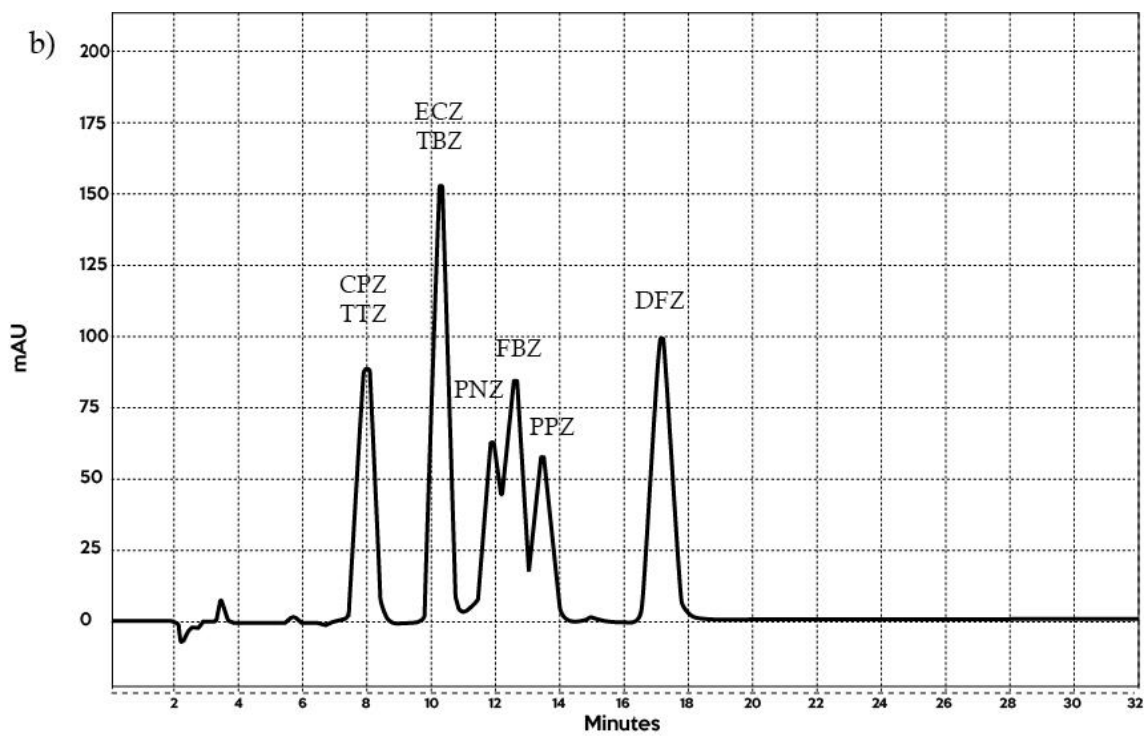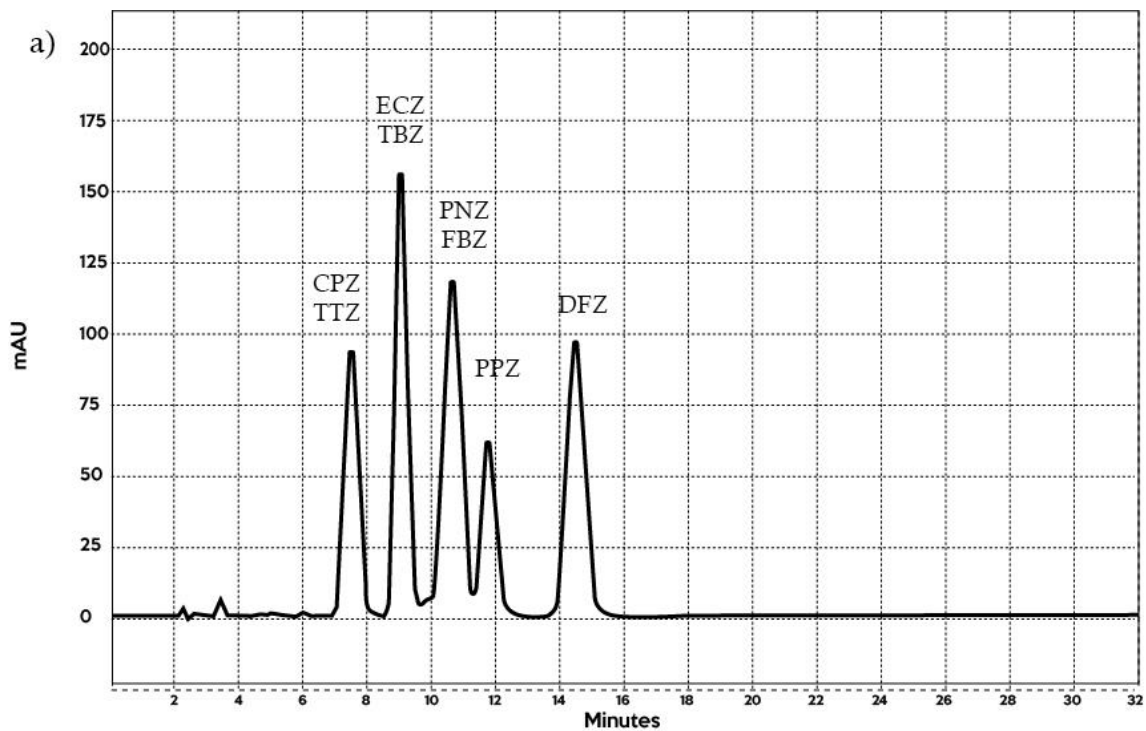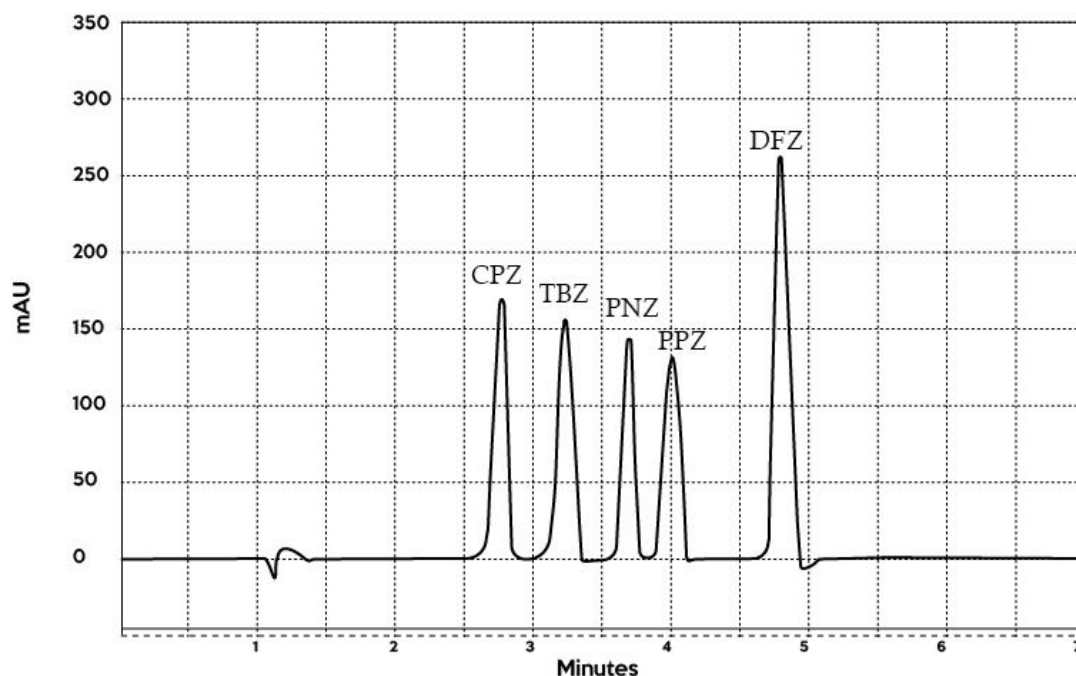

