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VALIDITY OF METHOD FOR DETERMINING DEOXYNIVALENOL THROUGH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH DETECTION IN THE ULTRAVIOLET IN WHEAT SAMPLES

(Validación de un método para la determinación de deoxinivalenol por cromatografía líquida de alta resolución con detección ultravioleta en muestras de trigo)

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Palabras clave: Trigo, harina de trigo, Deoxynivalenol, cromatografía líquida de alta resolución.

ABSTRACT

With the purpose of evaluating the wheat grain and wheat flour contamination by Deoxynivalenol (DON) in the municipality of Chapeco-SC, and standardize an useful method to detect this mycotoxin by High Performance Liquid Chromatography (HPLC), six samples of wheat grains from different storage location, and one sample of wheat grain from the flour milling industry were obtained during in the month of august 2008. Samples belong to a corporation from Chapeco-SC that stores and processes wheat grains, flour and wheat middlings, among other products. The extraction has been carried out with methanol: water (100: 100 v / v), filtered paper filter and applied in immunoaffinity column specific DON. After the wash with water column, the toxin was eluted with methanol. The detection and quantification Deoxynivalenol in samples was carrid out through the method of HPLC in the UV -visible with detection 244 nm. The 6 analyzed samples of wheat grain showed DON levels within 7.0 and 10.1 ppb, while the wheat flour contained 90.2 ppb.

DON contents in wheat grains and wheat flour are lower than the limits claimed by the studied corporated importers and the international l egislation.

RESUMEN

Con el objetivo de evaluar la contaminación por Deoxinivalenol (DON)en granos y harina del trigo en la municipalidad de Chapeco-SC y estandarizar un método de detecciòn para este micotoxina por cromatografía líquida de alta resolución (CLAR), se procesaron durante el mes de agosto de 2008, seis muestras de granos del trigo en diferentes situaciones de almacenamiento y una muestra de harina de trigo de un molino . Las muestras pertenecen a una cooperativa de Chapeco-SC que procesa y almacena granos y harinas de trigo entre otros productos. La extracción de la micotoxina se obtuvo con metanol: agua (100: 100 v / v), filtrado en papel filtro y aplicado a una columna de inmunoafinidad específica (DON). Después del lavado de la columna con agua, la toxina fue elucidada con metanol. La detección y cuantificación de Deoxinivalenol en las muestras se determinó por el método CLAR en el UV -visible con una longitud de onda de 244 nm. Las 6 muestras analizadas del grano, mostraron que el DON nivela entre 7,0 y 10,1 ppb, mientras la harina del trigo alcanzó las 90,2 ppb.

Los niveles de DON en los granos y harina de trigo tienen límites menores que los exigidos por las cooperativas importadoras estudiadas y la legislación internacional.

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INTRODUCTION

Mycotoxins are secondary metabolites produced by some species of filamentous fungi, when biological and environmental factors favorable exist, being capable to produce poisonous effects on animals and man (Peraica *et al.*, 2000; Sabino, 2008). The term mycotoxins cams from the Greek «Mykes» that means mushroom, and of the Latin word «Toxicum» that means poison, in other words, the expression grecco-latin «mykes toxicum» denotes that mycotoxin is the toxin produced by fungi (Scussel, 1998).

The contamination of the foods by mycotoxins can happen in the field during the development or maturation, during and after the crop, in the transport, in the storage or manufacture of the products, when favorable bioclimatic ateconditions exist, as the rain excess, damages for insects, and stress of the plant (Bennett & Richard, 1996; Gonçalves *et al.*, 2001; Sabino, 2008). It is important also to stand out that the relative humidity of the air, the content of humidity of the grains, the temperature, the light, the mechanical damages and the microclimate (storage atmosphere) they influence in the expression of those toxins in polluted foods for mycotoxigenics fungi in the storage places and stock. In Workshop on Mycotoxins and Ficotoxins accomplished in 1996 in Italy , sponsored by Food Agriculture Organization, they were mentioned as the five main toxins or «the big 5» the aflatoxins, ochratoxin, T-2 toxin, deoxynivalenol, and fumonisins (Scussel, 1998), meantime in the Brazilian and South American reality we have the patulin, trichothecenes and zearalenone in larger prevalence than the T-2 toxin (Fink-Gremmels, 2008). The trichothecene includes a group of more than 180 biologically chemically composed assets related produced predominantly by filamentous fungi of the goods ***Fusarium*** and ***Stachybotrys*** that grow on foods, animal rations, or in the environment (Pestka, 2007).

