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Simultaneous quantification of A-trichothecene mycotoxins in grains using liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry☆

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Abstract

An approach for simultaneous determination of the main type A-trichothecenes by liquid chromatography and atmospheric pressure chemical ionization mass spectrometry is described. Parameters for coupling of LC–MS such as cone voltage, nebulizing temperature and the LC flow-rate, were optimized to provide detection of mycotoxins with maximum sensitivity. Furthermore, the effects of cone voltage and temperature on the fragmentation pattern of the tested toxins were studied. Main type A-trichothecenes such as T-2 Toxin, HT-2 Toxin, acetyl T-2 Toxin, diacetoxyscirpenol, monoacetoxyscirpenol (15-acetoxyscirpenol) and neosolaniol were separated on a reversed-phase narrow bore C_{18} column, using a linear gradient and a flow-rate of 0.3 ml/min. Mass spectra were obtained in positive ion mode for confirmation and quantitation. The method involves extraction and purification of toxins by using multifunctional Mycosep columns. Deuterated T-2 Toxin was used as an internal standard. A linear working range between 80 and 500 μg/kg in matrix with an acceptable correlation coefficient was observed. The developed method was validated by using a blank oats sample. The detection limit in the matrix was found to be between 50 and 85 μg/kg in selected ion mode for all tested A-trichothecenes. Recovery data were found to be between 77 and 101%. Within run and day-to-day precision were determined as having comparable levels to those found using GC methods. Furthermore, the matrix effect was investigated by comparing the internal standard versus the external standard method in quantification studies. In addition, the developed method was applied for the analysis of naturally contaminated oats, maize, barley and wheat samples.

Keywords: Food analysis; Grain; Trichothecenes; Mycotoxins

1. Introduction

Research into contamination of food and feed with molds and their secondary metabolites, mycotoxins, has intensified in the last 20 years. The mycotoxin-producing genus *Fusarium* is an important group of fungi. Trichothecenes are a group of tetracyclic sesquiterpene alcohols produced by toxigenic *Fusarium* species, especially *F. sporotrichioide*,

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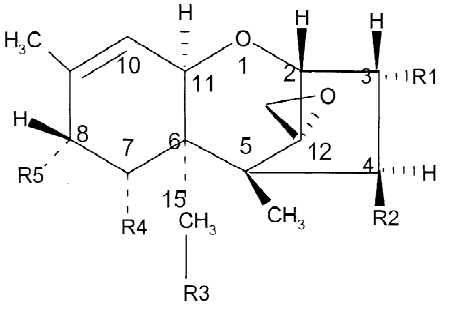
F. poae and *F. equiseti*. Trichothecenes are frequently detected in food and feeds, especially in cereals both before and after harvest [1–3]. Trichothecenes are divided into two groups, type A and type B. Nivalenol, deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol and fusarenone X are naturally occurring type B trichothecene mycotoxins, whereas T-2 Toxin (T-2), HT-2 Toxin (HT-2), acetyl T-2 Toxin (AcT-2); deacetoxyscirpenol (DAS), 15-acetoxyscirpenol (MAS) and neosolaniol (NEO) belong to the type A-trichothecenes (Fig. 1). In contrast to type B-trichothecenes, type A-trichothecenes do not contain a ketone at C8.

Trichothecenes have been found to be toxic. In particular, type A-trichothecenes such as T-2 Toxin, HT-2 and DAS have been reported to have extremely toxic effects on skin and mucous surfaces and can induce lesions on the mucosa of the mouth and oesophageal region in poultry and pigs. The most toxic trichothecene is T-2 Toxin, which has been reported to cause dermatitis of the nose and buccal commissures in pigs [2–4]. The immunotoxicity of A-trichothecenes, especially those of T-2 Toxin, has been investigated and they were found to be less immunotoxic than the B-trichothecenes [5].

Different methods for the analysis of A-trichothecenes have been reported based on TLC [6–9] and GC [6,10–17]. Especially high sensitivity can be reached by using capillary gas chromatography in combination with electron-capture detection (ECD) or mass spectrometry (MS) detection after derivatization of both A and B trichothecenes [10–20]. In particular, heptafluorobutylimidazole derivatization was performed for ECD [6,18]. Trimethylsilylation has also been used for both MS and flame ionisation detection (FID) of major trichothecene mycotoxins [6,19,20]. Immunological methods such as EIA and enzyme-linked immunosorbent assay (ELISA) have also been used [21–23]. However, commercially available kits based on immunological technique exist only for the T-2 Toxin.

In contrast to the B-trichothecenes, the A-trichothecenes do not have a conjugated double bond group and therefore are not amenable to ultraviolet detection if HPLC is used. As a consequence, the HPLC–UV technique in this case is less applicable than gas chromatography [6,24,25]. Owing to sensitivity limitations, HPLC methods for type A-trichothecenes require very effective clean-up procedures, appropriate derivatization and a more sensitive and selective detector. Different post-column strategies have been applied for fluorescence detection after



	R_1	R_2	R_3	R_4	R_5
Neosolaniol	OH	$OCOCH_3$	$OCOCH_3$	H	OH
Monoacetoxyscirpenol	OH	$OCOCH_3$	H	H	H
Diacetoxyscirpenol	OH	$OCOCH_3$	$OCOCH_3$	H	H
T-2 Toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
HT-2 Toxin	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
Acetyl T-2 Toxin	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$

Fig. 1. Structures of type A-trichothecenes.