Figure 5. Chromatogram of five triazole compounds using HPLC-DAD, 1.0 mL·min^{-1} flow and mobile phase 57:43 (v:v) acetonitrile and phosphate buffer 0.1 mol·L^{-1} pH 2.0

decrease of retention times. With a flow rate of 1.0 mL·min^{-1} the complete separation of CPZ, TBZ, PNZ, PPZ and DFZ was achieved in only 6 min (Fig. 5).

4.2.2 Concentration dependences

Linearity of response of the five pesticides that could be separated (CPZ, TBZ, PNZ, PPZ and DFZ) was tested by HPLC-DAD, using last optimum mobile phase consisting of 57% ACN and 43% of 0.1 mol·L^{-1} phosphate buffer, pH 2.0.

Concentration dependences were measured in the range from $1·10^{-4}$ mol·L^{-1} to the minimum detectable value (ca $1·10^{-7}$ mol·L^{-1}, depending on the compound), results are shown in Fig. 6. Linear regressions were made using concentrations between $1·10^{-4}$ mol·L^{-1} and $1·10^{-7}$ mol·L^{-1} and between $1·10^{-5}$ mol·L^{-1} and $1·10^{-7}$ mol·L^{-1}. Estimation of *LOQ* from the two calibration ranges resulted in values around $3.5·10^{-6}$ mol·L^{-1} and $1.1·10^{-6}$ mol·L^{-1}, respectively. For that reason, in Table 7 data are obtained between $1·10^{-5}$ mol·L^{-1} and $1·10^{-7}$ mol·L^{-1} having lower *LOQ* because of the higher standard deviation of the intercept when higher concentrations are included.
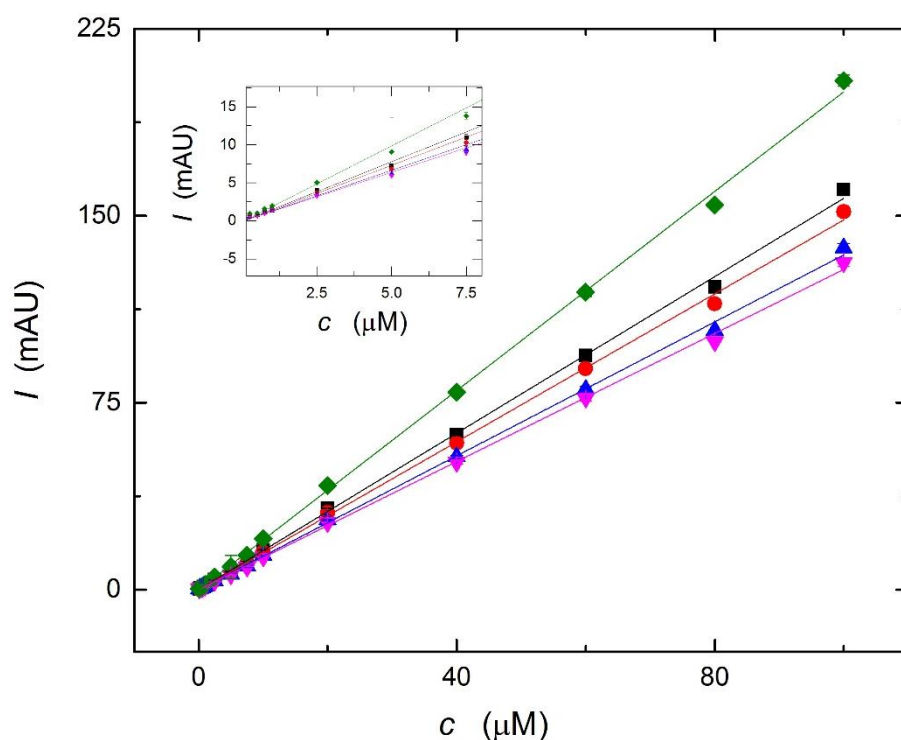
Figure 6. Concentration dependences using HPLC-DAD. CPZ in black squares, TBZ in red circles, PNZ in blue triangles, PPZ in pink cones, DFZ in green diamonds. Evaluated from peak heights, separation conditions as in Fig.5. In the insert, the lower concentrations calibration range can be seen.

Table 7. Parameters of concentration dependences and LOD and LOQ values for CPZ, TBZ, PNZ, PPZ and DFZ obtained by linear regression between $1 \cdot 10^{-5}$ mol$\cdot$L^{-1} and $1 \cdot 10^{-7}$ mol$\cdot$L^{-1}. Evaluated from peak heights, separation conditions as in Fig. 5.

Analyte	LDR /µM	Slope ± SD / mAU· µM^{-1}	Intercept ± SD / mAU	R^2/ ad.	LOD / µM	LOQ / µM
CPZ	0.1-100	1.526 ± 0.036	0.071 ± 0.163	0.996	0.32	1.07
TBZ	0.1-100	1.443 ± 0.037	0.038 ± 0.167	0.995	0.35	1.16
PNZ	0.1-100	1.315 ± 0.032	0.032 ± 0.145	0.995	0.33	1.10
PPZ	0.1-100	1.257 ± 0.032	0.057 ± 0.147	0.995	0.35	1.17
DFZ	0.1-100	1.947 ± 0.052	0.024 ± 0.240	0.994	0.37	1.23

The wide-range linearity of the developed HPLC-DAD method was demonstrated, approximately three orders of magnitude. Recalculation of molar to ppm units revealed detection limits shown in Table 8. Comparing to the MRL allowed by the UE (Table 2), TBZ and DFZ limits are higher than *LOQ* using this method. On the other hand, CPZ and

PNZ MRL limits are lower than *LOQ*. Nevertheless, in analysis of real samples a preconcentration step can be used so that the concentration limits achieved by HPLC-DAD meet the MRL limits for real samples.

Table 8. LOQ and LOD in ppm for CPZ, TBZ, PNZ, PPZ and DFZ obtained from the data in Table 7.

Analyte	*LOD* / ppm	*LOQ* / ppm
CPZ	0.09	0.31
TBZ	0.11	0.35
PNZ	0.09	0.31
PPZ	0.12	0.40
DFZ	0.15	0.50

4.2.3 Analysis of real samples

The samples were analyzed using optimized HPLC-DAD method with isocratic elution using a mobile phase 57:43 (*v:v*) ACN and 0.1 mol·L^{-1} phosphate buffer pH 2.0 with a flow rate of 1.0 mL·min^{-1} .

