Journal of Chromatography B, 963 (2014) 75–82

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous determination of major type B trichothecenes and deoxynivalenol-3-glucoside in animal feed and raw materials using improved DSPE combined with LC-MS/MS

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ARTICLE INFO

Article history:
Received 19 March 2014
Received in revised form 26 May 2014
Accepted 27 May 2014
Available online 4 June 2014

Keywords:
Mycotoxin
Deoxynivalenol-3-glucoside
Type B trichothecenes
Animal feed
LC-MS/MS
Simultaneous determination

ABSTRACT

A simple and reliable method for simultaneous determination of deoxynivalenol-3-glucoside and major type B trichothecenes (deoxynivalenol, nivalenol, fusarenon X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol and deepoxy-deoxynivalenol) in animal feed and raw materials has been developed and validated in this study. The method was based on an improved dispersive solid-phase extraction (DSPE) followed by analysis using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Also, matrix-matched calibration curve ($R^2 > 0.99$) was employed to minimize matrix effects and ensure accurate quantification. The recoveries during sample preparation process (including extraction and clean-up) ranged from 79.03% to 118.39%, with intra-day and inter-day relative standard deviation lower than 20% for all the analytes. The limit of quantification ranged from 5.0 μg/kg for deoxynivalenol to 13.6 μg/kg for fusarenon X. The validated method was successfully applied to the analysis of animal feed and corn. The pilot study showed that 37 out of 41 samples were contaminated with deoxynivalenol-3-glucoside at the levels of 6.0–121.0 μg/kg. Most of the type B trichothecenes were also found with the exception of fusarenon X, at the contaminated levels of 10.0–1382 μg/kg. To the best of our knowledge, this was the first scientific report on the co-occurrence of masked deoxynivalenol and type B trichothecenes in animal feed and raw materials.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by various fungal species, which can easily contaminate many cereal grains and the derived animal feed during cultivation or storage. Therefore, the co-occurrence of mycotoxins has aroused increasingly great attention worldwide in recent decades [1]. Among these toxic compounds, type B trichothecenes, mainly produced by *Fusarium* species such as *F. graminearum* and *F. culmorum*, are regarded as the most prevalent mycotoxin family [2]. So far, the best known and most frequently found compounds in this mycotoxin family are deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON) and fusarenon X (FUSX) (Fig. 1) [3].

DON is the most common trichothecene mycotoxin in cereal grains at global level. It can cause many adverse health effects on animals, such as feed refusal, diarrhea, emesis, decreased body weight gain, haematological disorders and immunosuppression [4,5]. DON is also regarded as the most important causative toxicant responsible for human red mould disease [6]. On the molecular levels, it can inhibit the synthesis of DNA, RNA and proteins. Both ADONs (3ADON and 15ADON) are the derivates of DON, with the hydroxyl at the C-3 or C-15 position being acetylated. ADONs possess equivalent or much stronger toxicity to animals than DON and can be rapidly deacetylated to DON in the digestive tract of organisms [5]. NIV and FUSX are also widely found along with DON in cereals. Although there is no sufficient toxicity data for NIV and FUSX, a few studies showed that some minor pathological changes could be observed in hens fed with 1 mg NIV/kg feed while no effects in chicken fed with 5 mg DON/kg feed [7]. In

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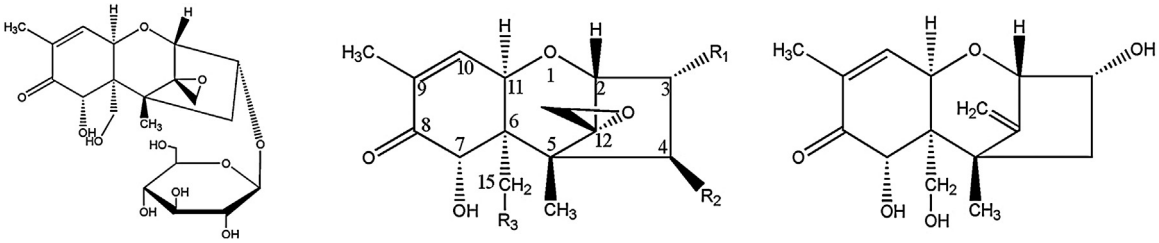
http://dx.doi.org/10.1016/j.jchromb.2014.05.053
1570-0232/



Type B trichothecenes	R_1	R_2	R_3
deoxynivalenol	-OH	-H	-OH
3-acetyldeoxynivalenol	-OAc	-H	-OH
15-acetyldeoxynivalenol	-OH	-H	-OAc
nivalenol	-OH	-OH	-OH
fusarenon X	-OH	-OAc	-OH

Fig. 1. Chemical structures of deoxynivalenol-3-glucoside and major type B trichothecenes.

addition, deepoxy-deoxynivalenol (DOM) is the major metabolism of DON *in vivo* and thus supposed to be not existed in naturally contaminated cereal grains. However, considering that some animal products containing DOM may be added into feed as the source of proteins, DOM is also co-analyzed in the present study.

