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Development of methods for voltammetric determination of biologically important substances

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Abstract

This Ph.D. thesis was focused on the development of methods for voltammetric determination of biologically important substances, namely plant growth regulators, especially paclobutrazol, daminozide and its degradation product *N*,*N*-dimethylhydrazine and fungicides, namely mefentrifluconazole, fluxapyroxad and metconazole. The determination was carried out in an alkaline environment using differential pulse voltammetry in combination with a working boron-doped diamond electrode. As supporting electrolytes, 0.07 mol L^{-1} NaOH with 33 % acetonitrile for paclobutrazol, pure Britton-Robinson buffer (pH 9) for daminozide and *N*,*N*-dimethylhydrazine and Britton-Robinson buffer (pH 9) with 33 % acetonitrile for mefentrifluconazole, fluxapyroxad and metconazole were selected for the analysis of the studied compounds. Quantitative analyses were carried out in these supporting electrolytes, and subsequently, the limit of detection and limit of quantification values were calculated to be 0.083 and 0.277 μmol L^{-1} for paclobutrazol, 0.44 and 1.46 μmol L^{-1} for daminozide, 0.063 and 0.209 μmol L^{-1} for *N*,*N*-dimethylhydrazine, 10.81 and 36.04 nmol L^{-1} for mefentrifluconazole, 13.98 and 46.61 nmol L^{-1} for fluxapyroxad and 32 and 107 nmol L^{-1} for metconazole, respectively. The developed methods were successfully applied to the analysis of mixtures of selected analytes as well as to their determination in commercial preparations.

Abstrakt

Tato disertační práce byla zaměřena na vývoj metod voltametrického stanovení biologicky významných látek, a to regulátorů růstu rostlin, zejména paklobutrazolu, daminozidu a jeho rozkladného produktu *N*,*N*-dimethylhydrazinu a fungicidů mefentriflukonazolu, fluxapyroxadu a metconazolu. Stanovení se provádělo v alkalickém prostředí s využitím diferenční pulzní voltametrie v kombinaci s pracovní borem dopovanou diamantovou elektrodou. Jako základní elektrolyty byly pro analýzu zájmových látek vybrány 0.07 mol L^{-1} NaOH s 33 % acetonitrilu pro paklobutrazol, čistý Britton-Robinsonův pufr o pH 9 pro daminozid a *N*,*N*-dimethylhydrazin a Britton-Robinsonův pufr o pH 9 s 33 % acetonitrilu pro mefentriflukonazol, fluxapyroxad a metconazol. V těchto základních elektrolytech byly v rámci kvantitativní analýzy dosaženy uspokojivé meze detekce a meze stanovitelnosti, které byly 0.083 a 0.277 μmol L^{-1} pro paklobutrazol, 0.44 a 1.46 μmol L^{-1} pro daminozid, 0.063 a 0.209 μmol L^{-1} pro *N*,*N*-dimethylhydrazin, 10.81 a 36.04 nmol L^{-1} pro mefentriflukonazol, 13.98 a 46.61 nmol L^{-1} pro fluxapyroxad, a 32 a 107 nmol L^{-1} pro metconazol. Navržené metody byly úspěšně aplikovány na analýzu vybraných směsí studovaných analytů a jejich komerčních přípravků.

Keywords

plant growth regulators, fungicides, voltammetric determination, differential pulse voltammetry, boron-doped diamond electrode

Klíčová slova

regulátory růstu rostlin, fungicidy, voltametrické stanovení, diferenční pulzní voltametrie, borem dopovaná diamantová elektroda

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Introduction

Nowadays, plant growth regulators and fungicides are an integral part of modern agriculture and horticulture. Plant growth regulators are mainly used to modify plants' size and appearance, improve the characteristics and quality of fruits and strengthen plants against adverse natural phenomena such as storms, hail, floods, droughts, etc. Fungicides are used to suppress and control fungal diseases [1-6].

In addition to their beneficial effects, plant protection products can cause environmental damage and be toxic to humans and animals if not handled according to current regulations. Since the possibility of human error always exists, there is a need to control the amount of these substances in environmental compartments and prevent their release.

This work focuses on developing methods for the voltammetric determination of commonly used plant growth regulators and fungicides. The substances for which methods have been developed include the plant growth regulator daminozide and its degradation product *N*,*N*-dymethylhydrazine, as well as commonly used fungicides, namely mefentrifluconazole, fluxapyroxad and metconazole. Also, in this paper, findings are presented that extend the already developed and described method for the voltammetric determination of the plant growth regulator paclobutrazol [7].

The development of methods has focused on analysing these substances and their commercially available preparations containing either one or a mixture of the studied analytes. Procedures for the determination of paclobutrazol and daminozide in enriched river and apple rinse water samples are also described.

1. Theoretical part

1.1 Plant protection products

Agriculture and horticulture have always been crucial sectors for the survival of humankind. In the past, adverse natural conditions, fungal diseases and pests could result in crop failure, leading to famine throughout a village or region. However, over the last 150 years or so, science has advanced to the point that all these negative effects can be prevented or at least reduced by using plant growth regulators and fungicides [1-6].

1.1.1 Plant growth regulators

Plant growth regulators are a large group of substances that include phytohormones, also called natural plant growth regulators, and plant growth retardants, which are synthetically developed substances. Phytohormones are directly produced by plants and were discovered in the 1920s. These substances can induce significant changes in the plant organism at very low concentrations without being phytotoxic. The first discovered phytohormone was indolyl-3-acetic acid (IAA), whose main effect was then considered to be the stimulation of plant growth elongation. Later, it was also found to stimulate root growth and cell division and to regulate fruit and leaf drop. With time, other groups of phytohormones have been discovered, namely cytokinins, gibberellins, abscisic acid, etc. The above-mentioned synthetic growth regulators are not related to phytohormones, but they can influence their metabolism and transport to varying degrees and, depending on the concentration, can both stimulate and inhibit plant growth and development [1-4]. Most of the synthetic plant growth retardants used in the world are inhibitors of gibberellin synthesis. The first gibberellin was obtained by fermentation of the phytopathogenic fungus *Giberella fujikuroi*, due to which abnormal elongation of shoot growth was observed in rice. Today, 140 gibberellins are known, but the most widely used are GA3 or gibberellic acid and a mixture of GA4 and GA7 [1, 2, 8, 9].

1.1.2 Fungicides

Nowadays, the use of fungicides is an integral part of agriculture and horticulture, as there is a need to prevent and protect cultivated commodities from various fungal pathogens both during cultivation and subsequent storage. Pathogens can lead to blight in wheat, sugar beet, spinach, chard and other essential crops. Also, the development of moulds in cereals causes loss of dry matter, degradation of lipids and proteins, unpleasant taste, etc. Cereals are most commonly attacked by pathogens of the genera *Fusarium*, *Alternaria*, *Penicillium* and *Aspergillus*, and grapes are most at risk from infections by *Botrytis cinerea*, *Plasmopara viticola* and *Uncinula necator*. According to their chemical group and mode of action, fungicides can be divided into triazoles, dicarboximides, benzimidazoles, dithiocarbamates, phenylamides, phthalimides, anilinopyrimidines, phenylpyrroles, phenoxyquinolines, QoI-fungicides and inorganic compounds [5, 6].

Some pathogens may develop resistance to a particular fungicide, and it may no longer be effective. This resistance is a significant problem for farmers and fungicide providers that must be solved. Therefore, new products are being developed to prevent this resistance, one of which is mefentrifluconazole [6, 10].

