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Determination of nivalenol and deoxynivalenol in wheat using liquid chromatography–mass spectrometry with negative ion atmospheric pressure chemical ionisation

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Abstract

A new, rapid and sensitive method has been developed for the determination of nivalenol (NIV) and deoxynivalenol (DON) by using HPLC in combination with an atmospheric pressure chemical ionization (APCI)-interface and a single quadrupole mass spectrometer. Different LC and MS parameters have been optimized prior to this in order to obtain better results and sensitivity. The effect of nebulizing temperature on the sensitivity and fragmentation of NIV and DON in an APCI interface was investigated. Also, the influence of the cone voltage on the fragmentation pattern was studied, which was shown to have a tremendous effect. Furthermore, the effect of modifiers such as ammonium acetate, acetic acid and ammonia on the ionisation yield of the above substances have been investigated. The extraction was carried out using acetonitrile–water. A two step purification was then applied on two different Mycosep clean up columns. We have used a modified, rapid and isocratic HPLC method combined with a negative ion APCI–MS for the separation and quantitative determination of NIV and DON in wheat extract. An RP C_{18} column was used for the separation of selected compounds in wheat extract with water–acetonitrile–methanol (82:9:9, v/v/v) at a flow-rate of 1 ml/min without a split. Calibration curves show good linearity and reproducibility. The detection limit and precision were determined for NIV and DON. Both compounds could be detected down to μg/kg level in wheat using selected ion monitoring of the $[M-H]^-$ ions and the main fragments.
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Keywords: Wheat; Food analysis; Nivalenol; Deoxynivalenol; Mycotoxins; Trichothecenes

1. Introduction

The occurrence of mycotoxins in agricultural commodities is a major health concern for livestock and humans. The most prominent genera of fungi that produce mycotoxins are *Aspergillus*, *Fusarium* and *Penicillium*. New mycotoxins are continually found to be produced by members of these genera. Nivalenol (NIV) and deoxynivalenol (DON) are naturally occurring type B trichothecene mycotoxins (Fig. 1) produced by several species of the fungus *Fusarium*. Most trichothecenes have been found to be highly toxic causing sublethal toxicosis in animals if occurring in feed. These mycotoxins can also cause clinical effects such as reduced weight gain, emesis and diarrhoea. Deoxynivalenol (vomitoxin) is the most common mycotoxin but also nivalenol has been reported to occur in cereals. Accurate, rapid, and efficient assays are necessary to determine the contamination of food and feed so that exposure to mycotoxins can be prevented.

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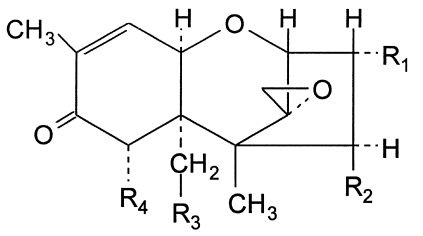

	R_1	R_2	R_3	R_4
Deoxynivalenol	OH	H	OH	OH
Nivalenol	OH	OH	OH	OH

Fig. 1. Structures of nivalenol and deoxynivalenol.

Many different methods for the determination of nivalenol and deoxynivalenol have been published based on TLC [1,2], GC [3–10] and HPLC [11–17]. The most common and sensitive method seems to be gas chromatographic separation with electron-capture or MS detection [3–10]. The use of HPLC with UV detection for the determination of NIV and DON has some sensitivity limitations due to the lack of characteristic UV absorption of these molecules. Also immunological methods such as enzyme immuno assay (EIA) and enzyme-linked immunosorbent assay (ELISA) have been developed [18–20] and are available on the market.

The mass spectrometer is a very sensitive, selective and specific detector for HPLC. The LC–MS analysis of DON and other *Fusarium* trichothecene mycotoxins was for the first time reported by Tiebach et al. [21] by using a direct liquid introduction interface. Dynamic fast atom bombardment (FAB) was also described for the identification of DON by Kostiainen et al. [22]. The LC–MS analysis of NIV has only been applied on a DLI interface in negative ion chemical ionisation mode [21]. No other LC–MS technique has been reported for the analysis of NIV. Voyksner et al. [23], Rajakylä et al. [24], Kostiainen et al. [25,26] and Tiebach et al. [27] have used a thermospray interface for the analysis of DON and some other mycotoxins. The thermospray LC–MS was one of the more favorable LC–MS interfaces in the early 1990, which was routinely used as described in the above papers. Thermospray interface was then overshadowed by other emerging soft ionisation techniques such as atmospheric pressure chemical ionization (APCI) and electrospray interfaces. Especially, the development of the APCI interface over the past few years allows a sensitive detection of small and polar compounds using the LC–MS interface. The introduction of this technique has expanded dramatically the role of mass spectrometry in the analysis of naturally occurring substances in food and feed [28]. The determination of DON in feed has been recently reported by Huopalahti et al. [29] by using an ion spray technique after supercritical fluid extraction.

We describe the use of the APCI interface for routine and simultaneous determination of nivalenol and deoxynivalenol in whole wheat flour for the first time. Different MS and LC parameters have been optimized and the influence of these parameters on the fragmentation patterns of NIV and DON in $APCI^-$ mode has been investigated.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile and methanol used in HPLC were purchased from Scharlau (Barcelona, Spain). Buffer salts of analytical grade were obtained from Merck (Darmstadt, Germany). Water was purified in a UPW2 system (F&L, Vienna, Austria). All solvents were filtered through a 0.2 μm filter to remove particles and were degassed using a Waters in-line degasser (Milford, MA, USA). Nivalenol and deoxynivalenol standards were purchased from Sigma (St. Louis, MO, USA).