At first, the blank extract, i.e. extract obtained from plants no treated by the pesticides, was injected. It can be seen in Fig. 7 that there are many signals in the obtained chromatogram, presumably complicating the analysis. This large matrix effect excludes the possibility of quantifying the pesticides in the sample based on the obtained calibration dependences (Fig. 6). Thus, standard additions method was used for quantification, using additions of concentrations between 0.75 and 2.0 µmol·L^{-1} of 1·10^{-4} mol·L^{-1} standard solution of pure analytes in ACN to the extract, which was injected

into the HPLC-DAD setup. The number of additions was 2-5 depending on the available volume of the extract.

Fig. 8 depicts chromatograms of the extracts obtained from tomatoes treated with CPZ (Fig. 8a), CPZ and TBZ (Fig. 8b), TBZ and PNZ (Fig. 8c) and CPZ, TBZ and PNZ (Fig. 8d).
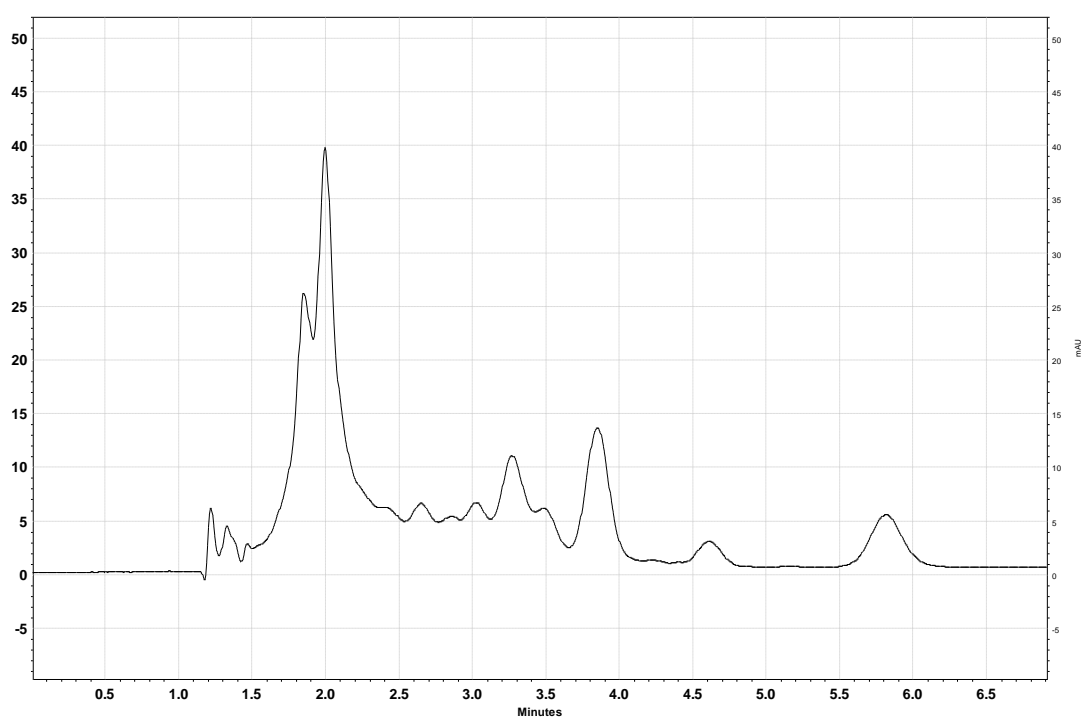

Figure 7. HPLC-DAD chromatogram of the peel extract of non-treated tomato.
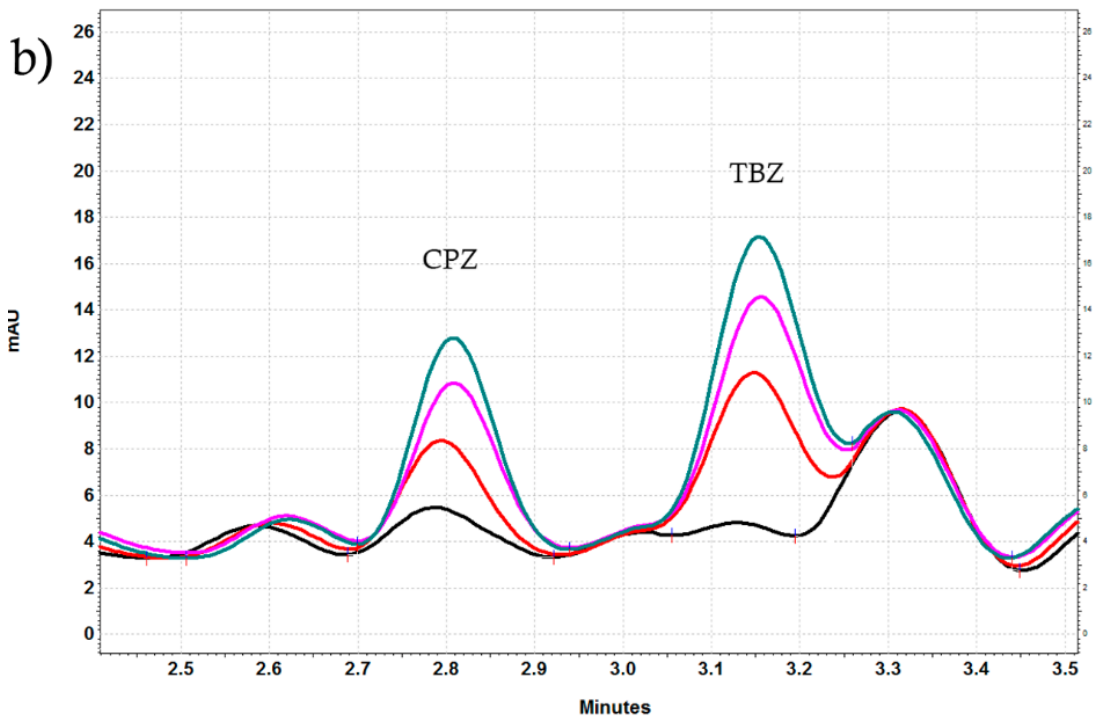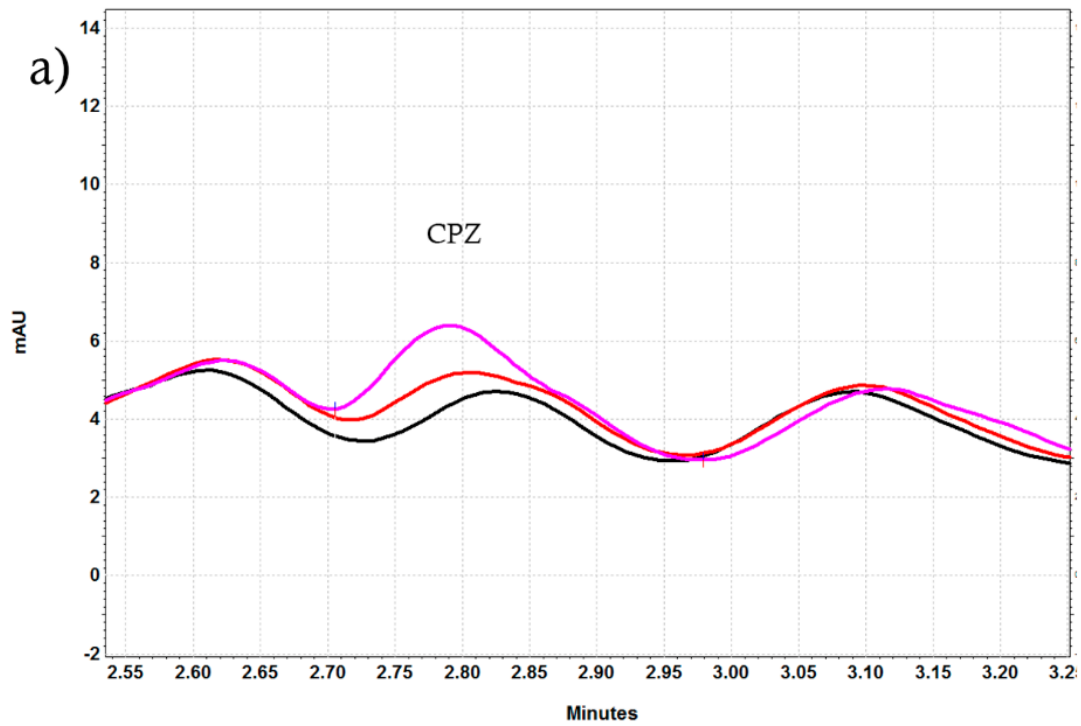
.

