7th IMEKOFOODS
Worldwide food trade and consumption: quality and risk assessment
25-27 October 2023, Maison-Alfort / Paris, France

METHOD FOR DETERMINATION OF DEOXYNIVALENOL IN WHEAT FLOUR

M. Multescu[1], I. E. Susman[1,2], A. Culetu[1*], D. E. Duta[1]

[1] National Institute of Research and Development for Food Bioresources – IBA Bucharest, 6 Dinu Vintila Street, 021102 Bucharest, Romania
[2] University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
*alinaculetu@gmail.com

Abstract
The objective of this study was to develop and validate a sensitive, fast and reliable HPLC method for determination of deoxynivalenol (DON) in wheat flour. The calibration curve demonstrated good linearity in the concentration range of 25–2500 µg/kg. The accuracy of the determination of DON exhibited a mean recovery rate of 104.3%. Consequently, the limits of detection and quantification were 14.55 µg/kg and 44.09 µg/kg, respectively.

Keywords: deoxynivalenol, wheat flour, validation, curve calibration, limit of detection, limit of quantification

1. INTRODUCTION

Mycotoxins represent secondary metabolites primarily synthesized by molds under specific conditions. These compounds are non-essential for fungal growth or reproduction processes but exhibit toxicity towards animals or humans. It is noteworthy that a single mold species can generate various types of mycotoxins, and conversely, distinct mold species may produce identical mycotoxins. While over 300 mycotoxins have been reported, the actual count of these compounds in nature may might be bigger [1].

Deoxynivalenol DON (also known as vomitoxin, dehydronivalenol, 12,13-epoxy-3,4,15-trihyroxytrichothec-9-en-9-one, RD-toxin, and 4-deoxynivalenol) is the most commonly detected trichothecene mycotoxin in cereal grains at very high concentrations [1]. The problem of DON contamination in cereal grains and their products is significant. DON is found in over 90% of all samples contaminated with mycotoxins, and its presence often signals the presence of other harmful substances [2]. Consuming contaminated foods or animal feeds made from oats, barley, wheat, corn, and other grains can pose a potential risk to human and animal health. Even basic cooking methods may not remove DON from these foods and feeds [3].

There is a high demand for rapid, sensitive, and precise methods to detect DON and its derivatives in foods, animal feeds, and human samples. These methods are crucial for toxicological analysis and assessing the risk of exposure. They are also necessary to enforce regulations set by governments and international organizations. The typical analytical process for DON involves extracting the toxin from samples using a solvent, then cleaning up the sample to remove any interference from its natural composition. The final step is detecting the target toxin using appropriate analytical methods [4].

Thus, it is crucial to establish and assess an analytical technique for quantifying the concentration of DON. Several methods have been developed for measuring DON, such as HPLC [5, 6, 7], LC/MS [8, 9, 10], gas chromatographic analysis [11], and GC-MS [12, 13], involving derivatization. These methods include clean-up processes like solid-phase extraction [14, 15] and immunoaffinity column clean-up [16, 17, 18]. Among these, using a multifunctional column for clean-up appears to be a straightforward and reliable approach.

The scope of the present work was to develop and validate a robust, fast and accurate HPLC based method that allows the determination of DON in wheat flour.

2. MATERIALS AND METHODS

2.1. Chemicals

Deoxynivalenol was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Ultrapure water and acetonitrile for chromatography (HPLC grade) were obtained from Honeywell (Charlotte, North Carolina, USA).

2.2. Preparation of standard solution of deoxynivalenol

A stock solution of DON (100 mg/kg) was prepared by dissolving 1 mg DON in 10 mL acetonitrile. Two working solutions (10 mg/kg and 1 mg/kg) were prepared by diluting the stock solution with acetonitrile. These solutions were further used to prepare the calibration solutions which were injected in the HPLC and the peak area was reported.

2.3. Preparation of wheat flour extract

An amount of 10 g wheat flour sample was extracted with 50 mL mixture of acetonitrile:water (84:16, v/v) on an orbital shaker (GFL, Burgwedel, Germany) for 30 min. The extract was filtered through filter paper and 20 mL of the filtered extract was passed through DONeX clean-up columns using the EluVac Vacuum Manifold system (LCTech GmbH, Obertaufkirchen, Germany). The columns were additionally washed with 10 mL acetonitrile:water (84:16, v/v). The fourth part of the eluate obtained was evaporated under vacuum to dryness in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) and reconstituted in 1 mL of mobile phase (water:acetonitrile = 92:8, v/v). The sample was measured by HPLC and DON quantification was done by external calibration.

