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Determination of deoxynivalenol and nivalenol by liquid chromatography and fluorimetric detection with on-line chemical post-column derivatization

Marilena Muscarella [a], Marco Iammarino [a], Donatella Nardiello [b,c], Carmen Palermo [b,c], Diego Centonze [b,c,*]

[a] Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Via Manfredonia 20, 71100 Foggia, Italy
[b] Dipartimento di Scienze Agro-ambientali, Chimica e Difesa Vegetale, Università degli Studi di Foggia, Via Napoli 25, 71100 Foggia, Italy
[c] Centro Interdipartimentale di Ricerca BIOAGROMED, Università degli Studi di Foggia, Foggia, Italy

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ABSTRACT

A rapid, sensitive and selective analytical method was developed for the quantitative determination of deoxynivalenol (DON) and nivalenol (NIV) in cereals intended for human and animal consumption. The method, based on liquid chromatography and fluorescence detection, involves an automated 2 channel post-column derivatization, performed with sodium hydroxide, methyl acetoacetate and ammonium acetate. The chromatographic separation was accomplished using a C18 column eluted in isocratic mode with a mixture of 0.01% acetic acid and acetonitrile. Optimal fluorescence detection was obtained by an excitation and emission wavelength of 360 nm and 470 nm, respectively. The sample preparation required a rapid extraction of mycotoxins with water and a purification step by hydrophilic–lipophilic balance column clean-up. Under the optimized experimental conditions, a complete separation of DON and NIV was obtained in less than 20 min. The on-line post-column derivatization ensures excellent results in terms of simplicity and sensitivity, with limits of detection down to 0.014 mg/kg. The proposed method was extensively validated and the analytical performances of linearity (correlation coefficient of 0.9998), selectivity, precision (intra-day precision lower than 8%) and recovery (ranging from 89% to 101%) were evaluated, demonstrating the method feasibility in accurate confirmation analyses.

1. Introduction

Trichothecenes are secondary metabolites produced by several fungal genera, but mainly by *Fusarium* species. Nowadays, more than 140 trichothecenes are known and according to their functional groups are commonly divided into four classes (A–D). Deoxynivalenol (DON) and nivalenol (NIV) mycotoxins, belonging to the group of trichothecenes B, are spread worldwide in cereals, such as wheat, corn, barley and oats [1–3]. These compounds have been known for a number of years to cause toxicosis in humans, as well as in farm animals, leading to food refusal, vomiting, anemia, hemorrhage and immune-suppression [4]. For DON, the European Union (EU) has set a maximum level (ML) of 0.75 mg/kg in cereals intended for direct human consumption, and of 1.75 mg/kg in unprocessed durum wheat, maize and oats [5]. Actually, for NIV no legal limits have been established, due to its lower toxicity than other trichothecenes. Nevertheless, NIV has been shown to cause a variety of toxic effects, including inhibition of protein synthesis and nucleic acids in vitro, increase of chromosomal aberrations frequency, and embryotoxicity in mice [6]. Therefore, the development of sensitive, reliable and fast method for the determination of both DON and NIV represents an important feature for the evaluation and management of risk to public health arising from dietary exposure to *Fusarium* toxins. Current analytical methods for trichothecene mycotoxins in cereals have been recently reviewed [7]. In the last decade, liquid chromatography coupled with tandem mass spectrometric detection gained more importance for multi-analyte mycotoxin determination, assuring accurate and sensitive analyses [8–13]. Anyway, in official control analysis, chromatographic methods based on fluorescence detection represent a valid alternative as confirmatory methods, which provide good results in terms of selectivity, instrumental costs and simplicity. In addition, compared to UV–visible adsorption, the fluorimetric detection assures higher sensitivity, which is essential for the analysis of baby foods for infants and young children, for which a more restrictive limit of 200 μg/kg has been set [5]. DON and NIV are not naturally fluorescent, and then a derivatization step is required for a sensitive detection at low concentration levels. In order to overcome drawbacks due to pre-column techniques, usually time-consuming and poorly reproducible, post-column derivatization is more suitable. To the best of our knowledge, a few applications of post-column

* Corresponding author at: Università degli Studi di Foggia Dipartimento di Scienze Agro-ambientali, Chimica e Difesa Vegetale Via Napoli 25, 71100 Foggia, Italy. Tel.: +39 0881 589104; fax: +39 0881 589101.
E-mail address: centonze@unifg.it (D. Centonze).