derivatization with anthracene-9 carbonyl chloride [26] or coumarin-3-carbonyl chloride [27,28]. Immunochromatography was another strategy that has been employed for the detection of these molecules [22]. On the other hand, coupling of HPLC and MS provides a great opportunity for the analysis of such substances. MS detection in HPLC eliminates the need for sample derivatization and moreover has the potential for the confirmation of interested analytes because of the structural and molecular mass information it provides. Using the single ion monitoring mode the appropriate sensitivity can be achieved by reducing background noise. The LC–MS analysis of some trichothecenes such as T-2 Toxin and T-2 tetraol has been reported by Voiksner [29] using a thermospray interface. The determination of T-2 Toxin, diacetoxyscirpenol, deoxynivalenol as well as their metabolites has also been described by Voiksner et al. [30]. Rajakylä et al. [31] reported on the use of thermospray for the determination of major mycotoxins, including T-2 Toxin, DAS and HT-2 Toxin. Kostiainen et al. [32–34] described the comparison of a thermospray interface with dynamic fast atom bombardment (FAB) and analysed T-2, HT-2, DAS, MAS and DON. The LC–MS methods noted here were reviewed briefly by Niessen [35]. Huopalahti et al. [36] reported on the application of an electrospray ionisation (ESI) interface for the analysis of some A- and B-trichothecenes using supercritical fluid for the extraction of these compounds. Recently an atmospheric pressure chemical ionisation (APCI) interface, combined with an ion trap mass spectrometer, was applied for the analysis of type A- and B-trichothecenes in positive ion mode [37]. Additionally, the use of an APCI interface in negative ion mode for the analysis of type B-trichothecenes in wheat samples was reported by our group [38]. Different analytical methods were reviewed by Langseth et al. [39] and recently by Krska et al., who compared all methods and steps, including LC–MS methods, for type A- and type B-trichothecenes [40]. Although GC–ECD or GC–MS after formation of fluorinated derivatives are the methods of choice in analysis of trichothecene mycotoxins, certain method problems as described in two earlier publications [40,41] were also observed.

In this publication we describe for the first time the use of HPLC–APCI-MS on a single quadruple MS for simultaneous determination of the main types of A-trichothecenes. The optimization of LC and MS parameters is reported and the influence of these parameters on fragmentation patterns and sensitivity of A-trichothecenes are investigated. Additionally the matrix effect is investigated by comparing internal and external quantification methods. The developed method was validated in oats and was then applied for quantification of type A-trichothecenes in oats, maize, barley and wheat samples for feed purposes.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium acetate (98%), ammonia, acetonitrile (LiChrosolv) and methanol (LiChrosolv) were purchased from Merck (Darmstadt, Germany). Water for the HPLC mobile phase was purified in a UPW2 system (F&L, Vienna, Austria). Solvents were filtered through a 0.2 μm filter to remove particles and were degassed using a Waters in-line degasser (Milford, MA, USA). All mycotoxin standards, including internal standard [$^2H_3$]T-2 Toxin (T-2-d_3), were purchased from Sigma (St. Louis, MO, USA). The blank oats samples were kindly supplied by Professor H. Pettersson (Swedish University of Agricultural Sciences, Uppsala, Sweden).

2.2. Chromatography

HPLC analysis was carried out using a Waters 626-LC pump and a Waters 717plus autosampler (Milford, MA, USA). For the separation of toxins a reversed-phase C_{18} 200 mm×2.1 mm I.D column (Hypersil, Runcorn, UK) packed with 5 μm ODS-2 was used and the column temperature was kept at 30 °C. A linear gradient consisting of 1 m*M* ammonium acetate–acetonitrile (80:20, v/v) changing to acetonitrile 100% (containing 1 m*M* ammonium acetate) in 5 min was used. After 3 min washing with acetonitrile, the column was then re-equilibrated with 1 m*M* ammonium acetate–acetonitrile (80:20, v/v) for 10 min. The analysis was carried out using a HPLC flow-rate of 0.3 ml/min and a run time of

18 min. Under these chromatographic conditions all investigated mycotoxins could be separated.

2.3. Mass spectrometry

A Platform II single quadrupole instrument and APCI interface equipped with a Pepperpot counter electrode (Micro Mass, Manchester, UK) was used for all mass spectrometry studies. The MS experiments were performed in the APCI positive mode. The MS parameters were optimized in the scan mode (m/z 250–600) by direct injection of each mycotoxin standard without the use of an analytical column. Nitrogen of pure quality was used as nebulizing and carrier gas (Messer Griesheim, Vienna, Austria). Carrier gas flow was set at 250 l/h and sheath gas flow was held at 125 l/h. For quantitative analysis after optimization, the source temperature was maintained at 100 °C and the APCI vaporizing temperature was kept at 300°C. Cone voltage was set at 20 V.

The quantitative determination of all compounds was conducted in the selected ion monitoring (SIM) mode by using the molecule ions, ammonium adducts or the main fragments. Dwell time of 0.3 s and span of 0.2 u were used for SIM detection.

2.4. Sample preparation

Samples were ground and were then passed through a 1-mm sieve (Pulverisette 19, Fritsch, Idar-Oberstein, Germany). An aliquot of 25 g was extracted with 100 ml of acetonitrile–water (84:16, v/v) by stirring the mixture for 90 min using a magnetic stirrer. The extract was filtered through a cellulose filter (Schleicher & Schuell, Dassel, Germany). Sample clean-up was performed in two steps using multifunctional Mycosep columns 227 and 216 (Romer Labs., Washington, MO, USA). An aliquot of 10 ml of crude extract was then transferred to the culture tube and pressed through the Mycosep column 227. In a further step, 6 ml of purified extract were applied to an equilibrated (90:10, v/v) Mycosep column 216 and eluted with 9 ml of acetonitrile–water (90:10, v/v). The cleaned-up extract was then evaporated under a stream of nitrogen at 60 °C in a heated aluminium block. The residue was then re-dissolved in 600 μl of mobile phase (1 m*M* ammonium acetate–acetonitrile 20:80, v/v) and 50 μl were injected into the LC–MS system. Since the A-trichothecenes have no characteristic absorption maxima, the concentration of compounds in standard solution could not be determined by spectrophotometer, therefore enough toxin was bought to weigh the standards.