Experimentally, the sharp oral exhibition of lower doses of the trichothecenes causes vomit, diarrhea and gastroenteritis, while, higher doses promote severe damages to the lymphoids and epithelials cells of the mucous membrane gastrointestinal, causing hemorrhage and shock. The bone marrow and the thymus are also objectives of these metabolites, that contribute to the immunosupression widespread (Pestka, 2007).

Among the trichothecenes of larger importance we can mention Deoxynivalenol (DON), also known as vomitoxin (Canady *et al.*, 2001), one of the trichothecenes more commonly found in grains of cereals, mostly in parts for million (ppm) (Wolf-Hall & Bullerman, 2006). All of the animal species experimentally appraised until the moment exhibited sensibility to the deoxynivalenol (Pestka, 2007), however enough experimental proofs of DON carcinogenicity don't exist; for that reason it was sent to Inter-Agency, 3 groups went for Research on Cancer (Iarc, 1993).

Epidemic studies demonstrate that foods most liable to be contaminated by DON are cereals, mainly grains as wheat, barley , oats, rye and corn and less frequently rice, sorghum and triticale, hay, straw and animal ration (Canady *et al.*, 2001; Scussel, 1998). Starting from 1990 the deoxynivalenol passed to be considered as a mycotoxin of high priority in control programs, however, just some countries determined allowable limits of this toxin in foods (FAO, 2004). The European Community, for instance, recommends DON values in the cereals products and the corn by-products destined to the animal feed in the order of 8 and 12 ppm, respectively.

Numerous analytical methods for thin layer chromatography (TLC), gaseous chromatography (GC) and high performance liquid chromatography (HPLC) have been established for analysis of the trichothecenes. The analytical methods commonly employed, mainly for laboratories of analyses for regulation of allowed maximum levels, are submitted to the validity and compared to certified reference methods, so that they can demonstrate that the chosen tool supply comparable, and exact results easily detected. In general, the objective of accomplishing the validity of a method, is to demonstrate that the proposed analytical method (using a specific head office, and different stages) it produces, satisfactory , exact and reproductible results for a certain property in study (Josephs, 2004).

Considering the contamination of foods with toxigenic fungi and mycotoxin production, is usually related to environmental conditions of cultivation and storage, continuous investigations of the occurrence become necessary (Oliveira *et al.*, 2002), because of the poisonous effects on man and animals, as well as, the agroeconomic losses, being important to detect and to quantify the present mycotoxins in foods and nutritious goods destined to human and animal consumption (Whitaker, 2006; Lazo & Sierra, 2008; Krska *et al.*, 2008). Knowing the importance of the cultivation of wheat in the Brazilian gross domestic product, besides being an important product of the Brazilian basic basket in the flour form and, mainly, for being one of the cereals liable to be contaminated by DON, this study was aimed to the standardization of a new , fast and robust method for detection and quantification of this mycotoxin for HPLC in wheat samples of grain and wheat flour, coming from an agricultural corporation of Chapeco, SC.

MATERIALS AND METHODS

The standard solution was prepared by using an analytical reagent of DON with 99% purity (Sigma-Aldrich,

St. Louis, MO, USA). For determination of the samples liquid chromatograph was used Varian (Palo Alto, California, USA) with detector of UVvis and column C18 Microsorb-MV of 250 x 4,6mm x 1/4 was used ‘’. During the operation water/methanol/acetonitrile in the proportion of 20/40/40% of 244 nm was used as mobile phase in the wave lenghth of 244 mL(Czerwiecki & Wilczyfiska, 2003).

