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Analytical approach for simultaneous determination of azoxystrobin, prothioconazole and trifloxystrobin in plant protection products

Sanja Lazić, Aleksandra Šušnjar, Slavica Vuković, Jelena Ećimović, Mitar Đukanović and Dragana Šunjka*

University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, Novi Sad, Serbia

SUMMARY

In this study, an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method with diode array detection (DAD) was developed and validated for simultaneous determination of azoxystrobin, trifloxystrobin and prothioconazole in plant-protection products. Chromatographic separation of the active substances was achieved using 0.1% acetic acid and acetonitrile (30:70 v/v) at a flow rate of 0.52 ml/min on a Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm × 1.8 µm) and UV detection at 210 nm. Validation was done by evaluating the linearity and precision of the method, repeatability of injections, accuracy, and limits of detection and quantification (LOD and LOQ). Under the conditions, correlation coefficients of linearity were 0.996-0.997, the precision of method, expressed as relative standard deviation, was lower than the modified Horwitz values, the accuracy of all individual substances was within the range of 94.61-107.35%, while repeatability of the injectons was satisfied with RSD of 0.94-1.35%. LOD and LOQ were 0.0063 mg/ml and 0.019 mg/ml for azoxystrobin, 0.0051 mg/ml and 0.015 mg/ml for prothioconazole and 0.0051 mg/ml and 0.015 mg/ml for trifloxystrobin, respectively. A simple, precise, accurate, and fast analytical method for simultaneous determination of the fungicides azoxystrobin, prothioconazole and trifloxystrobin can be proficiently used for their detection and quantification in formulated products. The developed and validated method was applied to real samples, confirming the method's applicability.

Keywords: pesticide formulations, active ingredients, analytical methods, reversed phase high-performance liquid chromatography (RP-HPLC/DAD), fungicides

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*Corresponding author: draganas@polj.uns.ac.rs

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INTRODUCTION

The main challenges to achieving high yields include a wide range of pests, weeds and diseases that cause damage and great losses, which can be prevented or controlled by plant protection products (PPPs). Without effective and quality PPPs, and their conscientious application, agricultural production would be severely compromised, resulting in seriously affected global food availability and agricultural economy.

Thus, for such plant protection to be implemented, it is necessary before application of PPPs to determine their quality, and this is achieved by analyzing the physicochemical properties and impurities of toxicological significance, and identification and determination of amounts of active substances in formulations (FAO & WHO, 2022). These characteristics need to be evaluated according to CIPAC (Collaborative International Pesticides Analytical Council) or AOAC (Association of Official Agricultural Chemists) standard methods. If official methods are not available, it is necessary to develop and validate a suitable method.

Diseases caused by phytopathogenic fungi represent a serious challenge for agricultural production worldwide. Controlling such diseases often requires the application of PPPs or some other agricultural practices, and their effective control is crucial for preserving agricultural productivity. Their timely identification and adequate control play a key role in ensuring a stable and sustainable food supply for the growing world population. However, the problem arises when several diseases occur simultaneously, and resistance is developed to frequently applied active substances. For this reason, mixing two or more active substances with different spectrums and modes of action is becoming increasingly common.

Hence, to expand the spectrum of action, reduce instances of pest resistance, replace multiple applications with a single one, and reduce the number of treatments, multi-pesticide PPPs are being introduced. A good example are PPPs based on two or three active substances, such as a multipesticide formulation based on azoxystrobin, prothioconazole and trifloxystrobin.

Azoxystrobin and trifloxystrobin are strobilurin fungicides, synthetic analogs of natural fungal metabolites (Clough, 1993). According to FRAC, these active substances belong to the fungicide group C, whose mode of action is described as inhibition of cellular respiration (Quinone Outside Inhibitors) (FRAC, 2023). It is reflected in binding to the external site of the cytochrome b-c1 complex (quinone), where coenzyme Q10 (ubiquinone), which is responsible for transferring electrons to proteins, would otherwise be bound. These systemic fungicides are transported by the xylem, and in some cases also act as contact fungicides with a wide range of action (Team of editors, 2020). They are registered for the control of fungi from the divisions Basidiomycota, Ascomycota, Deuteromycota and Oomycetes (Bartlett et al., 2002; Anonymous, 2023) (Table 1 and Table 2).