Figure 8. Chromatograms of peel extracts from tomatoes treated by (a) CPZ (b) CPZ and TBZ (c) TBZ and PNZ (d) CPZ, TBZ and PNZ. Black line corresponds to sample and following lines (red, pink, cyan, purple and green) with the standard additions.
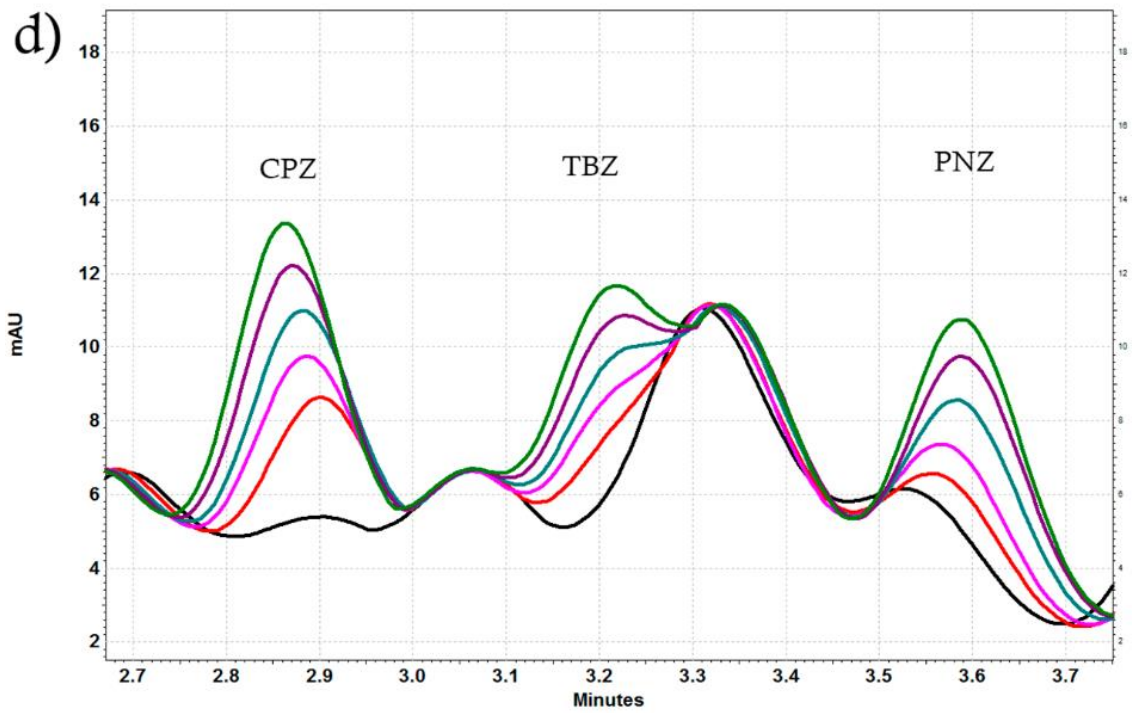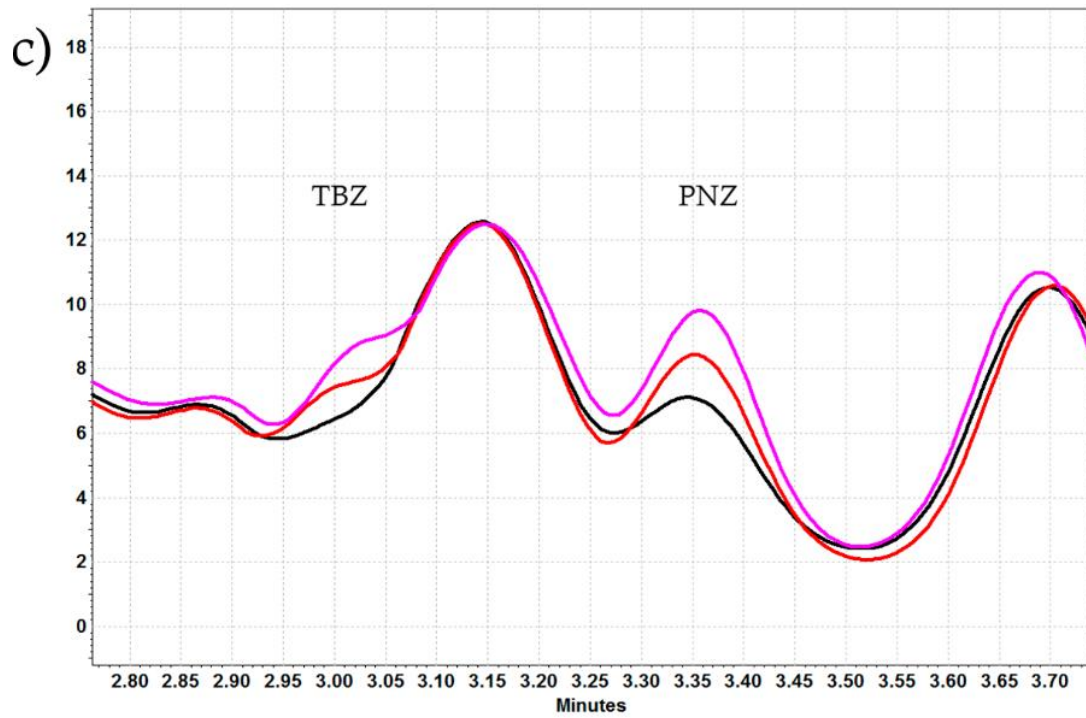
Figure 8. Continuation

Table 9. Molar concentrations ($c_{extraction}$) of CPZ, TBZ and PNZ in peel extracts from tomatoes treated by these pesticides individually or in mixture, calculated using standard addition method based on HPLC-DAD method and mg of pesticide per kilogram of tomato peel.