2.2. HPLC–MS equipment

The LC system used in the HPLC–MS experiments consisted of a Waters 626-LC pump and a Waters 717plus autosampler. Chromatographic separation and detection were performed on a Platform II instrument using an APCI interface equipped with a Pepperpot counter electrode (Micro Mass, Manchester, UK).

A C_{18} Hypersil BDS 5 μm analytical column, 250 mm×4.6 mm (Hypersil, Runcorn, UK), was used and column temperature was kept at 50°C. The isocratic mobile phase consisted of water–acetonitrile–methanol (82:9:9, v/v/v) with a flow-rate of 1 ml/min and a run time of 12 min without splitting.

All MS experiments were performed in the APCI negative mode. The optimization steps were carried out in scan mode (*m/z* 200–600) by using the analytical column. Nitrogen of pure quality as nebulizing and carrier gas was used (≥99.999%, AGA, Lidingoe, Sweden). Carrier gas flow was set at 250 l/h and sheath gas flow was held at 125 l/h.

The source temperature was kept at 100°C, the APCI vaporizing temperature was held at 400°C and cone voltage was set at 20 V. Quantitative determination of both compounds was applied in the single ion monitoring (SIM) mode and the following ions have been detected in a dwell time of 0.3 s and a span of 0.2 u.

Nivalenol (M_r 312) *m/z* 311, 281, 245, 205

Deoxynivalenol (M_r 296) *m/z* 295, 265, 249

2.3. Sample preparation

Sample clean-up steps were performed using multifunctional Mycosep columns 227 and 216 (Romer Labs., Washington, MO, USA). The extraction of DON and NIV was carried out by shaking the whole wheat flour (25 g) with 100 ml of acetonitrile–water (84:16, v/v) for 90 min. The extract was filtered and 8 ml was then transferred into the culture tube of the Mycosep column 227. The purification was performed by pushing the flange end of the cleanup column into the culture tube. Four milliliters of purified extract was then applied into a Mycosep column 216 and eluted with 12 ml of acetonitrile–water (90:10, v/v). The cleaned-up extract was then evaporated under a nitrogen stream at 60°C. Afterwards the residue was resolved in 400 μl of mobile phase (water–acetonitrile–methanol, 82:9:9, v/v/v) and 125 μl was then injected into the LC–MS system. The concentration of each compound in a standard solution was determined using a spectrophotometer after dissolving the mycotoxins in acetonitrile to ensure the accuracy of concentration.

3. Results and discussion

3.1. Optimization of APCI parameters

3.1.1. Effect of cone voltage

By applying a potential difference between the sampling cone and the skimmer in the intermediate pressure region, the collision-induced dissociation occurs in this region [30–33]. Figs. 2 and 3 show the results of increasing the cone voltage on the mass spectra of NIV and DON. The intensity of the deprotonated molecules $[M–H]^-$ and the adducts decreases by increasing the cone voltage, as expected. Even at a low cone voltage (10 V) by using a vaporizing temperature of 400°C, the mass spectra of both compounds consist of fragments and more intensive $[M–H]^-$ ions (*m/z* 295 for DON and *m/z* 311 for NIV). Also, adduct ions of tested substances could be observed, which indicate in both cases, the addition of two water molecules $[M–H+2H_2O]^-$ (*m/z* 331 for DON and *m/z* 347 for NIV). This fact indicates that the cone voltage plays a major role in the fragmentation and that the cleavage processes of molecules are not only due to thermal degradability of tested substances. When the cone voltage is increased, the kinetic energies of the ions are increased. It thus follows that higher amounts of energy are transferred into the internal energy of the compounds leading to more intense fragmentation. At cone voltages higher than 30 V, only fragment ions could be observed in the spectra of NIV and DON. For both molecules the loss of 30 mass units have been observed, which indicates the cleavage of the epoxy group and occurrence of *m/z* 265 (DON) and *m/z* 281 (NIV) fragments $[M–H–CH_2O]^-$. The other fragment ion in case of NIV occurs due to loss of a further two water molecules *m/z* 245 $[M–H–CH_2O–2H_2O]^-$. For deoxynivalenol the *m/z* 249 fragment could be observed, which indicates the loss of methane $[M–H–CH_2O–CH_4]^-$. Other fragment ions of *m/z* 205 for NIV and *m/z* 247 for DON have been observed, which could not be interpreted. The intensive fragmentation in APCI, for both selected mycotoxins, has the advantage of a diagnostic fingerprint and, thus, identifying the presence of compounds in the matrix. The quantitation could be done using fragment ions.