Table 1. Phytopathogenic fungi controlled by azoxystrobin

Plant species	Phytopathogenic fungi
Wheat/ Barley	*Erisiphe gramminis, Puccinia striiformis, Puccinia recondite, Rhynchosporium secalis, Fusarium spp., Septoria nodorum*
Sugar beet	*Cercospora beticola, Rhizoctonia solani*
Sunflower	*Sclerotinia sclerotiorum, Diaporthe helianthi, Phomopsis helianthi*
Oilseed rape	*Sclerotinia sclerotiorum*
Tomato/ Potato	*Phytophthora infestans, Aternaria solani*
Paprika	*Leveillula taurica*
Cucumber/ Watermelon/Melon/ Zucchini	*Pseudoperonospora cubensis, Alternaria cucumerina, Erysiphe cichoracearum, Cladosporium cucumerinum*
Cabbage	*Peronospora parasitica, Alternaria brassicae, Albugo candida*
Carrot	*Erysiphe heraclei, Alternaria dauci*
Strawberry	*Mycosphaerella fragariae, Podosphaera aphanis*
Raspberry	*Dydimella applanata*
Blackberry	*Kuehneola uredinis*
Grapevine	*Plasmopara viticola, Uncinula necator*

Prothioconazole is a fungicide in the group of triazolinthiones, which belongs to the G group according to FRAC, and affects sterol biosynthesis in membranes (FRAC, 2023). It is used as a systemic fungicide with protective, curative and eradicative effects (Team of editors, 2020), affecting ergosterol in the cell membrane, which plays a significant role in its structure and permeability, and is essential for cell growth (Parker et al., 2013). Plant species and phytopathogenic causing agents for which prothioconazole is registered are given in Table 3 (Anonymous, 2023).

For individual determination of azoxystrobin, the available method implies the use of gas chromatography (Dobrat, W. & Martijn, 2009), while the available methods for determination of trifloxystrobin and prothioconazole suggest the use of high-performance liquid chromatography (HPLC) (De Oliveira & Garvey, 2017; Partian & Garvey, 2021). However, methods for simultaneous determination of these fungicides are still lacking. The current study therefore aimed to develop and validate a method for simultaneous analysis of azoxystrobin, prothioconazole and trifloxystrobin in formulated products.

MATERIALS AND METHODS

Analytical standards of azoxystrobin (97%), prothioconazole (99.5%) and trifloxystrobin (98%) were obtained from Dr Ehrenstorfer (Augsburg, Germany). Acetonitrile (HPLC grade), ultrapure water and acetic acid were purchased from J.T. Baker (Netherlands). The plant protection product (SC formulation) used in this study contained azoxystrobin (111.9 g/l), prothioconazole (143 g/l) and trifloxystrobin (111.2 g/l).

Individual stock solutions of analytical standards of azoxystrobin, prothioconazole, and trifloxystrobin were prepared by dilution in acetonitrile and ultrasonic homogenization. Stock solutions were then used to obtain a series of mixture solutions in concentrations of 0.0218-0.5135 mg/ml for azoxystrobin, 0.0162-0.3815 mg/ml for prothioconazole and 0.0213-0.5016 mg/ml for trifloxystrobin (Table 4). All standard solutions were stored at 4 °C in the dark.

Table 2. Phytopathogenic fungi controlled by trifloxystrobin

Plant species	Phytopathogenic fungi
Wheat/ Barley	*Puccinia striiformis, Puccinia recondite, Erisiphe gramminis, Fusarium spp.*
Sugar beet	*Cercospora beticola*
Sunflower	*Phoma spp.*
Tomato	*Botrytis cinerea, Leveillula taurica*
Apple	*Venturia inaequalis, Podosphaera leucotricha, Colletotrichum spp.*
Cherry/Sour cherry	*Monilia laxa*
Blueberry	*Botrytis cinerea*