Sample	Peel mass / g	Pesticide	Applied amount of each azole (µmol/plant)	$C_{extraction}$ / µmol· L^{-1}	mg·kg^{-1} tomato peel
1	2.03569	CPZ	3.52	0.924	0.66
2	3.03874	CPZ	1.76	1.39	0.67
		TBZ	1.76	0.648	0.33
3	3.98458	TBZ	1.76	-	-
		PNZ	1.76	1.32	0.47
4	2.95496	CPZ	1.17	1.02	0.50
		TBZ	1.17	-	-
		PNZ	1.17	0.031	0.01

Different possible interactions with other species (e.g., metal ions, humic acids) may cause the losses on the pesticide additions. While peaks of CPZ and PNZ could be identified in all extracts of plants treated with them, TBZ was found only in one case, in the mixture with CPZ (Fig. 8b). The lack of TBZ signals may be fault of the mentioned interactions with other species or matrix effect, decreasing the *LOQ*. Also, PNZ concentration in sample 4, where it is present in the mixture with CPZ and TBZ, is very low, below *LOD* values. The concentration of each pesticide in extracted samples was calculated as well as the residual concentration on the tomatoes (mg·kg^{-1}). The obtained results are summarized in Table 9.

Comparing the results with MRL values listed in Table 3, CPZ in the samples had higher concentration than the allowed limits. For TBZ (in the case in which it is quantizable) and PNZ, the concentrations were lower than the MRL.

Using developed HPLC-DAD method is possible to separate and quantify CPZ, TBZ and PNZ even at concentrations lower than MRL allowed by the EU regulations. However, further work is needed to improve the resolution between the chromatographic peaks of the analyzed pesticides and to reduce the matrix signal that complicates the evaluation of peaks. These problems can be solved by developing more effective extraction and cleaning methods and different conditions in the HPLC-DAD avoiding the

presence of interfering compounds from matrix in extracts or their co-elution with detected triazole pesticides.

4.3 Electrochemistry of the azoles

Electrochemistry of these compounds is not well known and could be interesting to investigate it, in order to develop an alternative method for the detection of the pesticides. The structure of many azole pesticides contain halogens, double bonds or even epoxides likely to be oxidized, so an electrochemical response could be expected.

4.3.1 Pretreatment in BDD electrode

The oxidizability of triazole pesticides were tested in a wide positive potential range between +1.4 V to +2.5 V. Firstly, the effect of surface pretreatment on the cyclovoltammetric response of the inner-sphere redox marker $[Fe(CN)_6]^{3-/4-}$ in 1 mol·L^{-1} KCl was tested. At high positive potentials (<2.0 vs. Ag/AgCl) the surface of the BDDE electrodes becomes oxidized because of reaction of hydroxyl radicals which are formed due to the water decomposition following the next reaction (eq. 1):

$$H_2O \ \rightarrow \ H^+ + OH\cdot + e^- \qquad (1)$$
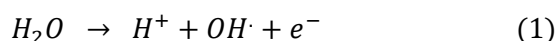
Hydroxyl, epoxide, carbonyl, carboxyl or other oxygen functional groups represented in Fig. 9 appear at the BDD surface and this can be regarded as O-terminated. This oxygen-containing groups hinder electron transfer at the electrolyte-electrode interface, frequently causing decreased sensitivity of the electrode.
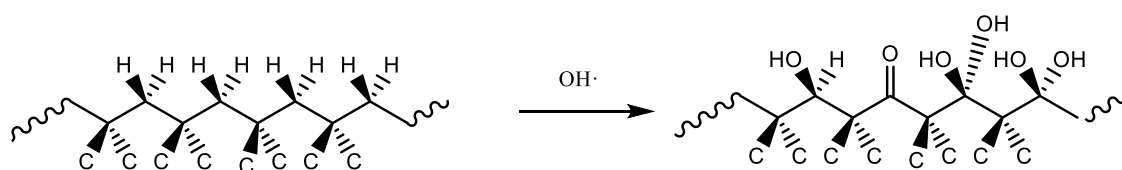
Figure 9. Oxidation reaction of the BDDE surface by hydroxyl radicals formed by water decomposition at highly positive potentials.

Cyclic voltammograms of $1 \cdot 10^{-4}$ mol·L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ were recorded using Eko-Trivo polarograph, in 1 mol·L^{-1} KCl as supporting electrolyte, BDDE as working electrode, Ag/AgCl 3 mol·L^{-1} KCl as reference electrode and platinum as auxiliary electrode. The potential was swept from -0.4V to 0.8V at a scan rate of 100 mV·s^{-1}. Using this inner sphere reversible redox system exchanging one electron, the difference between anodic and cathodic peak potentials (ΔE_p) has a theoretical value of 0.059 V.

Polishing the BDDE surface with alumina eliminates partially the oxygen groups, preferably those attached to sp^2 carbon. For that reason, CV were recorded after applying a high voltage of +2.4 V on the electrode during 1.5 hours to check the effect of oxidation on the electrode surface; then, the electrode surface was polished for 5 minutes with alumina and cleaning it with deionized water, and CV were recorded again. Fig. 10 shows the voltammograms registered in the two conditions.
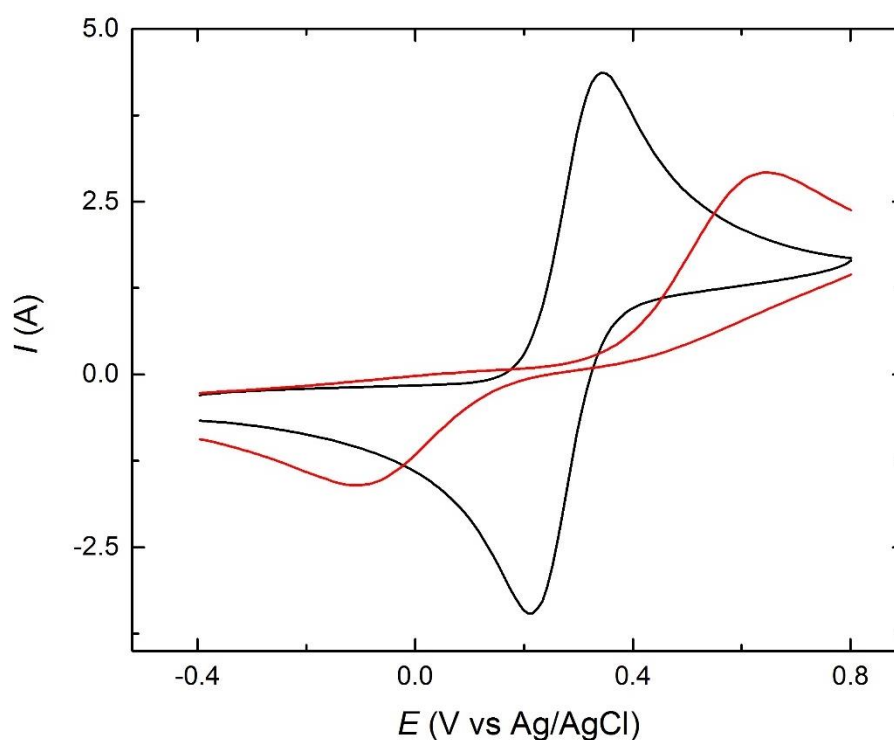
Figure 10. Cyclic voltammograms of $1 \cdot 10^{-4}$ mol·L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ between -0.4 V to +0.8 V at scan rate 100 mV·s^{-1} on BDDE. In red curve, BDDE after anodic activation at +2.4V for 1.5 hours. In black curve, BDDE polished for 5 minutes.