For the analysis of the linearity a solution of reference of 100 ppb of DON was prepared and starting from this, the concentrations of 20, 40, 60, 80 and 100 ppb, in methanol grade HPLC (Vetec, Rio de Janeiro, Brazil). The linearity was evaluated by Analysis of Variance (ANOVA)) (0.01 level). The test of Turkey was used for confirmation of the data. While, for the reprodutibilty analysis, tests intra-day and inter-days were accomplished to determine the precision of the proposed analytical method. Concentrations different from DON stardard were prepared (20, 40, 60, 80 and 100 ppb) and analyzed in it triplicates in three different days. The limit of detection (LOD) and limit of quantification (LOQ) of DON were ascertained using standard solutions, and calculated as 3 ó/*a* and 10 ó/*a*, respectively, where *a* is the coefficient of inclination of the calibration curve andó it is the standard deviation of the intercept of the straight line (EMEA, 2008). The precision and accuracy were certain for recovery test through the addition of a certain amount of the standard (60 ppb) in matrix. The test was accomplished in triplicates. And the robustness of the proposed method was tested moving the pH of the middle of dissolution of the standard solution in ± 0,1 units (for dissolution studies and stability) and the characteristics of the matrix (data not showed).

For the analysis of the wheat sample, six samples were obtained of wheat grains coming from different places, and a sample of wheat flour of the industry , both belonging to a corporation that stores and it industrializes wheat grains, wheat flour , among other products, in the municipal district of Chapeco-SC. Such samples were collected during the month of August of 2008, in six storage locations. The wheat samples were nominated in agreement with the origin storage location: storage location 1, storage location 5, storage location 6, storage location 7, storage location 8, and storage location 9, and the flour sample, just named as wheat flour . The wheat samples were collected with the grains in movement, in other words, during the shipment, discharge or transilage of the grains, in regular time intervals (15 minutes). Five samples of wheat collected from each storage location,w obtained being later reduced to a single samples containi 1 kg. The same procedure was adopted for the collection of the flour sample, before it distribution in final packing. In the laboratory, the lot samples were homogenized and reduced the bracket of 125 g for the quarteament technique, starting from the one which, we removed analytical sub-samples in enough amounts for accomplishment of the analyses to proceed described. And, for the wheat in grains, we proceeded to the trituration in industrial blender

DON extraction was made in the following way: brackets of 50 g of the samples (triturated wheat grains and wheat flour) were homogenized in béquer with a 200 mL methanol/water (100:100 v/v) solution for 5 minutes. Being later filtered in paper filter and stored in flask properly identified. Of the extracted filtrate, only 1,5 mL (= 0,3125 g of the sample in equivalence) they was applied in immunoaf finity columns DONtest-HPLC™ (V icam, Watertown, MA, USA), with syringe and disposable needle. Subsequently , the column was washed with 5,0 mL of ultrapure water and the toxin eluated with 1,2 mL of grade HPLC methanol, through the column. The eluate was collected and maintained in refrigerator for subsequent analysis of HPLC.

RESULTS

The chromatography conditions were adjusted starting from several injections of the standard solutions.

Figure 1. shows the chromatograms obtained for the solutions of 20, 40, 60, 80 and 100 ppb, according to the proposed method.

Retention times ranged from 2.7to 3.0 min. And, minimum and maximum the retention times were the same for all of the points. It was just verified the variation of the area of the chromatogram, that is related as the larger the area the higher will be the concentration.