3. Results and discussion

3.1. APCI mass spectra of A-trichothecenes

In contrast to B-trichothecenes [38], the response in positive ion mode for all investigated compounds was found to be distinctly more sensitive than in negative ion mode. The mass spectra of A-trichothecene mycotoxins in positive ion mode have very specific fragmentation patterns (Fig. 2). The mass spectra of T-2, MAS and AcT-2 show intensive fragmentation even at low cone voltages. As can be seen from Fig. 2, the registered mass spectra of tested toxins are almost identical to those noted by Rajakylä et al. [31] in thermospray (filament on), in plasmaspray mode [32], in fast atom bombardment [33] and in multiple MS (MS^n) [37]. Generally, it can be said that the APCI spectra in our experiments show more intensive fragmentations than thermospray (TSP) spectra [30–34].

For AcT-2, additionally loss of 101 u (isovaleryl) was noted. The mass spectra of T-2 show a relatively intensive ammonium adduct $[M+NH_4]^+$ compared to $[M+H]^+$ and fragment ion of 365 u for loss of isovaleryl side chain $[M+H-(CH_3)_2CHCH_2COOH]^+$ was noted. Additionally, for DAS the fragment ions 307 u as $[M+H-CH_3COOH]^+$, 349 u as $[M+H-H_2O]^+$ and a weak $[M+H]^+$ peak were observed. For NEO, similar mass spectra to DAS were observed but additionally loss of 78 u for $[M+H-H_2O-CH_3COOH]^+$ was registered. Furthermore, for MAS $[M+NH_4]^+$ and $[M+H]^+$ as well as 265 u as $[M+H-CH_3COOH]^+$ were obtained. The loss of the acetyl group could be observed for all tested molecules.

Berger et al. have reported strong ammonium adducts in all cases of trichothecenes with an acetyl group at C15 [37]. In our experiments, only for NEO, DAS, T-2 and AcT-2 could intensive ammonium adducts $[M+NH_4]^+$ be observed. However,