Table 10. Differences between CV anodic and cathodic peak potentials of $1\cdot10^{-4}$ mol·L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ after using it 1.5 hours at 2.4V and after polished the BDDE.

BDD electrode	ΔE_p / V
Oxidized	0.756
Polished	0.135

In Fig. 10, a decrease of both, cathodic and anodic signal height and an increase of the ΔE_p value (Table 10) can be recognized for oxidized surface (red curve) in comparison with the polished surface (black curve). This difference corresponds to hindered electron transfer because of the oxygen-containing groups on the O-terminated surface. After the polishing, a reduction of the ΔE_p is shown due to faster electron transfer. For that reason, BDDE pre-treatment using polishing was made before each working day.

4.3.2 Oxidizability of the triazoles in the HPLC-ED setup

Amperometry at constant potential was used to study the oxidizability of the triazole pesticides in the HPLC-ED setup. An overflow home-made wall-jet electrochemical cell system with polished BDD working electrode was used for that purpose. Firstly, hydrodynamic voltammograms were ploted. For that, injection of a mixture containing $1\cdot10^{-4}$ mol·L^{-1} of CPZ, TBZ, PNZ, PPZ and DFZ (Fig. 11) was made using previous optimized separation conditions. Other azoles were injected separately, because their retention times are similar to those of the previously mentioned pesticides and thus overlap of peaks was observed in chromatograms. FCZ was injected too in higher concentration of $1\cdot10^{-3}$ mol·L^{-1} because using the concentration of $1\cdot10^{-4}$ mol·L^{-1} the peak was not recognizable. The detection potential, E_{det}, was increased from +1.4 V to +2.6 V and the background current was measured. At higher potentials, the current steeply increases because of water decomposition reaction (eq. 1), and causes relative decline of the peak heights of studied triazoles (Fig. 11).

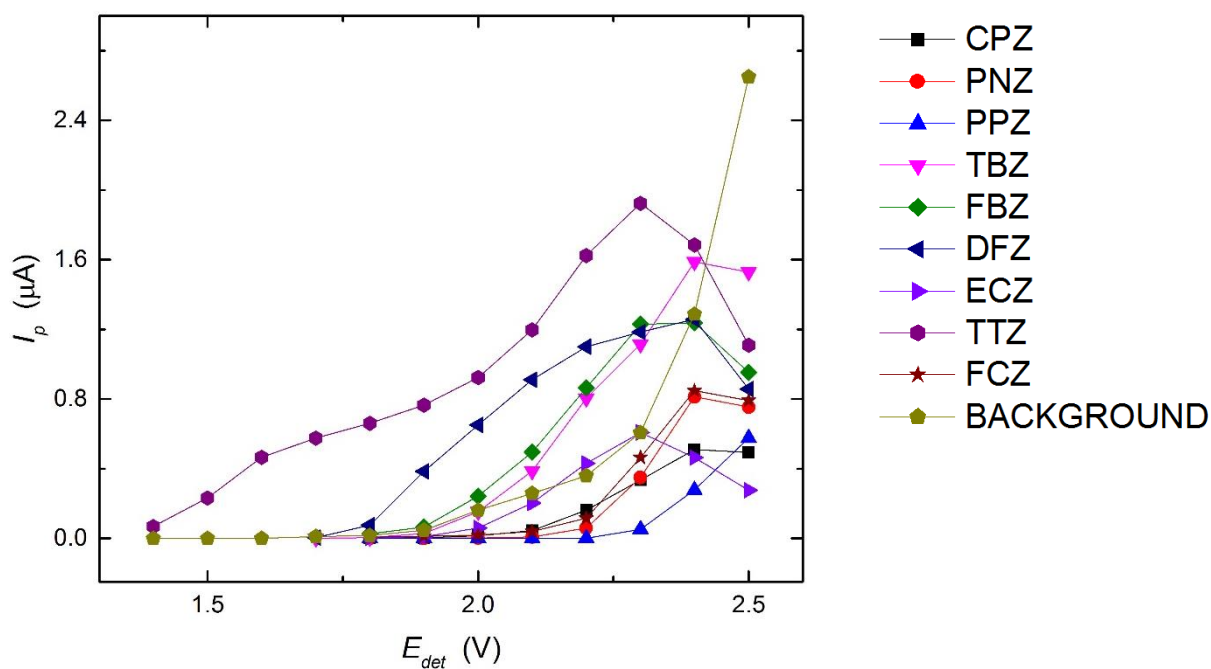*Figure 11. Hydrodynamic voltammograms of triazole pesticides measured on a polished BDDE in HPLC-ED system with condition as in Fig. 5 and FCZ concentration $1·10^{-3}$ mol·L^{-1}, other compounds $1·10^{-4}$ mol·L^{-1}.*
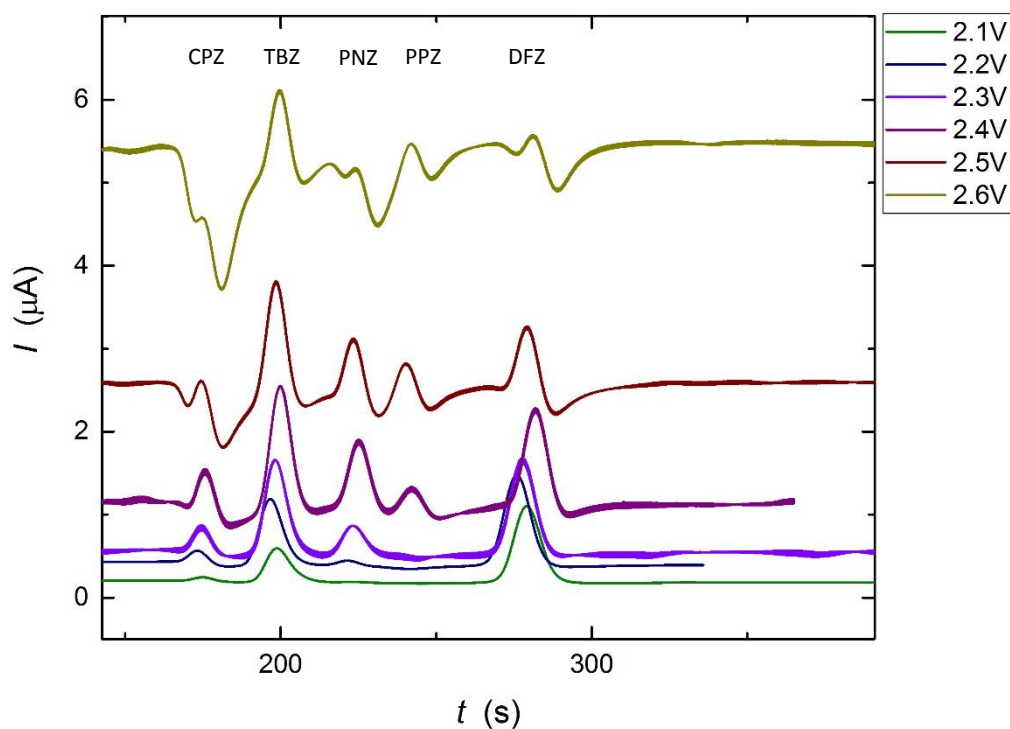
Figure 12. Chromatograms of CPZ, TBZ, PNZ, PPZ and DFZ (in order of appearance) at concentration $1·10^{-4}$ mol·L^{-1} obtained at different detection potentials. Separation conditions as in Fig. 5.