2.4. HPLC conditions

All HPLC measurements were performed on a 1260 Infinity II high performance liquid chromatography system with DAD detector (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) and with an Agilent Eclipse Plus C18 column, 4.6x150 mm, 5 µm and Guard Cartridges precolumn 4.6x12.5 mm, 5 µm. The mobile phase was water:acetonitrile = 92:8 (v/v) with a flow rate of 1 mL/minute. UV detection was performed at λ = 220 nm. The injection volume was 100 µL, the sample temperature was 20 °C and the column temperature set to 33 °C. The retention time for DON was 8 ± 0.4 min.

2.5. Validation parameters

The tests were performed for linearity, precision, accuracy, selectivity, sensitivity and uncertainty.

Precision was determined by repeatability and inter-laboratory reproducibility. The following parameters were reported: mean for concentration values, standard deviation in repeatability (*SD(r)*) and reproducibility (*SD(R)*), relative standard deviation in repeatability (*RSD(r)*) and reproducibility (*RSD(R)*) calculated as *SD/mean*100*. The repeatability (*r*) and reproducibility limit (*R*) were also considered, calculated as *2.8*SD*, for each case.

The sensitivity of the method was evaluated by limit of detection (LOD) and limit of quantification (LOQ). LOD is defined as the lowest concentration of analyte in a sample that can be detected under the conditions of an established analytical procedure.

LOQ of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated according to the following equations [20]: $LOD = 3.3 \cdot Sd/b$ and $LOQ = 10 \cdot Sd/b$

where: *Sd* is the standard deviation of the calibration curve and *b* is the slope of the calibration curve, calculated according to ISO 8466 [21].

3. RESULTS AND DISCUSSION

3.1. Linearity

The calibration curve for quantifying DON in wheat flour was linear over the concentration range of 25-2500 µg/kg (Table 1, Figure 1) and the coefficient of determination was higher than 0.999.

Table 1. Results for calibration curve for DON

DON concentration (µg/kg)	Area
25	4.343
50	8.278
100	15.097
250	39.074
500	78.410
1000	153.436
2500	383.812

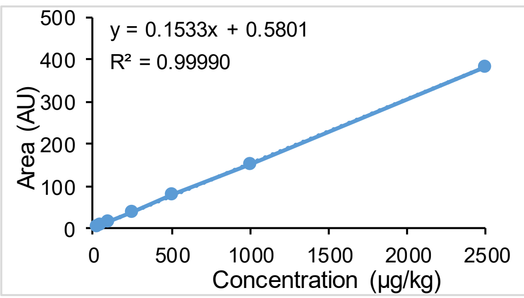

Figure 1. Calibration curve for DON

3.2. Precision

To determine the repeatability of the method, 6 parallel samples of wheat flour and 6 parallel samples of wheat flour fortified with 1000 µg/kg DON were analysed in the laboratory by a single analyst on the same instrument. The calculated parameters are presented in Tables 2 and 3. The value less than or equal to which the absolute difference between two test results obtained under repeatability conditions (repeatability limit) is 9.48 µg/kg. Acceptance imposed criteria for repeatability of the method are in accordance with the provisions of the Commission Regulation (EC) no. 401/2006 [22] as the values obtained for the relative standard deviation in repeatability conditions (RSD(r)) was ≤ 20%.

To determine the intra-laboratory reproducibility of the method, 4 samples of wheat flour and 4 samples of wheat flour fortified with 1000 µg/kg DON were analysed in the laboratory in the same day by 2 analysts using the same instrument. The results are presented in Tables 4 and 5. The value less than or equal to which the absolute difference between two test results obtained under intra-laboratory reproducibility conditions is 3.34 µg/kg. The relative standard deviation in intra-laboratory reproducibility (RSD(R)) was ≤ 40%, which is in conformity with Commission Regulation (EC) no. 401/2006 [22].

The applied method showed a good precision, which fulfils the performance criteria for DON [22].