Table 3. Phytopathogenic fungi controlled by prothioconazole

Plant species	Phytopathogenic fungi
Wheat/ Barley	*Erisiphe gramminis, Puccinia striiformis, Puccinia recondite, Rhynchosporium secalis, Fusarium spp., Septoria nodorum, Pyrenophora teres*
Sugar beet	*Cercospora beticola*
Sunflower	*Sclerotinia sclerotiorum, Botrytis cinerea, Phoma spp.*
Oilseed rape	*Sclerotinia sclerotiorum, Alternaria brassicae*

Table 4. Concentrations of azoxystrobin, prothioconazole and trifloxystrobin in a series of solutions

	azoxystrobin (mg/ml)	prothioconazole (mg/ml)	trifloxystrobin (mg/ml)
mix 1	0.51348	0.38148	0.50160
mix 2	0.36677	0.27249	0.35829
mix 3	0.18339	0.13624	0.17914
mix 4	0.13099	0.09732	0.12796
mix 5	0.06549	0.04866	0.06398
mix 6	0.02183	0.01622	0.02133

The Agilent 1100 Series with DAD detector and a reversed-phase Zorbax Eclipse XDB-C18 (50 mm × 4.6mm × 1.8μm) (Agilent Technologies, USA) were used for an HPLC analysis.

Applying the IAEA guidelines (IAEA, *2009*), validation was performed based on several analytical performance parameters: linearity, method precision, repeatability of injections, accuracy, and limits of detection and quantification (LOD and LOQ) (CIPAC, 1999).

Evaluation of the linear relationship between analyte concentration and the corresponding detector response is essential. The linearity should be tested at several concentration levels, and this assessment should confirm a linear growth of the detector's response with increasing analyte concentrations.

Precision and repeatability are parameters that are similar but refer to different aspects of the performance of the method being validated. Precision reflects the congruity of results obtained under modified and predicted conditions. It clarifies the ability of a method to give consistent results under different conditions (apparatus, analyst, environment, time) and it employs statistical parameters, such as the relative standard deviation of repeated measurements, as well as the Horwitz limit (European Commission, 2019).

On the other hand, repeatability indicates the method's precision in repeated measurements of the same sample under identical conditions (apparatus, analyst, environment, time). Also, it indicates the convenience of the method for repeated consecutive applications on the same sample, which ensures its applicability in routine analytical practice.

Accuracy is determined by comparing the obtained results with the results obtained using certified reference material. When certified reference material is not available, standard samples to which a known amount of analyte is added can be used. Accuracy is most often expressed as a percentage of analytical procedure „recovery" for a known additional amount of analyte in the sample, so it is essential for estimating in what percentage the method reflects the actual concentration of the analyte in a tested sample (ICH, 2022).

Finally, LOD and LOQ need to be determined. LOD is the lowest possible value that can be identified and not expressed as an exact value, while LOQ is the lowest value of an analyte that can be quantified and precisely determined (Zuas et al., 2016).

RESULTS AND DISCUSSION

HPLC analysis

A simultaneous analysis of azoxystrobin, prothioconazole and trifloxystrobin was performed using HPLC/DAD. During the process of method validation, different wavelengths, combinations of mobile phases, their flow rates and column temperatures were tested. The most effective for the analysis were the conditions shown in Table 5.

The appropriate separation and appearance of analytical signals of azoxystrobin, prothioconazole and trifloxystrobin were achieved under these conditions at all tested wavelengths (Figure 1). However, the best response was achieved at the wavelength of 210 nm (Figure 2), and it was therefore chosen for further analysis. The UV spectra of all three active substances were recorded at the same wavelength (Figure 3).

The applicability of the method in terms of specificity was demonstrated by example chromatograms (standard and formulation with active substance) (European Commission, 2019).

