Determination of mycotoxins by a liquid chromatography/time of flight mass spectrometry

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Abstract

A liquid chromatography/mass spectrometry (LC/MS) method based on time of flight MS (TOF-MS) with a real time reference mass correction technique using dual spray-ESI source was developed for the determination of Fusarium mycotoxins [fumonisinsB1(FMB1), fumonisinsB2(FMB2), fumonisinsB3(FMB3), zearalenone (ZEN), α-zearalanol (α-ZAL), β-zearalanol(β-ZAL), α-zearalenol(α-ZOL), β-zearalenol (β-ZOL)] and Aspergillus mycotoxins [ochratoxin A(OTA)] in corn, rice, and feed. Samples were cleaned-up with solid phase extraction(SPE) mini column. Detection of the mycotoxins was carried out in exact mass chromatograms with a mass window of 0.03 Da. Calibration curves were linear from 1 to 100 ng/mL. The limits of detection ranged from 0.1 to 6.1 μg/kg in foodstuffs analyzed in this study. The LCTOF-MS method was found to be suitable for the screening of multiple mycotoxins in foodstuffs.

Key words: mycotoxin, LCTOF-MS, dual spray-ESI

Introduction

Many crops are susceptible to fungal attack not only in the field but also during storage. These fungi may produce a toxic secondary metabolite, called mycotoxin. Major mycotoxins found in cereals are aflatoxins (AFs), ochratoxins, trichothecenes (TRs), fumonisins (FMs) and zearalenone (ZEN). Although these mycotoxins cause typical health problems in humans as well as animals with a sub-acute or acute dose, the chronic effects at low levels of exposure are of great concern such as those affecting immunotoxicity and resulting in the decrease of host resistance to infectious diseases and cancer. In order to assess the exposure to these mycotoxin, it is essential to develop a sensitive and reliable method. Liquid chromatography-triple quadrupole mass spectrometry (LC-QqQMS) has become so far, the most widely used technique for quantitation of mycotoxins in food. However, when a large number of compounds with different molecular weights are detected simultaneously using a quadrupole instrument, the demand for scanning over a wide mass range would result in decreased sensitivity. Also, when selected ion monitoring (SIM) with LC-MS or multiple reaction monitoring (MRM) modes with LC-QqQMS are used to increase sensitivity, it may in fact decrease with the number of analyses. Also, there may be a poorer confirmation of identity when these mass-specific acquisition modes are used because they do not acquire the full mass spectra.

In contrast to quadrupole or ion trap instruments, TOF-MS has the capability of detecting a wide mass range without losing significant sensitivity. Furthermore, TOF instruments provide enhanced full mass range spectra sensitivity and accuracy due to their high
resolution\(^1\). This also allows to distinguish among isobaric ions and increases confidence in the identification of analytes by giving as estimate of the molecular composition of each ion\(^2\)-\(^4\). And, the information obtained from TOF analysis has the additional advantage that quantitation can be performed on any mass observed in the acquired mass range\(^5\),\(^6\).

In this study we describe a rapid and reliable method utilizing LCTOF-MS method for OTA, fumonisins (FMB1, FMB2, FMB3), ZEN and its metabolites (ZAL, ZOL) in corn based feed, wheat and rice.

### Experimental

**Chemicals** The mycotoxins, ZEN, ZAL, ZOL, OTA, FMB1, FMB2 and FMB3 were purchased from Sigma Aldrich Japan (Tokyo, Japan). LC-MS grade acetonitrile, reagent grade formic acid and ammonium acetate were purchased from Wako Chemical (Osaka, Japan). Pure water was purified with a Milli-Q system (Millipore, Tokyo, Japan). Purine and hexakis (1H 1H, 3H-tetrafluoropentoxy) phosphazene mixture solution as lock mass compounds were obtained from Agilent Technologies (Santa Clara, CA, USA).

**Liquid Chromatography** An Agilent 1100 series liquid chromatograph system (Agilent Technologies, Waldbronn, Germany) including a vacuum solvent degassing unit, a binary high-pressure gradient pump, an automatic sample injector, a column thermostat, a photodiode array was used. LC separation was performed on a 150 x 2.1 mm I.D. column packed with 3.5 μm ZORBAX Eclipse XDB C18 (Agilent Technologies, Santa Clara, USA) at 40°C. The LC mobile phase was aqueous 10 mM ammonium acetate (A) 0.1% formic acid (B) and acetonitrile (C). The initial gradient condition was 75% B and 25% C changing linearly to 70% C in 20 min for FMs and the initial gradient condition was 80% A and 20% C changing linearly to 50% C in 20 min for OTA. Isocratic mode (55% A and 45% C) was performed to separate ZEN and its metabolites. The injection volume was 5 μL (FMs, ZEN, ZAL and ZOL) and 30 μL (OTA).