The influence of cone voltage on signal intensity

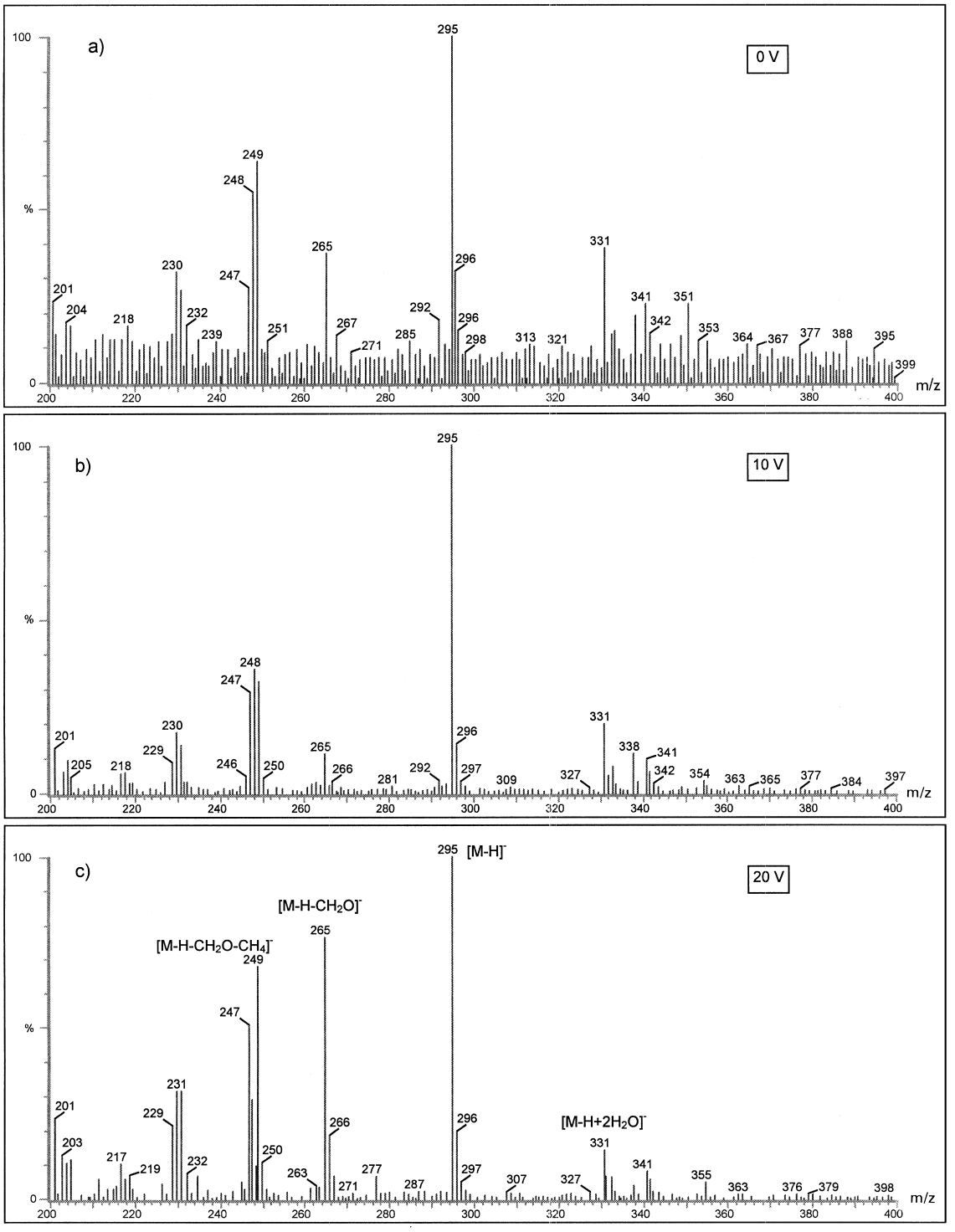

Fig. 2. Influence of different cone voltages on fragmentation pattern (a–e) of DON in the negative ionization mode.

