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Three liquid chromatographic methods for the analysis of aflatoxins in for different corn (*Zea mays*) matrices

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ABSTRACT

Liquid chromatographic analyses of aflatoxins (AFs) in corn, with post-column derivatization using a photochemical reactor for enhanced detection (PHRED) and a Kobra electrochemical cell system were compared with the pre-column derivatization method using trifluoroacetic acid (TFA). AFs in four different corn matrices were analyzed and validated in terms of the limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, and precision. The LOD and LOQ for the PHRED, Kobra, and TFA methods were 0.004–0.03, 0.01–0.05, and 0.03–0.17 ng/g, respectively, and 0.01–0.10, 0.02–0.14, and 0.11–0.51 ng/g, respectively. Accuracy expressed as average recoveries was 79–110% for PHRED, 70–109% for Kobra, and 77–133% for TFA. In the three derivatization methods, the mean recoveries of AFs were significantly different, at some but not all concentrations, between matrices of dehulled corn and corn with hull ($p < 0.05$). For dehulled yellow corn, the TFA method consistently gave slightly poor recovery values for AFs B1 and G1 than did the PHRED and Kobra methods. The values for the TFA methods were improved by using a modified cleanup procedure. These results indicate that PHRED and Kobra derivatization methods as well as TFA method comply with the analytical requirements for AF analyses in corns.

1. Introduction

Aflatoxins (AFs) are natural secondary metabolites produced by some molds, mainly *Aspergillus flavus* and *Aspergillus parasiticus*. They are contaminants of agricultural commodities, particularly under critical temperature and humidity conditions, before or during harvest, or because of inappropriate storage (Rustom, 1997; Sweeney and Dobson, 1998). AFs B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) can contaminate corn, wheat, rice, groundnuts, pistachios, cottonseed, copra, and spices. Corn (*Zea mays* L.) and its associated by-products are traditional staple foods and feed ingredients for humans and animals. Mycotoxins such as AFs, fumonisins, deoxynivalenol, and zearalenone are frequently detected in corn and corn products. In particular, AF contamination in corn probably generates the most concern since the AFs are potent liver toxins and carcinogens.

National and international institutions and organizations, such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO), and the Food and Agriculture Organization (FAO), have recognized the potential health risks to animals and humans posed by consuming AF contaminated food and feed (Manetta, 2011). To protect human and animal health, many countries have enacted specific regulations for mycotoxins in food and animal feed. In 2013, more than 100 countries worldwide had enacted regulations or detailed guidelines for the control of mycotoxins in food and animal feed. Most of these countries regulate the sum of the levels of the four most prominent types of AFs B1, B2, G1, and G2, or in combination with a specific limit for AFB1 in food. Currently, the European Commission set maximum levels for AFB1 (5.0 mg/kg) and total AFs (10.0 μg/kg) in all cereals and their products including corn (maize) and corn products. In contrast, for corn to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs, maximum levels for AFB1 and total AFs are 5.0 μg/kg and 10.0 μg/kg, respectively (European Commission, 2006a, 2006b).

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South Korea set regulatory limits for AFB1 (10.0 μg/kg) and total AFs (20.0 μg/kg) in all cereals and their products including corn and corn products (MFDS, 2015).

Considering the toxicity of AFs and the maximum acceptable limit set in food and feedstuffs, the analytical identification and quantification of AFs at very low levels must be carried out using reliable methods. The literature includes many reports on AF detection. There are many sensitive and specific methods, but simple and rapid methods are now also available. As new analytical technologies have developed, they have been quickly incorporated into mycotoxin testing strategies. Sometimes reports reflect advances in analytical science, but often modifications of existing methods to improve the analytical process are published.

Because AFs are naturally strongly fluorescent compounds, the high-performance liquid chromatography (HPLC) analysis of these molecules is most often achieved by fluorescence detection. Reversed-phase (RP) eluents quench the fluorescence of AFB1 and AFG1 (Kok, 1994). It is for this reason, to enhance the response of these two analytes, that chemical derivatization is commonly required; use is made of pre- or post-column derivatization with suitable fluorophores to improve detectability. The pre-column approach uses trifluoroacetic acid (TFA) with the formation of the corresponding hemiacetals (Akiyama et al., 2001), which are relatively unstable derivatives.

Post-column derivatization is based on the reaction of the 8,9-double bond of AFs with halogens. Initially, the post-column reaction used iodination (Shepherd and Gilbert, 1984); however, it has several disadvantages, such as peak broadening and the risk of iodine crystallization. An alternative method involves bromination by an electrochemical cell (Kobra cell) with potassium bromide (KBr) dissolved in an acidified mobile phase, or by the addition of bromide or pyridinium hydrobromide perbromide to a mobile phase, and using a short reaction coil at ambient temperature (Stroka et al., 2001; Senyuva and Gilbert, 2005; Brera et al., 2007). The bromination methods offer the advantages of being simple, rapid, and easy to automate, which improve reproducibility and ruggedness and reduce analysis time. A post-column derivatization method that is supposed to be analytically equivalent to iodination and bromination is the photochemical method. It is based on the formation of hemiacetals of AFB1 and AFG1, due to the UV radiation of the HPLC column eluate (Joshua, 1993; Waltking and Wilson, 2006).