**Mass spectrometry** Mass spectrometry was performed using an Agilent 1100 series MSD TOF instrument equipped with a dual spray-ESI source (Agilent Technologies, Santa Clara, USA). Nebulizer gas as well as drying gas (350°C) was nitrogen generated from pressurized air by a nitrogen generator (Airetech, Tokyo, Japan). The nebulizer gas, the drying gas, the capillary voltage, the fragmentor voltage and the vaporizer temperature were set at 50 psi, 6 L/min, 4000 V, 100 V and 350°C, respectively. The determination was performed by operating the MSD TOF in the positive ion mode (FMs, OTA) and negative ion mode (ZEN, ZOL and ZAL). Profiling mode mass spectra were acquired over the scan range m/z 100-1000 with a spectral acquisition rate of 0.8 s per spectrum and the mass resolution was from 3800 FWHM at m/z 121 to 10000 FWHM at m/z 922 in lock mass solution. The calibration of the spectral range was performed using an ESI calibrant solution (Agilent Technologies, Santa Clara, USA) and a fifth-order non-linear calibration curve was usually adopted. To perform the real time lock mass correction, lock mass solution including purine (C₅H₄N₄ at m/z 121.050873, 10 μM) and hexakis (1H 1H, 3H-tetrafluoropentoxy) phosphazene (C₁₈H₁₈O₆N₃P₃F₂₄ at m/z 922.009798, 2 μM) was introduced to dual spray-ESI source using a calibrant delivery system. Quantitative analysis was performed using the exact ion chromatograms of the base peak ions with 0.03 Da windows.

**Sample Preparation** All real sample extract was kindly gifted from some customers. The extraction and cleanup for mycotoxins from samples were carried out by typical procedure.

### Results And Discussion

**Fumonisins (FMB1, FMB2 and FMB3)** The appropriate performance of chromatographic
Fig. 1. Exact mass chromatograms and calibration curves of FMB1, FMB2 and FMB3. 1; FMB1, 2; FMB3, 3; FMB2. Concentration: 1 ng/mL

Fig. 2. Mass chromatograms and mass spectra of FMB1, FMB2 and FMB3 in popcorn. 1; FMB1, 2; FMB3, 3; FMB2.

separation and ionization was investigated. The separation of all FMs was performed by using 0.1 % formic acid solution because all FMs could not be eluted from the reversed phase.
column by neutral buffer solution such as ammonium acetate. Fig. 1 shows the exact mass chromatograms of all FMs using protonated molecular ion which was the base ion with 0.03 Da at 1 ng/mL and the calibration curves of all FMs. As a result, the signal to noise ration of FMB1, FMB2 and FMB3 were 30, 16 and 28, respectively. The calibration curve for each FMs was linear over the working range (1 to 100 ng/mL), respectively. Squared correlation coefficients ($r^2$) were higher than 0.999 for the seven point calibration curves. Fig. 2 show the exact mass chromatograms of FMs in popcorn which was purchased in the local market and calculated amount of each FMs was 11.3, 4.5 and 4.2 ng/g. Mass spectra and the accuracies obtained in the mass measurements of the protonated molecules of all detected FMs were also shown in Fig. 2. The relative mass errors obtained were less than 3 mg/kg. These accuracies provide highly reliable identification of the target species.

**Ochratoxin A** Mass chromatograms of Ochratoxin A at 1 ng/mL and 0.2 ng/mL using protonated molecule with 0.03 da were shown in Fig. 3. Signal noise ration of ochratoxin A at 1 ng/mL was 9 and $r^2$ of calibration curve was 0.999. This analytical performance in terms of linearity could compare very well with typical instrument used for quantitation purpose such as quadrupole mass spectrometer. The selectivity of LCTOF-MS relies on resolving power of the instrument on the m/z axis. The higher resolution provided by the instrument, the better the selectivity for qualification and quantification. Taking into account that the resolving power of a TOF-MS is in the range 5000-10000, it can discriminate between isobaric interferences within 0.05 Da mass difference (e.g., using an ion at 350 m/z). Fig. 4 show an example of the selectivity achieved by TOF-MS. When a narrow mass window is selected in the mass chromatogram for protonated molecule of ochratoxin A in rice extract, the interferences disappear leading to an enhanced signal-to-noise ratio.

![Fig. 3. Mass chromatograms, mass spectrum and calibration curve of ochratoxin A](image)
**Zealarenon and its metabolites**  
ZEN is easily metabolized to α and α and β-ZOL. Further reduction of the C11-C12 double-bond leading to α and β-ZAL was demonstrated. Therefore, in this study, ZEN and four metabolites were investigated. Fig. 5 show accurate mass
chromatogram of ZEN and metabolites. It was difficult to completely separated β-ZAL and β-ZOL. However, it was not necessary to separate these metabolites because the mass difference of them was 2 Da. The calibration curves of ZEN and its metabolites were created at five different concentration levels in the range from 50 to 1 μg/kg using I.S at 10 μg/kg. Linearity of all target compounds were over 0.998. To evaluate the effectiveness of developed method by LCTOF-MS, it was applied to the analysis of feed sample. Fig. 6 show mass chromatograms and mass spectra of ZEN and metabolites which were detected in food extract. The calculated amount of ZEN was 237 ng/mL and metabolites were in the range from 0.38 to 4.52 ng/g. Although these amounts of metabolites were below LOQ, mass spectra of ZOL could be acquired and accuracies of deprotonated molecules for these metabolites were 1.3 and 0.75 mg/kg.

Conclusion

A study to evaluate the usefulness of LCTOF-MS for quantitative analysis of mycotoxins in food was carried out. The results obtained showed a good analytical performance in terms of sensitivity, selectivity and linearity of the method. Furthermore, the resolving power, accurate mass measurement capability and full spectral sensitivity also LCTOF-MS attractive as a tool for identifying on-target “unknowns” compounds in complex matrices. Currently, the first choice is typically triple quadrupole MS for target-mycotoxin analysis. However, the introduction of LCTOF-MS will greatly improve mycotoxin analysis in food.

References