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Multitoxin analysis of trichothecenes, including NX toxins, in rice and wheat☆

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ABSTRACT

Fusarium species biosynthesize trichothecenes that contaminate food and poses health risks. Among these, NX toxins produced by *F. graminearum* and *F. culmorum*, are structurally similar to deoxynivalenol (DON). Accurate measurement of these mycotoxins in food is essential for food safety. We developed UPLC-QDA and UHPLC/PDA methods to simultaneously quantify nine trichothecenes, in particular NX2-M1, NX2, and NX3. In this study, two complementary analytical workflows, UPLC-QDA (single-quadrupole mass spectrometry) and UHPLC/PDA—were developed and validated for the simultaneous determination of nine trichothecenes in rice and wheat matrices. Sample cleanup was comparatively performed using two solid-phase extraction (SPE) procedures, OASIS® HLB and Mycosep® 227, to assess their performance in reducing matrix effects and improving recovery. The UPLC-QDA method enabled the reliable quantification of all target analytes, including the NX-series, with recovery values between 70 and 103 % and intra-day precision (RSD) below 13 %. Conversely, UHPLC/PDA was unable to detect NX2-M1 and NX3 due to their poor UV absorption. Mycosep® 227 exhibited superior cleanup efficiency and lower signal suppression compared to OASIS® HLB, particularly in wheat extracts. Detection limits ranged from 1–34 μg/kg for UPLC-QDA and 4–26 μg/kg for UHPLC/PDA. Overall, the study provides a validated and practical framework for the simultaneous quantification of both regulated and emerging trichothecenes, highlighting the necessity of MS-based detection for accurate determination of NX toxins in complex cereal matrices.

1. Introduction

Trichothecenes comprise a structural group of sesquiterpenoid mycotoxins produced mainly by fungi of the genera *Fusarium, Myrothecium* and *Stachybotrys*, with *Fusarium* species posing the greatest threat to global food systems due to their wide distribution and impact on cereal crops (Desjardins A.E., 2006; Foroud et al., 2019). These mycotoxins are characterized by a tetracyclic 12,13-epoxytricothec-9-ene core structure and are classified into type A and type B trichothecenes based on the functional group at the C-8 position. Type A compounds, including T-2 and HT-2, contain hydroxyl, ester, or alkyl groups at the C-8 position, while Type B compounds, such as DON and NIV, feature a ketone group. The recently identified NX series toxins constitute a subgroup of Type A trichothecenes but differ structurally due to the absence of the carbonyl group at C-8 (Varga et al., 2015; Aitken et al., 2019). Trichothecene contamination is particularly widespread in wheat (*Triticum aestivum*) and rice (*Oryza sativa*), two of the world's most important staple crops with global production in 2023 estimated at approximately 510 million tons for rice and 800 million tons for wheat (FAO and CIMMYT). In wheat, *F. graminearum* and *F. culmorum* are the main causative agents of *Fusarium* head blight (FHB), a disease that not only reduces grain yield and quality but also causes the accumulation of mycotoxins in harvested kernels (Blandino et al. 2012; Haidukowski et al. 2022; McMullen et al., 2012; Tu et al., 2023). By contrast, *F. fujikuroi* and related species more

☆ **Key Contribution:** Development and evaluation of UPLC-QDA and UHPLC/PDA methods for the simultaneous determination of nine mycotoxins, including the NX toxins.