Table 5. Conditions for HPLC-DAD determination of azoxystrobin, prothioconazole and trifloxystrobin

Mobile phase	0,1% acetic acid: acetonitrile
Mobile phase ratio	30:70
Column temperature	20 °C
Flow	0.520 ml/min
Wavelength	254 nm, 210 nm, and 230 nm
Injected volume	1 μl
Retention time of azoxystrobin	1.793min
Retention time of prothioconazole	2.403min
Retention time of trifloxystrobin	4.191min

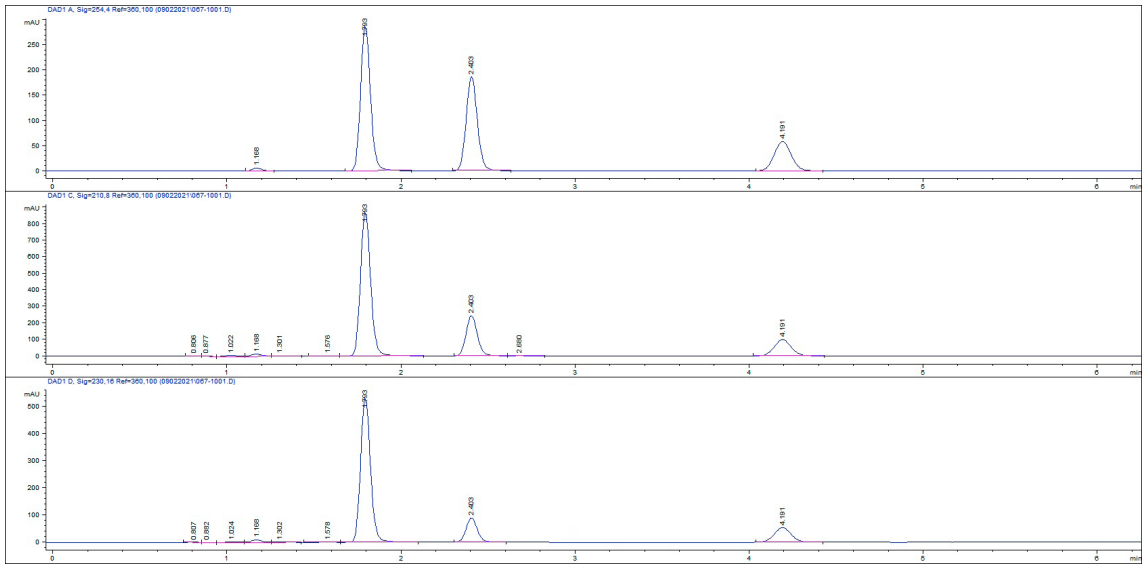

Figure 1. Chromatogram of the mixture of analytical standards of azoxystrobin (0.183385 mg/ml), prothioconazole (0.136243 mg/ml) and trifloxystrobin (0.179143 mg/ml) in acetonitrile at different wavelengths under conditions specified in Table 5 - azoxystrobin (1.793 min), prothioconazole (2.403 min) and trifloxystrobin (4.191 min)

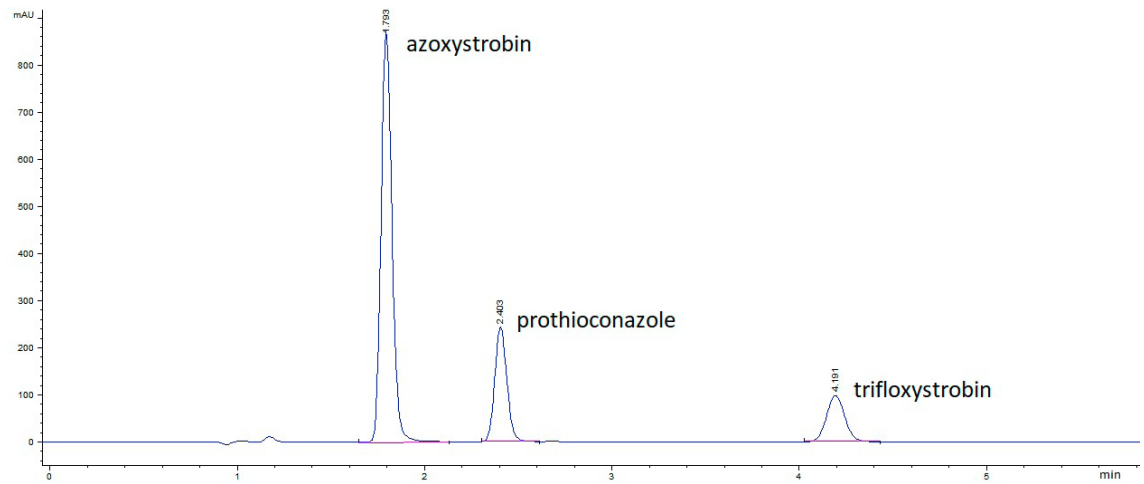

Figure 2. Chromatogram of the mixture of standards of azoxystrobin (0.183385 mg/ml), prothioconazole (0.136243 mg/ml) and trifloxystrobin (0.179143 mg/ml) in acetonitrile at the wavelength of 210 nm

