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# Deoxynivalenol and nivalenol analysis in soybean and soy flour

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## Abstract

A rapid and accurate method of quantifying deoxynivalenol (DON) and nivalenol (NIV) in soybean and soy flour is described. The samples were extracted with acetonitrile:water (84:16, v/v) and cleaned through a solid-phase extraction (SPE) column. The mycotoxins were separated, detected and quantified by reversed-phase high performance liquid chromatography (HPLC) with UV detection (220 nm) using water:methanol (88:12, v/v) as mobile phase. Characteristics of this in-house method such as accuracy, precision and detection and quantification limits were defined by means of a recovery test with spiked soybean and soy flour samples. The detection limit (LOD) was 0.1 µg/g for DON and 0.2 µg/g for NIV, based on a signal-noise ratio 3:1. Quantification limit (LOQ) was established as three times the detection limit.

**Keywords**: HPLC, *Fusarium*, mycotoxins

## 1. Introduction

Soybean (*Glycine max* L.) is the main source of protein throughout the world used both as food and feedstuffs (FAO, 2004; Hepperly, 1985). This commodity is important for the Argentinean economy, with an average production of 40.5 million tons per year. Our country ranks third among soybean world exporters. Most of the production is exported to the European Union as seed, oil and flour (SAGPyA, 2007).

Hygienic safety of soybean and by-products depends on contamination by microscopic fungi. *Fusarium* rot of soybeans is described in the literature and different *Fusarium* species have been isolated from this commodity (Pitt and Hocking, 1999). These strains are known to produce a broad spectrum of toxins including trichothecenes of A- and B-types (DeNijs *et al*., 1996). Among B-type trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) are important mycotoxins produced mainly by *Fusarium graminearum* Schwabe [teleomorph = *Gibberella zeae* (Schwein.) Petch] (Glenn, 2007). DON is the most distributed *Fusarium* mycotoxin and occurs worldwide in crops from temperate regions. The major physiological response to DON in animals is loss of appetite, with pigs being the most sensitive animals to this toxin. DON is also reported as an immunosuppressive mycotoxin affecting cellular immune response by acting directly on bone marrow, spleen, lymphoid tissues, thymus and intestinal mucosa (Rotter *et al.*, 1996). An advisory level of 0.9 µg/g of DON in finished feeds was suggested by the European Union (EC, 2006). Nivalenol (NIV) also occurs in cereals and has been found extensively in Japan and Korea, and at relatively low levels in samples from Europe, Southern Africa and South America (Placinta *et al*., 1999), but is of concern because NIV is more toxic to animal tissue than DON (Ryu *et al*., 1988).

Numerous methods for measuring DON and NIV have been developed including HPLC (Josephs *et al*., 2001; Lauren and Greenhalgh, 1987; Shirai *et al*., 2000), LC/MS (Berger *et al.*, 1999; Plattner, 1999), gas chromatography analysis (Scott *et al*., 1989) and GC/MS (Scott *et al*., 1981; Sugita-Konsihi *et al*., 2006; Tanaka *et al*., 2000) with derivatisation, including solid-phase extraction clean-up (Malone *et al*., 1998; Shirai *et al*., 2000; Tanaka *et al*., 2000; Trucksess *et*

*al*., 1998) and immunoaffinity column clean-up (Dietrich *et al*., 1995; Cahill *et al.*, 1999). Among these methods, using a multifunctional column clean-up procedure seems to be very simple and reliable (Trucksess *et al*., 1998).

Richardson *et al*. (1985) suggested that soybean products present a mycotoxic hazard which requires attention. At present there is little information about the natural occurrence of *Fusarium* toxins in soybean and by-products in Argentina. The aim of this work was to describe a method for DON and NIV determination in soybean and soy flour by using HPLC analysis coupled with a multifunctional column clean-up.

## 2. Materials and methods

### Chemicals and materials

Acetonitrile, methanol (both HPLC grade) and hexane were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). DON and NIV toxins were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Clean-up Mycosep$^{TM}$ 225 columns were obtained from Romer (Romer Labs. Inc., Union, MO) and paper filter (Whatman no. 4) was obtained from Whatman (Maidstone, UK).

### Standard preparation and calibration curve

DON and NIV stock solutions were prepared by dissolving the solid standard in methanol to obtain concentrations of 500 and 200 µg/ml, respectively (stock I). Stock II standard solutions were obtained by appropriately diluting the stock I solutions of DON and NIV in methanol to achieve a final concentration of 100 µg/ml for each toxin. Combined DON/NIV working standard solutions for HPLC calibration curve were prepared by dissolving adequate amounts of the stock II solutions in water:methanol (95:5), previously evaporated to dryness under nitrogen stream. Four mixed working solutions to perform calibration were prepared at concentrations of 4.0/2.0, 2.0/1.0, 1.0/0.5 and 0.5/0.25 µg/ml of DON/NIV respectively. The calibration curves showed good linearity for both mycotoxins ($R^2$=0.9984 for NIV and $R^2$=0.9980 for DON).