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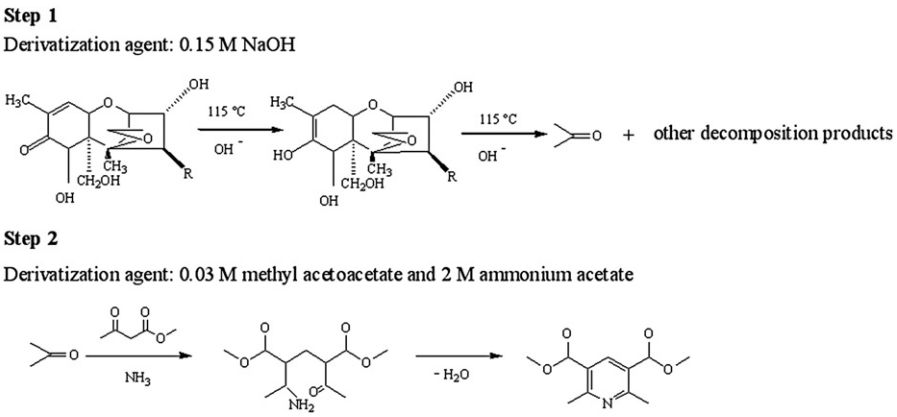

Fig. 1. Chemical derivatization process of deoxynivalenol (R=H) and nivalenol (R=OH).

derivatization have been reported for the determination of trichothecene mycotoxins [14,15]. The proposed derivatization process was based on a modified Hantzsch synthesis by reaction of type B-trichothecenes with NaOH, methyl acetoacetate and ammonium acetate [15]. As shown in Fig. 1, when heated under the influence of alkali, DON and NIV breaks down into several sub-products accompanied by the release of formaldehyde. The generated formaldehyde reacts with methyl acetoacetate and ammonium to form a fluorescent dihydropyridine derivative.

A detailed study of the experimental parameters influencing the derivatization reaction of DON and NIV was described [15]. Nevertheless, the proposed chromatographic conditions do not satisfy the recent European requirements in terms of selectivity [16–18]; in fact, the chromatograms of real samples show a lot of interfering peaks in the retention time-window of the analyte. Moreover, a very time-consuming sample clean-up was reported, which consisted of a double solid phase extraction. A few years ago [14], different clean-up procedures, based on immunoaffinity chromatography, were described exclusively for DON, but a full method validation, as currently required by recent European rules for the official control methods [16–18], was missing.

In the present work, the on-line chemical derivatization 2 channel-process was used and the development of a rapid and automated method by reverse phase liquid chromatography and fluorescence detection is described for the quantitative determination of DON and NIV in cereals for human and animal consumption. Separation experimental conditions as well as the sample extraction, and clean-up were carefully evaluated, shooting for the development of a fast and selective method for high throughput applications in risk-assessment studies and control analyses. Also, the proposed method was submitted to a validation procedure, in agreement with the European directives [16–18] to assess accuracy, sensitivity, reproducibility and ruggedness.

2. Materials and methods

2.1. Chemicals and working standard solutions

Standards of deoxynivalenol (99.4%) and nivalenol (98.6%) were supplied by Sigma-Aldrich (Steinhem, Germany). Water, acetic acid, methanol and acetonitrile of LC grade were purchased from Baker (Deventer, Holland). Ammonium acetate ($\geq$97%), sodium hydroxide ($\geq$98%) and methyl acetoacetate (99%), were purchased from Sigma-Aldrich. A 100 mg/mL stock solution of deoxynivalenol and nivalenol was prepared in acetonitrile and stored at -20 °C up to 12 months. Working standard solutions were prepared by dilution in mobile phase and stored at -20 °C when not in use; these solutions were stable for at least 3 months.

2.2. Sample preparation

A 5-g portion of the sample (durum wheat and maize) was suspended in 15 mL of water and vortexed for few minutes. After centrifugation at $2112 \times g$ for 10 min at 4 °C and filtration on cellulose acetate (0.80 μm, Minisart® CE 0120/CE, Sartorius Stedim Biotech GmbH), an aliquot of 3 mL of the extract was transferred on the top of an hydrophilic–lipophilic balance (HLB) cartridge (6 mL, OASIS® HLB, Waters), previously activated by 6 mL of water. After washing with 3×6 mL of 0.01% acetic acid, the fraction containing the mycotoxins was eluted with 3 mL of methanol. After evaporating to dryness at 40 °C under a nitrogen stream (Dubnof Bath BSD/D), the residue was solubilised in 1 mL of mobile phase, filtered on cellulose acetate (0.2 μm, Minisart® CE 0297/CE, Sartorius Stedim Biotech GmbH) and then injected. The overall analytical procedure involved no dilution factor. The stability of the purified extracts was high enough to allow autosampler overnight injections.