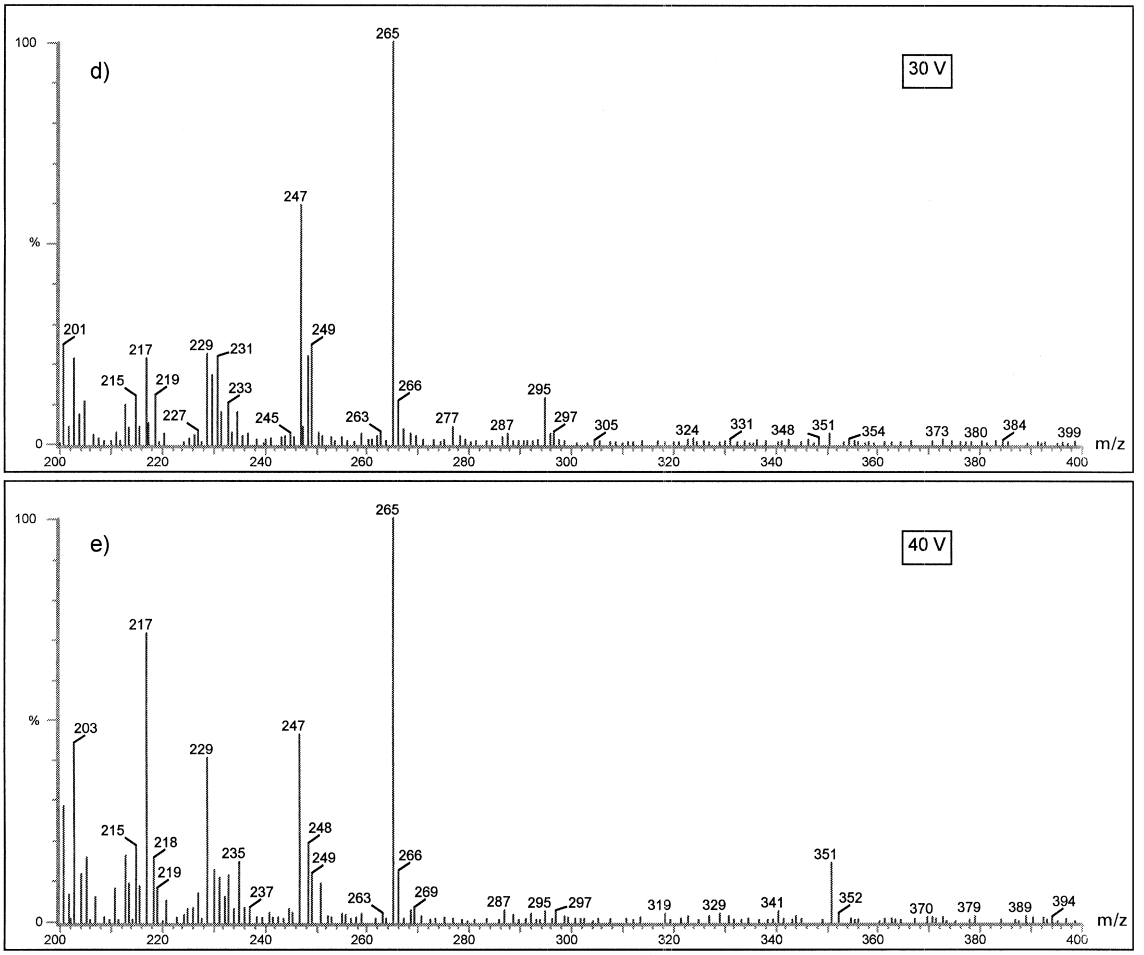

Fig. 2. (*continued*)

of both compounds has also been studied and it has a tremendous effect as shown in Fig. 4. At low (10 V) and high (40 V) voltages, the signal-to-noise ratios of the total ion current were deteriorated, so that an optimum could be reached at a cone voltage of 20 V. By increasing the cone voltage the intensity of the total ion current of both compounds decreases.

3.1.2. Effect of vaporizer temperature

Under thermospray conditions the ionisation is carried out at a temperature of about 250°C. No strong thermal degradation has been reported in thermospray studies [23–27]. Using the APCI interface much higher temperatures (>300°C) have to be applied for a better ionisation. The increase of the vaporizing temperature leads to a reduction of the aerosol droplet sizes and yields an improvement in the evaporation of the solvent and, therefore, a better ionization efficiency. Varying the vaporizer temperature between 300 and 500°C, the optimal temperature for a maximum response was found for NIV at about 400°C and for DON at 350°C (Fig. 5). The intensities of the deprotonated molecules $[M–H]^-$ and the adduct ions $[M–H–2H_2O]^-$ decrease as the vaporizer temperature is increased. In addition, the intensive fragmentation also seems to be a result of the thermal degradation, which takes place in parallel to the collision induced dissociation.