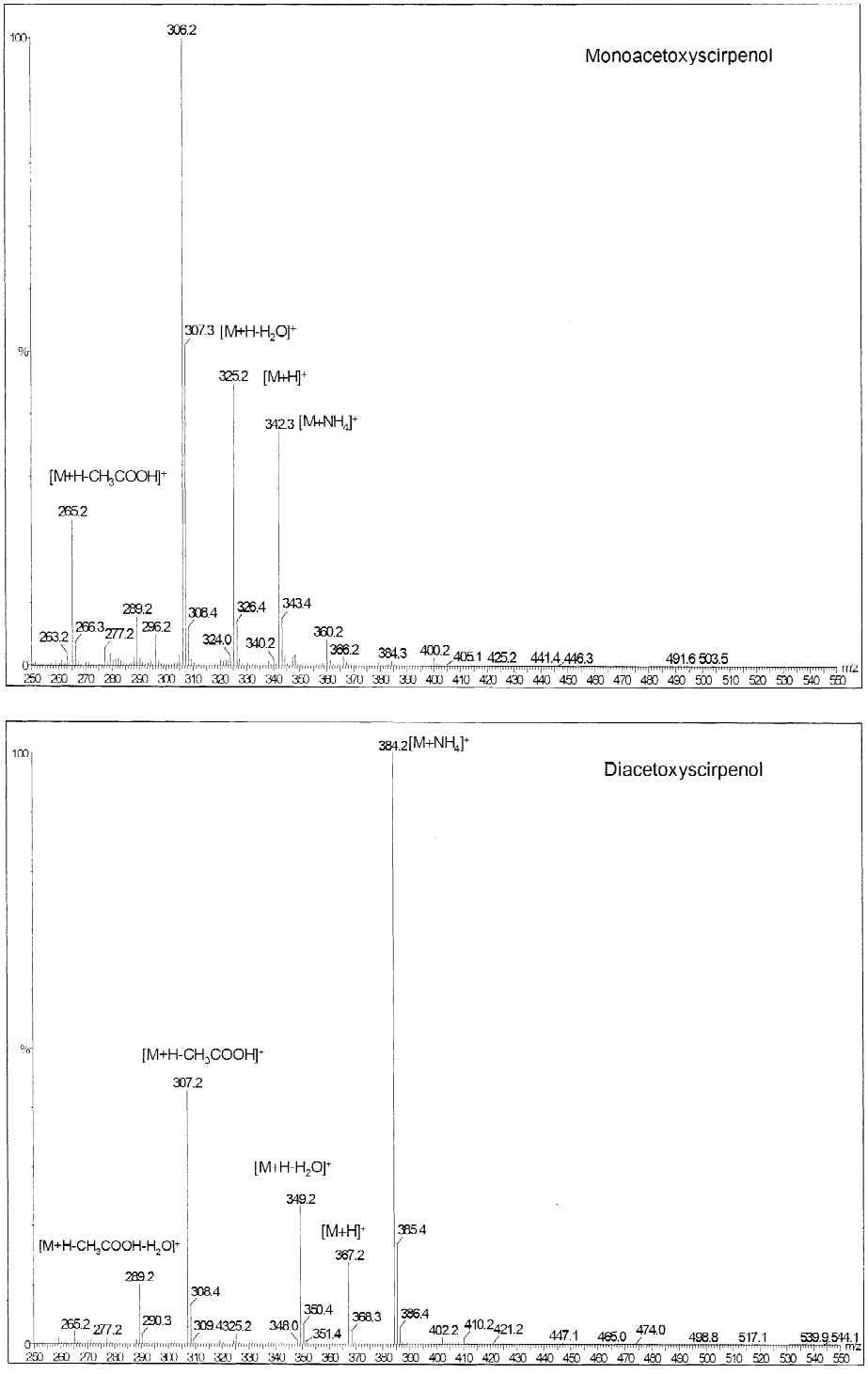


Fig. 2. APCI mass spectra of tested type A-trichothecenes in positive ionisation mode.

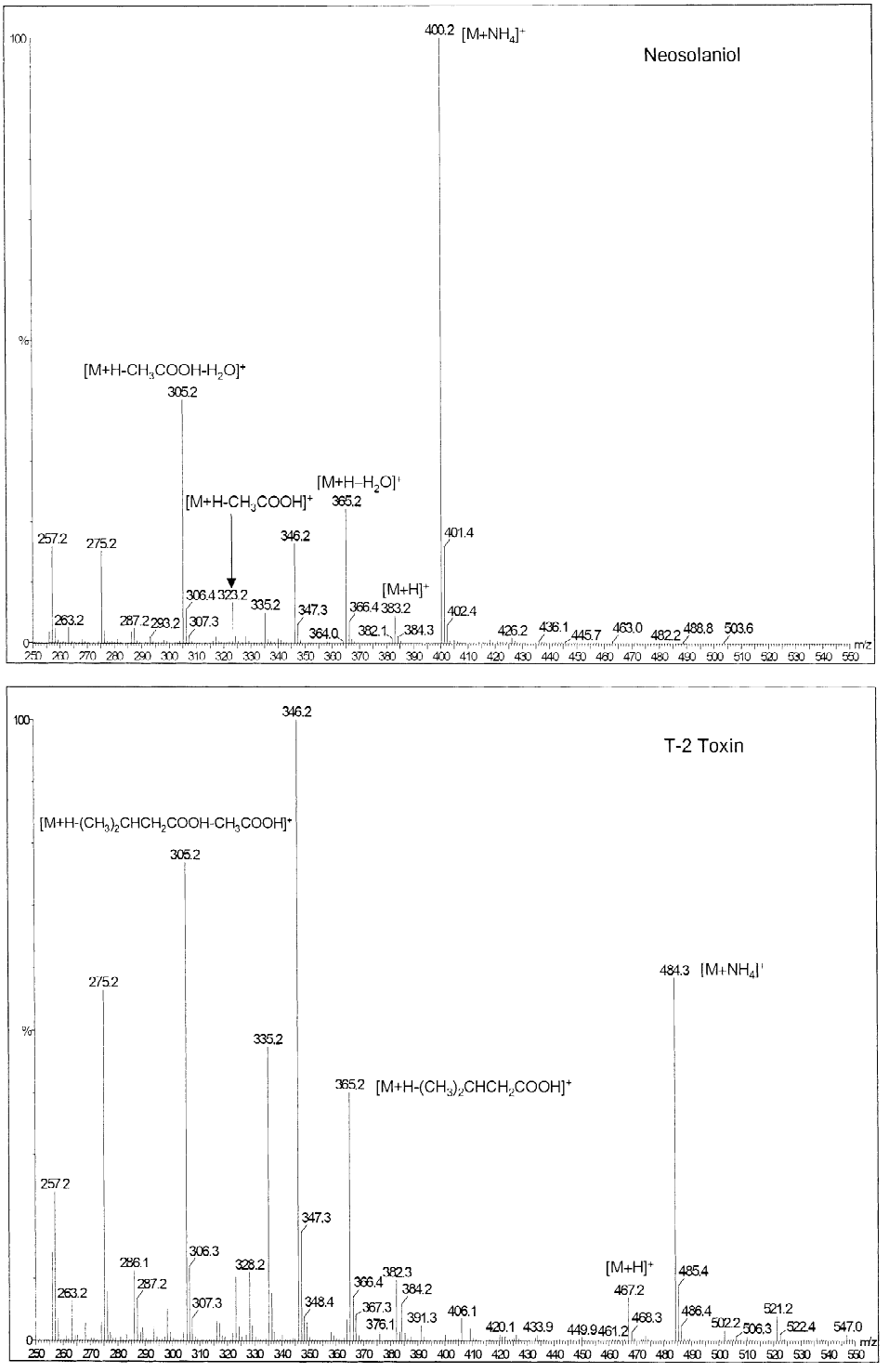


Fig. 2. (*continued*)