2.3. Apparatus and method

Chromatographic separations were performed on a LC system, Agilent Technologies SL 1200 Series (Waldbronn, Germany) consisting of a binary pump, a thermostated autosampler, a column compartment and a fluorescence detector. Chromatographic separations were performed on a Eurospher C18 column (150 mm × 4.0 mm i.d., particle size 5 μm) from Knauer (Berlin, Germany), in isocratic mode at 0.4 mL/min. The mobile phase consisted of 0.01% acetic acid/acetonitrile, 90:10 (v:v). The injection volume was 20 μL. On-line post-column chemical derivatization was performed by using a commercially available system supplied by LabService Analytica S.r.l. (Bologna, Italy), and consisting of two double-piston pumps, operating at 0.25 mL/min, and a thermostatable 2-channel post-column derivatization unit (Pickering PCX 5200), set at 115 °C, equipped with a 1.2 and 1.6 mL knitted reaction coils. The derivatization agent (A) was 0.15 M NaOH, the derivatization agent (B) was a solution composed of 0.03 M methyl acetoacetate and 2 M ammonium acetate. Fluorescence detection was performed at the excitation and emission wavelengths of 360 and 470 nm, respectively. The system was interfaced, via a network chromatographic software (Agilent ChemStation), to a personal computer for control of instruments, data acquisition and processing.

2.4. Validation procedure

The linearity test was performed by three series of analyses on three different days, by injecting standard solutions of DON and NIV at concentrations of 0.25, 0.50, 1.0, 2.0, and 4.0 mg/L. The method selectivity was tested by the analysis of 20 independent blank samples of cereals (durum wheat and maize), found negative by ELISA screening. Precision and recovery were determined by performing tests on two sets of blank wheat bran samples (six replicates each) fortified with DON and NIV, at five concentration levels: 0.375 mg/kg, 0.750 mg/kg (maximum level set for DON in cereals intended for direct human consumption), 1.250 mg/kg, 1.750 mg/kg (maximum level of DON in unprocessed durum wheat, maize and oats) and 2.625 mg/kg. The experiments were performed in twelve different days with the same instruments but different operators and instrumental calibrations.

3. Results and discussion

3.1. Optimization of chromatographic conditions and sample clean-up

Different stationary phases, elution programs and mobile phases have been proposed in order to separate type B trichothecenes by reverse-phase liquid chromatography [19–24]. The use of a polar-modified C18 column turned out very useful to perform the separation of DON and NIV by using, as the eluent, a diluted acetic acid solution containing a small percentage of acetonitrile, and operating in isocratic mode; acetonitrile is more appropriate than methanol that can contain traces of formaldehyde. Therefore, such a mobile phase resulted particularly suitable for a sensitive fluorescence detection of DON and NIV, following the derivatization reaction. Moreover, a complete separation (see Fig. 2) in less than 20 min of run time was achieved.

The sample preparation protocol was carefully optimized in terms of analysis throughput, solvent mixtures, and solid phase purification procedures. DON and NIV are water soluble toxins, and the extraction process from cereal-based samples is usually performed using water solutions with the presence of an organic solvent [25–29]. Compared to aqueous acetonitrile and methanol solutions with percentages up to 50% (v/v), the extraction with whole water gave the best results in terms of DON recovery (higher than 97%). Afterwards, in an attempt to improve the sample purification from matrix interfering peaks and to get the optimal compromise between clean extracts and high recoveries, performances of different clean-up cartridges were compared. Cartridges with hydrophilic and lipophilic retention characteristics (OASIS® HLB, Waters), C18 (Bond Elut-C18, Varian), immunoaffinity columns (DONtest WB™, Vicam), silica-gel (Silica

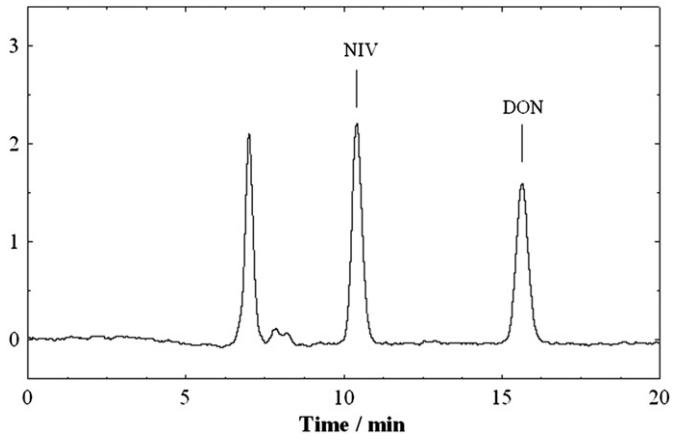

Fig. 2. Chromatogram of a mixed standard solution containing DON and NIV at a concentration of 1 mg/L.