Fig. 12 presents the chromatograms of CPZ, TBZ, PNZ, PPZ and DFZ mixture where the background current increase is visualized as well as the decrease of the peak heights. At detection potentials higher than +2.6 V the peaks of triazole pesticides could not be recognized.

Based on the hydrodynamic voltammograms, the minimal detection potentials, E_{det}, which have to be applied to see the signals of the particular triazoles in HPLC-EDD are summarized in Table 11.

Table 11. Minimal E_{det} (V) for oxidation of the studied triazole compounds estimated using HPLC-ED in Fig. 5 conditions.

Compound	Concentration (mmol·L^{-1})	Minimal E_{det} (V) for oxidation
CPZ	0.1	2.2
PNZ	0.1	2.3
PPZ	0.1	2.3
TBZ	0.1	2.0
FBZ	0.1	1.9
DFZ	0.1	1.6
FCZ	1.0	2.2
ECZ	0.1	2.0
TTZ	0.1	1.4

Based on the E_{det} values, triazole pesticides can be divided in four groups: TTZ oxidizable at $E_{det} \geq$ +1.4 V, DFZ at $E_{det} \geq$ +1.6 V, FBZ, TBZ and ECZ at $E_{det} \geq$ +1.9 V − +2.0 V and PNZ, CPZ , FCZ and PPZ at $E_{det} \geq$ +2.2 V − +2.3 V. Obviously, TTZ is the most easily oxidizable compound: oxidation starts at +1.4 V and possesses two signals, one with maxima at ca +1.7 V and the second at ca + 2.3 V. Presumably, the first peak is due to oxidation of the double bond in the structure of triticonazole (see Table 1). The origin of the second oxidation signal, as well as the origin of the signals of all other triazole pesticides is questionable. In general, they possess halogen/s (chlorine or fluorine) substituted in their benzene rings, thus the oxidation could be connected with electron removal from

the aromatic ring forming a radical cation. The difference in oxidation potentials is given by their different structures, e.g. type of halogen, structure of the rest molecule attached to the aromatic ring, directly influencing electron density in the aromatic ring. Mechanism of oxidation will be the subject of other study. FCZ could not be detected using HPLC-DAD but in HPLC-ED its signal was registered at 1.5 min using a flow rate of 1.0 mL·min^{-1}, being among the worst oxidizable compounds; using FCZ concentration of $1·10^{-4}$ mol· L^{-1} the peak was not observed and it appeared only for concentration of $1·10^{-3}$ mol·L^{-1}.

4.3.3 Concentration dependences

The concentration dependences were measured for the mixture of CPZ, TBZ, PNZ, PPZ and DFZ at detection potential, E_{det}, of +2.3 V and +2.4 V because of reasonable peak height with respect to background current. Optimized conditions for HPLC separation were used, i.e. isocratic elution with mobile phase consisting of ACN : 0.1 mol·L^{-1} phosphate buffer pH 2.0, 57:43 (*v:v*), and flow rate of 1.0 mL·min^{-1}. Signal-concentration linearity was tested using concentrations between $1·10^{-4}$ mol·L^{-1} and $1·10^{-5}$ mol·L^{-1}. Measuring four times each concentration; linear regression, *LOD* and *LOQ* were evaluated (Fig.14, Table 12) for each detection potential.

The linearity of the concentration dependences is good, as confirms the coefficient of determination R^2 nearing to one. For the two detection potentials, using +2.3 V lower *LOQ* was achieved and slightly better correlation. However, the intercept interval does not include the 0, probably because blank correction was not done.
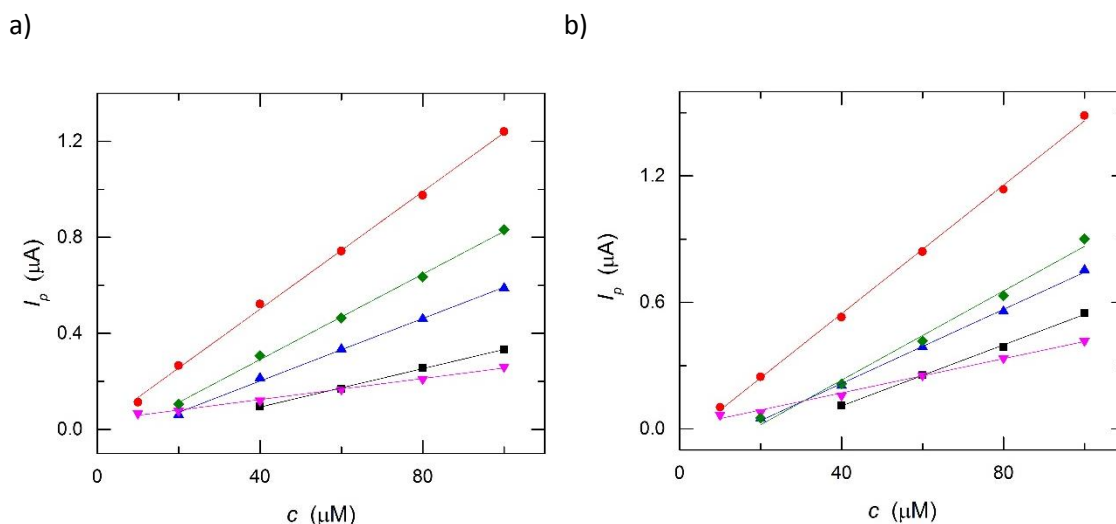
a)   b)

Figure 13. Concentration dependences for CPZ, TBZ, PNZ, PPZ and DFZ evaluated using HPLC-ED (conditions as in Fig.5). CPZ in black squares, TBZ in red circles, PNZ in blue triangles, PPZ in pink cones and DFZ in green diamonds. Evaluated using peak heights at two detection potentials a) +2.3 V b) +2.4 V

Table 12. Parameters of concentration dependences and LOD and LOQ values for CPZ, TBZ, PNZ, PPZ and DFZ obtained using by linear regression evaluated using peak heights obtained by HPLC-ED and separation conditions as in Fig. 5. for two detection potentials: + 2.3 V and + 2.4 V.

