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## 論 文 内 容 の 要 旨

This thesis presents the development, validation, and application of analytical methods for determination of fungal metabolite/mycotoxin levels in grains. Analytical methods are mainly focus on four carcinogenic secondary fungal metabolites such as aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA) and sterigmatocystin (STC), and a primary fungal metabolite ‘ergosterol (ERG)’. In addition, the relationship between ERG and mycotoxins (AFs, OTA and ZEA) was investigated whether any correlation exists or not.

Unlike AFs, STC is not a well-studied mycotoxin owing to the lack of sensitive and reliable analytical methods. As a precursor of the carcinogen $AFB_1$, STC exerts the same carcinogenic and mutagenic effects. After observing the cross-reactivity of antibody for AFs, a new clean-up method was developed for analysing STC in grains using a commercially available immunoaffinity column (IAC) for sample preparation. After developing IAC clean-up method, STC was determined by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS).

At first, STC was extracted with acetonitrile (84 %) and the extract was purified by IAC. Purified sample was analysed either by LC-MS or GC-MS. Using LC-MS analysis, the limit of detection (LOD) for STC was 1.0 μg/kg in grains. The calibration curve was linear in the range of 3.0-150 μg/kg, with a coefficient of determination of 0.999. Average recovery of STC within the range of 5.0-100 μg/kg was 83.2-102.5 %, with a relative standard deviation of repeatability (RSDr) of 0.24-6.5 %. Moreover, a sensitive and reliable GC-MS method using on-column injection was developed to determine STC in grains without derivatization. The matrix effect was investigated in three different grains, and an insignificant matrix effect (< 15 %) was observed after IAC clean-up. The LOD of the method was 2.4 μg/kg. The calibration curve was linear in the range of 8-120 μg/kg in grains, with coefficient of determination of 0.998. Good recovery (93.2 %) was obtained in maize with a low RSDr of

less than 10 %. Both LC-MS and GC-MS methods are successfully applied in STC pre-treated grains to determine STC contamination at low levels.

Fungal biomarker ERG has been used as a useful indicator to know fungal invasion and give possible signal for mycotoxin contamination in grains. Therefore, a simple, rapid and sensitive GC-MS method using on-column injection was developed and validated in grains. Matrix matched calibration curves were constructed to compensate for matrix effects. The LOD of the method was 40 µg/kg. The calibration curve was linear in the range of 0.2 to 20 mg/kg with coefficient of determination of 0.999. Acceptable recoveries of maize (98-110 %) and wheat (96-110 %) samples were obtained at three spiking levels, with an RSDr of less than 8 % in maize and 7 % in wheat. Then, the developed method was successfully applied to 37 marketed grains for ERG determination.

Only a few reports have been published for simultaneous determination of AFs, OTA, and ZEA levels in maize. Therefore, a simultaneous determination of these three agriculturally important mycotoxins was developed and validated using HPLC with a fluorescence detector. The LOD of the method was determined to be 0.025 µg/kg for $AFB_1$, 0.0125 µg/kg for $AFB_2$, 0.05 µg/kg for $AFG_1$, 0.025 µg/kg for $AFG_2$, 0.5 µg/kg for OTA and 15 µg/kg for ZEA in maize. Calibration curves for mycotoxins ($AFB_1$, $B_2$, $G_1$, $G_2$; OTA; ZEA) showed linearity within the tested ranges with coefficient of determination in excess of 0.999. The mean recoveries were $AFB_1$ (76 %), $AFB_2$ (83 %), $AFG_1$ (80 %), $AFG_2$ (85 %), OTA (90 %), and ZEA (89 %), with RSDr of 0.6-4.9 %. The developed method was successfully applied to 139 maize samples for simultaneous determination of mycotoxin levels.

To correlate fungal/mycotoxin contamination with ERG content, the relationship between ERG and mycotoxins (AFs, OTA and ZEA) was investigated in maize samples collected from four geographic locations. For this experiment, a simple HPLC with a UV detector was used for ERG analysis. ERG was not significantly correlated with AFs among 139 maize samples analysed. However, a significant correlation ($r^2 = 0.82$) was observed between ERG and ZEA. The co-occurrences of AFs and ZEA were found in 47 % of total samples. Half of the total samples (50 %) contained more than two mycotoxins. Results indicate that mycotoxin contaminants in maize are within the EU limits if ERG levels are less than 3 mg/kg. This indication could be a useful indicator to understand fungal invasion and, on a merely qualitative basis, mycotoxin contamination in grains.