Under the described experimental conditions, a graph of the calibration curve was built for this method, and in the Table 1, the values obtained in the validity of the method are shown. The analysis of the variance (ANOVA) of absorbances demonstrated that DON analysis

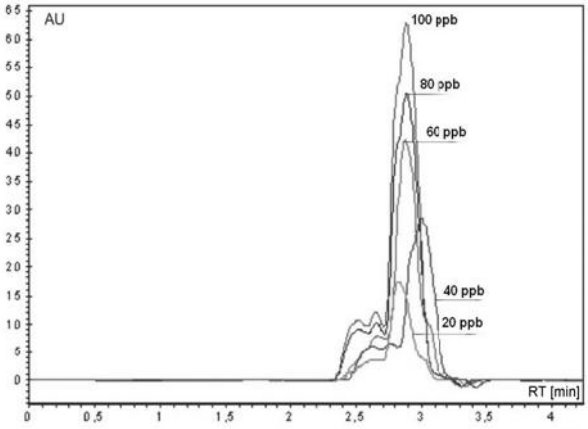

Figure 1. Chromatograms of the solutions standard of DON (20, 40, 60, 80 and 100 ppb), according to the method proposed in the wavelength of 244 nm

Table 1. Analysis of Variance (ANOVA) of the areas unit To get DON calibration curve through the proposed method. Turkey test was used for confirmation of data (0.001 level).

Variations for units	Degrees of liberty	Sum square	Mean square	Distribution of the variance	Value p
Treatments	1	17850.07	17850.07 59.03	<0.001	
Blocks. 14		8073.60	576.69	1.91	<0.001
Error	3	02.54			
Turkey (1 and 2)				10.87	<0.001

for this method revealed linearity (correlation coefficient equivalent to 0.9947) (figure 2). The value of F=59,0, using 0.001 level, therefore, is no significant, indicating there is no linearity deviation, as it can be observed in the Table 1. The calibration curve was prepared plotting the values of the area of the peaks of the solutions of references of the tested concentrations (20 to 100 ppb). Area and concentration were submitted to the test of ANOVA, to find the linearity of the method. The calibration equation and the correlation coefficient were found like y = 0.16008x + 1.61713 (r = 0.9947) (y = b.x + a; where *y* is area units of DON peaks, *b* is the inclination of the straight line, *a* is intercept and *x* is the concentration of the solution measured in ppb). The correlation coefficient was of 0.9947, indicating an excellent linearity of the method (Figure 2).

The reproductibility of the method is expressed in the intra-day and inter-day tests. The values found for intra-day were from 0.66% to 0.97% and for interday rehearsals were from 1.18% to 1.51%, what indicates a great reproductibility. While, the values of LOD and LOQ for the mentioned method were 0.11 ppb (LOD) and 0.27 ppb (LOQ). Values, these that would not exclude this method of being used for DON determination, for they are extremely low

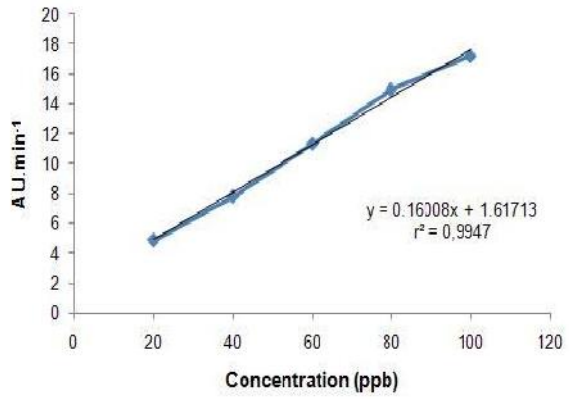

Figure 2. Graph of the points of DON calibration curve, according to the proposed method. The used points were: 20, 40, 60, 80, 100 ppb. The curve was built in the program Galaxie Chromatography Workstation version 1.9.