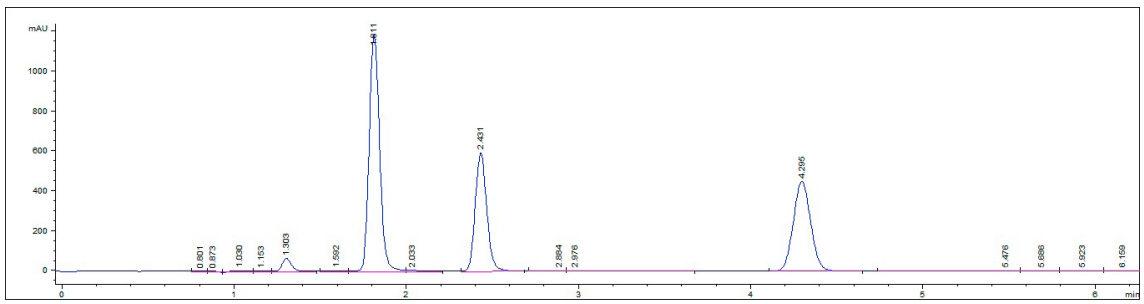

Figure 3. Chromatogram of PPP in acetonitrile - azoxystrobin (1.611 min), prothioconazole (2.431 min) and trifloxystrobin (4.295 min)

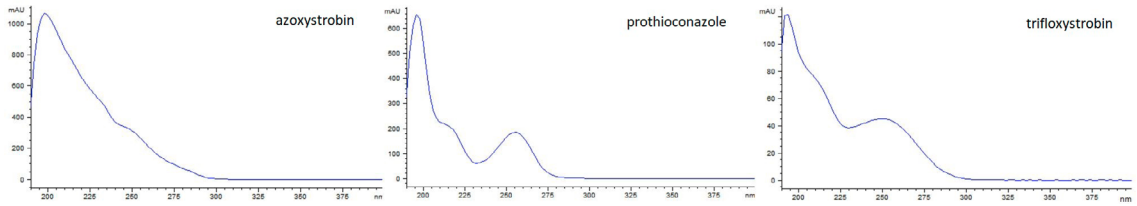

Figure 4. UV spectra of analytical standards of azoxystrobin, prothioconazole and trifloxystrobin in acetonitrile at the detector wavelength of 210 nm

Validation of method

The chromatographic conditions were checked by examining the linearity of the detector response, precision, repeatability and accuracy of the method, and by determining the LOD and LOQ, and the dependence of the peak area on the injected volume.

The linearity of detector response was determined at six concentration levels. Analytical and statistical data obtained by the linear regression method are shown in Table 6.

A good linearity of detector response was achieved within the range of tested concentrations of azoxystrobin, prothioconazole and trifloxystrobin (Table 6). The obtained values indicate that the increase in contents of the introduced compounds linearly follows the increase in area of analytical signal. In the regression equation, the correlation coefficient of linear dependence for the analyzed compounds was 0.996-0.997, which indicates a high sensitivity of this method for determination of azoxystrobin, prothioconazole and trifloxystrobin since the acceptable coefficient of determination is ≥0.98 (European Commission /3030/99).

The repeatability of injections of azoxystrobin, prothioconazole and trifloxystrobin was established by injecting 1 µl of the mixture of standard solutions of these compounds seven times (European Commission /3030/99). The values of the obtained peak areas for the tested compounds are shown in Table 7.

The relative standard deviation (RSD%) values of 0.94, 1.35 and 1.07 for azoxystrobin, prothioconazole and trifloxystrobin, respectively, indicate that good repeatability of determination of these compounds was achieved by the applied method (RSD≤2).