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commonly affect rice, causing the disease Bakanae; however, recent studies have reported the presence of DON and other trichothecenes in rice products, particularly under warm and humid storage or growing conditions (Wiemann et al 2013). The toxicological profile of trichothecenes includes cytotoxic, immunosuppressive, proinflammatory, and emetogenic effects, with severity depending on toxin structure and exposure route. Among them, T-2 toxin and HT-2 toxin are the most acutely toxic, followed by NIV and DON (Authority (EFSA) et al., 2017; Polak-Śliwińska e Paszczyk 2021; Wu et al., 2014). Regulatory bodies such as the European Commission have established maximum allowable levels for DON in cereals and cereal-based products under Commission Regulation (EC) No. 1881/2006, Regulation (EU) 2023/915, and Regulation (EU) 2024/1022, and have recently introduced binding maximum levels for the sum of T-2 and HT-2 toxins in cereals and cereal-based foods under Commission Regulation (EU) 2024/1038. In contrast, nivalenol is not yet regulated in the EU, despite its frequent co-occurrence with DON and potential additive toxicity (Knutsen et al 2017). Outside the EU, other jurisdictions such as the United States Food and Drug Administration (FDA) have issued advisory levels, rather than enforceable limits for DON, recommending no more than 1000 μg/kg in finished wheat products intended for human consumption (FDA, 2010). Japan and China have set national limits for deoxynivalenol (DON) in cereal products. In China, T-2 toxin is regulated in complete feed under GB 2761–2017, while Japan has no official maximum level for T-2 in food. However, T-2 is monitored through national surveys, and risk management is guided by dietary and regional assessments (Ono, 2007; MHLW, 2002). A particularly concerning development in recent years is the identification of NX trichothecenes, a novel subclass of Type A toxins produced by certain *F. graminearum* chemotypes. NX-2, the predominant compound, lacks the C-8 carbonyl group found in DON, resulting in altered polarity and potential evasion of conventional analytical detection methods (Chen et al., 2022) NX-3, the de-acetylation product of NX-2 is found in plants infected with NX-2 producing strains, toxicological data indicate that it compounds may possess equal or greater toxicity than DON in some biological systems (Woelflingeder et al., 2020; Pierron et al., 2022). NX2-M1, a non-toxic rearrangement product of NX-2, is more polar than DON and may behave differently in chromatographic systems (Varga et al., 2018). Given the increasing global cultivation of wheat and rice, particularly in regions vulnerable to *Fusarium* infection due to climate variability, the emergence of structurally novel and potentially more toxic trichothecenes poses a serious challenge to food safety. This highlights the need for expanded monitoring, updated detection protocols, and a reassessment of current regulatory thresholds to account for emerging risks associated with NX toxins. Due to the potent toxicity and structural diversity of trichothecenes, reliable detection and quantification in food and feed products are critical for public health protection. Detection and quantification methods include ultra high-performance liquid chromatography (UHPLC) with Photodiode Detector Array, ultra-performance liquid chromatography (UPLC) coupled with mass spectrometry (MS) or photo diode detector array (PDA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have become the standards for multi-mycotoxin analysis due to their sensitivity, selectivity, and ability to detect multiple trichothecenes simultaneously (Malachová et al. 2014). These analytical techniques are generally effective for common Type A and Type B trichothecenes such as DON, T-2, and HT-2 toxins. However, the emergence of structurally novel compounds—like the NX trichothecenes—has raised concerns about the efficacy of existing detection protocols. NX2-M1, NX-2 and NX-3, due to their altered polarity and lack of the characteristic ketone functional group at C-8 found in Type B trichothecenes, may evade detection in conventional LC-DAD-MS workflows (Varga et al., 2018). As a result, new method development, including optimization of chromatographic separation, is essential to accurately detect these compounds in complex cereal matrices (Warth et al., 2013; Oplatowska-Stachowiak et al., 2018, AFSCA 2021]. The analytical determination of trichothecenes in cereal matrices is particularly challenging due to their chemical diversity, low concentrations in naturally contaminated samples, and matrix complexity. Sample clean-up is therefore a critical step in trichothecene analysis to enhance selectivity, reduce matrix effects, and improve quantification accuracy, particularly in the context of multi-residue analysis. In this context, solid-phase extraction (SPE) technics, in particular Oasis® HLB and Mycosep® 227 clean-up columns, have demonstrated high analyte recovery and compatibility with trichothecene structures (De Girolamo et al., 2020; Weingaertner et al., 1997). These methods have been useful for cleaning rice cultures, a substrate used to study *Fusarium* mycotoxin biosynthesis and wheat samples for monitoring mycotoxins in food systems. As the emergence of new trichothecene analogues, such as NX toxins, continues, the analytical methodologies used to detect them must also evolve. The development and validation of accurate, sensitive, and matrix-compatible analytical methods remain essential for effective mycotoxin surveillance, regulatory compliance, and risk assessment in global food systems. This study aims to develop and validate two complementary analytical methods for the simultaneous quantification of nine trichothecene mycotoxins: nivalenol (NIV), deoxynivalenol (DON), 3-acetyl-DON (3-AcDON), 15-acetyl-DON (15-AcDON), NX2-M1, NX-2, NX-3, T-2, and HT-2 toxins. The selected methods are ultra-performance liquid chromatography coupled with electrospray ionization mass spectrometry (UPLC-QDA) and UHPLC with photo diode Detector Array (UHPLC/PDA). These target compounds represent both established and newly identified emerging trichothecenes. To improve sample preparation, two solid SPE techniques, Oasis® HLB and Mycosep® 227 clean-up columns, were evaluated for their performance in rice cultures and wheat matrices. The goal is to establish robust, sensitive, reliable, and reproducible workflows that improve trichothecenes detection, support regulatory compliance, and strengthen food safety risk assessments in response to emerging mycotoxin threats.

2. Materials and methods

2.1. Chemicals and materials

Analytical-grade methanol (MeOH) and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy). UPLC grade. Acetonitrile was obtained from Merck (Merck KGaA, German). Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, MA, USA). Standard nivalenol (NIV), deoxynivalenol (DON), calibrant solutions in acetonitrile 3-acetyl deoxynivalenol (3Ac-DON), 15-acetyl deoxynivalenol (15Ac-DON) Biopure™ and Mycosep® 227 were purchased Romer Lab (Romer Labs Inc., Tulln, Austria). Purification of NX-2, NX-3 and NX2-M1 is described in Varga et al. 2015 and Varga et al. 2018., respectively. Ammonium acetate for mass spectrometry, (CH_3COONH_4) > 99.0 %, T-2 toxin and HT-2 toxin, sodium chloride (NaCl) were purchased from Sigma–Aldrich (Milan, Italy). paper filters (Whatman No. 4) from Whatman (Maidstone, UK). Solid-phase extraction columns OASIS® HLB 3 mL, 60 mg (Waters, Milan, Italy). The chemical structures of all trichothecene standards analyzed in this study (NIV, DON, 3-AcDON, 15-AcDON, NX2-M1, NX-2, NX-3, T-2, and HT-2) are shown in Fig. 1.

2.2. Preparation of standard solutions

NIV, DON, T-2 and HT-2 stock solutions (1 mg/mL each) were prepared by dissolving solid commercial toxins in acetonitrile (HPLC grade). 3-AcDON, 15-AcDON were purchased at 100 μg/mL in acetonitrile. NX2-M1, NX-2 and NX-3 100 μg/mL in methanol were dried under nitrogen and redissolved in acetonitrile. Mixed standard solutions 10 μg/mL (working standard solution 1, WS1) and 1 μg/mL (working standard solution 2, WS2) for spiking purposes were prepared by diluting adequate amounts of the stock solutions in acetonitrile. Standard solutions for UHPLC calibration curve were prepared by