Table 2. Repeatability for 6 parallel samples of wheat flour

Injection no.	Area	Concentration (µg/kg)
1	9.340	55.719
2	8.929	53.031
3	9.230	54.996
4	9.300	55.455
5	8.439	49.827
6	8.077	47.499
Mean (µg/kg)	-	**52.75**
SD(r) (µg/kg)	-	**3.39**
RSD(r) (%)	-	**6.42**
r (µg/kg)	-	**9.48**

Table 3. Repeatability for 6 parallel samples of wheat flour fortified with 1000 µg/kg DON

Injection no.	Area	Concentration (µg/kg)
1	160.333	1042.294
2	160.698	1044.677
3	160.459	1043.114
4	160.534	1043.608
5	160.730	1044.885
6	160.406	1042.767
Mean (µg/kg)	-	**1043.56**
SD(r) (µg/kg)	-	**1.04**
RSD(r) (%)	-	**0.10**
r (µg/kg)	-	**2.92**

Table 4. Intra-laboratory reproducibility for 4 samples of wheat flour

Injection no.	Area	Concentration (µg/kg)
1	10.925	66.075
2	11.034	66.787
3	10.716	64.707
4	10.640	64.207
Mean (µg/kg)	-	**65.44**
SD(R) (µg/kg)	-	**1.19**
RSD(R) (%)	-	**1.82**
R (µg/kg)	-	**3.34**

Table 5. Intra-laboratory reproducibility for 4 samples of wheat flour fortified with 1000 µg/kg DON

Injection no.	Area	Concentration (µg/kg)
1	157.640	1024.699
2	157.805	1025.774
3	157.751	1025.420
4	158.106	1027.741
Mean (µg/kg)	-	**1025.91**
SD(R) (µg/kg)	-	**1.30**
RSD(R) (%)	-	**0.13**
R (µg/kg)	-	**3.64**

3.3. Accuracy

Accuracy was determined from recovery studies. A known amount of wheat flour was spiked with 1000 µg/kg DON and the recovery percentage (R, %) of the method was established from 6 repeated analysis of the fortified sample. The average recovery of the

DON determination method was 104.3%, falling within the provisions of Commission Regulation (EC) no. 401/2006: 70 to 120 %.

3.4. Selectivity
Selectivity was verified by chromatographic separation of DON in the presence of other components from the wheat flour matrix.

3.5. Sensitivity
LOD and LOQ obtained were 14.55 μg/kg and 44.09 μg/kg, respectively. Considering the fact that the maximum limit allowed by the legislation in force for DON in cereal flour is 750 μg/kg [19], the method developed for DON determination using HPLC is sensitive (detection limit and quantification limit are very low).

3.6. Uncertainty
The uncertainty was estimated, according to the EURACHEM/CITAC Guide [23] and ISO/IEC Guide 98-3:2008 [24]. The uncertainty of results for DON determination in wheat flour was ± 10.2%.

4. CONCLUSIONS
The proposed analytical method was successfully applied for determination of DON content in wheat flour samples. The method's primary advantage lies in its user-friendly nature, as it minimizes solvent usage, making it both cost-effective and environmentally friendly, in line with the principles of green analytical chemistry. Additionally, this approach significantly reduces analysis time, making it a time-saving solution.

5. REFERENCES
[1] R. Ran, C. Wang, Z. Han, A. Wu, D. Zhang, J. Shi. Determination of deoxynivalenol (DON) and its derivatives: Current status of analytical methods. Food Control 2013, 34, 138-148.

[2] P. Sobrova, V. Adam, A. Vasatkova, M. Beklova, L. Zeman, R. Kizek. Deoxynivalenol and its toxicity. Interdiscip. Toxicol. 2010, 3(3), 94-99.

[3] J. J. Pestka, A.T. Smolinski. Deoxynivalenol: toxicology and potential effects on humans. J. Toxicol. Environ. Health Part B, 2005, 8(1), 39-69.

[4] G. S. Shephard. Determination of mycotoxins in human foods. Chem. Soc. Rev. 2008, 37(11), 2468-2477.

[5] R. D. Josephs, R. Schuhmacher, R. Krska. International interlaboratory study for the determination of the Fusarium mycotoxins zearalenone and deoxynivalenol in agricultural commodities. Food Addit. Contam. 2001, 18, 417-430.