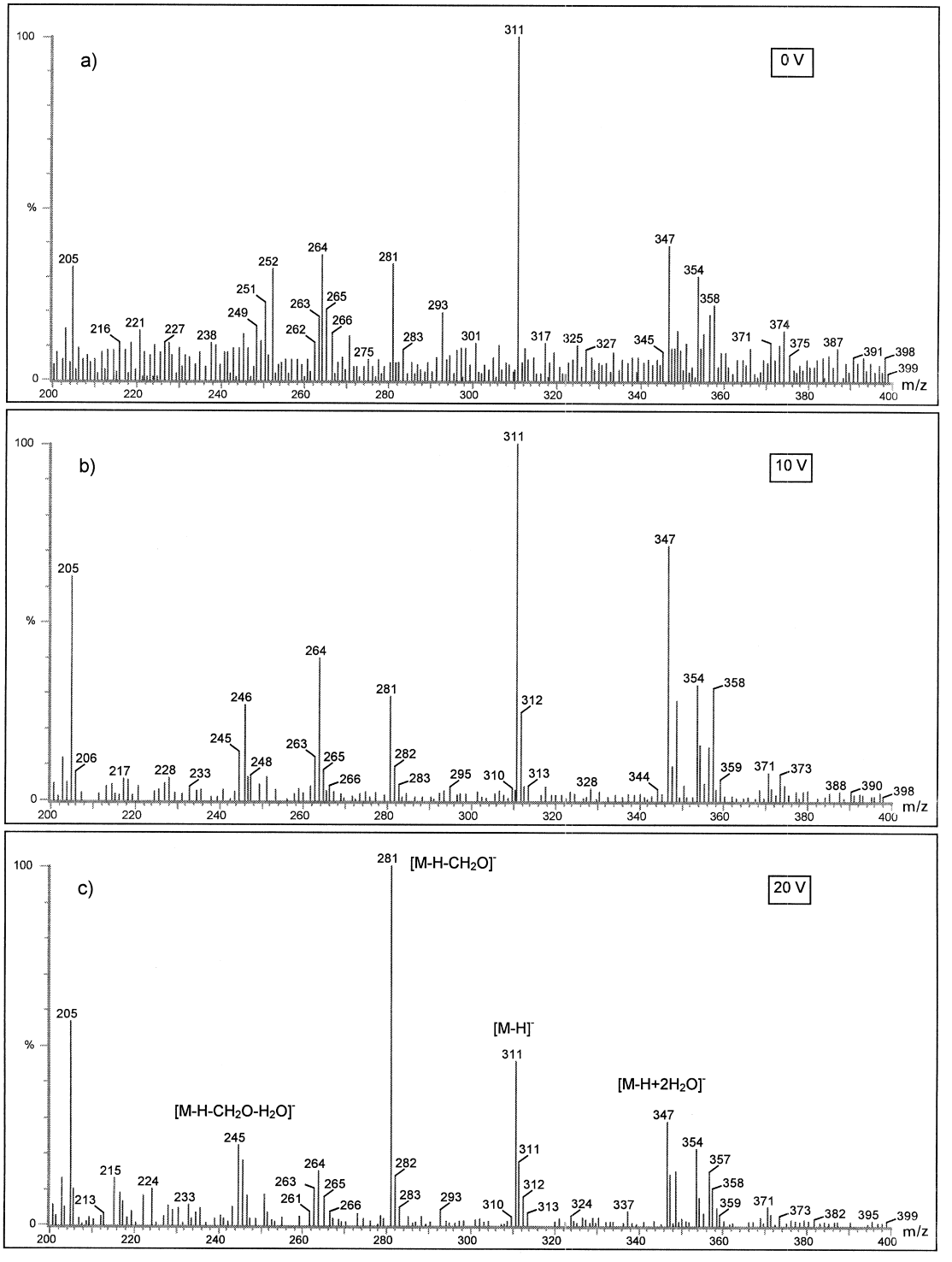

Fig. 3. The effect of increasing the cone voltage on the fragmentation pattern (a–e) of NIV in the negative ionization mode.

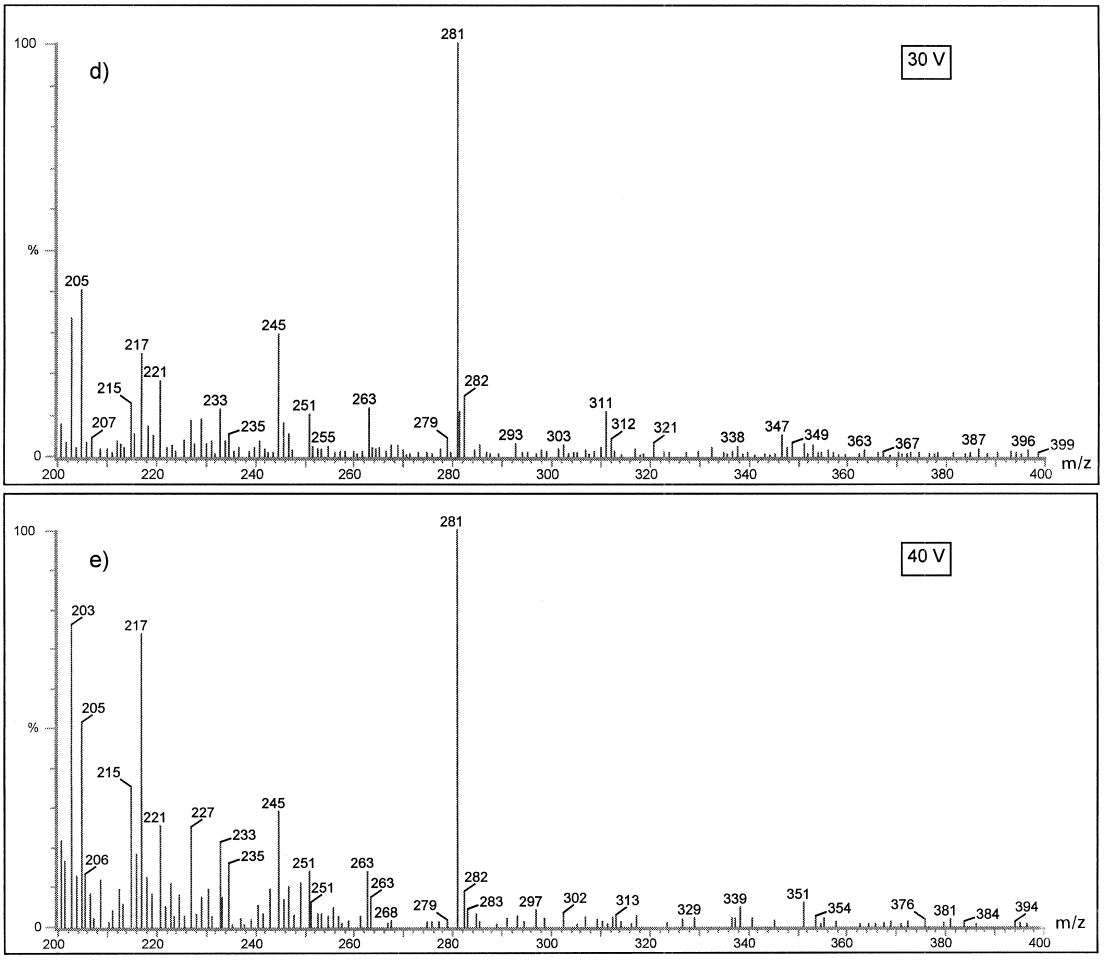

Fig. 3. (*continued*)

3.1.3. Influence of buffers

Addition of a mobile phase modifier as a reactant, acetate ions, or as deprotonization agents, such as ammonia, has been reported to improve the chemical ionization and therefore the ion abundance [33]. In this study the effect of acetic acid and ammonium acetate as well as ammonia on the sensitivity of the APCI interface has been studied. In contrast to the earlier reports, we could observe a decrease in the signal-to-noise ratio by the use of modifiers in the HPLC mobile phase. In the case of ammonium acetate the signal was suppressed strongly and the detection limit was reduced. The addition of ammonia, as a deprotonization agent, had no significant effect on the signal intensity. The addition of acetic acid to the mobile phase seems to have a negative effect on the signal-to-noise ratio (Fig. 6). When buffers were added into the HPLC mobile phase, adduct ions of either acetate or ammonia have been observed in all experiments.