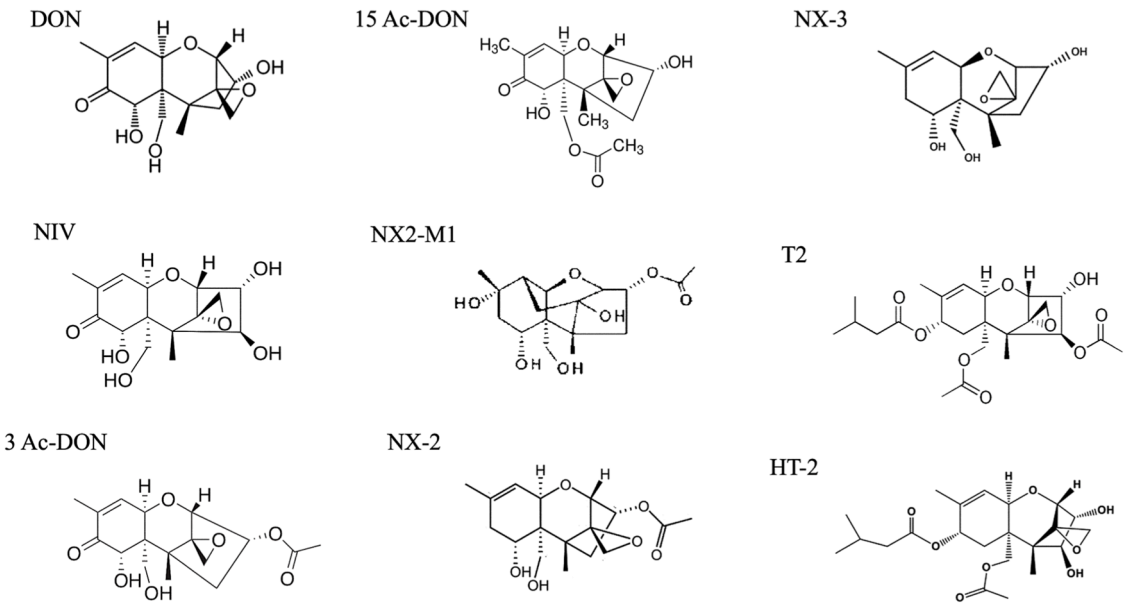

Fig. 1. Chemical structures of the nine trichothecenes analyzed, including the NX series (NX2-M1, NX-2, NX-3).

redissolving aliquots in water/methanol (80:20, v/v) previously evaporated to dryness under nitrogen stream. Standard solutions for UPLC calibration curve were prepared by adding aliquots to a final extract after purification to avoid matrix effects, then evaporated to dryness under nitrogen stream and dissolved with water/methanol (80:20, v/v).

2.3. Apparatus

The UPLC apparatus consists of a Waters Acquity UPLC® system (Milford, MA, USA) equipped with a binary solvent manager, a sample manager, a column heater and a QDA/MS detector. The analytical column was an Acquity UPLC® BEH C18 (2.1 mm × 100 mm, 1.7 μm, Waters, Milford, MA, USA) preceded by an Acquity UPLC™ column in-line filter (0.2 μm). The chromatographic separation was performed by a gradient elution using solvent A) H_2O + 0.5 % glacial acetic acid + 1mM ammonium acetate and B) MeOH + 0.5 % glacial acetic acid + 1mM ammonium acetate as follows: the initial composition of the mobile phase was A) 90 %, B) 10 % kept constant for 6 min, then solvent B was linearly increased to 90 % in 14 min, and kept constant for 5 min. From 25.1 to 28 minutes, the proportions of A and B are 0 % and 100 %, respectively. Following this, from 28 to 32 minutes, the proportions revert to 90 % A and 10 % B. The run concludes at 32 minutes. The flow rate of the mobile phase was 0.3 mL/min. The column was kept at a temperature of 40 °C; the detector was set at different m/z ratio depending on mycotoxin (Table 1). Data acquisition and instrument control were performed by Empower™ 2 Software (Waters). With this method, the retention times were 2.23, 3.77, 3.80, 4.20, 10.96, 11.07, 11.36, 15.39, 16.32 min. for NIV, NX2-M1, NX-3, DON, 3-AcDON, 15-AcDON, NX-2, HT-2 and T-2 respectively. NX2-M1 and NX-3 were not detected by UV detection. UPLC-QDA, a single-quadrupole mass spectrometer, was operated in positive electrospray ionization mode. The cone voltage (20 V) and SIR acquisition parameters were optimized for each analyte to maximize sensitivity and ensure reproducibility of quantitative data. The UHPLC/PDA apparatus was an Agilent 1290 series (Agilent, Waldbronn, Germany) equipped with a binary pump, autosampler, column thermostat set at 30 °C and a diode array detector set at 203 nm and 220 nm. The analytical column was a UHPLC RRHD Eclipse Plus C18 (2,1 × 100 mm, 1,8 μm, Agilent, Waldbronn, Germany) preceded with in-line filter 1290 infinity II (0.3 μm). UHPLC method was carried out in binary gradient mode with a flow rate of 0.3 ml/min (solvent A: 100 % H_2O) and (solvent B: 100 % Methanol) as follows: the initial composition of the mobile phase was A) 85 %, B) 15 % kept constant for 10 min. After an initial time of 10 min at 85 % A, the proportion of B increased to 90 % in 20 min and kept constant for 5 minutes. The total run concludes in 40 minutes. With this method, the retention times were 1.68, 5.69, 8.39, 19.52, 19.72, 26.56, 28.07 min. for NIV, DON, NX2, 3-AcDON, 15-AcDON, HT-2 and T-2 respectively. The mobile phase composition, flow rate, and column temperature were systematically optimized to achieve the best resolution of the nine trichothecenes. Optimal separation was obtained using a methanol/water gradient containing 0.5 % acetic acid and 1 mM ammonium acetate, which improved peak shape and reduced baseline noise.

Table 1
Selected ion recording (SIR) parameters for the UPLC-QDA detection of trichothecene mycotoxins. Each analyte is listed with its abbreviation, monitored mass-to-charge ratio (m/z), and cone voltage applied (20 V for all compounds).