+2.3V	LDR / µM	Slope ± SD / µA·µM⁻¹	Intercept ± SD / µA	R² / ad.	LOD / µM	LOQ / µM
CPZ	40-100	0.0040±0.0001	-0.6670±0.0064	0.999	4.81	16.0
TBZ	10-100	0.0123±0.0002	0.0110±0.0137	0.998	4.00	13.3
PNZ	20-100	0.0065±0.0002	-0.0587±0.0102	0.998	4.68	15.6
PPZ	10-100	0.00219±0.0001	0.0371±0.0044	0.995	6.05	20.2
DFZ	20-100	0.0089±0.0002	-0.0652±0.0139	0.998	4.69	15.6

+2.4V	LDR / µM	Slope ± SD / µA·µM⁻¹	Intercept ± SD / µA	R² / ad.	LOD /µM	LOQ / µM
CPZ	40-100	0.0072±0.0002	-0.1804±0.0141	0.998	5.85	19.5
TBZ	10-100	0.0153±0.0003	-0.0635±0.0158	0.999	3.11	10.4
PNZ	20-100	0.0088±0.0002	-0.1368±0.0124	0.998	4.22	14.1
PPZ	10-100	0.0041±0.0002	0.0083±0.0091	0.993	6.75	22.5
DFZ	20-100	0.0106±0.0006	-0.1921±0.0367	0.989	10.42	34.7

LOD and LOQ was recalculated in ppm for +2.3 V and the values are shown in Table 13. Nevertheless, this LOQ is not low enough to determine the triazole pesticides in the tomato real samples. For that reason, a comparison of tomato sample concentrations obtained by HPLC-DAD and HPLC-ED setup could not be performed.

Table 13. LOQ and LOD in ppm for CPZ, TBZ, PNZ, PPZ and DFZ obtained from the data in Table 12.

Compound	LOD / ppm	LOQ / ppm
CPZ	1.4	4.7
TBZ	1.2	4.1
PNZ	1.3	4.4
PPZ	2.1	6.9
DFZ	1.9	6.3

5. CONCLUSIONS

In this work, the separation conditions were optimized for detection of triazole pesticides using HPLC-DAD system with reversed stationary phase and isocratic mode of elution. Separation of the nine selected azole pesticides (CPZ, PNZ, PPZ, TBZ, FBZ, DFZ, FCZ, ECZ, TTZ) was not achieved because of similar retention times of some of them. At least, separation of five of these compounds (CPZ, TBZ, PNZ, PPZ and DFZ), was achieved using Kromasil Eternity-5 PhenylHexyl column, 1.0 mL·min^{-1} flow rate and mobile phase 57:43 (*v:v*) acetonitrile and 0.1 mol·L^{-1} phosphate buffer of pH 2.0. Linearity was proven in a wide range that allows to detect concentrations lower than the maximum residue level of these pesticides in food allowed by EU when an efficient extraction technique is employed.

Oxidizability of azole pesticides was found at high potentials using a BDD electrode. However, the lower sensitivity of this detector does not allow to use HPLC-ED as an analytical method for detection or quantification of triazole pesticides in real matrices. *LOD* and *LOQ* for both methods are summarized in Table 14, using HPLC-DAD they are at least 10 times lower than for HPLC-ED

Table 14. Different LOD and LOQ for HPLC-DAD and HPLC-ED.

Compound	HPLC-DAD		HPLC-ED	
	LOD / ppm	*LOQ* / ppm	*LOD* / ppm	*LOQ* / ppm
CPZ	0.09	0.31	1.4	4.7
TBZ	0.11	0.35	1.2	4.1
PNZ	0.09	0.31	1.3	4.4
PPZ	0.12	0.40	2.1	6.9
DFZ	0.15	0.50	1.9	6.3

Fluconazole determination using HPLC-DAD was problematic because it does not absorb sufficiently in UV region. Further, it oxidation needs very positive potentials and high concentrations to observe signals in HPLC-ED. For that reason, other methods of detection have to be found for this compound.

Because of the possible dangerous exposure of humans to triazolic pesticides and the scientific uncertainties about their interaction between them, more researches have to be made, providing us the achievement of safe and reliable methods that will avoid the endanger of human beings.

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9. Reglamento (UE) nº 1004/2013 de la Comisión, de 15 de octubre de 2013, por el que se modifican los anexos II y III del Reglamento (CE) no 396/2005 del Parlamento Europeo y del Consejo en lo relativo a los límites máximos de residuos de ciproconazol, ciprodinilo, fluopiram, 8-hidroxiquinolina, nicotina, pendimetalina, pentiopirad y trifloxistrobina en determinados productosTexto pertinente a efectos del EEE.

10. Reglamento (UE) nº 89/2019 de la Comisión, de 18 de enero de 2019, que modifica los anexos II, III y V del Reglamento (CE) n.o 396/ 2005 del Parlamento Europeo y del Consejo por lo que respecta a los límites máximos de residuos de bromadiolona, etofenprox, paclobutrazol y penconazol en determinados productos.

11. Reglamento (UE) nº 750/2010 de la Comisión, de 7 de julio de 2010, que modifica los anexos II y III del Reglamento (CE) no 396/2005 del Parlamento Europeo y del Consejo por lo que respecta a los límites máximos de residuos de determinados plaguicidas en determinados productos.

12. Reglamento (UE) nº 491/2014 de la Comisión, de 5 de mayo de 2014, en lo relativo a los límites máximos de residuos de ametoctradina, azoxistrobina, cicloxidim, ciflutrina, dinotefurán, fenbuconazol, fenvalerato, fludioxonil, fluopiram,

flutriafol, fluxapiroxad, glufosinato de amonio, imidacloprid, indoxacarbo, MCPA, metoxifenozida, pentiopirad, espinetoram y trifloxistrobina en determinados productos.

13. Reglamento (UE) nº 34/2013 de la Comisión, de 16 de enero de 2013, por el que se modifican los anexos II, III y IV del Reglamento (CE) no 396/2005 del Parlamento Europeo y del Consejo por lo que respecta a los límites máximos de residuos de 2-fenilfenol, ametoctradina, las cepas DSM 14940 y DSM 14941 de Aureobasidium pullulans, ciproconazol, difenoconazol, ditiocarbamatos, folpet, propamocarb, espinosad, espirodiclofeno, tebufenpirad y tetraconazol en determinados productos.

14. Reglamento (UE) 978/2011 de la Comisión. de 3 de octubre de 2011. por el que se modifican los anexos II y III del Reglamento (CE) no396/2005 del Parlamento Europeo y del Consejo en lo que respecta a los límites máximos de residuos en determinados productos.

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