Both plant growth regulators and fungicides can pose a risk to the environment and people who come into contact with them, so it is very important to handle them according to current regulations and follow safety instructions.

1.2 The studied substances

1.2.1 Paclobutrazol

Paclobutrazol, (2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol, abbreviated PBZ, is a widely known plant growth regulator used since 1985. The main effect of PBZ is inhibition of gibberellin production, which subsequently leads to retardation of elongation growth [11, 12]. Other effects of PBZ are improvement of storage capacity, quality and size of fruits (e.g., apples). Moreover, PBZ-treated plants better tolerate adverse natural conditions such as drought or increased soil salinity [12-14]. PBZ is considered an environmentally stable compound in aquatic and soil environments, with a half-life in soil of 43-618 days under anaerobic conditions. However, potential releases of PBZ to the environment, especially to water, should be strictly monitored as PBZ has acute (H400) and chronic (H410) toxicity to aquatic organisms [15, 16].

1.2.2 Daminozide

Daminozide (4-(2,2-Dimethylhydrazin-1-yl)-4-oxobutanoic acid), abbreviated DMZ, is a plant growth regulator used since 1963 since it was registered in the USA for use on potted chrysanthemums [17]. With time, its use has expanded to other commodities such as apples, peaches, cherries, seed potatoes, field beans, groundnuts and ornamentals [17, 18]. DMZ was used a lot, mainly in apple and peach cultivation, because it contributed to increasing flower development, fruit size and color, reducing vegetative growth and improving storage properties [18, 19]. However, since 1989, its use on food crops has been banned due to suspected carcinogenicity. It was found that residues of DMZ could be found in apples and apple products up to one year after application of this product. The biggest problem is that when, e.g., apple juice is heated, DMZ degrades to produce the much more toxic *N*,*N*-dymethylhydrazine (UDMH), classified as a "probable human carcinogen", group B2 [17-21]. During studies on rats, DMZ was found not to cause developmental or reproductive toxicity. Also, no mutagenicity was demonstrated for DMZ together with UDMH [17]. DMZ is almost non-toxic to mammals, freshwater fish, and birds and moderately toxic to aquatic invertebrates. Regarding the environmental fate of DMZ, it degrades rapidly in soil but is considered stable to hydrolysis [17].

1.2.3 Mefentrifluconazole

Mefentrifluconazole ((2RS)-2-(4-(4-chlorophenoxy)-α,α,α-trifluoro-o-tolyl)-1-(1H-1,2,4-triazol-1-yl)propan-2-ol), abbreviated MFZ, is a representative of DMI-fungicides, whose main effect is inhibition of sterols demethylation, especially of ergosterol. MFZ has high fungicidal activity and is used to protect cereals, fruits, and vegetables against fungal diseases [22, 23]. As described above, one of the problems in using fungicides is the development of resistance to these fungicides in fungal pathogens. Many of the DMI-fungicides face this problem. However, it has been found that this resistance does not occur with MFZ in certain cases. For example, MFZ is very effective against many triazole-resistant isolates of the pathogen *Zymoseptoria tritici* and other pathogens [24]. MFZ, like most triazoles, is a chiral compound and exists in the form of two enantiomers, namely *S*-(+)-MFZ and *R*-(-)-MFZ. In one study, *R*-(-)-MFZ was found to be a much more effective inhibitor of ergosterol biosynthesis than *S*-(+)-MFZ. Still, the commercial formulation contains a racemic mixture of both these enantiomers [22, 25]. Regarding short- and long-term exposure, the target organ for MFZ is the liver, but as long as the NOAEL of 3.5 mg/kg bw per day is not exceeded, no adverse effects should be observed. MFZ has low acute toxicity when taken orally, dermally and inhaled and is not considered to be immunotoxic, neurotoxic, genotoxic, or teratogenic but has been classified as a skin sensitizer (H317). MFZ is highly persistent in aqueous and soil environments with a half-life (DT50) of 104 to 477 days (soil) and has a high sorption capacity [26]. MFZ is classified as " very toxic to aquatic life" (H400) and " very toxic to aquatic life with long-lasting effects" (H410), so its releases to the aquatic environment should be strictly monitored [27, 28].

1.2.4 Fluxapyroxad

Fluxapyroxad (3-(difluoromethyl)-1-methyl-N-[2-(3,4,5-trifluorophenyl)phenyl] pyrazole-4-carboxamide), abbreviated FPX, is a potent SDHI-fungicide, whose action is based on the inhibition of succinate dehydrogenase (SDH) in complex II of the fungal mitochondrial respiratory chain. FPX is used to control and suppress fungal diseases in fruit trees, vegetables, cereals, legumes, cotton, and others [29,30]. As reported in one study, in addition to its fungicidal effects, FPX also contributed to improved photosynthesis rate, increased leaf greening and water use efficiency [31, 32]. As in the case with MFZ, the target organ for FPX is the liver; moreover, it has been classified as an "unlikely carcinogen", Group 4. FPX is a very persistent substance with a DT50 of 213 to 1827 days for soil and a DT50 of 420 to 731 days for aquatic systems. As FPX is moderately to slightly mobile and is also classified as H400 and H410, it is necessary to prevent its release into the environment [29, 33].

1.2.5 Metconazole

Metconazole (5-[(4-chlorophenyl)methyl]-2,2-dimethyl-1-(1,2,4-triazol-1-ylmethyl) cyclopentan-1-ol), abbreviated MCZ, is another representative of DMI-fungicides. MCZ was developed in 1992 in Japan by Kureha Corporation. It is currently registered in 40 countries, including the EU, to control a wide range of crops, including canola, wheat, corn, soybean and others [34, 35]. MCZ is also a chiral substance and exists as two pairs of diastereoisomers and two pairs of enantiomers, namely *rac*-MCZ, *cis-1R,5S*-MCZ, *cis-1S,5R*-MCZ, *trans-1S,5S-* MCZ and *trans-1R,5R*-MCZ. These enantiomers differ in their toxicity to certain living organisms (e.g., *Chlorella pyrenoidosa*) as well as in their bioactivity against certain pathogens, namely, *Fusairum graminearum* and *Alternaria triticina* [35, 36]. The acute toxicity of MCZ is low to minimal for all exposure pathways, and it is not expected to be carcinogenic, neurotoxic, or mutagenic but is suspected of reproductive toxicity, particularly harm to the unborn child (H361d). The main pathway of degradation of MCZ in the environment is aerobic metabolism in soil, DT50 from 192.5 to 660 days. MCZ is considered a moderately mobile substance and is stable to hydrolysis and moderately to slightly degradable by aqueous photolysis; DT50 in water was 1-15 days and in sediments, 116-814 days. A release of MCZ to environmental compartments could endanger non-target organisms such as mammals and birds, vascular and non-vascular aquatic plants, freshwater and marine fish (MCZ is classified as "toxic to aquatic organisms with long-term effects" (H411)) and invertebrates [37].

1.2.6 Methods for the determination of plant growth regulators and fungicides

The most commonly used method for the determination of plant growth regulators and fungicides is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [15, 20, 26, 34]. Depending on the nature of the sample being analysed, this method can be supplemented with additional sample pre-cleaning and extraction procedures and enhanced using a different non-mass detector. Voltammetric methods for directly determining the studied analytes have not yet been described.