Mycotoxin	Abbreviation	SIR (m/z)	Polarity
NIV	[NIV+Na]+	335	ESI +
DON	[DON+H]+	319	ESI +
NX2-M1	[NX2-M1+H]+	343	ESI +
NX-2	[NX-2+H]+	325	ESI +
NX-3	[NX-3+H]+	283	ESI +
3-AcDON	[3AcDON+H]+	339	ESI +
15-AcDON	[15AcDON+H]+	339	ESI +
HT-2 toxin	[HT2+Na]+	442	ESI +
T-2 toxin	[T-2+NH_4]+	484	ESI +

2.4. Sample Preparation

The samples were finely ground using an MLI 204 laboratory mill (Buhler S.p.A., Milan, Italy). The test samples (10 g) spiked with WS1 and WS2 to evaluate method performance. Then the samples were extracted with 50 mL acetonitrile/water (84/16, v/v) + 1 % acetic acid for 90 min on an orbital shaker. The extracts were filtered through filter paper (Whatman No. 4) and then divided into two methods: A) 8 mL of filtrate was submitted to clean-up through a Mycosep® 227 column. 2.5 mL of purified extract was transferred to a 4 ml vial; B) 2.5 mL of filtrate (equivalent to 0.5 g sample) was evaporated to dryness at 50°C under a stream of air. The residue was reconstituted with 100 μL of methanol and then 900 μL of water was added. The OASIS® HLB column was conditioned with 2 mL methanol, equilibrated with 2 mL methanol/water (10:90, v/v) and then the reconstituted sample extract was passed

through the column. The column was washed with 1 mL methanol/water (20:80, v/v) and finally, the toxins were eluted with 1 mL methanol. After purification method A and B, 10 µL of the extract was injected into the UPLC-QDA system and UHPLC/PDA system. (Weingaertner et al., 1997; De Girolamo et al., 2020)

2.5. Method validation

The analytical method for the determination of the nine trichothecenes in rice and wheat samples was validated in-house according to the EC Regulation 401/2006 and 519/2014 and the AOAC Requirements for Single Laboratory Validation of Chemical Methods (AOAC, 2016). The linearity range of the method was tested by preparing 7 different standard solutions (calibrants) at the concentration of 50, 75, 100, 250, 500, 750 and 1000 ng/mL, containing each mycotoxin in the 50-1000 ng/mL concentration range. Matrix matched calibration curves were also prepared. Seven different matrix-matched calibrants (in the range 5-1000 µg/kg) were obtained by spiking the eluted extracts of blank rice or wheat samples with an appropriate amount of each mycotoxin stock depending on the desired levels chosen to 5, 25, 50, 100, 250, 500 and 1000 µg/kg. Calibrants were prepared in water/methanol (80/20, v/v and analyzed in triplicate. Coefficients of determination (R^2) were calculated for both standard and matrix-matched calibration graphs (Suppl_TableS1). To assess any possible matrix-interfering effect on trichothecenes detection, the signal suppression/enhancement values (SSE%), defined as the percentage ratio of the matrix-matched calibration slope to the solvent calibration slope, were calculated to confirm the efficiency of the method in separating matrix-interfering compounds from target mycotoxins. The LOD (limit of detection) and LOQ (limit of quantification) values, expressed as µg of trichothecenes/kg of matrix, were estimated as the concentration of analyte which provided a signal-to-noise ratio (S/N) higher than 3 and 10, respectively. Table 3a–3b summarizes the LOD and LOQ values for each analyte (i.e., DON, NIV, NX2-M1, NX-2, NX-3, 3AcDON, 15AcDON, T-2 and HT-2). Precision was evaluated by repeatability (intra-day RSD%) calculated from three replicates per concentration level (100, 500, and 1000 µg/kg). RSD values ranged from 2–13 % (acceptable per EC Regulation 401/2006). The recovery values were calculated by spiking rice and wheat samples at 100, 500 and 1000 µg/Kg, while the accuracy and repeatability (relative standard deviation, RSD%) of the method were determined by analyzing three replicates for each spiking level. Carry-over was assessed by injecting solvent blanks immediately following the highest concentration standard, confirming the absence of significant residual signal (response $<$ 20 % of the LOQ) and demonstrating the efficacy of the system wash procedure. Short-term autosampler stability was verified by re-injecting prepared QC samples at regular intervals throughout the analysis period; the mean responses remained within $\pm$ 15 % of the initial value, ensuring the reliability of results throughout the sequence. Selectivity was confirmed by analyzing blank matrix and solvent injections, which showed no co-eluting peaks at the analyte's retention time, assuring the absence of interferences.

3. Results

3.1. Quantification of trichothecenes in rice and wheat

3.1.1. Linearity and matrix effect

The linearity study was performed by analyzing the nine trichothecenes in mobile phase and in rice and wheat matrix (Table 2a–Table 2b). The compounds were analyzed in matrices by spiking sample extracts of a blank (mycotoxin free) rice and wheat samples. Good linearity (lack of fit test with $p>0.05$) and good determination coefficient $R^2 \geq 0.990$ was recorded in the range from 5 to 1000 µg/kg for all mycotoxins (Suppl_tableS1). As detailed above, these calibration curves in matrix (rice and wheat) were obtained by spiking the eluted fraction of blanks samples with known amounts of each analyte. To evaluate the matrix

Table 2a
Calibration parameters for trichothecenes quantified by UPLC-QDA of rice samples. For each toxin, the matrix-matched calibration equation, the coefficient of determination (R^2), and the signal suppression/enhancement (SSE%) are reported. The calibration of each toxin was performed in the range of 5 to 1000 µg/kg using 7 experimental points, in triplicates.