3.1.4. Optimization of the HPLC flow-rate

The HPLC flow-rate of the mobile phase was varied from 0.5 to 1 ml/min, keeping all other parameters constant in order to optimize the signal response. For both tested substances, the optimum of HPLC flow-rate was found to be between 0.9 and 1 ml/min. The optimal flow-rate for signal intensity depends on the chemical properties of each indi-

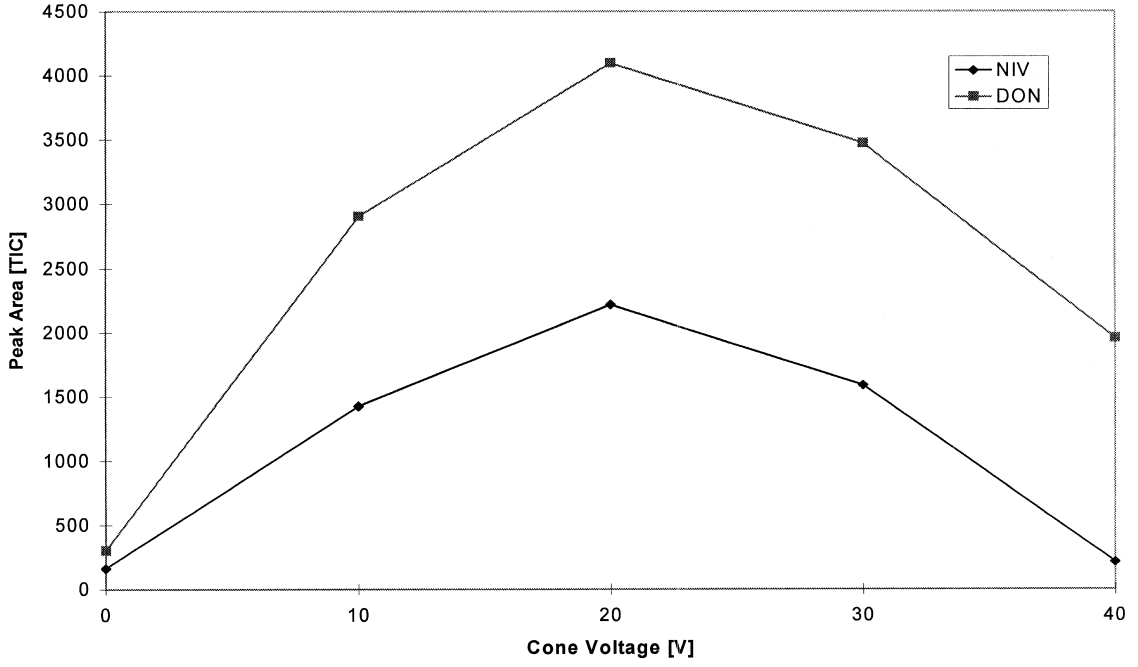

Fig. 4. The effect of cone voltage on signal intensity of NIV and DON.

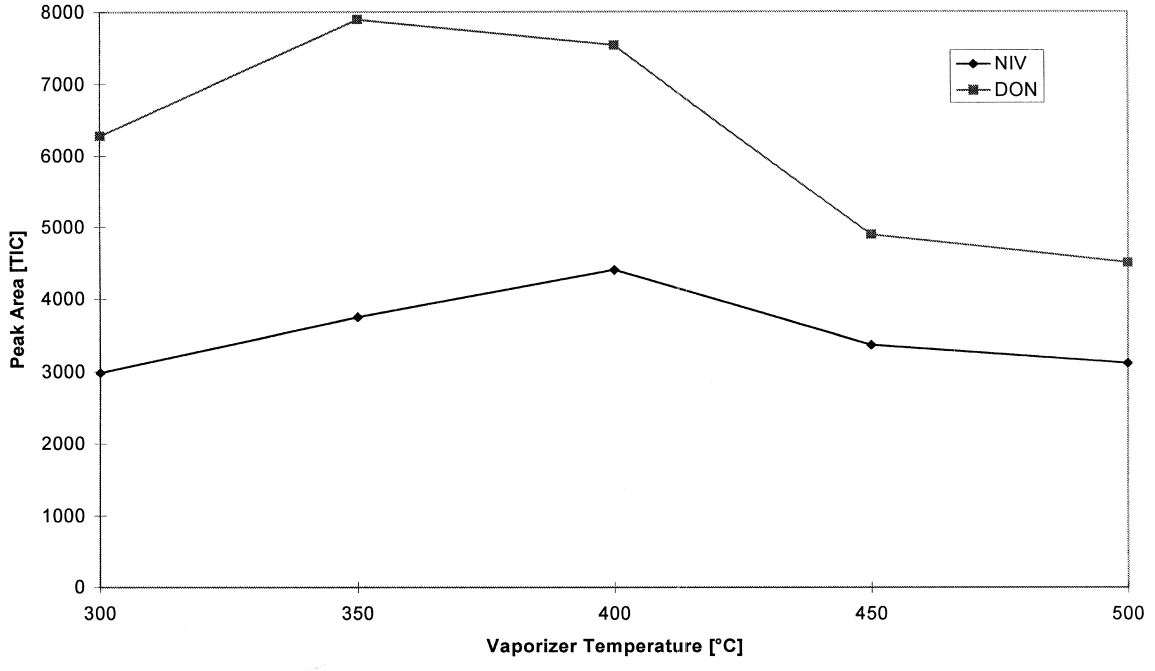

Fig. 5. Dependence of NIV and DON response on the APCI vaporizer temperature.