1.3 Voltammetric methods in electroanalysis

The main voltammetric method used in this work is differential pulse voltammetry (DPV). As is evident from the name, it is a pulse method based on the insertion of voltage pulses on a linearly increasing potential. In DPV, the current reading is taken twice before the pulse is inserted and at the end of the pulse; then, these currents are subtracted according to the formula $\Delta I = I_2 - I_1$, and the value obtained is plotted on a graph as a function of potential. The output of the DPV measurement is a voltammogram in the form of a peak. The height of this peak (or the magnitude of the current) is directly proportional to the concentration of the studied analyte. DPV is one of the most sensitive pulse methods; with its use, very low limits of detection and limits of quantification can be achieved. Therefore, this method is used to analyse trace concentrations of important compounds in environmental compartments [38-41].

Another voltammetric method used in this work is cyclic voltammetry (CV), which is based on the principle of continuous change of the insertion potentials at the electrode-solution interface and measurement of the resulting current. In CV, the observed current is a function of potential and time, and the working electrode's potential is linearly dependent on time. The CV method is very useful in determining the character of the electrochemical reaction undergoing the analyte under study and also in determining the controlling process occurring at the electrode [38-41].

Boron-doped diamond electrode (BDDE) is widely used in modern voltammetric analysis. Due to its wide potential window, BDDE can be used for analysis of both very positive and negative signals in aqueous and organic environments. BDDE provides a stable electrochemical response, which is essential for reliable analysis. Another advantage is its inertness, as the studied substances are complex organic compounds. Because of all these benefits, BDDE has been selected as a working electrode for the development of voltammetric methods for the determination of plant growth regulators and fungicides [38-41].

2. Experimental part

2.1 Chemicals

Standard solutions of PBZ with a concentration from 4.5 to 6.0 mmol L^{-1} (Sigma-Aldrich, Germany) were prepared by weighing and transferring the calculated amount of analytical standard into a 25 ml flask and adding acetonitrile (ACN) of purity p.a. (Ing. Petr Švec-PENTA s.r.o, Czech Republic). Standard solutions of DMZ (Sigma-Aldrich, Germany) with a concentration of 0.01 mol L^{-1} and UDMH (98 %, Sigma-Aldrich, Germany) with a concentration of 0.025 mol L^{-1} were prepared by weighing (for UDMH by measuring) the respective amounts into a 25 ml flask and adding 0.01 mol L^{-1} NaOH (Ing. Petr Švec-PENTA s.r.o., Czech Republic) for DMZ and 0.1 mol L^{-1} H_2SO_4 (Ing. Petr Švec-PENTA s.r.o., Czech Republic) for UDMH. Standard solutions of MFZ with a concentration from 0.2 to 4 mmol L^{-1}, FPX with a concentration from 0.08 to 4 mmol L^{-1}, and MCZ with a concentration from 0.3 to 4 mmol L^{-1} were prepared by weighing and transferring the calculated amount of the respective isolated substance (the isolation procedure is described in Chapter 2.3) into a 25 ml flask and adding ACN. ACN was also used as part of the supporting electrolyte.

The analysed commercial products were B-NINE®SG containing 850 g DMZ/1000 g (Arysta LifeScience Registrations Great Britain LTD., Great Britain), Revysol containing 75 g L^{-1} MFZ, Revytrex containing 66.7 g L^{-1} MFZ and 66.7 g L^{-1} FPX, Revystar XL containing 100 g L^{-1} MFZ and 50 g L^{-1} FPX, Caramba containing 60 g L^{-1} MCZ, Librax containing 42 g L^{-1} MCZ and 62.5 g L^{-1} FPX (BASF Ltd, Czech Republic) and Sergadis containing 300 g L^{-1} FPX (AgroBio Opava s.r.o., Czech Republic).

Solutions of real samples of the studied substances were prepared by weighing (or measuring) the calculated amount of the respective preparation into a 25ml volumetric flask and filled to the mark with ACN (or deionised water for DMZ).

A solution of 0.1 mol L^{-1} NaOH, used as a component of the supporting electrolyte during PBZ determination, was prepared by weighing an appropriate amount of solid and then dissolving it in distilled water. Britton-Robinson (BR) buffer solutions were prepared by mixing the acidic (0.04 mol L^{-1} solutions of H_3PO_4, H_3BO_3, and CH_3COOH) and alkaline (0.2 mol L^{-1} NaOH) components using a pH meter to obtain the desired pH value. Borate buffer with a concentration of 1.0 mol L^{-1} was prepared in a 100 mL volumetric flask by diluting 6.183 g of H_3BO_3 in deionised water and alkalinised to pH 9 with 1.0. mol L^{-1} NaOH solution. The strongly acidic ion-exchange resin Amberlite® IRC120 H in the H^+ cycle (SUPELCO, Sigma Aldrich, Germany, total volume capacity 1.92 mEq ml^{-1}) was used to isolate and concentrate DMZ, UDMH and PBZ from water.

2.2 Instrumentation

All voltammetric measurements were performed using Autolab PGSTAT 128N potentiostat/galvanostat (Metrohm Autolab, Utrecht, The Netherlands), operated with NOVA 1.11 software. The measurement cell consisted of three electrodes, namely a working – boron-doped diamond electrode (BDDE) (Windsor Scientific LTD, active surface 7.07 mm^2, inner diameter 3 mm, resistance 0.075 Ω cm with B/C ratio at deposition 1000 ppm), a reference - saturated Ag|AgCl|KCl electrode and an auxiliary electrode in the form of platinum wire (both electrodes from Monokrystaly, Czech Republic). The Denver TB 124 balance (Denver Instruments, USA) was used for weighing chemicals. Bandelin Sonorex ultrasonic baths (Schalltec Gmbh & Co. KG, Germany) were used to homogenise solutions. The pH measurements were performed using an ORION STAR A221 pH meter (Thermo Fisher Scientific, USA). Independent analysis was performed using an Agilent 1260 Infinity II Prime LC System HPLC analyzer equipped with a Macherey - Nagel EC 250/4 Nucleosil 120-5 C18 separation column (250 mm length, 4 mm inner diameter and 5 µm particle size) with a diode array detector (DAD). The mobile phase was a mixture of CH3CN/H2O (90/10, v/v). All measurements were carried out at laboratory temperature (23 ± 2 °C).

2.3 Procedures

The parameters for CV were as follows:

- for PBZ a potential (E) ranged from an initial potential (E_{in}) of 0 mV to an upper vertex potential ($E_{\text{upper vertex}}$) of +2600 mV, then switched to a lower vertex potential ($E_{\text{lower vertex}}$) of −200 mV and ended at E_{fin} potential of 0 mV, the scan rate (v) ranged from 10 to 100 mV s^{-1};
- for DMZ and UDMH: $E_{\text{in}} = E_{\text{fin}} = +600$ mV, $E_{\text{lower vertex}} = +500$ mV, $E_{\text{upper vertex}} = +2200$ mV, v = 20-350 mV s^{-1} (for DMZ), 20-200 mV s^{-1} (for UDMH);
- for MFZ and FPX: $E_{\text{in}} = E_{\text{fin}} = 0$ mV, $E_{\text{lower vertex}} = -800$ mV, $E_{\text{upper vertex}} = +2200$ mV, v = 10-100 mV s^{--1};
- for MCZ: $E_{\text{in}} = E_{\text{fin}} = 0$ mV, $E_{\text{lower vertex}} = -500$ mV, $E_{\text{upper vertex}} = +2300$ mV, v = 10-100 mV s^{-1}.