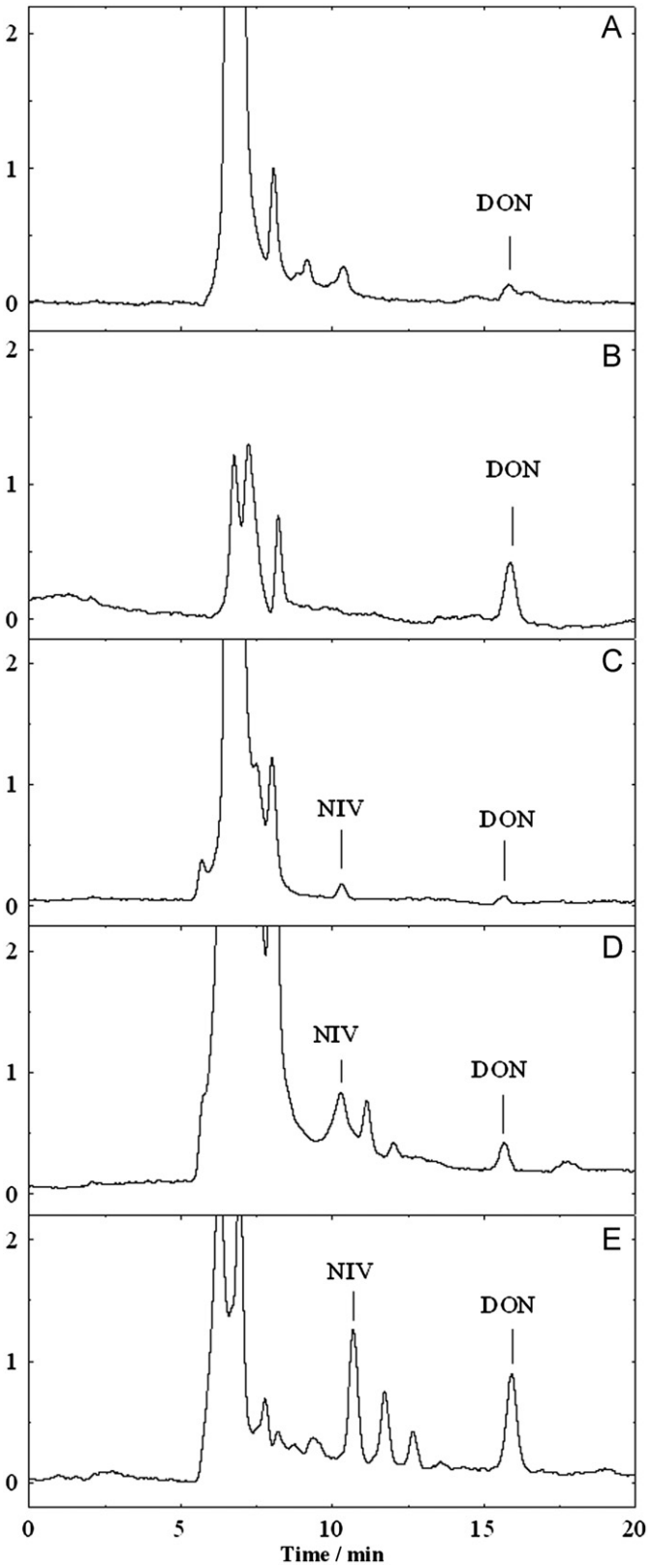

Fig. 3. Chromatograms of a spiked wheat sample with DON and NIV at 1.75 mg/kg, and purified by SPE columns: (A) silica-gel (Applied Separations); (B) immunoaffinity DONtest WB™ (Vicam); (C) multifunctional trichothecene EP (R-Biopharm); (D) C18 Bond Elut (Varian); (E) OASIS® HLB (Waters).