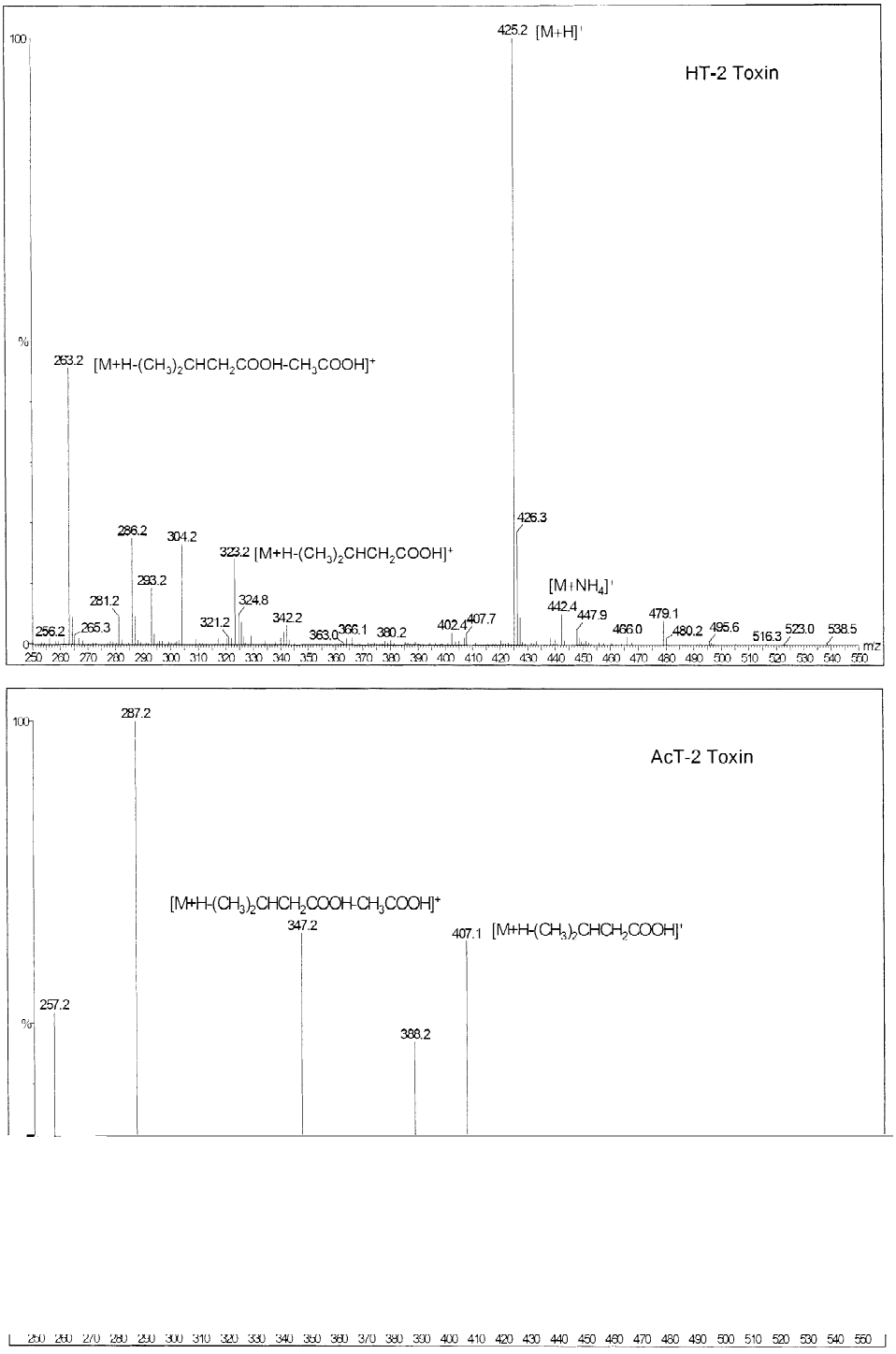


Fig. 2. (*continued*)

in contrast to the mentioned work [37], for HT-2 an intensive $[M+H]^+$ base peak but only a weak $[M+NH_4]^+$ was noted.

Generally we observed intensive ammonium adducts only for those molecules with ester groups at both C15 and C4. On the other hand, in the case of HT-2 and MAS, which do not have two ester groups, an intensive $[M+H]^+$ could be registered. The formation of $[M+NH_4]^+$ should be due to the presence of ester groups at both positions, which, it is suggested, have a tendency towards higher ammonium adduction [31]. Therefore, we think the formation of $[M+NH_4]^+$ adducts depends only on the number of acetyl groups, not on their position. This is in accord with the observation of Rajakylä et al. [31]. Consequently, the ionisation mechanism in our APCI source seems to be very similar to those in the thermospray filament on source.

3.2. Evaluation of the APCI interface parameters

The response in APCI-MS detection has experimentally been shown to be strongly dependent on the interface parameters. Therefore the main parameters were preliminarily optimized in order to obtain maximum sensitivity for all tested mycotoxins. This was achieved by repetitive injections of standard solution (20 μl) into the LC–MS system. Generally the best response was observed for DAS followed by NEO. Response of T-2 and AcT-2 were similar in intensity, whereas HT-2 and MAS show the lowest response.

Different cone voltages were applied and the effect on sensitivity and fragmentation patterns of tested substances were investigated. Fig. 3 shows the influence of cone voltage on the changes of response. For all tested compounds, except HT-2, the best sensitivity was observed at 20 V. Only in the case of HT-2 was an optimum reached at a cone voltage of 30 V. The mass spectra in all cases show an intensive fragmentation pattern and a decrease of base peaks at higher cone voltages, as expected. Fig. 4 shows the effect of changing vaporizer temperature, which was varied between 200 and 600 °C. For NEO, HT-2 and T-2, a maximum was reached at 300 °C and for MAS at 400 °C, whereas DAS and AcT-2 show the best sensitivity at 600 °C. The effect of temperature on fragmentation was studied: similar to the cone voltage effect, generally higher temperatures lead to intensive fragmentation and decrease of protonated molecules and the adduct ions.