The parameters for DPV were as follows:

- For PBZ: $E_{\text{in}} = +1200$ mV, $E_{\text{fin}} = +2300$ mV, modulation amplitude (A) = +30 mV, modulation time (t) = 60 ms, v = 40 mV s^{-1};
- For DMZ, UDMH, MFZ, FPX, and MCZ: $E_{\text{in}} = +500$ mV, $E_{\text{fin}} = +2200$ mV (+2300 mV for MCZ), A = +50mV, t = 80 ms, v = 40 mV s^{-1}.

Before each measurement, electrochemical pretreatment of the working electrode surface was performed by applying a potential of +2000 mV, −200 mV and +2000 mV, each for 10 s. The limit of detection (LOD) and quantification (LOQ), respectively, was calculated as threefold (tenfold in the case of LOQ) of the standard deviation of the intercept divided by the slope of the respective linear concentration dependence.

Parameters for comparative analysis were as follows: injection volume was equal to 20 μl, mobile phase flow rate - 1 ml min^{-1}, DAD detector wavelength - 220 nm, analysis time - 5 min, analysis was repeated five times. The calibration curve method was used to evaluate the results.

Excess UDMH from the mixed sample (DMZ and UDMH) was eliminated by boiling in 1.0 mol L^{-1} NaOH solution for a few minutes.

Pure MFZ, FPX, and MCZ were obtained by isolation from the respective preparations. 10 ml of Revysol (for MFZ), Sercadis (for FPX), or Caramba (for MCZ) and 100 ml of chloroform were measured into an Erlenmeyer flask. The resulting heterogeneous mixture was stirred for 24 hours. The mixture was then filtered through a celite pad. Subsequently, the filtrate was evaporated. The filtrate was subjected to flash chromatography using a mobile phase of hexane/ethyl acetate (5 % to 40 % ethyl acetate). In the case of Sercadis, FPX was obtained as a white crystalline substance after evaporation. In contrast, in the case of Revysol and Caramba, a mixture of MFZ and MCZ, respectively, and C10-C13 hydrocarbons was obtained as a white waxy substance after evaporation. To remove residual hydrocarbons, the crude MFZ and MCZ were washed with hexane on a frit and then dried. Standards of MFZ, FPX and MCZ were obtained as white crystalline substances with a purity of> 99 %, as determined by gas chromatography with mass spectrometry (GC-MS) analysis. The nuclear magnetic resonance (NMR) results were in agreement with the literature.

During the analysis of real water samples with an admixture of DMZ and PBZ, a concentration technique based on analyte isolation using a strongly acidic Amberlite® IRC120 H catex in an H^+ cycle was used. In the case of DMZ, cations were removed from the water by adding EDTA under stirring for 10 min, and then DMZ was adsorbed with 1.0 g of catex for 1 h under stirring at 700 rpm at laboratory temperature. The ionex was then separated and extracted with 5 ml of 1.0 mol L^{-1} NaOH at 70 °C with stirring at 500 rpm for 20 min. The obtained extract was subsequently analysed voltammetrically. In the case of PBZ, the analyte was isolated from a volume of 250 ml using 0.1 g of catex from river water. The retention was carried out for 3 h, stirring at 700 rpm at laboratory temperature. Then, the ionex was separated and subsequently extracted with a mixture of 0.5 ml of 1 mol L^{-1} NaOH and 1 ml of acetonitrile for 5 min under boiling under reflux condenser and stirring at 500 rpm. The resulting solution was also analysed voltammetrically.

3. Results and discussion

3.1 The search for a suitable supporting electrolyte and pH value

The first step in developing methods for the determination of the studied analytes was searching for a suitable composition of supporting electrolyte and pH value.

In the master thesis [7], a suitable supporting electrolyte was recommended for the determination of PBZ, namely 0.07 mol L^{-1} NaOH with 33% ACN.

For DMZ, the experiments were performed in a pure BR buffer environment with a pH ranging from 2.16 to 10.16. The most suitable environment for the analysis of DMZ and UDMH was found to be alkaline, especially BR buffer with pH 9. In this environment, a linear dependence of peak height (I_p) on concentration (c), the highest sensitivity of response (for DMZ), and the optimum peak shape were obtained.

Three supporting electrolytes were tested for MFZ, FPX, and MCZ, namely, pure BR buffer, BR buffer with 33% ACN and BR buffer with 67% ACN. The pH values ranged from 2.1 to 11.19 for MFZ, from 2.03 to 11.02 for FPX and from 2.06 to 10.10 for MCZ. After evaluation of the measured data, it was found that pure BR buffer (aqueous environment) was unsuitable for the determination of the studied fungicides over the whole range of pH tested. Thus, for example, the potential of peak maxima (E_p) of MFZ and MCZ in aqueous acidic to slightly acidic media shifted significantly towards more positive potentials as a function of c. As for FPX, an unstable response was observed in the aqueous acidic environment with a decrease in peak at higher concentrations. This decrease may be due to the poor solubility of the analyte. In addition, the I_p of MFZ, FPX and MCZ increased non-linearly as a function of c over the whole range of pH tested in aqueous media. When the analyte determination was performed in a BR buffer medium with 33% ACN, a linear dependence of I_p on c was obtained, but only in a slightly alkaline to alkaline medium; moreover, the shift of E_p as a function of c was minimal. In the BR buffer medium with 67% ACN, the peaks of MFZ, FPX, and MCZ were located at the most positive potentials. Moreover, the dependence of I_p on c for MFZ was significantly nonlinear.

For the voltammetric determination of MFZ, FPX and MCZ, a BR buffer with 33% ACN and pH 9 was chosen since, for these analytes in this environment, the dependence of I_p on c was linear, a stable and reproducible response was achieved, moreover, the E_p shift as a function of c was minimal.

3.2 Voltammetric behaviour of studied substances

The next step was to investigate the voltammetric behaviour of the studied analytes using CV in selected supporting electrolytes. It can be seen from Figure 1 that all the studied substances undergo anodic oxidation. According to the shape of the backward curve, it can be further concluded that for all analytes, the electrochemical reaction is irreversible.