To check the precision and accuracy of the method, recovery studies were considered, through the method of standard addition. The percentage of recovery of the standard added for the samples was made throuth calculations as: Recovery % = (Ct-Cu) / Ca x 100 where Ct is the total concentration of the found analyte; Cu is the concentration of the present analyte in the reference solution; and Ca is the concentration of the analyte of the standard. Results are shown in the table 2, 0.01 level being used. The recovery percentage was of 100.91% in the mobile phase in subject, indicating that this method has precision and accuracy , due to the fact of reproducing repetitive and close results to the real, because there was not a marked variation accentuated in the theoretical value of the practical (variation of 0.37%).

The variation of pH in half of dissolution of the standard in ±0,1 unit didn't cause any significant effect in the area in the chromatogram, being shown a method that can be used for samples with different pH strips, as the case of urinary samples that presents different concentrations of hydrogen ions.

In relation to the analysis of the samples, samples A1 and A2 used in the standardization of the extraction technique and deoxynivalenol purification were analyzed by HPLC according to the proposed method, showing the presence of the toxin in study (Figure3), because by comparing with the chromatograms of the standard

Table 2. Statistical analysis of the results obtained by the proposed method, in the assay with a standard solution of DON containing 60 ppb.

Statistic Parameter	Value found in the proposed method
Mean ± SD	60.07± 0.41
Recovery (%)	100.91
Test-t significance	0.09
Test-F de significance	1.03

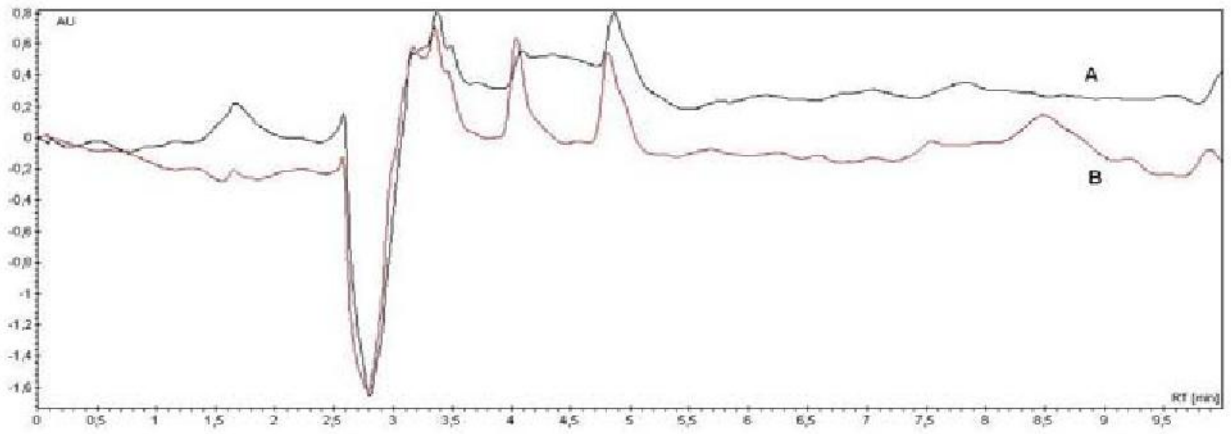

Figure 3. Chromatograms of samples A1 and A2, analyzed by HPLC according to the method proposed for quantification of DON. (A) Sample 1. (B) Sample 2.