The precision of the method was determined by injecting 1 µl of the PPP solution in acetonitrile five times, and the relative standard deviation (RSD%) was 0.79, 0.98, and 1.27 for azoxystrobin, prothioconazole and trifloxystrobin, respectively (Table 8). The results are acceptable when the experimental RSDr values are below those obtained by the modified Horwitz equation (CIPAC, 1999).

The Horwitz limit value was calculated based on the equation %RSDr=2(1-0.5 log C), which was modified to %RSDr=%RSDr×0.67. The relative standard deviation (RSD) of reproducibility of azoxystrobin, prothioconazole and trifloxystrobin determination using the peak area is well below the modified Horwitz limit values of 2.00, 1.93 and 2.01% (Table 9).

Table 6. Linearity parameters for azoxystrobin, prothioconazole and trifloxystrobin determination by HPLC/DAD

	Peak area		
	azoxystrobin	prothioconazole	trifloxystrobin
mix 6	476.85	146.1	86.35
mix 5	1058.25	319.35	189.1
mix 4	2234.7	899.25	501.3
mix 3	3322.25	1114.7	663.55
mix 2	6021.5	2210.35	1340.85
mix 1	7965.2	3217.1	1987.35
Regression equation	y = 15444 + 215.7	y = 8391.x – 12.19	y = 3953.x – 30.18
Section	215.7	12.19	30.18
Slope	15444	8391.x	3953.x
Correlation coefficient	**0.996**	**0.997**	**0.997**

The accuracy of the method was assessed using the standard addition method, i.e. by adding a known amount of the analyte to the sample (Table 10). The appropriate known amounts of analytical standards of azoxystrobin, prothioconazole and trifloxystrobin were added to PPP samples in which the content of active substances was previously determined. After the analysis, the expected concentrations were compared with the concentrations obtained using the described method. The high agreement between values obtained in the procedure (94.61-107.35%) and actual values confirms the accuracy of the applied method for determination of azoxystrobin, prothioconazole and trifloxystrobin, given that the acceptable value is between 90 and 110 % of target concentration (European Commission /3030/99).

Table 7. Repeatability of determination of azoxystrobin, prothioconazole and trifloxystrobin

Concentration	0.51348	0.38148	0.5016
	azoxystrobin	prothioconazole	trifloxystrobin
Peak area	3149.0	954.3	591.1
	3100.9	948.8	578.1
	3167.6	979.0	572.5
	3170.1	978.4	573.9
	3106.1	955.4	581.8
	3114.5	952.4	577.1
	3115.0	951.3	577.2
Mean value	3131.9	959.9	578.8
SD	29.6	12.9	6.1
RSD	**0.94**	**1.35**	**1.07**

Table 8. Precision of determination of azoxystrobin, prothioconazole and trifloxystrobin

	azoxystrobin	prothioconazole	trifloxystrobin
Peak area	5031.2	2815.2	3137.4
	5052.4	2869.1	3220.3
	5137.7	2852.5	3193.2
	5073.9	2826.7	3157.6
	5084.6	2800.8	3122.6
Mean value	5075.9	2832.9	3166.2
SD	40.2	27.7	40.19
RSD	**0.79**	**0.98**	**1.27**

Table 9. Horwitz limit values for the active substances azoxystrobin, prothioconazole and trifloxystrobin

		γ=1.0996 g/cm^3			*Horwitz* value RSDr	modified *Horwitz* value
	g/l	am/γ/10	C	logC	2(1-0.5logC)	RSDr × 0.67
azoxystrobin	111.9	10.18	0.10	-0.99	2.99	2.00
prothioconazole	143.0	13.00	0.13	-0.89	2.89	1.93
trifloxystrobin	111.2	10.11	0.10	-1.00	3.00	2.01

Table 10. Accuracy of determination of azoxystrobin, prothioconazole and trifloxystrobin in PPPs

Active substances	I mg/ml	I Rec.%	II mg/ml	II Rec.%	Mean Rec.%	SD
azoxystrobin	0.10269	94.61	0.03668	95.91	95.26	0.92
prothioconazole	0.07629	101.23	0.02725	99.32	100.28	1.35
trifloxystrobin	0.10032	107.35	0.03583	100.96	104.16	3.19