In general, living plant can conjugate mycotoxins to certain functional groups such as sugars, amino acids and sulfate as part of their defense against xenobiotics. These bound compounds generally escape routine analysis, and are so-called masked mycotoxins [8]. Deoxynivalenol-3-glucoside (D3G) is the most famous masked mycotoxin arising from the conjugation of DON to glucose. This compound has been detected in various cereal crops as well as barley-based products like malt and beer, with the contaminated levels even exceeding those of free DON [9–12]. In addition, it is demonstrated that *Fusarium* head blight (FHB) have a close relationship with D3G/DON ratio. With the frequent outbreaks of FHB in recent decades, the D3G concentration may increase in future as consequence of plant's self-protecting detoxification process [13]. The toxicological data of D3G on animals is still scarce, but some evidences indicated that D3G could be *in vivo* transferred into DON by human lactic acid bacteria and reactivated the toxicity of DON [12,14]. Accordingly, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has regarded D3G as a potential source to dietary DON exposure [15].

To avoid the potential health risks caused by the intake of *Fusarium* toxins, the European Commission set the guidance values for DON at 900 μg/kg in complementary and complete feeding stuffs for pigs, and 8000 μg/kg in feed ingredients (cereals and cereal products) [1]. In China, the maximum limits for DON in swine and poultry feed are set at 1000 μg/kg and 5000 μg/kg, respectively [16]. Considering the toxicity and metabolism of both ADONs, the JECFA has set the provisional maximum tolerable daily intake (PMTDI) of 1 μg/kg body weight for total DON and ADONs [17]. No regulatory limits for FUSX, NIV and D3G have been established in animal feed and raw materials due to the insufficient survey and toxicological data.

To more accurately and legally monitor the co-occurrence of these mycotoxins in animal feed and raw materials, robust and sensitive analytical method is urgently needed. So far, several analytical methods using LC-MS/MS have been developed for the determination of D3G and major type B trichothecenes in cereal and derived food [11,18–20]. Most of these methods used solid–liquid extraction, followed by clean-up with various cartridges including solid-phase extraction (SPE) column, multifunctional clean-up column or immuno-affinity column (IAC). However, these approaches were usually time-consuming and expensive which limited their extensive application, especially when large amounts of samples had to be analyzed. More importantly, the recoveries of D3G were too low (50–70%) as reported in these studies. The aim of this study is to develop a simple sample treatment procedure based on improved dispersive solid-phase extraction for simultaneous determination of D3G and major type B trichothecenes in animal feed and raw materials. With the merits of high-throughput, desirable sensitivity and recovery, the developed method was successfully applied to the analysis of swine feed, poultry feed and corn.

2. Experimental

2.1. Chemicals and standards

The solid standards of DON, 15ADON, 3ADON, NIV, FUSX and certified calibration solution of DOM (50 μg/mL) were purchased from Sigma–Aldrich (St. Louis, MO, USA). D3G (50 μg/mL) and [$^{13}C_{15}$]-DON as internal standard (25 μg/mL) were obtained from Biopure (Tulln, Austria). Sodium chloride, anhydrous magnesium, sulfate ammonium acetate and acetic acid (AA) were supplied by Aladdin (Shanghai, China). The absorbents including primary secondary amine (PSA), graphitized carbon black (GCB), cleanert silica and C18 were obtained from Agela (Tianjin, China). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were provided by Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was obtained from Millipore (Bedford, MA, USA). All other chemicals and solvents used were of analytical grade.

2.2. Preparation of standard solutions

The stock standard solutions of DON, 15ADON, 3ADON, NIV and FUSX were separately prepared by dissolving solid portions in ACN at 100 μg/mL. Whereas for DOM, D3G and [$^{13}C_{15}$]-DON, the certified calibration solutions were used as stock standard solutions. All stock standard solutions in amber glass vial were stored at −20 °C except [$^{13}C_{15}$]-DON, which was stored at 4 °C. From the individual stock standard solutions, a combined working solution except