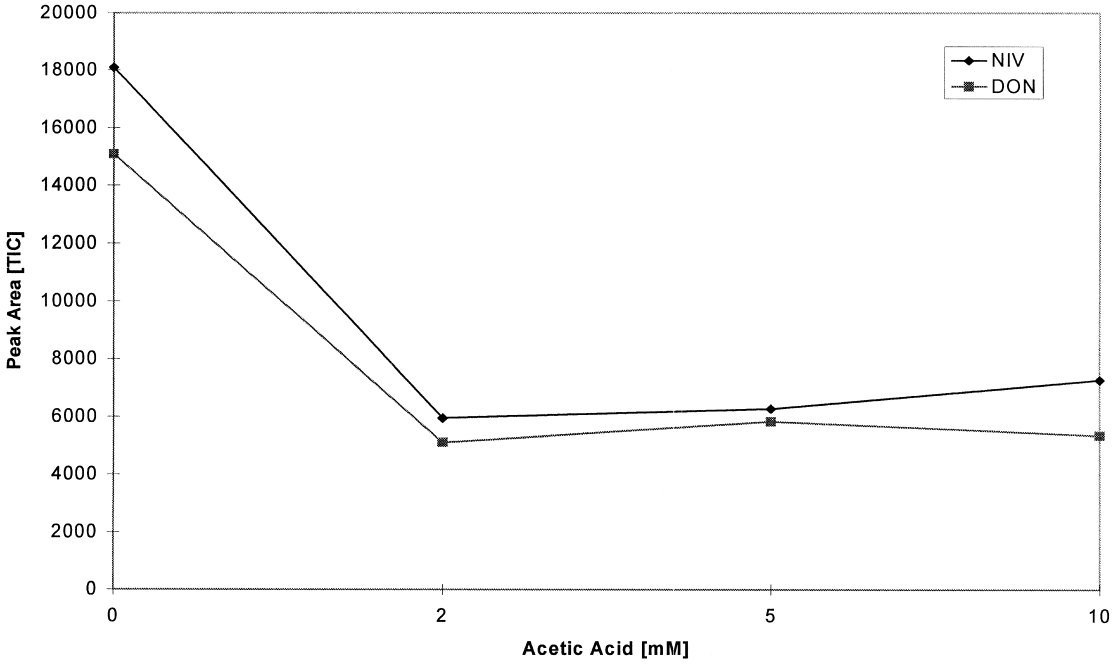

Fig. 6. The influence of acetic acid concentration in the mobile phase on the signal intensity of NIV and DON.

vidual compound as well as on the interface design and the geometry of the interface.

3.1.5. Quantitative analysis

Both spiked and naturally contaminated samples as well as blank wheat samples have been analyzed. Wheat samples were extracted and cleaned up using multifunctional columns as described in Section 2.3. The use of the second clean-up column provided more clean extracts, which has the advantage of reducing of the cleaning intervals of the source. The quantitative determination was performed using SIR of appropriate deprotonated molecules and main fragment ions. No carry over in the LC–MS system has been observed. Figs. 7 and 8 show the LC–APCI–MS chromatograms of a blank and spiked wheat sample in the SIM mode. Relative poor ionisation abundance of the tested mycotoxins, if compared to zearalenon in the APCI mode [34], was compensated for by the injection of a high amount of the sample extract. No negative effects on peak shape has been observed due to the use of high temperature separation, which led to a reliable and acceptable peak shape in the SIM chromatogram. The use of high column temperature has the advantage of shorter analysis time, reducing back pressure and yielding better peak shape. The dwell time for the SIM mode has also been optimized, which was a very important parameter for higher sensitivity and better peak shape.

The calibration curves show good linearity and reproducibility in a range between 50 and 1000 μg/kg of each mycotoxin in wheat, which corresponds to 12.5–250 ng of each mycotoxin injected (r^2=0.9939 for DON and r^2=0.9991 for NIV). The sensitivity of the APCI interface seems to be better for deoxynivalenol than for nivalenol. The detection limits of standards without a matrix were lower than that in wheat extract. In a matrix, the quantitation limit for DON was about 40 μg/kg wheat, whereas for NIV 50 μg of mycotoxin/kg wheat could be determined in the SIM mode. In addition, the proposed DON level in feed is about 300 μg/kg, whereas for NIV a 500 μg/kg level has been suggested.

Because of either loss of analyte during clean up or incomplete extraction of the trichothecenes the extraction and clean up are very important steps in