Table 11. Analyses of azoxystrobin, prothioconazole and trifloxystrobin in PPPs

Fungicide and type of formulation	Amount in PPP declared by manufacturer	Measured concentrations
Prothioconazole, EC	250 g/l	252.7±0.3 g/l
Azoxystrbin, SC	250 g/l	250.4±0.1 g/l
Trifloxystrobin, WG	500 g/l	501.6±0.5 g/l
Prothioconazole + azoxystrbin, SC	150 g/l +250 g/l	152.4±0.4 g/l +251.1±0.5 g/l
Prothioconazole + trifloxystrobin, SC	175 g/l +150 g/l	175.2±0.3 g/l +150.4±0.2 g/l

Based on the described method validation, this HPLC protocol can be successfully used for determining the exact contents of all three active substances in PPPs.

Analyses of PPPs

The developed analytical method was applied to determine the contents of active substances in formulated products. Five commercially available plant protection products were analyzed using the method and compared with reference values (Table 11). The calibration curve method and an external standard were applied. Based on the regression equations of calibration curves, the amounts of active substances in the measured mass of samples were calculated. The obtained results confirmed an agreement between the measurement concentrations and reference values given by the manufacturer.

In this study, an analytical method for simultaneous determination of azoxystrobin, prothioconazole and trifloxystrobin in pesticide formulations by HPLC–DAD was developed and validated. Parameters such as linearity, precision and accuracy confirmed its reliability. Thus, the method can be used to analyze products containing the three active substances, and equally successfully those that contain a combination of two or a single one of them. Conditions were set to a lower flow rate of the mobile phase, as the applied short column requires, and uniform separation of analytical signals was obtained at the wavelength of 210 nm. Using the short column, it was managed to develop a method which is fast, simple, accurate and economical in solvents consumption.

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Analitička metoda za istovremeno određivanje azoksistrobina, protiokonazola i trifloksistrobina u sredstvima za zaštitu bilja

REZIME

U ovoj studiji, razvijena je i validovana izokratska metoda za istovremeno određivanje azoksistrobina, trifloksistrobina i protiokonazola u sredstvima za zaštitu bilja primenom reverzno-fazne visokoperformantne tečne hromatografije (RP-HPLC/DAD). Hromatografsko razdvajanje aktivnih supstanci postignuto je upotrebom 0,1% rastvora sirćetne kiseline i acetonitrila (30:70 v/v) kao mobilne faze, pri protoku od 0,52 ml/min. Korišćena je Zorbax Eclipse XDB-C18 (50 mm x 4.6 mm x 1.8 µm) kolona, a detekcija je vršena na talasnoj dužini od 210 nm. Validacija metode obuhvatala je procenu linearnosti i preciznosti metode, ponovljivosti injektovanja, tačnosti metode, kao i limita detekcije i kvantifikacije (LOD i LOQ). Pod navedenim uslovima postignuti koeficijent korelacije linearnosti kretao se 0.996-0.997. Preciznost metode, izražena kao relativna standardna devijacija (RSD), bila je niža od modifikovanih Horwitz-ovih vrednosti. Tačnost određivanja svake pojedinačne aktivne supstance kretala se 94,61-107,35%, dok je ponovljivost injektovanja bila zadovoljavajuća sa RSD vrednostima 0,94-1,35%. Limiti detekcije i kvaktifikacije iznosili su 0,0063 mg/ml i 0,019 mg/ml za azoksistrobin, 0,0051 mg/ml i 0,015 mg/ml za protiokonazol, 0,0051 mg/ml i 0,015 mg/ml za trifloksistrobin. Stoga, razvijena i validovana jednostavna, precizna, tačna i brza analitička metoda za istovremeno određivanje fungicida azoksistrobina, protiokonazola i trifloksistrobina se može uspešno primenjivati za njihovo pojedinačno, ali i istovremeno određivanje u formulisanim proizvodima. Daljom primenom opisane metode na realne uzorke, potvrđena je njena namena.

Ključne reči: formulacije pesticide, aktivne supstance, analitičke metode, reverzno-fazna visokoperformantna tečna hromatografija (RP-HPLC/DAD), fungicidi