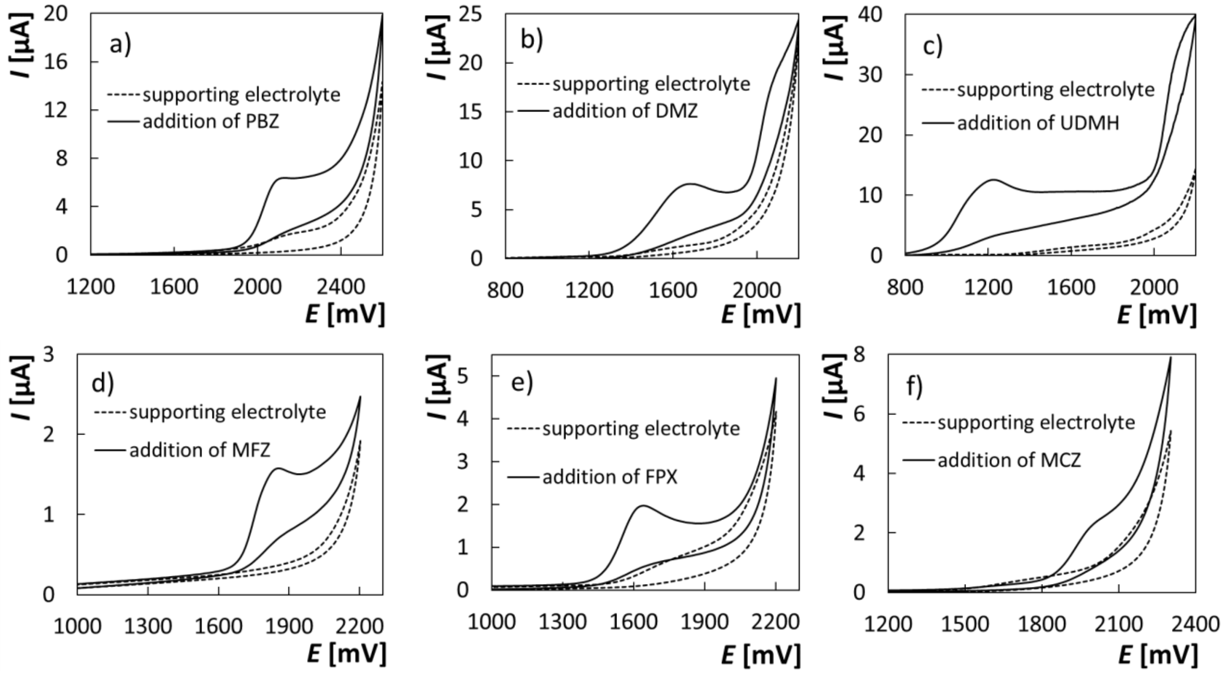

Figure 1: Cyclic voltammograms of supporting electrolyte and PBZ (a), DMZ (b), UDMH (c), MFZ (d), FPX (e) and MCZ (f)

(supporting electrolytes: 0.07 mol L^{-1} NaOH with 33 % ACN (a), BR buffer (pH 9) (b, c), BR buffer (pH 9) with 33% ACN (d-f); c_{PBZ} = 98.8 µmol L^{-1}, c_{DMZ} = 0.267 mmol L^{-1}, c_{UDMH} = 0.46 mmol L^{-1}, c_{MFZ} = 12.74 µmol L^{-1}, c_{FPX} = 74.06 µmol L^{-1}, c_{MCZ} = 66.29 µmol L^{-1}; $E_{in} = E_{fin}$ = 0 mV (a, d-f), +600 mV (b, c); $E_{lower\ vertex}$ = −200 mV (a), +500 mV (b, c), −800 mV (d, e), −500 mV (f); $E_{upper\ vertex}$ = +2600 mV (a), +2200 mV (b-e), +2300 mV (f); v = 40 mV s^{-1} (a-f); method - CV)

3.3 Scan rate study

To determine the controlling process of the electrode reaction, a study of the dependence of the current response on the scan rate was performed for each analyte on BDDE. The scan rate (v) values ranged from 20 to 350 mV s^{-1} for DMZ, from 20 to 200 mV s^{-1} for UDMH and from 10 to 100 mV s^{-1} for PBZ, MFZ, FPX and MCZ. From the measurements, the dependencies of I_p on v, I_p on $v^{1/2,}$ and log (I_p) on log (v) were plotted, the latter also being described by Equations 1 (PBZ), 2 (DMZ), 3 (UDMH), 4 (MFZ), 5 (FPX) and 6 (MCZ). After evaluating the measured data, it was found that for

DMZ and UDMH the electrode reactions are controlled by kinetics. The driving process for PBZ, MFZ, FPX and MCZ was found to be diffusion with the influence of kinetics.

$$\log (I_\mathrm{p}\ [\mu\mathrm{A}]) = (0.4274 \pm 0.0070) \log (v\ [\mathrm{mV\ s^{-1}}]) + (0.099 \pm 0.012),\ r = 0.9990 \quad (1)$$

$$\log (I_\mathrm{p}\ [\mu\mathrm{A}]) = (0.208 \pm 0.013) \log (v\ [\mathrm{mV\ s^{-1}}]) + (0.442 \pm 0.026),\ r = 0.\ 9855 \quad (2)$$

$$\log (I_\mathrm{p}\ [\mu\mathrm{A}]) = (0.172 \pm 0.013) \log (v\ [\mathrm{mV\ s^{-1}}]) + (0.598 \pm 0.025),\ r = 0.9853 \quad (3)$$

$$\log (I_\mathrm{p}\ [\mu\mathrm{A}]) = (0.2126 \pm 0.0070) \log (v\ [\mathrm{mV\ s^{-1}}]) - (0.358 \pm 0.011),\ r = 0.9978 \quad (4)$$

$$\log (I_\mathrm{p}\ [\mu\mathrm{A}]) = (0.\ 311 \pm 0.023) \log (v\ [\mathrm{mV\ s^{-1}}]) - (0.259 \pm 0.038),\ r = 0.9891 \quad (5)$$

$$\log (I_\mathrm{p}\ [\mu\mathrm{A}]) = (0.444 \pm 0.014) \log (v\ [\mathrm{mV\ s^{-1}}]) - (0.452 \pm 0.023),\ r = 0.9962 \quad (6)$$

3.4 Quantitative determinations of the studied substances

In the selected supporting electrolyte and with the DPV parameters specified in the experimental section, the concentration dependencies for each analyte were measured using BDDE as a working electrode:

- For PBZ, the linear dependence was measured over the concentration range from 0.521 to 52.1 µmol L^{-1}, shown in Figure 2a and described by Equation 7. For a narrower concentration range of PBZ, in particular, for c_PBZ from 0.521 to 5.21 µmol L^{-1}, the values of LOD = 0.083 µmol L^{-1} and LOQ = 0.277 µmol L^{-1} were calculated.
- For DMZ, anodic curves were measured over the concentration range from 21.79 to 174.32 µmol L^{-1}, shown in Figure 2b. It can be seen from the figure that over the whole concentration range, the I_p increases linearly as a function of c_DMZ; this dependence is described by Equation 8. The LOD and LOQ values for concentration range from 1.69 to 13.52 µmol L^{-1} were calculated and were 0.44 and 1.46 µmol L^{-1}, respectively.
- For UDMH, the concentration range was from 50.45 to 403.59 µmol L^{-1}. The obtained anodic curves are shown in Figure 2c. The linear dependence of I_p on c_UDMH for the above concentration range is described by Equation 9. For the concentration range from 0.499 to 2.994 µmol L^{-1}, the values of LOD and LOQ were 0.063 and 0.209 µmol L^{-1}, respectively.
- For MFZ, the linear dependence of I_p on c_MFZ was obtained over the concentration range from 0.051 to 12.75 µmol L^{-1}, which is described by Equation 10. As an example, the anodic curves of MFZ measured over a narrower concentration range, namely from 0.068 to 6.79 µmol L^{-1}, are shown in Figure 2d. The LOD and LOQ values were 10.81 and 36.04 nmol L^{-1}, respectively, for the MFZ concentration range from 0.051 to 2.55 µmol L^{-1}.
- For FPX, the studied concentration range was from 0.1 to 13.6 µmol L^{-1}, and the obtained linear dependence of I_p on c_UDMH is described by Equation 11. The values of LOD = 13.98 nmol L^{-1} and LOQ = 46.61 nmol L^{-1} were calculated for a concentration range from 0.1 to 4.08 µmol L^{-1}. Figure 2e shows the anodic curves of FPX measured in the concentration range from 0.136 to13.6 µmol L^{-1}.