Gel, Applied Separations), and multifunctional cartridges, based on charcoal, celite and ion-exchange resin (Trichothecene EP Columns, R-Biopharm) were used in this study. As can be noted from Fig. 3, the best results were achieved with OASIS columns that showed a more selective purification and higher average recoveries. The optimized clean-up procedure, based on simple sequential steps of cartridge loading, washing and elution, allowed an effective reduction of the overall analysis time, which was less than 40 min, including the chromatographic separation.

3.2. Method validation

Validation of the analytical methods is essential to provide accurate results with a high within- and inter-laboratory reproducibility, which are very important parameters in monitoring and risk-assessment studies, as well as in official controls. In agreement with Decision 657/2002/EC [16] and Regulation 882/2004/EC [17], which describe the analytical parameters to be tested to assure the method reliability, method validation parameters such as selectivity, linearity, detection and quantification limits, precision, recovery, ruggedness have been evaluated. The results of the validation procedure have been compared to the reference data indicated by Regulation 401/2006/EC [18], which concerns the methods of sampling and analysis for the official control of mycotoxins in foodstuffs.

3.2.1. Selectivity towards interferences

For the assessment of the method selectivity, durum wheat and maize samples were processed by the proposed method. The comparison of typical chromatograms obtained for blank and spiked samples (see Fig. 4) evidenced that the proposed method is able to distinguish the analytes from other matrix components, since in the retention time-window of interest ($\pm 2.5\%$ of the retention time of each trichothecene) no interfering peaks were observed.

3.2.2. Calibration curves and limits of detection and quantification

A good linearity was found for DON and NIV in the range 0.25–4.0 mg/L, with correlation coefficients higher than 0.9998. The goodness-of-fit of the data to the calibration curve is obtained in terms of response factor distribution (signal-to-concentration ratio, y_i/x_i) whose reference range is $(y/x)_{mean} \pm 10\%$. Linearity has been also checked by an F test for Lack-of-Fit that has been performed by using all the calibration datasets ($n=15$ data pairs, $c=5$ calibration points, $j=3$ replicates at each calibration point). The experimental F values (0.048 for DON and 1.073 for NIV), calculated by the lack-of-fit and pure error sums of squares, divided by the corresponding degrees of freedom, respectively 3 and 10, were clearly lower than the $F_{crit}(0.99; 3; 10)$ value of 6.55. Furthermore, any systematic instrumental bias can be ruled out since the confidence interval of intercept includes the zero value at 95% confidence level ($v=4$). The calibration parameters evaluated for each mycotoxin are reported in Table 1. LOD values (signal-to-noise ratio of 3) were 0.014 mg/kg and 0.011 mg/kg for DON and NIV, respectively. These values are noticeably lower than the maximum residue limits established either for cereals or baby foods; in the latter case a limit of 0.200 mg/kg has been set for DON.

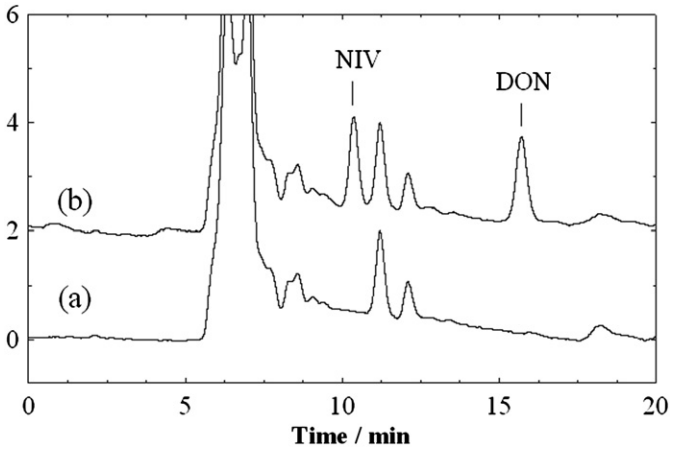

Fig. 4. Chromatograms of blank (a) and spiked (b) maize samples with DON and NIV at 1.5 mg/kg.

Table 1
Calibration parameters obtained by injections of trichotecene standard solutions.

	$y=a+bx$[a]				
	$a \pm SD$	$b \pm SD$	r[b]	LOD[c]	LOQ[c]
DON	-2.1 ± 1.0	52.2 ± 0.5	0.9998	0.014	0.047
NIV	-2.2 ± 1.2	59.7 ± 0.6	0.9998	0.011	0.037

[a] y is the signal in luminescence unit (LU) and x is the value of concentration in mg/L.
[b] Correlation coefficient.
[c] Detection and quantification limits (mg/kg) estimated from the chromatogram of durum wheat at a signal-to-noise ratio of 3 and 10, respectively.