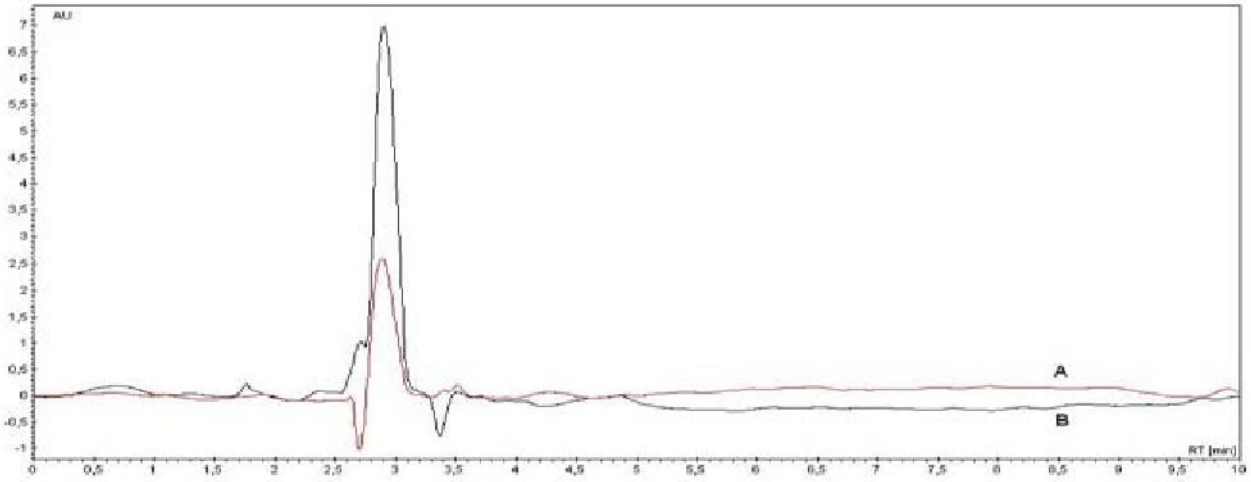

Figure 4. Chromatograms of sample A3 with addition of 60 ppb of DON (A) and of sample A4 with addition of 100 ppb of DON (B), analyzed by HPLC according to the proposed method.

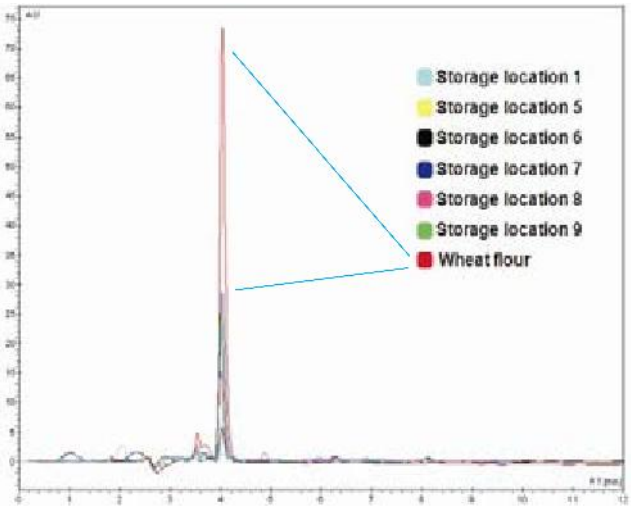

Figure 5. Chromatograms of the samples of the storage locations and of the wheat flour analyzed by HPLC according to the proposed method

solutions of DON, retention peak is not observed in approximately 3 minutes. While, in samples A3 and A4 where it was added to the vial 60 ppb and 100 ppb of DON (100 µL) respectively, the presence of the toxin is observed with retention peak in approximately 3 minutes in the cromatograms (Figure 4).

In the Figure 5, it can be observed that the toxin was detected in all of samples. And, starting from the area of peak obtained of each sample, DON concentration was calculated by the calibration equation (Table 3).

DISCUSSION

The reproductibility of the retention's times, the efficiency of the analytical method employed in this work and the linearity of the studied composition was appropriate and efficient for determination of DON levels, because this standardization allowed an analysis in a reduced time, emphasizing a new experimental condition of analysis.

Table 3. Correlation of the values of the area of peak and concentration of DON (in ppb) in samples in the study, according to the proposed method.

Area	Storage location	DON (ppb)
0.2 1		0.82
0.3 5		0.88
0.2 6		0.82
0.3 7		0.88
0.5 8		0.92
0.6 9		0.97
16,0 6	Wheat flour	9.20

Source: data of the research.