For the optimization of HPLC flow-rate the mobile

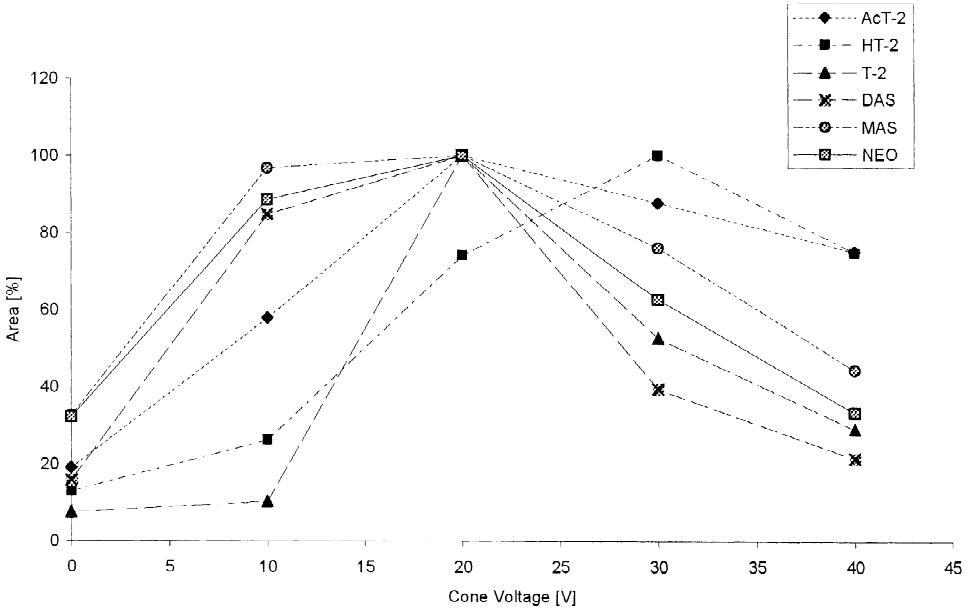


Fig. 3. The influence of cone voltage on signal intensity of type A-trichothecenes.

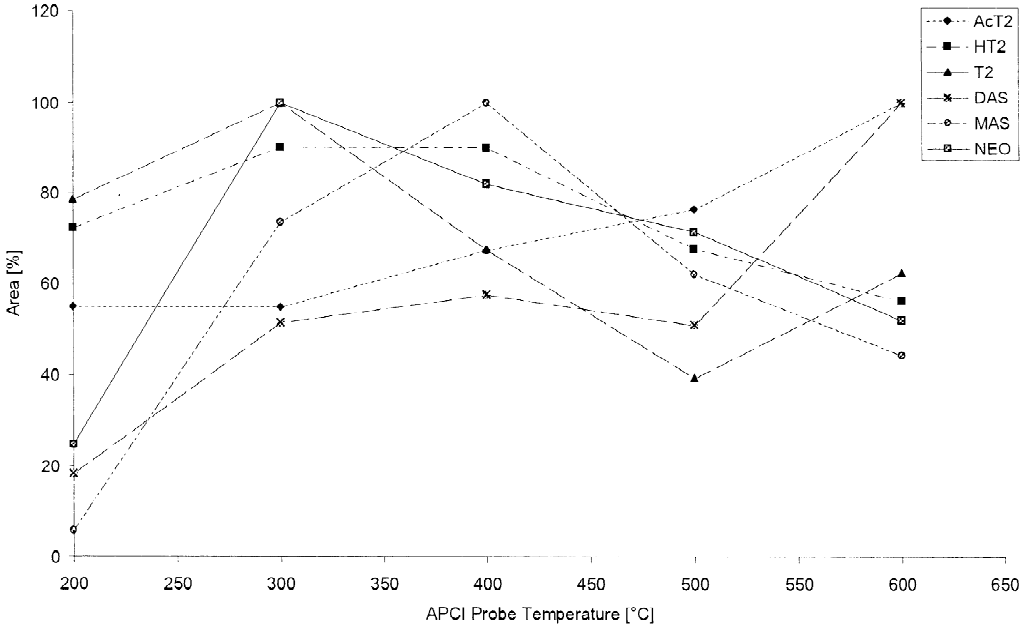


Fig. 4. Effect of APCI vaporizer temperature on the MS signal of type A-trichothecenes.

phase was changed between 0.3 and 1 ml/min. As can be seen in Fig. 5, differing behaviour of tested substances could be observed. T-2, HT-2 and AcT-2 show a better sensitivity in the lower range at 0.3 ml/min, whereas MAS shows the best sensitivity at 0.8 ml/min. Similar to type B-trichothecenes [38],