[6] D. R. Lauren, R. Greenhalgh. Simultaneous analysis of nivalenol and deoxynivalenol in cereals by liquid chromatography. J. Assoc. Off. Anal. Chem. 1987, 70, 479-483.

[7] Y. Shirai, Y. Ono, K. Akimoto. Simultaneous determination of deoxynivalenol and nivalenol in grain by high performance liquid chromatography with multifunctional clean up column for purification. Res. Rep. Animal Feed. 2000, 26, 1-9.

[8] R. D. Plattner. HPLC/MS analysis of fusarium mycotoxins, fumonisins and deoxynivalenol. Nat. Toxins, 1999, 7, 365-370.

[9] U. Berger, M. Oehme, F. Kuhn. Quantitative determination and structure elucidation of type A- and B-trichothecenes by HPLC/ion trap multiple mass spectrometry. J. Agric. Food Chem. 1999, 47, 4240-4245.

[10] R. D. Voyksner, W. M. Hagler WM, S. P. Swanson. Analysis of some metabolites of T-2 toxin, diacetoxyscirpenol and deoxynivalenol by thermospray high-performance liquid chromatography-mass spectrometry. J. Chromatogr. A 1987, 394, 183-199.

[11] P. M. Scott, G. A. Lombaert, P. Pellaers, S. Bacler, S. R. Kanhere, W. F. Sun, P. Y. Lau, D. Weber. Application of capillary gas chromatography to a survey of wheat for five trichothecenes. Food Addit. Contam. 1989, 6, 489-500.

[12] T. Tanaka, A. Yoneda, S. Inoue, Y. Sugiura, Y. Ueno. Simultaneous determination of trichothecene mycotoxins and zearalenone in cereals by gas chromatography-mass spectrometry. J. Chromatogr. A 2000, 882, 23-28.

[13] P. M. Scott, P. Y. Lau, S. R. Kanhere. Gas chromatography with electron capture and mass spectrometric detection of deoxynivalenol in wheat and other grains. J. Assoc. Off. Anal. Chem. 1981, 64, 1364-1371.

[14] M. W. Trucksess, S. W. Page, G. E. Wood, T. H. Cho. Determination of deoxynivalenol in white flour, whole wheat flour, and bran by solid-phase extraction/liquid chromatography: Interlaboratory study. J. AOAC Int. 1998, 81, 880-886.

[15] B. R. Malone, C. W. Humphrey, T. R. Romer, J. L. Richard. One-step solid-phase extraction cleanup and fluorometric analysis of deoxynivalenol in grains. J AOAC Int. 1998, 81, 448-452.

[16] P. M. Scott, M. W. Trucksess. Application of immunoaffinity columns to mycotoxin analysis. J. AOAC Int. 1997, 80, 941-949.

[17] R. Dietrich, E. Schneider, E. Usleber, E. Martlbauer. Use of monoclonal antibodies for the analysis of mycotoxins. Nat. Toxins 1995, 3, 288-293.

[18] L. M. Cahill, S. C. Kruger, B. T. McAlice, C. S. Ramsey R. Prioli, B. Kohn. Quantification of deoxynivalenol in wheat using an immunoaffinity column and liquid chromatography. J. Chromatogr. A 1999, 859, 23-28.

[19] European Commission. Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006.
[20] IUPAC. Nomenclature in Evaluation of Analytical Methods including Detection and Quantification Capabilities. Pure & Appl. Chem., 1995, 67, 1699-1723.
[21] ISO 8466-1:2021. Water quality — Calibration and evaluation of analytical methods. Part 1: Linear calibration function. ISO, Geneva.
[22] European Commission. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.
[23] EURACHEM/CITAC Guide. Quantifying Uncertainty in Analytical Measurement, 3rd ed., 2011, UK.
[24] ISO/IEC Guide 98-3:2008. Uncertainty of Measurement - Part 3: Guide to the Expression of Uncertainty in Measurement (GUM:1995). ISO, Geneva.

Acknowledgments

The funding for this project was provided by the Ministry of Research, Innovation, and Digitalization under Program 1 - Development of the national R&D system, Subprogram 1.2 - Institutional performance - financing projects for excellence in RDI, with the contract number 17 PFE/2021.