With the respective analysis of the results found, that are aided in the main analytical parameters, this work presents the possibility of using it in the routine laboratory as an alternative for DON determination through the method of HPLC-UV

In the present study DON concentrations in wheat grains varied frrom 0.82 to 9.20 ppb. Lower concentrations were found by Furlong *et al.* (1995) during a study accomplished in two cultivation of wheat in 10 producing places in the State of Sao Paulo where to DON it was present in four samples, in concentrations that varied from 0.57 to 0.50 µg.g.

In many other studies the amount of DON in wheat grains or wheat by-product, the detected concentrations were very superior in the present papers. Oliveira *et al.* (2002), for instance, evaluated DON incidence during the years 1998 and 2000 in wheat flour, bran and available bread-making products in the trade of Minas Gerais, and they found the toxin in 32 of the 47 analyzed samples, in a concentration strip from 40 to 1,205 µg.kg.

Dilkin *et al.* (2003) evaluated DON presence in 928 samples of cereals, being 357 of wheat, all obtained from producers and agricultural companies of the South area of Brazil in the period of January 1998 to June 2003. DON was present in 67 samples with medium and maximum concentrations of 1,670.3 and 15,957.0 µg.kg respectively

Calori-Domingues *et al.* (2007) researched DON in 100 wheat samples, being 50 of national wheat (coming from the States of Sao Paulo, Parana and Rio Grande do Sul) and 50 from imported wheat (Argentina and Paraguay). Of the total appraised samples, 94% of the national wheat and 88% of the imported wheat exhibited DON contamination with medium levels of 332 and 90 µg.kg, respectively. Of the samples of national wheat, two presented larger levels of contamination than 1,250 µg.kg (maximum allowed limits for the European Community) and such samples were coming from the State of Parana. The maximum level of contamination of the imported wheat was of 349 µg.kg.

The great majority of studies accomplished with wheat flour reveal values very superior to DON 9.20 ppb found in our study. Araújo *et al.* (2004), analyzed 78 wheat flour samples of factories and grocery stores collected in whole Brazil and it was found deoxynivalenol in 27 samples, with found medium levels of 283.94 µg.kg and maximum of 794.0 µg.kg levels. In a study accomplished by Lamardo *et al.*, (2006) in the trade of Sao Paulo, 42 wheat samples and wheat flour analyzed were acquired and analyzed for DON determination, and 19 samples (45%) they were polluted with tenors among 82 to 1,500 µg.kg. Baraj & Badiale-Furlong (2003), evaluated 112 wheat flour samples marketed in the city of Rio Grande (Rio Grande do Sul) and verified that only 2 samples were polluted with DON in levels of 128 and 323 µg.kg. In the wheat flour sample levels of DON were also detected, because, besides the wheat, the toxin can be found in wheat by-products such an, flour, breads, cookies and masses (Almeida, 2006). In the wheat flour levels were detected from superior DON to the values found in the wheat in grains. This because, accordin to food that one wants to produce (bread, cookie, among others), the used wheat flour should present a chemical composition, that provides technological or nutritional qualities to the product. To satisfy such requirements wheat grains, are used of different cultivate, classes, types, and even of different nationalities. What explains the deoxynivalenol concentration to be more elevated in the flour than in the wheat in grains in the analyzed samples.

The results of this work indicated that the proposed method is efficient for detection and DON quantification in wheat in grains and wheat flour, because as their indicators of merit presented a medium recovery of 100.91% for DON; dear precision for the coefficient of variation of 0.9947 and detection limit in the sample of 0.11 ppb. Comparing our method to that in the existing literature, it can be verified that there was a reduction of 75% in the time of analysis, a fact that makes it as the fastest method of validity to quantify of DON (Janes & Schuster, 2001; He *et al.*, 2009). In relation to the the other analytical parameters, the results obtained in this validity were similar to the protocols described by Cahill *et al.,* (1999) and Czerwiecki & Wilczyfiska (2003).

The present study showed that all of the samples (100%) collected were polluted with deoxinivalenol. The detected levels varied from 0.82 to 0.97 ppb for the wheat in grains, and 9.20 ppb for the wheat flour.

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