Analytical Methods

A multi-analyte LC–MS/MS method for the analysis of 23 mycotoxins in different sorghum varieties: The forgotten sample matrix

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ABSTRACT

An LC–MS/MS method was developed and validated for the detection and quantification of 23 mycotoxins in different varieties of sorghum. The method performance characteristics were as follows: suitable linearity ranges for all 23 mycotoxins with p-value >0.05; limits of detection (1.2–50 µg/kg), limits of quantification (2.5–100 µg/kg), repeatability (RSDr, 7–22%), intermediate precision (RSDR, 14–44%) and apparent recovery (0.2–11%, expressed as bias).

The method was applied to analyze 10 samples obtained from retail shops in Belgium ($n = 8$) and Germany ($n = 2$). Nine of the 10 samples (90%) were positive for the following mycotoxins: aflatoxin B_1 (50 µg/kg), alternariol monomethyl ether (<LOQ – 79 µg/kg), alternariol (303–357 µg/kg), diacetoxyscirpenol (<LOQ – 91 µg/kg), fumonisin B_1 (<LOQ – 95 µg/kg), fumonisin B_2 (<LOQ), fumonisin B_3 (<LOQ), T2 (<LOQ) and zearalenone (<LOQ).

1. Introduction

Sorghum (*Sorghum bicolor*) is the fifth most important cereal crop in the world, after wheat, rice, maize and barley and the second most important crop (after maize) in sub-Saharan Africa (FAO, 1994). It constitutes the main grain food for over 750 million people who live in semi-arid tropics of Africa, Asia and Latin America (Codex Alimentarius Commission., 2011). It belongs to a genus consisting of about 28 species of grasses but only one species namely *S. bicolor* is cultivated as grain for human consumption and animal feed. This crop has been neglected for some time, being replaced by maize as a staple food commodity in many rural settlements (Bandyopadhyay, Kumar, & Leslie, 2007). However, its rising industrial relevance as a raw material for food and agricultural industries has seen its re-emergence in world markets. As of 2007, sorghum production in Africa increased significantly even to the detriment of rice and wheat production (FAOSTAT, 2010). The renewed focus on sorghum is also because it is one of the most drought tolerant crops and its efficient use of water makes it the crop of choice to boost food security in drought stricken regions.

Nigeria is the world's largest producer of sorghum, followed by India, USA, Ethiopia and Argentina (FAOSTAT, 2012). With regard to production as a continent, sub-Saharan Africa is the largest producer with more than 26 million tonnes produced annually. In many parts of the world, sorghum is used in food items such as porridge, unleavened bread, cookies, cakes, malted beverages and soft drinks. It is anticipated that more white sorghum based products will debut in North America (US Grain Council, 2010). The crop is unique for having significant amounts of tannins which occur in the pigmented testa (Makun, Gbodi, Akanya, Salako, & Ogbadu, 2009). White grain food sorghums do not have tannins but could contain low amounts of phenolic acids. The white/cream/yellow types are the types used mostly (up to 75% in Nigeria) for human food, while the brown/red varieties are mostly used for indigenous beverages and drinks after processing. The cream and yellow grain types are intermediates with low tannin concentrations (contains no testa) in the pericarp (Daiber & Taylor, 1995), while the red and brown varieties are rich in tannins. Polyphenols (phenolic acids, tannins, flavonoids), which are abundant in sorghum, enhance the grain resistance to pests and microbial infestation (Audilakshmi, Stenhouse, Reddy, & Prasad, 1999).

Regardless of its inherent resistance to pests and microbes, fungal contamination constitutes a major biotic constraint to an increase in sorghum production worldwide. It is estimated that annual economic losses in Asia and Africa due to mold infestation are in excess of US $130 million (Chandrashekar, Bandyopadhyay, & Hall, 2000). While *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* species are common natural inhabitants of the semi-arid and

tropical climate of Africa, Asia and Middle East; *Fusarium* species are widespread in Europe and USA with *Alternaria* and *Fusarium* species being frequent in the Oceania region. Mycotoxigenic strains of these fungal species have been isolated from different sorghum varieties (Chandrashekar et al., 2000).

Mycotoxins are toxic fungal secondary metabolites and are common contaminants of food and feed commodities worldwide (Turner, Sabrahmanyam, & Piletsky, 2009). Based on classification of the compounds, over 400 mycotoxins are known today, with the aflatoxins, fumonisins, ochratoxins, zearalenone (ZEA) and the trichothecenes being the most widely investigated, due to their frequent occurrence and their severe adverse effects on human and animal health. In view of the increasing demand for this crop and the threat to its production and utilization by fungi and their toxins, there is need to understand the prevalence of natural contaminants such as mycotoxins on this grain. Unfortunately, there is limited information on mycotoxins in sorghum, which is not commensurate to the escalating economic value of this cereal.

Few analytical methods have been reported (Ayalew, Fehrmann, Lepschy, Beck, & Abate, 2006; Elegbede, 1978; Ghali, Khlifa, Ghorbel, Maaroufi, & Hedilli, 2008; Ghali et al., 2009; Kaaya & Warren, 2005; Makun et al., 2009; Matumba, Monjerezi, Khonga, & Lakudzala, 2011; Salifu, 1978; Sashidhar, Ramakrishna, & Ramesh, 1992) for the detection of mycotoxins in different sorghum varieties. Most of the reported methods were either limited to one or few analytes or the use of less reliable detection technique such as thin layer chromatography (TLC) or enzyme-linked immunosorbent assay (ELISA) (Ghali et al., 2009; Makun et al., 2009; Sashidhar et al., 1992). The first reported mycotoxin contamination was in sorghum in samples originating from Nigeria (Elegbede, 1978; Salifu, 1978). In Asia, the first study was reported in India (Sashidhar et al., 1992).