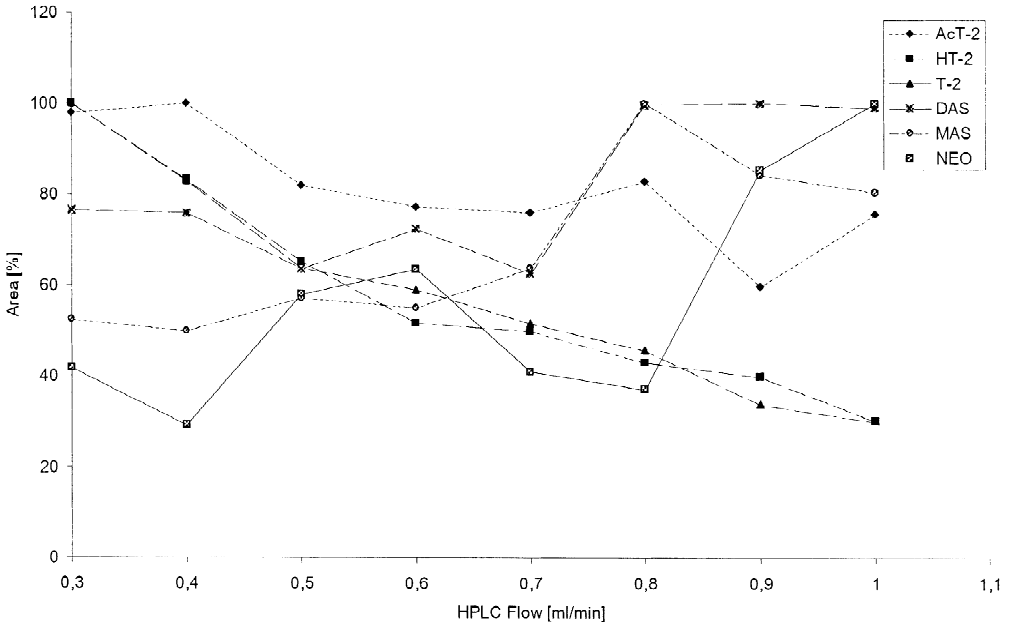


Fig. 5. Influence of HPLC flow-rate on signal intensity of type A-trichothecenes.

DAS and NEO have the best response at 1 ml/min. Since a narrow bore HPLC column was used for the separation of toxins in quantitative experiments, mobile phase flow was set to 0.3 ml/min.

Ammonium acetate in low concentration (1 m*M*) was added to achieve better retention time stability. Especially for MAS, higher retention time fluctuation was observed when ammonium acetate was not used. The selected conditions for the actual LC–MS chromatographic runs were relative to the above noted variables and chosen as a compromise.

3.3. Determination of A-trichothecenes, matrix effect investigation, linearity, sensitivity and precision of the LC–APCI-MS method

The use of an appropriate chromatographic condition prior to mass spectrometric analysis was necessary in order to determine all type A-trichothecenes in one step and in such a complex matrix. Under isocratic conditions, it was not possible to separate the toxins. Therefore a gradient system was developed, which is able to separate all tested toxins. A typical total-ion chromatogram is shown in Fig. 6 illustrating the baseline separation of a standard solution of A-trichothecene. Additionally, the use of a second clean-up Mycosep column 216 was necessary to remove matrix compounds and therefore avoid any possible matrix effects. The validation of the method was carried out using oats. For other matrices such as maize, barley and wheat, a mixture of toxin standards at a level of 80 and 200 μg/kg was added to each analysed matrix to ensure the accuracy of tested compounds in samples. Moreover, the intensive fragmentation as an in-source collision-induced dissociation (CID) has the advantage of allowing verification of each analyte in the biological matrix.

3.4. Calibration graphs

Calibration graphs were established using blank oat samples spiked with amounts of trichothecenes ranging from 80 to 500 μg/kg. Internal standards (deuterated T-2 Toxin) were added to each sample and the ratios of analyte peak area to internal standard peak area were calculated. For all tested mycotoxins calibration graphs show good linearity and reproducibility in the matrix. Table 1 shows the calibration equations (r^2) and their correlation coefficients for the tested compounds.

3.5. Limit of quantification (LOQ)

The limit of quantification was defined as the minimum concentration of the analyte in oats giving a signal-to-noise ratio of at least 10:1 under the assay conditions. In our experiments the detection limit of the instrument, injecting standard solution without matrix, was comparable to the results obtained by Berger et al. But when analysing compounds in the matrix, differences to the results reported by Berger et al. were observed. As can be seen in Table 1, quantification limits for T-2, AcT-2, DAS and NEO were observed at the 50 μg/kg level. For HT-2 Toxin a higher quantification limit with 85 μg/kg and 60 μg/kg for MAS were noted. Comparing the achieved quantification limits with the results published previously for NEO, DAS, HT-2, T-2 [37], differences were observed, which could be explained by various interface design and of course two different instrument types, ion trap and quadrupole.

3.6. Precision, accuracy and recovery

Intra-day and inter-day precision were assessed by replicate determination of a toxin mixture in spiked oat samples at the 200 μg/kg level. Results shown in Table 1 are comparable to those reported in the literature [17,37]. The precision values were determined in four series with double injections. The intra-assay relative standard deviation values were between 5.7% for NEO and 15.9% for AcT-2. The day-to-day relative standard deviation calculated from these results for examined compounds was between 9.3% for T-2 Toxin and 25.2% for MAS. To determine the recovery of the complete method, methanolic standard solutions were added to the matrix extract. The recovery values reported by Berger (LC–MS) and Schollenberger et al. (GC–MS) at a level of 500 μg/kg are in good agreement with those of our method at 200 μg/kg.

