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Co-isolation of deoxynivalenol and zearalenone with sol–gel immunoaffinity columns for their determination in wheat and wheat products

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ABSTRACT

The paper describes a sample clean-up method for the co-isolation of deoxynivalenol (DON) and zearalenone (ZON), two mycotoxins naturally co-occurring in wheat. The method is based on immunoaffinity columns prepared by co-immobilising anti-DON and anti-ZON antibodies in a porous sol–gel glass. The main task in developing the method consisted in finding a loading medium allowing retention of both analytes as well as a common elution medium for the dissociation of both antigen–antibody complexes formed. This can be achieved by co-extracting DON and ZON with ACN–water (60:40, v/v), reducing the acetonitril concentration to 2.5% before loading an aliquot of the diluted sample extract onto the DON/ZON column.The columns are washed with 5 ml of MeOH–water (10:90, v/v) before DON and ZON are co-eluted with 4 ml of ACN–water (50:50, v/v). Concentrations of DON and ZON are determined with HPLC-UV and HPLC-fluorescence detection, respectively. The sample clean-up method was shown to be applicable to wheat and wheat products, e.g., cornflakes, milk wheat mash and rusk. Spiking experiments (spike level 500 μg DON/kg and 50 μg ZON/kg) resulted in recovery rates from 82% to 111%.

1. Introduction

In a recent survey on the incidence of mycotoxins in feed and feed raw materials more than 50% of the samples derived from European countries were found to contain mycotoxins [1]. The most frequently detected mycotoxins were deoxynivalenol (DON), zearalenone (ZON) and T-2 toxin [1]. DON and ZON are predominantly produced by the *Fusarium* species *F. graminearum* and *F. culmorum* [2].

DON belongs to the so-called trichothecenes, a large group of structurally related mycotoxins with a sesquiterpenoid structure. DON is known to be harmful to both humans and animals by inhibiting the synthesis of DNA, RNA and proteins [3,4]. In vivo studies have shown that low doses of DON cause vomiting, diarrhoea and gastroenteritis whereas higher doses severely damage lymphoid and epithelial cells of the gastrointestinal mucosa, resulting in hemorrhage and endotoxemia [5]. Consumption of DON contaminated feed leads to reduced feed intake, feed refusal and growth retardation of the animals, causing tremendous economic losses worldwide. All animal species investigated so far are susceptible to DON, pigs being the most sensitive one [6].

20 years ago Tanaka et al. already pointed at the worldwide contamination of agricultural products with Fusarium mycotoxins [7]. A recent study carried out to assess the dietary intake of Fusarium toxins by the population of the European Union reported that wheat and wheat containing products, e.g., bread and pasta, are the major source of intake for DON [8]. In contrast to animals the toxic effects of DON on humans are, however, not well-known [9].

Since DON and ZON are produced by the same *Fusarium* species these two mycotoxins frequently co-occur in grain. In contrast to DON, the acute toxicity of ZON, a resorcylic acid lactone, is very low. ZON is, however, known to cause estrogenic effects in humans and animals by binding to the natural estrogen receptor [10,11]. Consumption of feed contaminated with ZON causes reproductive disorders in mammals, e.g., alteration of the morphology of the uterus and infertility [11]. In male pigs, ZON has been reported to decrease the serum testosterone level, the weight of testes and spermatogenesis [10]. ZON is frequently detected in maize, barley and wheat and products thereof [7].

In order to protect the health of humans and animals, many countries have established regulations for DON and ZON, e.g.,

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maximum limits in food and feed as well as tolerable daily intake (TDI) values for humans. Highly selective and sensitive analytical methods have to be applied to ensure that DON and ZON concentrations comply with the legal regulations. In the last years several review articles discussed the applicability of analytical methods to determine mycotoxins in complex matrices such as food, feed or biological samples, covering either trichothecenes [12,13], Fusarium toxins [14] or mycotoxins in general [15–20].

The methods developed so far can be divided into screening methods and methods enabling the accurate quantification of mycotoxins. Immunoassays play the most important role among the screening methods. Several immunoassay based methods have already been developed to determine DON [21–23] or ZON [24,25] in food and feed.

Quantitative determination of DON is frequently carried out by gas chromatography with either electron capture [26,27] or mass spectrometric detection [28,29] or high performance liquid chromatography (HPLC) with either ultraviolet (UV) detection [30,31], fluorescence detection after post-column derivatisation [32–34] or mass spectrometry (MS) detection [35–38]. For the determination of ZON, the most important analytical methods are HPLC with fluorescence [39,40] or MS [38,41] detection.

Sample pre-treatment plays an important role in trace analysis, in spite of the use of selective separation and sensitive detection methods. Recent papers, however, report on the simultaneous determination of 33 [42], 39 [43] or even 87 [44] mycotoxins and mycotoxin metabolites in one run after only very simple sample pre-treatment steps – after initially extracting the analytes aliquots from the unpurified diluted extracts are injected into the LC–MS/MS system. These methods make maximal use of the extreme selectivity of the MS/MS system but their simplicity is achieved by a compromise with their sensitivity. Because of the different structures of the mycotoxin classes the extraction conditions cannot be optimal for each of the mycotoxins. Incomplete extraction of some analytes and/or matrix effects caused by co-eluting compounds [44] hamper the application of so-called "dilute-and-shoot" methods for the accurate quantification of each of the analytes.