Toxins	OASIS® HLB matched calibration curve	Mycosep® 227 matched calibration curve	Mobile Phase calibration curve	SSE (%) OASIS® HLB	SSE (%) Mycosep® 227
NIV	y = 8.04x + 4.43	y = 6.54x - 1.34	y = 9.66x + 2.76	86	63
DON	y = 26.3x + 19.0	y = 24.3x + 27.9	y = 37.2x + 8.50	74	73
NX2-M1	y = 5.12x + 0.08	y = 6.60x + 1.5	y = 7.31x + 3.20	67	87
NX-2	y = 5.65x + 0,26	y = 5.43x + 1.93	y = 6.81x - 0.18	83	83
NX-3	y = 2.14x + 2.25	y = 1.83x + 0.33	y = 1.68x - 0.50	116	121
3-AcDON	y = 4.39x - 0.21	y = 5.48x + 1.23	y = 6.03x - 2.77	76	99
15-AcDON	y = 7.23x + 1.29	y = 7.99x + 0.90	y = 6.62x – 5.82	100	99
HT-2	y = 4.74x - 0.10	y = 3.44x + 0.01	y = 3.63x + 0.72	126	92
T-2	y = 25.7x + 4.98	y = 22.1x + 6.43	y = 20.9x - 0.19	127	111

Table 2b
Calibration parameters for trichothecenes quantified by UPLC-QDA of wheat samples. For each toxin, the matrix-matched calibration equation, the coefficient of determination (R^2), and the signal suppression/enhancment (SSE%) are reported. The calibration of each toxin was performed in the range of 5 to 1000 µg/kg using experimental points, in triplicates.

Toxins	OASIS® HLB Matched calibration curve	Mycosep® 227 matched calibration curve	Mobile Phase calibration curve	SSE (%) OASIS® HLB	SSE (%) Mycosep® 227
NIV	y = 8.04x + 4.43	y = 9.68x + 5.55	y = 9.66x + 2.76	86	104
DON	y = 37.2x + 27.1	y = 47.8x + 23.3	y = 37.2x + 8.50	106	133
NX2-M1	y = 3.16x + 1.03	y = 3.63x + 1.84	y = 7.31x + 3.20	43	50
NX-2	y = 3.90x – 1.27	y = 4.38x - 1.46	y = 6.81x - 0.18	51	62
NX-3	y = 1.05x + 0.02	y = 1.20x – 0.23	y = 1.68x - 0.50	68	76
3-AcDON	y = 4.10x + 2.04	y = 4.67x + 0.92	y = 6.03x - 2.77	76	84
15-AcDON	y = 5.40x + 2.37	y = 5.88x + 1.01	y = 6.62x – 5.82	77	81
HT-2	y = 2.39x - 0.26	y = 2.27x – 0.11	y = 3.63x + 0.72	63	60
T-2	y = 15.4x + 7.75	y = 14.9x + 4.75	y = 20.9x - 0.19	79	75

effect, we compared the slopes of the calibration curves obtained in matrix with those prepared in mobile phase. A matrix effect was confirmed by the signal suppression/enhancement (SSE) values observed for both matrices (Table 2a and Table 2b). Specifically, SSE ranged from 63 % to 127 % for rice samples and from 43 % to 133 % for wheat samples, following purification with either OASIS® HLB or Mycosep® 227. These results indicate a clear matrix effect for the toxins, particularly in wheat samples. Notably, the matrix effect was less pronounced when using the Mycosep® 227 purification step. Most SSE values fell outside the acceptable range of 100 ± 20 %, as defined by EU Regulation 2021/808, highlighting the importance of using matrix-matched calibration curves for accurate quantification of these trichothecenes.

3.1.2. Determination of LOD and LOQ

Tables 3a and b show LOD and LOQ values determined for the none trichothecenes. Table 3a refers to the LOD and LOQ values determined for rice and wheat samples using quadrupole detection, while Table 3b lists the same values using PDA. LOD and LOQ values for trichothecenes in rice samples detection were lower than 2.5 μg/kg, using both OASIS® HLB or Mycosep® 227 clean-up, while LOD and LOQ values in wheat samples detection were less than 5 μg/kg. Considering these results, it can be concluded that the method is sensitive, using both OASIS® HLB and Mycosep® 227 purification steps. and LOD and LOQ values in matrix guarantee the detection of the nine trichothecenes at low concentration levels.

3.1.3. Mycotoxin Recovery in Rice and Wheat: A Comparative Study

Artificially contaminated rice and wheat samples with different standard concentrations of the study aimed to compare two purification methods—OASIS® HLB and Mycosep® 227 —used to evaluate the recovery rates of various trichothecene mycotoxins (NIV, DON, NX2-M1, NX-2, NX-3, 3-AcDON, 15-AcDON, HT-2, and T-2 toxins) at three different concentration levels: 100, 500, and 1000 μg/Kg in triplicate. The analyses were carried out using ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-QDA) and with an Agilent 1290 Infinity II system equipped with a photo diode Array detector (PDA). The Mycosep® 227 purification method on rice samples (Table 4a) demonstrated excellent performance, with generally high and consistent recovery values across the full range of tested concentrations. Chromatograms obtained by UPLC-QDA and PDA, ensuring clear separation and reliable quantification in rice and wheat matrices purified with Mycosep 227® and OASIS®HLB (Suppl_FigureS2A and Suppl_FigureS3A). The signal intensity reflects the high sensitivity of the method, featuring a clean baseline and favorable signal-to-noise ratios for all targeted compounds. DON, one of the most widespread and regulated mycotoxins, also showed higher recoveries with the OASIS® HLB method, reaching an average of 94.3 % and peaking at 103 % at 100 μg/Kg. The average recovery of NIV was 81.7 %, with relatively low variability across the different spiking levels: 83 % at 100 μg/Kg, a slight increase to 84 % at 500 μg/Kg, and a slight decrease to 78 % at 1000 μg/Kg. These values fall well within the acceptable range according to international mycotoxin analysis guidelines and demonstrate good extraction efficiency and repeatability, especially at low and medium concentrations. The slight drop at the highest concentration may be attributed to possible sorbent saturation or sample matrix interference. The NX-series mycotoxins (NX2-M1, NX-2, and NX-3) showed high and consistent recovery rates, indicating the method's strong ability to retain and release these compounds. NX-3 achieved a 100 % recovery at both 500 μg/Kg and 100 μg/Kg, while NX2-M1 maintained recovery values between 83 % and 87 % across all levels. Recovery for NX-2 was slightly lower, being in the range of 71-74 %. The acetylated forms of DON (3-Acetyl-DON and 15-Acetyl-DON) showed recovery rates between 72 % and 77 %, confirming the method's suitability even for these less stable molecules. The HT-2 and T-2 toxins, which belong to type A trichothecenes, exhibited excellent performance across all concentrations. HT-2,