- For MCZ, oxidation curves were measured over the concentration range from 0.44 to 44.1 µmol L^{-1} see Figure 2f, Equation 12. For the concentration range from 0.44 to 4.41 µmol L^{-1}, values of LOD = 32 nmol L^{-1} and LOQ = 107 nmol L^{-1} were calculated.

$$I_p\ [\mu A] = (0.03399 \pm 0.00014)\ (c\ [\mu mol\ L^{-1}]) + (0.0071 \pm 0.0034),\ r = 0.9999 \qquad (7)$$

$$I_p\ [\mu A] = (0.008952 \pm 0.000059)\ c\ [\mu mol\ L^{-1}] - (0.0285 \pm 0.0065),\ r = 0.9999 \qquad (8)$$

$$I_p\ [\mu A] = (0.010449 \pm 0.000069)\ c\ [\mu mol\ L^{-1}] - (0.038 \pm 0.017),\ r = 0.9999 \qquad (9)$$

$$I_p\ [\mu A] = (0.03941 \pm 0.00018)(c\ [\mu mol\ L^{-1}]) - (0.00289 \pm 0.00090),\ r = 0.9998 \qquad (10)$$

$$I_p\ [\mu A] = (0.008769 \pm 0.000042)(c\ [\mu mol\ L^{-1}]) + (0.00053 \pm 0.00024),\ r = 0.9998 \qquad (11)$$

$$I_p\ [\mu A] = (0.014817 \pm 0.000084)(c\ [\mu mol\ L^{-1}]) + (0.0084 \pm 0.0017),\ r = 0.9997 \qquad (12)$$

To verify the reliability and accuracy of the proposed methods, repeatable quantitative determinations were performed for each analyte at several concentration levels, as listed in Table 1. The analysis was repeated five times for each analyte and concentration using the standard addition method. From the reported recovery and RSD values, it can be concluded that the proposed methods provide accurate and reliable results for the whole range of concentrations tested.

Table 1: Results of quantitative determinations of different concentrations of the studied analytes

Analyte	Added [µmol L^{-1}]	Found [µmol L^{-1}]	Recovery [%]	RSD (n = 5) [%]
PBZ	7.59	7.46±0.228	92.8-104.0	4.62
	1.91	1.86±0.054	92.9-103.6	4.41
	0.75	0.72±0.034	90.9-104.5	6.15
DMZ	21.79	21.83±0.940	96.3-105.9	4.33
	7.24	7.13±0.260	94.2-101.7	3.62
UDMH	50.45	50.12±0.818	97.8-101.7	1.63
	16.82	16.94±0.956	93.0-107.8	5.71
MFZ	1.28	1.27±0.038	95.6-102.3	3.02
	0.26	0.25±0.009	93.7-102.7	3.78
	0.05	0.05±0.002	94.1-103.9	4.15
FPX	0.50	0.49±0.020	95.4-105.8	4.06
	0.10	0.10±0.003	99.2-106.2	2.73
MCZ	4.41	4.27±0.101	94.3-100.2	2.38
	0.44	0.45±0.017	96.6-105.9	3.82

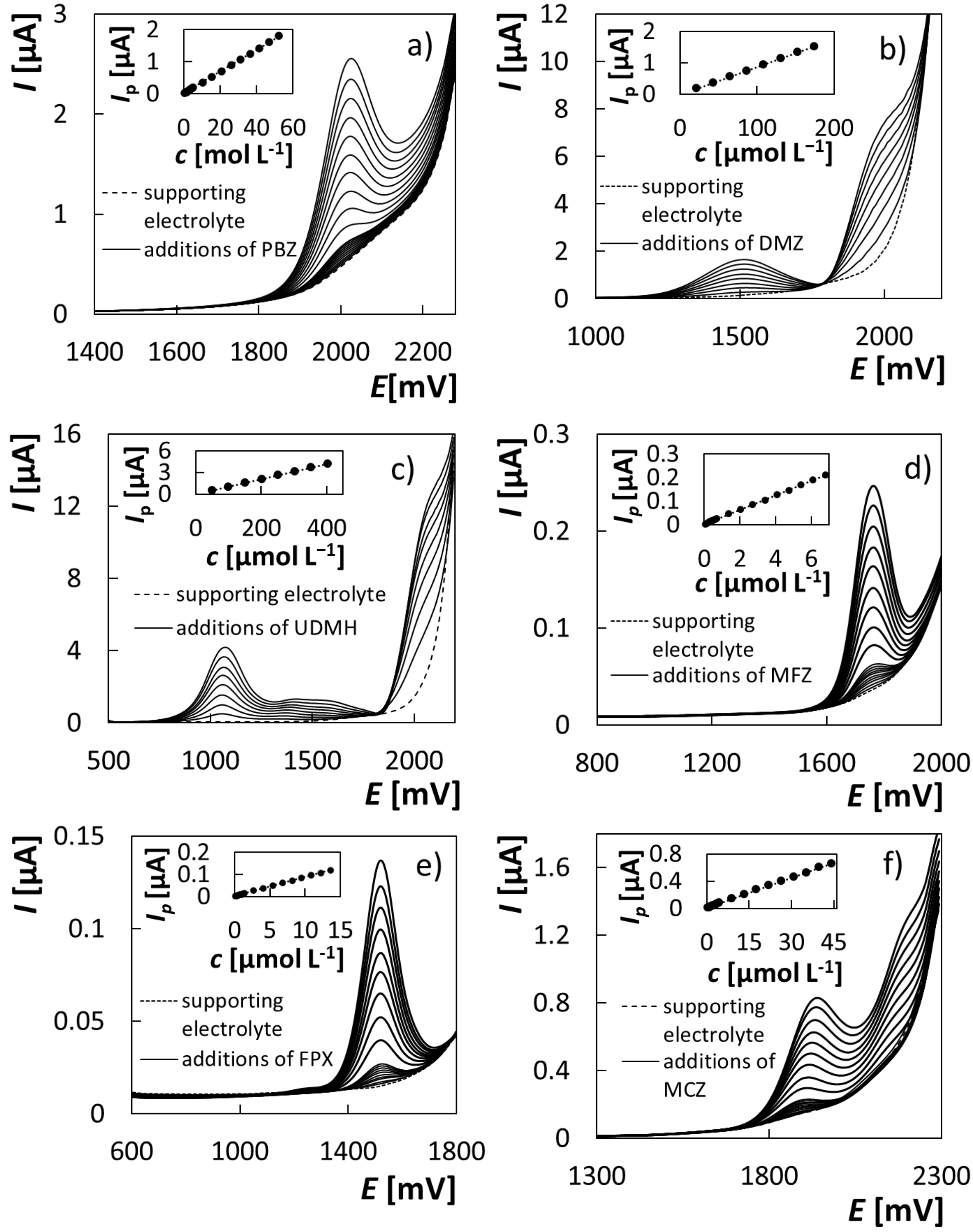

Figure 2: Anodic curves of the studied analytes and dependencies of I_p on c

(supporting electrolytes: 0.07 mol L^{-1} NaOH with 33 % ACN (a), BR buffer (pH 9) (b, c), BR buffer (pH 9) with 33% ACN (d-f); c_{PBZ} = 0.521-52.1 µmol L^{-1}, c_{DMZ} = 21.79-174.32 µmol L^{-1}, c_{UDMH} = 50.45-403.59 µmol L^{-1}, c_{MFZ} = 0.0679-6.79 µmol L^{-1}, c_{FPX} = 0.136-13.6 µmol L^{-1}, c_{MCZ} = 0.44-44.1 µmol L^{-1}; E_{in} = +1200 mV (a), +500 mV (b-f); E_{fin} = +2300 mV (a, f), +2200 mV (b-e); A = +30 mV (a), +50 mV (b-f); t = 60 ms (a), 80 ms (b-f); v = 40 mV s^{-1} (a-f); method - DPV)

3.5 Analysis of model mixtures of the studied analytes

Since most of the studied analytes are major components of several mixed commercial products, a study of model mixtures was carried out to investigate their inter-behaviour in mixtures.