Due to its high molecular selectivity, immunoaffinity chromatography is one of the most frequently applied clean-up methods in mycotoxin analysis. A number of papers have demonstrated that immunoaffinity columns prepared by entrapping the antibodies in porous sol–gel glass have several advantages compared to columns prepared by covalently binding the antibodies to a solid support material [45–59]. The potential of sol–gel bioaffinity columns for sample clean-up was recently addressed in a review article [60].

In one of our recent studies we prepared immunoaffinity columns by entrapping anti-DON antibodies by the sol–gel method and investigated their applicability to the clean-up of food and feed samples [61]. The sol–gel immunoaffinity columns prepared by our group proved to be as selective as commercial DON immunoaffinity columns prepared by covalently binding the antibodies to the solid support material but were superior with regard to production costs, storage stability and reusability.

To our knowledge, the potential of sol–gel immunoaffinity columns prepared by co-entrapping antibodies raised against different analytes has not been investigated so far.

The present paper describes the preparation of immunoaffinity columns for the simultaneous isolation of DON and ZON by co-immobilising anti-DON and anti-ZON antibodies in a porous sol–gel glass. We demonstrate the potential of the co-immobilisation of several antibodies in sol–gel glasses by applying these columns in the development of a clean-up procedure for both co-occurring mycotoxins in wheat and wheat products.

2. Experimental

2.1. Reagents and materials

Purified monoclonal anti-DON (1.0 mg/ml phosphate-buffered saline (PBS)) and purified monoclonal anti-ZON antibodies (8.3 mg/ml PBS) were provided by Zhongming Zheng (Department of Chemical and Environmental Engineering, National University of Singapore, Singapore). Rabbit immunoglobulin G (IgG), deoxynivalenol (DON), zearalenone (ZON) and a standard solution containing ZON, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) were obtained from Sigma (Vienna, Austria). A certified DON standard solution (100.8 μg/ml in acetonitrile (ACN)) was obtained from Biopure (Tulln, Austria). ACN and methanol (MeOH), both gradient grade for HPLC, were purchased from Fisher Scientific (Leicestershire, UK). Tetramethoxysilane (TMOS) was from Fluka (Buchs, Switzerland). Wheat samples were obtained from the Austrian Agency for Health and Food Safety. Food products were purchased in local food stores.

2.2. Instrumentation

Three different HPLC systems were used in the present study. HPLC systems 1 and 2 were used to determine DON and ZON, respectively, HPLC system 3 was applied to verify the identity of DON and ZON.

HPLC system 1 consisted of a high pressure gradient pump (Model L-7100, Merck, Darmstadt, Germany), a column thermostat (Model bfo-04 dt, W.O. electronics, Langenzersdorf, Austria) and a six-port injection valve (Model 7161, Rheodyne) equipped with a 100 μl stainless steel injection loop. DON was detected with a UV detector (Model L-4200, Merck) at 220 nm. Peaks were integrated using the McDacq software (Bischoff, Leonberg, Germany).

HPLC system 2 was composed of a high pressure gradient pump (Model L-6200, Merck), a column thermostat (Model 655 A–52, Merck) and a six-port injection valve (Model 7161, Rheodyne) equipped with a 100 μl stainless steel injection loop. ZON was detected with a fluorescence detector (Model F-1080, Merck) at 330/460 nm. Peaks were integrated using the Stratos version 3.0 software (Polymer Laboratories, Darmstadt, Germany).

HPLC system 3 consisted of a HP 1100 gradient pump, a HP 1100 autosampler (Agilent, Vienna, Austria) and a UV-detector (Agilent) set at 220 nm. A HCT plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) with an electrospray interface was used for detection in the negative mode. The selected temperature for the heated capillary was 300 °C. The dry-gas flow was set at 10 L/min. The parent ions of DON and ZON $[M-H]^-$ of 295 and 317 m/z, were used in MS/MS studies. The mass range in scan mode was 100–400 m/z, the scan speed was 8100 $m/z\,s^{-1}$. Pure nitrogen as nebulising and carrier gas was produced in a Nitrox Dominick hunter N_2-generator, UHPLCMS 18 (Alltech, Deerfield, Ireland).

Centrifugation was carried out with a Sigma centrifuge (Model 4K 10, Vienna, Austria).

2.3. Standard solutions and buffers

Stock solutions of DON and ZON were prepared by dissolving 10.0 mg of either DON or ZON in 10.0 ml of ACN. Working solutions of DON and ZON were prepared by diluting the stock solutions with bidistilled water or PBS. Stock solutions were stored at −20 °C, working solutions at 4 °C. PBS, pH 7.6, was prepared by dissolving 12.46 g $Na_2HPO_4.2H_2O$, 1.56 g $NaH_2PO_4.2H_2O$ and 8.5 g NaCl in 1 L bidistilled water.