Table 2
Reproducibility and recovery data for the determination of DON and NIV in spiked durum wheat samples.

Fortification Level (mg/kg)	Recovery (%)[a]	RSD (%)[b]		Reference value (%)	
		Intra-day ($n=6$) RSD_r	Inter-day ($n=12$) RSD_R	RSD_r	RSD_R
DON					
0.375	99 ± 7	7	8	≤ 20[c]	≤ 40[c]
0.750	101 ± 7	6	14		
1.250	97 ± 5	5	8		
1.750	100 ± 7	6	11		
2.625	101 ± 6	5	12		
NIV					
0.375	89 ± 5	4	11	12[d]	18[d]
0.750	91 ± 3	3	6	11[d]	17[d]
1.250	94 ± 9	7	13	10[d]	16[d]
1.750	90 ± 7	8	11	10[d]	15[d]
2.625	96 ± 7	6	14	9[d]	14[d]

[a] Mean value $\pm$ SD. Six replicates at each fortification level for each working session ($n=12$).
[b] Within-laboratory relative standard deviation under repeatability (RSD_r) and reproducibility (RSD_R) conditions.
[c] RSD_r and RSD_R reported in Reg. (EC) No. 401/2006 for deoxynivalenol determination.
[d] RSD_r evaluated as two-thirds of RSD_R, calculated by Horwitz equation, as reported in Decision 657/2002/EC.

3.2.3. Precision and recovery

The method was tested for accuracy, intra- and inter-day assay within-laboratory reproducibility, and the relevant data determined for each mycotoxin by spiked durum wheat samples are summarized in Table 2. Precision data have been previously processed by the Shapiro–Wilk test to verify the normal distribution. Afterwards, ANOVA one way test was performed in order to verify the homogeneity of the concentration mean values determined in two different days. By comparison with the reference values, as indicated in the Regulation (EC) No. 401/2006, relative standard deviations obtained for intra-day (RSD_r) and inter-day (RSD_R) analyses

demonstrated that the method can be considered valid for the quantification of DON and NIV in cereal samples.

Recovery percentages were evaluated by comparing the concentration of spiked samples, determined by the calibration regression line, with the nominal fortification level. Recoveries ranging from 97% to 101% for DON and from 89% to 96% for NIV were obtained. These results are in agreement with Regulation (EC) No. 401/2006 that establishes recoveries in the range 60–110% for DON concentrations in the range 100–500 μg/kg, and 70–120% for levels higher than 500 μg/kg. Moreover, these performance parameters as well as detection and quantitation limits are comparable or even better than those obtained by mass spectrometry based methods [9,10].

3.2.4. Ruggedness (major changes)

The method ruggedness under conditions of major changes has been assessed by using the Youden experimental design [30] performed for maize samples spiked with DON and NIV at a fortification level of 1.75 mg/kg. The Youden experimental design requires eight independent experiments: four with the validation matrix (durum wheat) and four with the alternative matrix (maize). Analysis of fortified maize samples gave a calculated standard deviation of difference (S_{Di}) of 0.22 mg/kg for DON and 0.24 mg/kg for NIV. These values were not significantly different (2 tails *F* test, at 7 and 11 degrees of freedom, 95% confidence level) from the estimated method precision (S_R=0.20 mg/kg and 0.18 mg/kg for DON and NIV, respectively), then the variation of the matrix has no effect on the analytical performances and, consequently, the method is also applicable to maize analysis.

4. Conclusions

A rapid and reliable method was developed for the simultaneous determination of DON and NIV in cereals for human and animal consumption. The on-line post column derivatization ensures high sensitivity and reproducibility and provides, compared to pre-column derivatization techniques, an increase of peak efficiency, and an improvement of the automation degree, since the post-column derivatization step is automatically controlled. The selectivity of the derivatization process allowed to simplify clean-up steps, avoiding extensive sample pre-treatments. The results of the method validation, performed according to Regulation (EC) No. 882/2004, demonstrate the method conformity with provisions of Regulation (EC) No. 401/2006, in terms of precision and recovery. Such an accurate and efficient method guarantees a fast analytical response in a short time, which is especially valuable in monitoring and in the official analyses, in particular for baby foods, whose DON maximum content has been set at 200 μg/kg. The method performance parameters such as detection and quantitation limits, recovery and precision were comparable or even better than those obtained by mass spectrometry based methods.

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