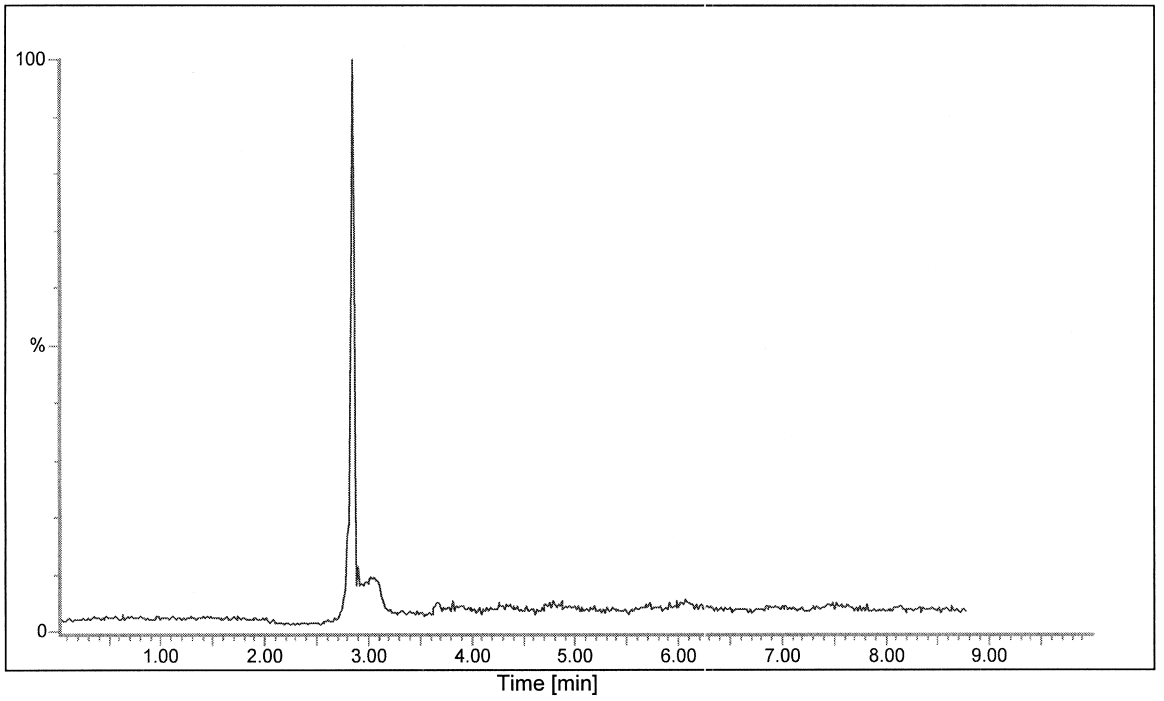

Fig. 7. LC–APCI$^-$–MS chromatogram of a blank wheat extract in SIM mode.

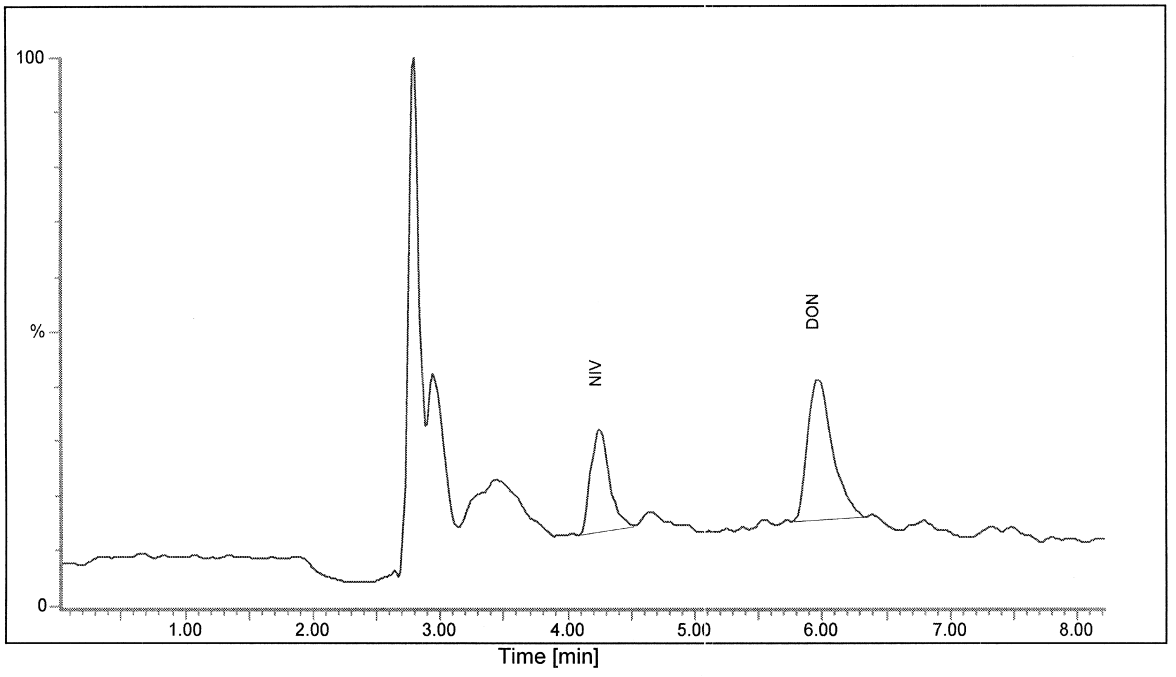

Fig. 8. LC–APCI$^-$–MS chromatogram of nivalenol and deoxynivalenol in spiked wheat extract (100 μg/kg wheat) in SIM mode.

mycotoxin analysis and the cause of poor recovery for some trichothecenes [14,16]. The recovery of the method ($n=3$) was about 70%±10 for NIV and 86%±8 for DON. A within-run precision ($n=5$) was determined for NIV and DON which had an excellent RSD for both compounds at the 500 μg/kg level (4.0% for NIV and 3.5% for DON). For 50 μg/kg ($n=5$) calibrant the RSD was about 7.7% for NIV and about 6.5% for DON.

4. Conclusions

The described LC–APCI–MS method for the analysis of nivalenol and deoxynivalenol seems to be a powerful tool, especially for the analysis of these mycotoxins in biological matrices, due to its high specificity for the tested compounds. The use of this technique has the great advantage of eliminating laborious derivatization and is more sensitive and selective than the other LC methodologies such as HPLC–UV.

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