Table 3a
UPLC-QDA Limit of detection (LOD, S/N=3) and limit of quantification (LOQ, S/N = 10) calculated for DON, NIV, NX2-M1, NX-2, NX-3, 3AcDON, 15AcDON, T-2, HT-2 in rice and wheat samples.

	μg toxins/kg matrix OASIS® HLB samples								
	NIV	DON	NX2-M1	NX-2	NX-3	3-AcDON	15-AcDON	HT-2	T-2
LOD	8	2	23	30	28	15	10	25	3
LOQ	26	5	75	100	92	51	32	84	11
	μg toxins/kg matrix Mycosep® 227 samples								
	NIV	DON	NX2-M1	NX-2	NX-3	3-AcDON	15-AcDON	HT-2	T-2
LOD	5	1	14	17	34	7	6	18	1
LOQ	17	5	45	55	112	22	20	58	5

Table 3b
UHPLC/PDA Limit of detection (LOD, S/N=3) and limit of quantification (LOQ, S/N = 10) calculated for DON, NIV, NX-2, 3AcDON, 15AcDON, T2, HT2 in rice and wheat samples.

	µg toxins/kg matrix OASIS® HLB samples						
	NIV	DON	NX-2	3-AcDON	15-AcDON	HT-2	T-2
LOD	**13**	16	22	13	20	26	26
LOQ	**43**	54	73	43	64	87	87
	µg toxins/kg matrix Mycosep® 227 samples						
	NIV	DON	NX-2	3-AcDON	15-AcDON	HT-2	T-2
LOD	4	4	18	9	12	11	20
LOQ	13	12	59	31	38	36	66

for instance, maintained recovery values between 77 % and 80 %, while T-2 ranged between 75 % and 79 %, confirming the robustness and reliability of the Mycosep® 227 method for these analytes. The OASIS® HLB method, widely used for rapid mycotoxin purification, also demonstrated generally good performance, although some differences emerged when compared to Mycosep® 227, particularly at lower concentrations. As shown in Table 4b, the acetylated forms of DON (3-AcDON and 15-AcDON) achieved excellent recovery rates, especially at 1000 µg/Kg, with values reaching 80–82 %. The HT-2 and T-2 toxins reached recovery rates in the range 86 % to 100 %, showing that the method remains highly effective for type A trichothecenes, especially at 500 and 100 µg/Kg. However, the NX-series mycotoxins showed less uniform performance. NX2-M1, NX-2, and NX-3 show consistent recoveries of approximately 70–72 %. NX3, for example, dropped to a 70 % recovery at 100 µg/Kg, compared to 100 % with Mycosep® 227. This suggests a greater sensitivity of the OASIS® HLB method to analyte concentration, particularly for certain compounds. Among the tested compounds, T-2 toxin exhibits the highest recovery (97-100 %), followed by HT-2 (86-91 %) and the acetylated derivatives of DON (3-AcDON and 15-AcDON), with recoveries between 78 % and 84 %. DON showed recoveries from 70 % and 103 %, while NIV showed values in the range 77-97 %. Overall, the data indicates consistent and reliable performance of the Mycosep® 227 cleanup procedure, particularly for NX2-M1 and NX-3, which showed reproducible recoveries across all spiking levels. The calculated RSD% values calculated for all spiking levels in rice samples were very low, in the range 2-13 %, except for 15-AcDON at the lowest spiking level that showed a precision egual to 25 %. Anyway, in all cases these repeatability values were acceptable being in line with EC Regulation 401/2006, 519/2014. Based on the results from the analysis of rice sample recoveries, identical recovery analyses were also carried out for wheat. The wheat chromatograms are overlaid with rice using UPLC-QDA (Suppl_FigureS3B). As a rice recovery analysis, the Mycosep® 227 purification method [Table 4b] demonstrated excellent performance, with generally high and consistent recovery values across the full range of tested concentrations. Chromatograms in wheat samples show good separation as well rice using UHPLC/PDA (Suppl_FigureS2B). Starting with the data related to DON, optimal recovery was found, reaching an average of 93 % and peaking at 102 % at 500 µg/Kg. The analysis performed on NIV yielded spiking results comparable to those obtained from rice samples. The average recovery of NIV was 81.7 %, with relatively low variability across the different spiking levels: 83 % at 100 µg/Kg, a slight increase to 84 % at 500 µg/Kg, and a slight decrease to 78 % at 1000 µg/Kg. High recovery rates for NX-series mycotoxins (NX2-M1, NX-2, and NX-3) were observed, mirroring those in rice spiking samples. However, this was not uniformly true across all cases. NX3 achieved a 100 % recovery at both 500 µg/Kg and 100 µg/Kg, while NX2-M1 maintained recovery values between 83 % and 87 % respectively at 100 µg/Kg and 500 µg/Kg. Recovery for NX-2 was significantly higher, in particular at 1000 µg/Kg to achieve 94 % recovery rate. The acetylated forms of DON (3-Acetyl-DON and 15-Acetyl-DON) showed recovery rates between 74 % and 77 %, but it shows a 100 % recovery increase at 500 for both 3-Acetyl-DON and 15-Acetyl-DON. The HT-2 and T-2 toxin exhibited excellent performance across all concentrations. Unlike the spiked rice samples, HT-2 showed recovery values of 70 % at 1000 µg/Kg and 77 % at 100 µg/Kg, but also a recovery below 70 % at 500 µg/Kg. Instead, T-2 ranged between 77 % and 95 %, demonstrating better recovery in wheat samples compared to rice. The OASIS® HLB method, also employed for wheat, demonstrated commendable performance. As evidenced in Table 2, the acetylated forms of DON (3-AcDON and 15-AcDON) achieved excellent recovery percentages, especially at 500 µg/Kg, with values reaching 78 % - 89 %. The HT-2 and T-2 toxins yielded recovery rates ranging from 81 % to 100 %, underscoring the method's sustained high efficacy for type A trichothecenes. NX2-M1, NX-2, and NX-3 exhibited consistent recoveries of approximately 70–95 %. NX-3, for instance, saw its recovery drop to 70 % at 100 µg/Kg, in contrast to the 100 % achieved with