Because of the paucity of information on the occurrence of mycotoxin in sorghum samples, in March 2011, the Codex Committee on Contaminants in Foods (The Hague, NL) opened a discussion paper on "mycotoxin in sorghum grain". One of the agreed resolutions was to set up a Codex Trust Fund with the objective of providing information (data) on mycotoxin contamination in sorghum from four sub-Saharan African (pilot) countries. To generate such data, a multi-mycotoxin liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the determination of 23 mycotoxins in different varieties of sorghum was developed and validated according to the guidelines specified in Commission Decision 2002/657/EC (EC, 2002a) and Commission Regulation 401/2006/EC (EC, 2006b).

2. Materials and methods

2.1. Standards

Standards namely deoxynivalenol (DON), deepoxy-deoxynivalenol (DOM), zearalenone (ZEA), aflatoxin B_1 (AFB_1), aflatoxin B2 (AFB_2), aflatoxin G_1 (AFG_1), T2-toxin (T2), aflatoxin G_2 (AFG_2), alternariol (AOH), ochratoxin A (OTA), ZEA, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), sterigmatocystin (STE), were purchased from Oskar Tropitzsch (Marktredwitz, Germany). 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), neosolaniol (NEO), alternariol methyl ether (AME), Altenuene (ALT) and roquefortine C (ROQ C) were purchased from Biopure (Tulln, Austria). Fumonisin B_3 (FB_3) was obtained from Promec Unit (Tygerberg, South Africa). Nivalenol (NIV), HT2 toxin (HT2) and fusarenon-X (FUS-X) were purchased from Fermentek (Jerusalem, Israel). Diacetoxyscirpenol (DAS) and zearalanone (ZAN) were obtained from Sigma Aldrich (Bornem, Belgium). NIV and NEO were obtained as solutions (100 µg/mL) in acetonitrile. FB_2 and FB_3

standards (1 mg) were prepared in 1 mL acetonitrile/water (50/50, v/v). Stock solutions of DON, 3-AcDON, 15-AcDON, FUS-X, AFB_1, AFB_1, AFG_1, AFG_2, HT2, T2, ALT, OTA, ZEA, FB_1, STE, FB_2, AOH, AME, ZAN and ROQ C were prepared in methanol at a concentration of 1 mg/mL. All stock solutions were stored at −18 °C except FB_2 and FB_3, which were stored at 4 °C. From the individual stock standard solutions, a standard mixture was prepared at the following concentrations: AFG_1, AFB_2, DAS, OTA and STE (1 ng/µL); AFG_2, AFB_1 and T2 (2 ng/µL); AME, NEO, ROQ C, HT2, ZEA (2.5 ng/µL); ALT and FB_1 (2.5 ng/µL); DON, FB_2 and FB_3 (10 ng/µL); 3-AcDON (12.5 ng/µL); FUS-X, 15-AcDON (25 ng/µL); NIV (40 ng/µL). The standard mixtures were prepared in methanol, stored at −18 °C and renewed every 3 months.

2.2. Reagents and materials

Acetic acid (LC–MS/MS grade) was supplied by Merck (Darmstadt, Germany). GracePure aminopropyl (NH_2) solid phase extraction (SPE) cartridges were obtained from Grace Discovery Sciences (Lokeren, Belgium). LC–MS grade methanol, HPLC grade methanol and n-hexane were purchased from VWR International (Zaventem, Belgium). Dichloromethane and ethyl acetate were purchased from Acros Organics (Geel, Belgium). Ammonium acetate was supplied by Grauwmeer (Leuven, Belgium). Ultrafree-MC centrifugal filter devices (0.22 µm) of Millipore (Millipore, Brussels, Belgium) were used. Water was purified on a Milli-Q Plus apparatus (Millipore, Brussels, Belgium).

2.3. Samples

A total of 10 samples were obtained from retailers in Belgium and Germany. While the samples from Belgium ($n = 8$) were of African origin, the samples from Germany ($n = 2$) were cultivated in Europe and all comprised of red sorghum samples. Method optimization and validation were performed using sample material that was tested free from the 23 target mycotoxins. The absence/presence of the target analytes in the tested samples were confirmed by comparing with a matrix matched calibration sample. The method was initially developed and validated for the red sorghum variety and then cross-validated for the white and other sorghum varieties.

2.4. Sample preparation protocol

All samples were milled to between 0.5 and 1 mm particle size. Homogenized samples were accurately weighed (3 g) and fortified with internal standard ZAN and DOM at 50 and 333 µg/kg respectively and left to equilibrate in the dark for 15 min. Extraction was carried out with 20 mL of methanol/ethyl acetate/water (70/20/10, v/v/v) for 40 min on an end-over-end shaker (Exacta, Delhi, India); followed by centrifugation for 15 min at 3170g. The supernatant was transferred into a new tube. Defatting was performed by adding 10 mL of n-hexane to the supernatant and the solution was agitated for 15 min on an end-over-end shaker. The hexane (upper) phase was discarded while the lower phase was subjected to a solid phase extraction (SPE). The next steps in sample preparation consisted of sample clean-up using amino SPE cartridges. Because the fumonisins (FB_1, FB_2 and FB_3) and ochratoxin A (both with carboxylic acid functional groups) binds strongly to the resin of the amino SPE cartridges, the defatted extracted was split into two parts and subjected to different clean-up procedures. A 5 mL portion of the defatted extract was transferred to a clean glass tube while the rest of the extract (approximately 12.5 mL) was later subjected to SPE step. From the 5 mL portion, 2.5 mL was added to a 10 mL solution of dichloromethane/formic acid (95/5, v/v), vortexed and centrifuged at 4000g for 10 min. Meanwhile the