3.7. Matrix effect

The use of an internal standard has been reported

Table 1
Intra- and inter-day precision expressed as relative standard deviation (RSD), limits of quantification (LOQ), recovery of each toxin in spiked oats with 200 μg/kg of each toxin and the calibration graphs and their correlation coefficients (r^2) of tested compounds in the matrix

Compound	Intra-day precision (RSD, %) 200 ng/g	Inter-day precision (RSD, %) 200 ng/g	LOQ (ng/g)	Recovery (%) 200 ng/g	r^2	Calibration equation $y = ax + b$[a]
T-2 Toxin	7.4	9.3	50	77.4	0.9933	$y = 0.0003x - 0.0054$
HT-2 Toxin	10.7	19.4	85	90.2	0.9915	$y = 0.0008x - 0.0104$
Acetyl T-2 Toxin	15.9	15.11	50	101.1	0.9880	$y = 0.0008x - 0.002$
Diacetoxyscirpenol	5.4	10.78	50	86.5	0.9960	$y = 0.0098x - 0.0869$
Monoacetoxyscirpenol	12.1	25.2	60	95.1	0.9928	$y = 0.0048x - 0.055$
Neosolaniol	5.7	13.7	50	91.5	0.9990	$y = 0.0038x + 0.016$

[a] x is the injected concentration in ng and y is the quotient of analyte peak area to peak area of internal standard.

to be the best way to eliminate matrix effects during ion formation [42–45]. Additionally, the use of deuterated internal standard has the advantage of eliminating the variation of detector response. In our experiment the two internal and external calibration methods were compared by determining the concentrations of all tested mycotoxins with and without internal standard. The recoveries for each toxin were determined by spiking the oats with an appropriate amount of toxin, worked up and analysed by LC–APCI-MS. The two series of data with and without internal standard were comparable and no significant difference could be observed between the two sets of data. These indicate neither negative nor positive ion suppression and therefore no matrix effect with the investigated toxins. Nevertheless, ones should be mentioned regarding the factors affecting the reproducibility of the response when using the LC–MS in the analysis of complex matrices such as problems due to dirtiness of the APCI source, cone and the focusing hexapoles at the front of the instrument. The use of a stable isotope labelled internal standard is a great advantage and can provide more reliable data in terms of reproducibility of response. Furthermore it can compensate for the sensitivity drift of the LC–MS system within a day. Fig. 7 shows the LC–APCI-MS chromatograms of a spiked oats sample in SIM mode.

The developed LC–MS method was further applied for determination of type A-trichothecenes in naturally contaminated grains. Mainly T-2 Toxin and HT-2 Toxin could be found, in six samples ($n = 20$). Only traces of DAS in two oats samples could be detected. Other A-trichothecene mycotoxins were not detectable in analysed maize ($n = 5$), barley ($n = 5$) and wheat ($n = 5$) samples. This is in accordance with the reports published previously, regarding the occurrence of A-trichothecenes in grain [46,47].

4. Conclusions

The present study shows that six A-trichothecenes can be analysed in a single HPLC run, using an LC–APCI-MS system. A very simple and time saving clean-up procedure based on multifunctional Mycosep columns yields clean reconstituted extracts. These sample preparation methods are reliable, quick and rugged. The developed method has been demonstrated to be selective and sensitive for the analysis of A-trichothecenes in several types of grains. The possibility of simultaneously detecting and confirming main type A-trichothecenes in different grains at levels as low as 50–85 μg/kg illustrates that LC–APCI-MS is an attractive and worthwhile method. Additionally, the use of internal standard can provide better robustness and improved reproducibility of results. However, the combination of HPLC with mass spectrometry would provide additional advantages and would eliminate the time consuming and difficult procedure of derivatization procedures required by both HPLC and GC. In sample preparation, selective component enrichment and clean up is necessary when using either LC–MS or GC–MS for the analysis of mycotoxins in complex matrices.

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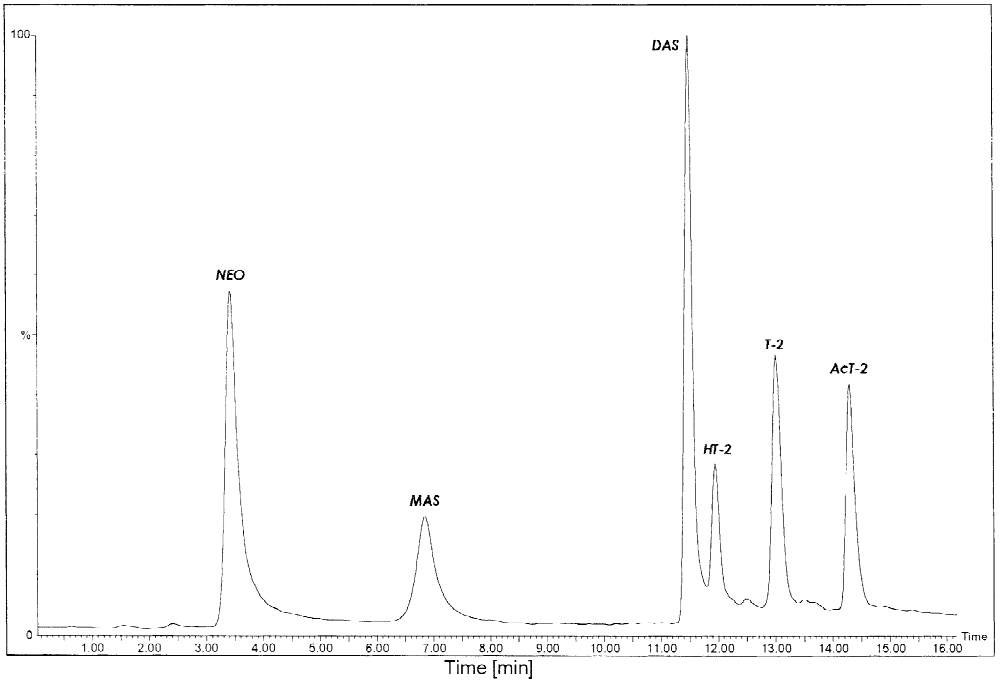

Fig. 7. TIC chromatogram (LC–APCI$^+$-MS) of separation of a toxin mixture solution containing 500 ng for each toxin in the scan mode (for details see Section 2.2).

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