### Sample preparation and clean-up procedure

About 1 kg of sample was finely milled with a Romer mill (Romer, Union, MO, USA) and mixed well. Subsamples (about 100 g) were taken and stored at -20 ºC until analysis. Twenty five grams of sample were extracted with 2 g NaCl, 40 ml of hexane and 100 ml of acetonitrile:water (84:16, v/v) by shaking in a rotary shaker at high speed for 30 min. The extract was cooled down to 4 ºC and filtered through filter paper Whatman Nº 4. Clean-up was carried out on a solid-phase extraction column. The filtrate (5 ml) was transferred to a culture tube, and it was slowly pressed into the tube with the rubber flange end turned down until 3 ml of the extract had passed through the column. The purified extract (2 ml) was transferred to a vial and evaporated to dryness under nitrogen stream at 60 ºC.

### Liquid chromatography analysis

The dried residue was redissolved in 400 µl of water: methanol (88:12, v/v), homogenised in a vortex mixer and injected into the HPLC system by full loop injection technique (Hewlett Packard model 1100 pump, Palo Alto, CA, USA; Rheodyne manual injector with a 50 µl loop, Rheodyne, Cotati, CA, USA). Chromatographic separations were performed on a stainless steel $C_{18}$ reversed-phase column (150x4.6 mm i.d., 5 µm particle size; Luna-Phenomenex, Torrance, CA, USA) connected to a precolumn Security Guard (20x4.6 mm i.d., 5 µm particle size, Phenomenex). The mobile phase was water: methanol (88:12, v/v) at a flow rate of 1.5 ml/min. The mycotoxins were detected by UV (Hewlett Packard model 1100 programmable UV detector, Palo Alto, CA, USA) at 220 nm and quantified by a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01, Palo Alto, CA, USA). The mycotoxin levels were calculated by comparing the area of the chromatographic peak of the samples with those of the standard calibration curve.

### Recovery experiment

Recovery experiments were performed in triplicate by spiking blank soybean and soy flour with DON and NIV at levels of 1.0, 1.5 and 2.0 µg/g by diluting aliquots of the stock solutions with the appropriate volumes of methanol. Spiked samples were left for 1 h, to allow solvent evaporation prior to extraction.

## 3. Results and discussion

Numerous methods have been developed for the determination of DON and NIV in different agricultural commodities, however there is no available technique for determining these mycotoxins in soybean and by-products using HPLC. The method applied in this study was based on the method described by Trucksess *et al.* (1998) with some modifications. One modification was the addition of NaCl and hexane to improve toxin extraction and to obtain a cleaner extract that significantly reduced both noise and number of peaks in the chromatogram. Another change was the cool down of the extract at 4 ºC previous to the clean-up step. This change allowed precipitation of interfering matrix components as reported by Schollenberger *et al.* (2006) in the modified method for zearalenone and α- and β-zearalenole in cereals, corn plants, corn silage and feedstuffs.

Results of the recovery experiments on spiked soybean and soy flour are reported in Table 1. Within the spiking range 1.0-2.0 µg/g for DON, mean recoveries were 88.5 and 89.7% for soybean and soy flour, respectively, with a within-laboratory relative standard deviation (RSDr) <7%. Analysis of samples spiked with NIV (range 1.0-2.0 µg/g) gave a mean recovery of 70.6 and 91.4% for soy flour and soybean, respectively, with relative standard deviations <3%. The recovery percentages for both toxins were similar to those obtained by Schollenberger *et al.* (2007) in soy foods. These authors reported recoveries of 74% and 81% for DON and NIV, respectively in soybean samples spiked at a level of 0.2 µg/g. Certified reference materials were not used to establish method characteristics because they were not available at the time of this study.

**Table 1. Results of the recovery experiments for DON and NIV.**

| | Soybean | | Soy flour | |
|---|---|---|---|---|
| **Spiking level (µg/g)** | **Recovery ± SD[1] (%)** | **RSD[2] (%)** | **Recovery ± SD[1] (%)** | **RSD[2] (%)** |
| DON | | | | |
| 1.0 | 83.7±2.9 | 3.5 | 93.5±3.4 | 3.6 |
| 1.5 | 89.3±5.4 | 6.0 | 93.3±9.2 | 9.8 |
| 2.0 | 93.3±3.8 | 4.1 | 82.3±9.4 | 11.4 |
| mean | 88.5±4.8 | 5.5 | 89.7±6.4 | 7.0 |
| NIV | | | | |
| 1.0 | 88.7±1.2 | 1.3 | 63.8±2.4 | 3.8 |
| 1.5 | 91.9±4.3 | 4.7 | 75.0±3.1 | 4.1 |
| 2.0 | 93.6±0.6 | 0.6 | 73.2±7.1 | 9.7 |
| mean | 91.4±2.5 | 2.7 | 70.6±1.9 | 2.7 |

[1] SD = standard deviation (n=3).
[2] RSD = relative standard deviation.