In the Food Code published by the Ministry of Food and Drug Safety in Korea (MFDS, 2015), TFA derivatization is the only method adopted as an analytical method for AFs using HPLC. Hence, the aim of the present study was to compare the official Korean pre-column derivatization method using TFA, as well as the widely used reference method in many countries, with two post-column derivatization methods—a photochemical reactor for enhanced detection (PHRED) and a Kobra electrochemical cell system—for the determination of AFs in four different corn matrices. The HPLC methods coupled with fluorescence detection (HPLC-FLD) with immunoaffinity cleanup were validated in terms of the limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, and precision.

2. Materials and methods

2.1. Samples

The types of corn samples analyzed were yellow corn and waxy corn, dehulled yellow corn and dehulled waxy corn, depending on the variety of corn and the processing conditions. A minimum sample size of 1 kg was used. All samples were finely ground using a high-speed blender; a ground sample would pass through a No. 20 sieve. All samples were stored at −18 °C in aluminum zipper bags.

2.2. Materials

A standard stock solution for total AFs was used, specifically, an 'Aflatoxin Mix' from Sigma-Aldrich (St. Louis, MO). This is a 'ready-to-use' product, supplied in a vial. It contains the following: AF 2600 ng/mL (1000 ng/mL of aflatoxin B1 and G1, and 300 ng/mL of aflatoxin B2 and G2). Methanol (MeOH, HPLC grade), acetonitrile (ACN, LC grade), and HPLC-grade water for extraction were purchased from Burdick & Jackson (Muskegon, MI). Sodium chloride (NaCl) and Tween 20 were obtained from Junsei (Tokyo, Japan). KBr, TFA, and nitric acid (HNO_3) were purchased from Sigma-Aldrich. Cellulose filter paper No.4, glass microfiber filters (GF/A and GF/B) and 0.22-μm PVDF syringe filters were obtained from Whatman (Maidstone, UK). AflaTest® WB immunoaffinity columns (IACs) were purchased from VICAM (Watertown, MA).

2.3. Sample preparation

Samples were analyzed using HPLC-FLD, according to the Korean Food Code (MFDS, 2015). Briefly, a 25-g sample was placed in a 250-mL beaker with 100 mL of MeOH:water (70:30, v/v) containing 1% NaCl and then blended for 5 min in a high-speed blender (ULTRA-TURRAX®; IKA Werke, Staufen, Germany). After extraction, the sample was filtered through a filter paper (Whatman No. 4). A 10-mL volume of filtrate extract was then diluted with 30 mL of water containing 1% Tween 20. After filtration through a GF/A filter, 20 mL of the filtrate were passed through an IAC at a flow rate of one drop per second. The IAC was washed with 10 mL of water and dried by rapidly passing air through. The toxins were eluted into screw-cap amber glass vials with 3 mL of ACN for TFA derivatization. The eluate was evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen. The dried residues were derivatized as follows: 200 μL of TFA were added, residues were left to stand for 15 min in a place where they were protected from direct UV light, and then they were diluted with 800 μL of ACN:water (20:80, v/v). The derivatized sample was vortexed for 30 s and then filtered through a 0.22-μm membrane into HPLC vials for autoinjection. The filtered solution (10 μL) was injected into the HPLC. Because AFs are subject to light degradation, all analytical work must be adequately protected from daylight. Therefore, all the procedures were carried out under subdued light and protected from direct UV light. For derivatization using the Kobra cell and the PHRED instrument, the eluate was first dried, redissolved with 1 mL of MeOH:water (1:1, v/v), and then filtered through a 0.22-μm PVDF syringe filter.

2.4. Instrumentation and conditions

2.4.1. TFA derivatization

Chromatographic analysis was performed with an Agilent 1260 Infinity Quaternary LC system (Agilent Technologies, Santa Clara, CA), comprising pumps, an autosampler, a column oven, and a fluorescence detector. AFs were monitored at λ_{ex} = 360 nm and λ_{em} = 450 nm. The RP column of Synergi Hydro-RP (250 mm × 4.6 mm, 4 μm; Phenomenex, Torrance, CA) was operated at 40 °C. The mobile phase comprised ACN:water (25:75, *v/v*) and the flow rate was 1.0 mL/min. The injection volume of sample was 10 μL.

2.4.2. PHRED cell (post-column photochemical derivatization cell)

Chromatographic analysis was performed on an Agilent 1260 Quaternary LC system (Agilent Technologies) with a post-column photochemical derivatization reactor. This reactor (Aura Industries, New York, NY) with a UV lamp (λ = 254 nm) and a knitted