Table 4a
. Quantitative content (μg/Kg) of trichothecenes detected in three spiked samples at different concentrations of standard solution (100, 500, and 1000) using OASIS® HLB and Mycosep® 227 clean-up prior to analysis in rice samples.

Spiking Level (μg/Kg)	*Recovery, % (RSD, %, n=3)*																	
	NIV		**DON**		**NX2-M1**		**NX-2**		**NX-3**		**3-AcDON**		**15-AcDON**		**HT-2**		**T-2**	
	Mycosep® 227	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**	**Mycosep® 227**	**OASIS® HLB**
1000	78 (10)	77 (4)	78 (3)	70 (5)	83 (0)	70 (5)	71 (1)	72 (1)	83 (2)	71 (5)	73 (5)	80 (5)	74 (2)	82 (4)	78 (5)	86 (7)	75 (1)	97 (2)
500	84 (10)	97 (9)	102 (7)	80 (4)	87 (1)	71 (6)	73 (1)	76 (1)	100 (3)	73 (3)	72 (5)	84 (8)	75 (5)	83 (4)	80 (1)	87 (11)	79 (2)	100 (3)
100	83 (0)	83 (0)	103 (5)	103 (5)	83 (9)	71 (5)	74 (4)	71 (8)	100 (8)	70 (4)	74 (13)	78 (10)	77 (13)	83 (4)	77 (7)	91 (4)	77 (1)	100 (13)

Table 4b
Quantitative content (μg/Kg) of trichothecenes detected in three spiked samples at different concentrations of standard solution (100, 500, and 1000) using OASIS® HLB and Mycosep® 227 clean-up prior to analysis in wheat samples.

Spiking Level (μg/Kg)	*Recovery, % (RSD, %, n=3)*																	
	NIV		DON		NX2-M1		NX-2		NX-3		3-AcDON		15-AcDON		HT-2		T-2	
	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB	Mycosep® 227	OASIS® HLB
1000	78 (10)	70 (0)	78 (3)	70 (0)	73 (0)	73 (2)	94 (1)	72 (1)	83 (2)	71 (1)	76 (6)	80 (1)	75 (4)	82 (3)	70 (8)	86 (2)	83 (5)	97 (2)
500	84 (10)	84(3)	102 (7)	100 (4)	87 (1)	95 (6)	92 (6)	91 (1)	100 (3)	74 (2)	99 (9)	78 (4)	100 (7)	89 (1)	70 (1)	81 (8)	95 (3)	90 (1)
100	83 (0)	83 (0)	100 (3)	100 (2)	83 (9)	71 (5)	74 (4)	71 (8)	100 (8)	70 (4)	74 (13)	78 (10)	77 (13)	83 (4)	77 (7)	91 (4)	77 (1)	100 (13)

Mycosep® 227. This implies a heightened sensitivity of the OASIS® HLB method to analyte concentration, particularly for specific compounds. Regarding NX2, a notable difference was observed at 500 μg/Kg, where recovery attained 95 %. DON displayed recoveries from 70 % to 100 %, while NIV presented values in the 70-84 % range. The obtained wheat recovery data underscore the consistent and dependable performance of the purification procedure for both Mycosep® 227 and OASIS® HLB, particularly concerning the entire NX series, which demonstrate reproducible recoveries across multiple spiking levels. The calculated RSD % values for all spiking levels in rice samples remained remarkably low, ranging from 0-13 %. All performance data presented herein were derived from spiked (artificially contaminated) matrices. While these results demonstrate method capability, it is recognized that the inclusion of at least one naturally contaminated sample or a certified reference material would be necessary to fully illustrate fitness-for-purpose under real-world co-contaminants. A limitation of this is the inability to utilize a naturally contaminated matrix for analysis.

4. Discussion

The developed analytical method demonstrated excellent linearity for all nine trichothecene mycotoxins in both rice and wheat matrices, with coefficients of determination (R^2) exceeding 0.990. The lack-of-fit test confirmed model adequacy ($p > 0.05$), fulfilling the typical validation requirements for mycotoxin analysis. These outcomes are consistent with previous reports emphasizing the necessity of matrix-matched calibration to compensate for matrix effects (Spanjer et al., 2008; Li et al., 2023). Matrix effects, expressed as signal suppression/enhancement (SSE), varied considerably between matrices, particularly in wheat (43 %–133 %), indicating stronger ionization suppression/enhancement than in rice. Several SSE values fell outside the EU-recommended range of 100 ± 20 % (European Commission, 2021), reinforcing the importance of matrix-matched calibration. The stronger matrix effects in wheat are likely due to its more complex and protein/starch-rich composition, which affects ionization efficiency during electrospray ionization (ESI) (Kruve et al., 2015).