In the case of DMZ, it is not a mixed sample in the true sense of the word, but a mixture of DMZ and its degradation product UDMH, since due to the degradation of DMZ, it must be assumed that real samples containing DMZ, will also contain UDMH. To determine how the presence of UDMH in a sample affects the determination of DMZ and conversely, model mixtures of these two substances were analysed. It was found that the determination of UDMH is not interfered by the presence of DMZ, for model mixtures with molar concentration ratios $c_{\mathrm{UDMH}}/c_{\mathrm{DMZ}}$ ranging from 0.29 to 2.92. The recoveries of UDMH determination ranged from 94.92 to 102.26 %. When DMZ was determined in the mixed sample, a significant effect of UDMH on the shape of the DMZ peak was observed, resulting in a large error of determination, namely for $c_{\mathrm{UDMH}}/c_{\mathrm{DMZ}}$ from 1.15 to 15.33, the recoveries ranged from 106.7 to 333.3 %, respectively. UDMH can be removed from a 1 mol L^{-1} NaOH solution by a short boiling. Experimentally, a relationship was obtained between the required boiling time used to eliminate UDMH from the solution and the ratio of current responses at the maxima of both analytes, $I_{\mathrm{max,\,UDMH}}/I_{\mathrm{max,\,DMZ}}$. This dependence can be described by Equation 13:

$$t\ [\mathrm{min}] = -\,0.0388\,x^2 + 1.2397x - 1.8788, \qquad (13)$$

where x represents $I_{\mathrm{max,UDMH}}\,/I_{\mathrm{max\ DMZ}}$, and t is the elimination/boiling time.

After eliminating UDMH in the above way, the recovery of DMZ determination was equal to 101.3%, 93.3%, 100% and 107.4% for UDMH: DMZ ratio of 1.15, 3.45, 6.66 and 15.33, respectively.

Next, model mixtures of MFZ and FPX, which are the main components of Revytrex and Revystar XL products, were analysed. A great advantage was that the peaks of the analytes were at different potentials, namely FPX at +1527 mV and MFZ at +1769 mV, which should allow their simultaneous determination. A total of 5 model mixtures were analysed with molar ratios given in Table 2. It was found that the two analytes do not affect each other's peak shape, height, or position, so they can be reliably and reproducibly determined simultaneously, as can be seen from the table.

MCZ was determined in the model mixtures along with FPX, as they are the main components of the Librax product. The peaks of these substances were also located at a sufficient distance, namely FPX at +1467 mV and MCZ at +1860 mV. The molar ratios of c_{FPX}:c_{MCZ} of the studied mixtures, together with the results of these analyses, are also presented in Table 2. From the given data, it can be concluded that both substances can be reliably determined in all studied model mixtures.

Table 2: Results of determination of selected studied analytes in model mixtures

$c_{\mathrm{FPX}}:c_{\mathrm{MFZ}}$	Analyte	Added [μmol L^{-1}]	Found [μmol L^{-1}]	Recovery [%]	RSD (n = 5) [%]
0.98:1	FPX	6.27	6.48±0.210	100.0-106.7	3.25
	MFZ	6.41	6.54±0.176	99.6-104.7	2.52
1.96:1	FPX	12.54	12.72±0.446	97.3-104.2	3.59
	MFZ	6.41	6.41±0.075	98.4-100.8	1.26
0.53:1	FPX	5.01	5.14±0.052	101.6-103.7	1.06
	MFZ	9.53	9.84±0.352	97.9-105.3	3.67
3.18:1	FPX	7.50	7.42±0.236	95.5-101.4	3.14
	MFZ	2.36	2.24±0.075	91.5-96.8	3.23
0.35:1	FPX	2.49	2.54±0.026	101.1-103.2	1.19
	MFZ	7.14	7.39±0.126	102.1-105.3	1.53
$c_{\mathrm{FPX}}:c_{\mathrm{MCZ}}$					
1:1	FPX	4.80	4.73±0.097	96.3-100.2	2.05
	MCZ	5.47	5.67±0.246	98.9-107.9	4.34
2:1	FPX	7.19	7.29±0.172	99.2-103.9	2.35
	MCZ	4.13	4.13±0.108	97.1-102.2	2.62
1:2	FPX	3.59	3.65±0.148	96.9-103.6	4.05
	MCZ	8.22	8.65±0.095	104.4-106.6	1.09
3:1	FPX	7.19	7.11±0.344	95.3-104.3	4.84
	MCZ	2.74	2.82±0.067	100.7-105.5	2.36
1:3	FPX	2.39	2.51±0.017	104.2-105.4	0.69
	MCZ	8.22	7.95±0.329	97.0-104.3	3.95

3.6 Analysis of real samples of the studied analytes

The next step was to apply the proposed method to the analysis of real commercial preparations. The analysis of the commercial product Toprex, containing PBZ, is presented in detail in the master thesis [7]. For the remaining studied analytes, a total of 7 preparations were analyzed, the names and declared contents of which are given in Table 3. Preparations containing only one substance, such as B-NINE (DMZ), Revysol (MFZ), Sergadis (FPX) and Caramba (MCZ), were analyzed first. The records of these analyses are shown in Figure 3. It can be seen from this figure that no interfering effect on the determination of DMZ and FPX was observed. In the case of the Revysol and Caramba analysis, it can be seen from Figures 3c and 3d that, in addition to the pure MFZ and MCZ, there is probably some impurity present that is reflected by a small peak in front of the MFZ and MCZ curves, respectively. Despite this fact, it was possible to find a suitable way of evaluating the obtained curves and to determine the content of the analyte reliably. When mixed products were analysed, interferences were usually observed that affected the peak shape, as was the case with Revytrex, Revystar XL and Librax. In the case of Revytrex and Revystar XL, a reliable way of evaluating the obtained curves was found, and the values found for both analytes were in agreement with those declared, see Table 3. As for the Librax analysis, only MCZ was reliably determined. Found FPX values did not correspond to the declared amount. To verify the reliability of the obtained results, most of the commercial products were analysed using the independent HPLC/DAD method. From the data presented in Table 3, it can be concluded that, except for FPX in Librax, the observed values agree with the results of the DPV analysis as well as with the declared content. In the case of the determination of FPX in Librax, using HPLC/DAD, it was found that in addition to the two main components (MCZ and FPX), there were two other small peaks. Moreover, FPX was found to be less than the declared content, so it could be its degradation products that affected its peak, and thus, the determination using the DPV method.

Spiked river and rinse water samples were also analysed for PBZ and DMZ. The concentration procedure used during the analysis of these samples is presented in the experimental section. From the result shown in Table 3, we can conclude that the proposed methods can be reliably used to analyse real samples such as river water and apple rinse water.