The linear regression equations for the recovery test of both DON and NIV shows $R^2$ values >0.99, indicating good linearity. A typical chromatogram obtained from spiked soybean sample is shown in Figure 1. The chromatogram shows a characteristic profile with no interference at the retention time of NIV (4.6 min) and DON (10.1 min).

The detection limit of the method was 0.1 µg/g for DON and 0.2 µg/g for NIV, based on a signal-noise ratio 3:1 and the quantification limit was established as three times the detection limit (0.3 µg/g for DON and 0.6 µg/g for NIV). The detection limit for DON was similar to the result obtained by Sugita-Konsihi *et al.* (2006) using HPLC-UV detection method coupled to a multifunctional clean-up column on wheat samples.

According to current methodology available for detection of DON and NIV there is only one report performed by Schollenberger *et al.* (2007) based on the use of the gas chromatography/mass spectrometry (GC/MS) method. These authors reported a detection limit of 0.007 and 0.014 µg/g for DON and NIV, respectively. The technique described in the present work, showed a detection limit higher than that reported with GC method, but is very simple and has the capacity to detect levels nine times lower than the lowest value recommended for the European Commission Directive (2006/576/ECC) for DON in feedstuffs (0.9 µg/g).

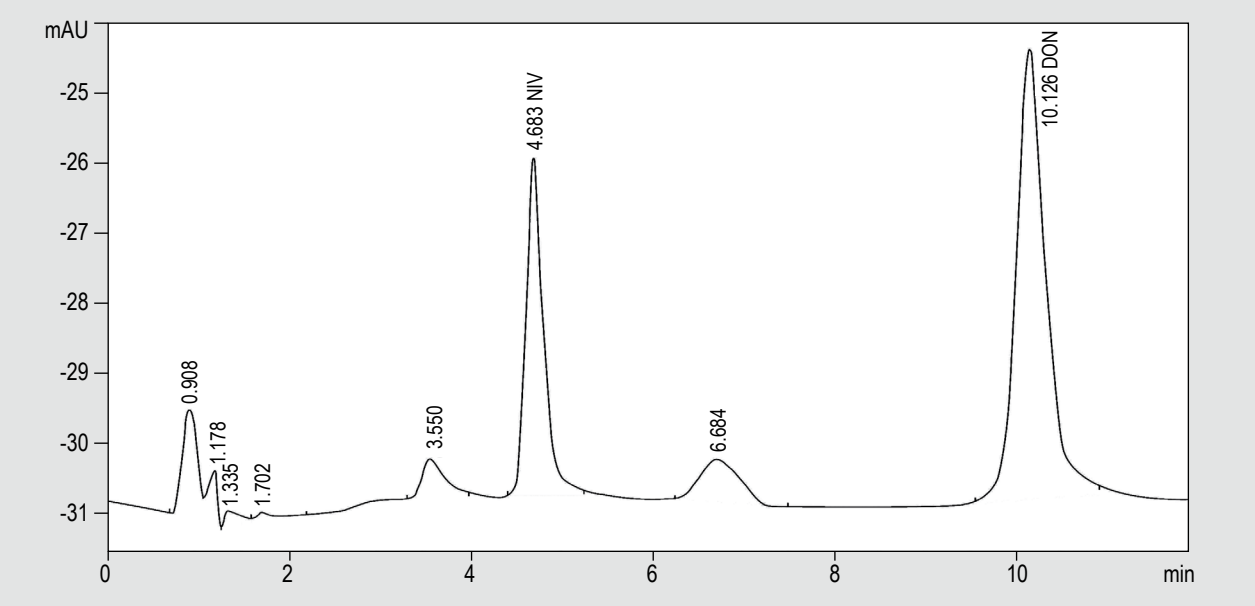


**Figure 1. HPLC chromatogram of spiked soybean sample with 2 µg/g for DON and 1 µg/g for NIV.**

## 4. Conclusion

The method described in the present study has proved to be quick and easy. The use of multifunctional clean-up columns for purification of the extract was convenient for time-saving and a reduction in clean-up steps. Although no collaborative validation of the method presented herein has been performed, the procedure adopted in this study has proved to be accurate and precise for determining DON and NIV on the two substrates evaluated.

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