A notable trend was that Mycosep® 227 consistently reduced matrix effects more effectively than OASIS® HLB, suggesting a more efficient removal of co-extracted interferences. Similar findings have been reported in other cereal matrix studies (Desmarchelier et al., 2010). These observations demonstrate that the Mycosep® 227 cleanup procedure provides a cleaner extract and a more stable response, which is essential for achieving accurate quantification, especially in complex matrices.

Both purification protocols yielded low detection limits, with LOD values ≤ 35 μg/kg for all target analytes in rice and wheat. These sensitivity levels are substantially below the maximum limits established by European legislation for deoxynivalenol (DON) and the sum of T-2 + HT-2 in cereals (European Commission, 2006). Such performance confirms the method's applicability for regulatory monitoring and routine control of trichothecene contamination.

The UPLC-QDA detection system provided significant advantages over the UHPLC/PDA setup, particularly for type A trichothecenes and emerging NX-type toxins (NX2-M1, NX-3). The latter possess weak UV absorbance ($\lambda max = 196$ nm, $\log \varepsilon = 3.81$ in ethanol), rendering them undetectable by PDA-based systems (Varga et al., 2015). The QDA detector enabled reliable identification and quantification regardless of chromophore strength, supporting prior evidence that mass spectrometric detection is indispensable for emerging and masked mycotoxins with poor UV activity (Uhlig et al., 2013). While UHPLC/PDA remains an accessible and cost-effective platform for routine screening of well-characterized toxins such as DON and its acetyl derivatives, its limitations in detecting NX2-M1 and NX-3 highlight the need for integrating MS-based techniques into comprehensive mycotoxin surveillance programs. As the occurrence and toxicological significance of NX-type trichothecenes continue to emerge, sensitive MS-based tools will be essential for ensuring both regulatory compliance and public health protection.

Recovery experiments confirmed the robustness and reliability of both cleanup approaches across spiking levels (100–1000 μg/kg). Mycosep® 227 consistently yielded higher and more stable recoveries, particularly for type A trichothecenes (NX-series, T-2, and HT-2 toxins). Recoveries were generally within the 80–110 % range, with minimal variability. The excellent recovery of DON (up to 103 % with OASIS® and 94.3 % on average with Mycosep® 227) agrees with previously reported LC-MS/MS data (Sulyok et al., 2024).

A slight decrease in nivalenol (NIV) recovery at the highest fortification level (78 % at 1000 μg/kg) may be attributed to sorbent saturation or competitive binding within the cleanup phase, consistent with previous observations (Koesukwiwat et al., 2014). OASIS® HLB produced satisfactory recoveries overall but exhibited higher variability, particularly for low-level and NX-type mycotoxins. For example, NX-3 recovery decreased to 70 % at 100 μg/kg, whereas Mycosep® 227 maintained 100 % recovery. This difference likely reflects the incomplete removal of polar matrix components by OASIS® HLB, which may interfere with trace-level detection (Sørensen et al., 2009).

Repeatability, expressed as relative standard deviation (RSD%), remained below 20 % for all analytes and levels, except for 15-AcDON at 100 μg/kg (25 %). All values complied with the European Commission criteria for repeatability and reproducibility under Regulations 401/2006 and 519/2014, confirming the method's precision. The lower variability observed with Mycosep® 227 further highlights its superior cleanup efficiency and reduced analyte–matrix interaction, in line with best practices for multiresidue mycotoxin analysis (Spanjer et al., 2008; Pereira et al., 2014).

Nevertheless, all values complied with the European Commission criteria for repeatability under Regulation 401/2006 and 519/2014, validating the method's precision. The lower variability in Mycosep® 227 - treated samples may reflect its more targeted cleanup efficiency and reduced analyte-matrix interaction, a factor critical for method reproducibility (Spanjer et al., 2008). Overall, the results confirm that both purification strategies are viable, but Mycosep® 227 provides superior matrix cleanup and higher analytical precision, particularly for low-abundance and matrix-sensitive mycotoxins. Combined with the enhanced sensitivity and selectivity of the UPLC-QDA system, the method ensures reliable quantification of both regulated and emerging trichothecenes, reinforcing its suitability for compliance monitoring and risk assessment within cereal-based food chains.

In conclusion, our multi-residue method is primarily designed for high-throughput screening and targeted surveillance of a large panel of analytes across complex matrices (rice and wheat). For this application, robustness, speed, and breadth of coverage are prioritized. While LOQ values may not represent the absolute lowest achievable levels, they are sufficiently low to detect non-compliant samples with high confidence, as they consistently fall well below the Maximum Residue Limits (MRLs) set by European Union (EU) regulations for the vast majority of target compounds. This study specifically highlights the necessity of the UPLC-QDA method for emerging NX toxins, which are undetectable by conventional UHPLC/PDA due to their poor UV absorption. Regarding purification, Mycosep® 227 consistently emerged as the superior cleanup strategy, providing reduced matrix effects and more consistent recoveries, particularly for the NX series in these complex matrices, compared to OASIS® HLB. As a practical implication for routine monitoring in rice and wheat, we conclude that the combination of Mycosep® 227 cleanup followed by UPLC-QDA detection provides the most robust, sensitive, and reliable framework for the simultaneous quantification of all nine trichothecenes.

Human and animal rights

The authors declare that the work described has not involved experimentation on humans or animals.

Informed consent and patient details

The authors declare that the work described does not involve patients or volunteers.

Author contributions

All authors attest that they meet the current International Committee of Medical Journal Editors (ICMJE) criteria for Authorship.

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CRediT authorship contribution statement

Vito D'Ascanio: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alessandro Annunziato:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daria Carella:** Methodology, Investigation, Formal analysis, Data curation. **Gerlinde Weisenberger:** Writing – review & editing, Visualization. **Antonia Susca:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Data curation. **Miriam Haidukowski:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Antonio Moretti:** Writing – review & editing, Visualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2026.101709.

Data availability

Data will be made available on request.

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