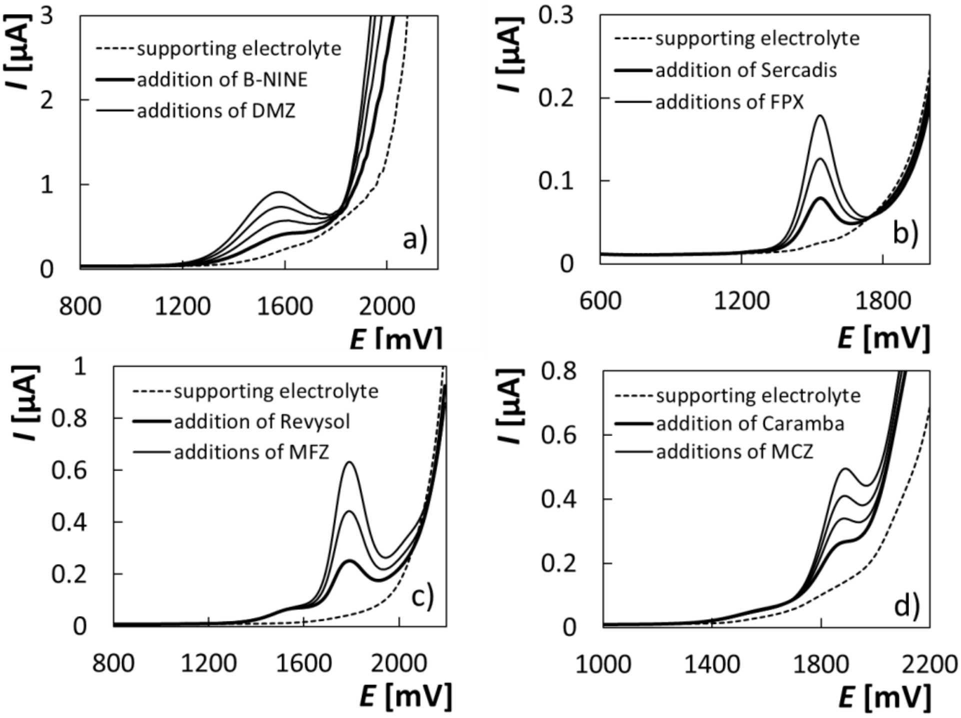

Figure 3: DP voltammograms of B-NINE (a), Sercadis (b), Revysol (c) and Caramba (d)

(supporting electrolytes (V = 15 mL): BR buffer (pH 9) (a), BR buffer (pH 9) with 33% ACN (b-d); $V_{\text{B-NINE}}$ = 30, V_{Sercadis} = 20 µL, V_{Revysol} = 10 µL, V_{Caramba} = 30 µL; V_{MFZ}= 30 µL, V_{FPX} = 20 µL, V_{MCZ} = 20 µL, c = 4 mmol L^{-1}; V_{DMZ} = 30 µL, c = 0,01 mol L^{-1}; E_{in} = +500 mV (a-c); E_{fin} = +2200 mV (a-c), +2300 mV (d); A = +50 mV; t = 80 ms; v = 40 mV s^{-1} (a-d); method - DPV)

Table 3: Results of determination of studied analytes in real samples using DPV and HPLC/DAD methods.

Commercial product/sample	Analytical method	Analyte	Declared [g L^{-1}]	Found [g L^{-1}]	Recovery [%]	RSD [%]
B-NINE$^{®SG}$	DPV	DMZ	850	831 ± 27.00	93.5–105.9	4.82
Spiked river water			4*	4.06 ± 0.13*	95.0–106.2	4.91
Spiked rinsing water 1			2*	2.04 ± 0.05*	97.0–106.0	3.93
Spiked rinsing water 2		UDMH	19.6*	19.48 ± 0.21*	97.9–102.0	1.64
Revysol	DPV	MFZ	75	75.05±1.48	98.5-103.2	1.97
	HPLC/DAD			75.83±1.72	97.6-103.2	2.27
Sercadis	DPV	FPX	300	302.30±3.39	99.5-101.7	1.12
	HPLC/DAD			301.62±4.60	98.1-102.1	1.52
Revytrex	DPV	MFZ	66.7	66.67±3.44	96.5-105.6	5.16
		FPX		65.25±2.64	94.5-104.6	4.04
	HPLC/DAD	MFZ		66.90±2.65	95.4-104.8	3.95
		FPX		66.82±2.45	97.2-105.8	3.67
Revystar XL	DPV	MFZ	100	99.88±2.58	97.5-104.0	2.58
		FPX	50	50.82±2.26	97.1-106.0	4.44
	HPLC/DAD	MFZ	100	102.18±3.47	97.7-106.5	3.40
		FPX	50	50.61±1.53	98.5-105.6	3.02
Caramba	DPV	MCZ	60	61.58±1.84	99.2-105.0	2.99
Librax	DPV	MCZ	45	43.79±2.03	94.2-101.6	4.63
		FPX	62.5	113.39±4.43	176.5-188.6	3.84
	HPLC/DAD	MCZ	45	45.18 ± 1.19	97.8-105.2	2.63
		FPX	62.5	54.95 ± 0.90	86.8-90.5	1.63
Spiked rinsing water 1	DPV	PBZ	20*	19.40±0.45*	93.2-100.0	3.51
			0.40*	0.38±0.01*	91.7-98.7	4.31

* value in [µmol L^{-1}]

Conclusion

This Ph.D. thesis was focused on the development of methods for voltammetric determination of biologically important substances, namely plant growth regulators, especially PBZ, DMZ and its degradation product UDMH and fungicides, especially MFZ, FPX and MCZ. The electrochemical method DPV, in combination with a working BDD electrode, was used for the determination of the above substances.

First, a suitable supporting electrolyte composition and pH value were searched for. The medium for voltammetric determination of PBZ, namely 0.07 mol L^{-1} NaOH with 33% ACN was recommended in the master thesis [7]. For DMZ and its degradation product UDMH, pure BR buffer at pH 9 was chosen as the supporting electrolyte. For MFZ, FPX and MCZ, BR buffer with 33 % ACN at pH 9 was chosen. In these supporting electrolytes, the studied substances gave a well evaluable, sensitive and stable current response, moreover, linear I_p on c dependencies were obtained.

Using CV, it was found that all the studied analytes undergo irreversible anodic oxidation. Based on the study of the dependence of current response on scan rate, it was found that the controlling process of the electrode reaction for DMZ and UDMH is kinetics and for PBZ, MFZ, FPX and MCZ it is diffusion influenced by kinetics.

During quantitative analyses of the studied compounds, LOD and LOQ values were calculated and were 0.083 and 0.277 µmol L^{-1} for PBZ, 0.44 and 1.46 µmol L^{-1} for DMZ, 0.063 and 0.209 µmol L^{-1} for UDMH, 10.81 and 36.04 nmol L^{-1} for MFZ, 13.98 and 46.61 nmol L^{-1} for FPX and 32 and 107 nmol L^{-1} for MCZ, respectively. To verify the reliability and accuracy of the proposed methods, repeatable determinations of different concentrations of the studied analytes were performed. After evaluation of the results, it was found that the recommended procedures for voltammetric determination of both growth regulators and fungicides gave correct and well-repeatable results. For most of the analytes, the RSD did not exceed 5%.

The proposed methods were used for the analysis of mixtures of selected analytes, namely DMZ along with UDMH, MFZ along with FPX and FPX along with MCZ. During the determination of DMZ in mixtures with UDMH, it was found that UDMH significantly affects the DMZ result already at concentration ratios higher than 1:1. Therefore, a UDMH elimination procedure was devised, which is based on a short boiling of the sample in a 1 mol L^{-1} NaOH environment. Repeatable and reliable results were obtained during the analysis of all these mixtures.

Finally, the proposed methods were applied to real samples. In total, seven commercially available preparations and four samples of enriched water were analysed. The determined amounts of the analytes, with the exception of FPX in Librax, were in agreement with the declared contents and were also confirmed by an independent method using HPLC/DAD.

Based on the above considerations, it can be concluded that the developed methods provide reliable results for the analysis of the analytes themselves and their mixtures and for the analysis of real commercial preparations.

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