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Determination of deoxynivalenol in organic and conventional food and feed by sol–gel immunoaffinity chromatography and HPLC–UV detection☆

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ABSTRACT

The paper describes the determination of deoxynivalenol (DON) in 55 wheat food and feed samples, 26 from conventional and 29 from organic production. Immunoaffinity columns prepared by entrapping anti-DON antibodies by the sol–gel method were used for sample clean-up. DON was quantified by high performance liquid chromatography (HPLC) and ultraviolet (UV) detection. In general, the incidence of DON contamination was rather low. In eight samples (14.5%) the DON concentration was above the LOQ (380 ng/g), in six samples (10.9%) DON was detected but could not be quantified (>LOD (200 ng/g), <LOQ). In seven conventional samples (two pasta, two cookie, two snack and one feed sample) but only in one organic sample (a snack) the DON concentration was >LOQ. The data indicate both a higher incidence of DON contamination and higher DON concentrations in food and feed samples from conventional than in those from organic production.

1. Introduction

Deoxynivalenol (DON) is one of the most frequently detected mycotoxin contaminants in wheat and wheat products from northern temperate regions, i.e. Europe and North America. It is a secondary metabolite produced by fungi from the *Fusarium* genus. *Fusarium graminearum* and *Fusarium culmorum* are the most important *Fusarium* ear blight pathogens of wheat, producing higher levels of DON than other *Fusarium* species [1]. DON belongs to the trichothecenes, which are esters of sesquiterpenoid alcohols containing a tricyclic ring system. DON is known to bind to eukaryotic ribosomes, inhibiting protein synthesis and thus leading to a variety of toxic effects [2]. In animals, acute exposure to high DON doses causes vomiting, diarrhea and gastrointestinal hemorrhage, with chronic exposure leading to reduced weight gain and anorexia [3]. So far, the toxic effects of DON on humans are not well-known, but epidemiological studies showed that the symptoms in humans are similar to those observed in animals. *In vitro* studies with human cells indicate that DON may also affect the human immune system [4]. In order to reduce the exposure of humans and animals, the European Commission has set maximum levels for DON in food and feed [5].

Several studies have already been carried out to investigate the parameters which influence the infection of grain with *Fusarium* species. High humidity at crop flowering is known to favour the growth of *Fusarium* species [6]. Appropriate field management (i.e. crop rotation, removal of residual fungal material) is a recognized mean to control DON contamination [7]. Some fungicides applied in conventional type of cultivation are known to be effective against *Fusarium* species [7]. The influence of different agricultural practices on the contamination of food and feed with DON is, however, still controversial. Some papers report higher incidence and/or higher DON concentrations in food/feed from organic production, whereas other researchers published opposing results [8–13].

Numerous analytical methods have already been developed for the determination of DON in food and feed, gas chromatography with electron-capture [14,15] or mass-spectrometry (MS) detection [8,12,16,17] and high performance liquid chromatography with ultraviolet (UV) [11,18] or MS detection [19–21] being the most common ones. Recent review articles focus on the determination of mycotoxins by LC–MS [22,23] or deal with mycotoxin analysis in general [24,25].

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In spite of the use of selective separation and sensitive detection methods, the complexity of food and feed matrices makes it necessary to apply selective sample pre-treatment steps. Solid-phase extraction with charcoal/alumina columns is frequently used to purify food and feed extracts containing DON [8,19,20,26]. By taking advantage of the selectivity of the interactions between antigens and antibodies, immunoaffinity chromatography is one of the most efficient sample pre-treatment methods to remove interfering matrix compounds and simultaneously enrich the analyte.

Commercial DON immunoaffinity columns, prepared by covalently binding the antibody to a solid support material, are commonly used for sample clean-up in DON analysis [11,18,26,27]. In the present paper, however, we used a different type of immunoaffinity columns developed in our laboratory. These sol–gel immunoaffinity columns offer several advantages compared to columns prepared by other immobilization techniques. A recent review discusses important aspects of sol–gel immunoaffinity columns, such as selectivity, binding capacity, column-to-column reproducibility, stability and re-usability [28]. Several papers have already demonstrated the applicability of sol–gel columns in the clean-up of food and feed samples [29–32]. One of our papers describes the characterization of sol–gel DON immunoaffinity columns and their applicability to the determination of DON in food and feed [33]. Recently, we demonstrated the applicability of sol–gel immunoaffinity columns containing both anti-DON and anti-zearalenone (ZON) antibodies to the co-isolation of both mycotoxins from wheat and wheat products [34].

The aim of the present paper was to determine DON concentrations in food and feedstuffs based on wheat to compare the influence of conventional and organic production. The analytical method included sample clean-up with sol–gel DON immunoaffinity columns and quantification of DON by HPLC–UV detection.

2. Experimental

2.1. Reagents and materials

Purified monoclonal anti-DON antibodies (1 mg/ml phosphate-buffered saline (PBS)) were provided by Zhongming Zheng (Department of Chemical and Environmental Engineering, National University of Singapore, Singapore). Deoxynivalenol (DON) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) and methanol (MeOH), both gradient grade for HPLC, were purchased from Fisher Scientific (Leicestershire, UK). Tetramethoxysilane (TMOS) was from Fluka (Buchs, Switzerland). Food products were bought in local supermarkets. Feed samples were collected from several feed mills. Ground and sealed samples were stored at 4 °C.

2.2. Standard solutions and buffers

A stock solution of DON was prepared by dissolving 10.0 mg of DON in 10.0 ml ACN. The actual concentration of the solution was determined using a spectrophotometer set at 217 nm (ε = 6825 l mol^{-1} cm^{-1}). Working solutions of DON were prepared by diluting the stock solution with bidistilled water. The DON stock solution was stored at −20 °C, working solutions at 4 °C. Phosphate-buffered saline (PBS), pH 7.6, was prepared by dissolving 12.46 g $Na_2HPO_4 \cdot 2H_2O$, 1.56 g $NaH_2PO_4 \cdot 2H_2O$ and 8.5 g NaCl in 1 l bidistilled water.

2.3. Instrumentation

Food and feed samples were ground in a mechanical mortar (type MM 2000, Retsch, Haan, Germany). For centrifugation, a Sigma centrifuge (Model 4K 10, Vienna, Austria) and an Eppendorf centrifuge (Model 5424, Hamburg, Germany) were used.

Two HPLC systems were used, HPLC system 1 to determine DON concentrations in food and feed samples, HPLC system 2 to verify the identity of DON. HPLC system 1 consisted of a high pressure gradient pump (Model L-7100, Merck), 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 a HP Series 1100 (Agilent, Darmstadt, Germany), consisting of an in-line degasser (G1379A), a binary pump (G1312A), an autosampler (G1367A, WP) and a UV detector (G1314A, VWD). The HPLC system was connected with a HCT esquire mass spectrometer (Bruker Daltonics, Bremen, Germany) using an electrospray (ESI) interface in negative mode. Ionisation was accomplished at 300 °C. The nebulizer gas was set at 30 psi and the dry gas at 10 l/min. The capillary voltage was set at 4 kV. Pure nitrogen (as nebulizing gas) was produced with a Parker Balston generator from Tewksbury (MA, USA).

2.4. HPLC phase systems

In HPLC system 1, a Phenomenex, Synergi 4u Polar-RP, 80 Å, 250 mm × 4.6 mm i.d., 4 μm (Phenomenex, Aschaffenburg, Germany) was used as analytical column. The mobile phase consisted of bidistilled water–ACN–MeOH (80:10:10, v/v/v). All separations were carried out at 25 °C applying a flow-rate of 1 ml/min.

In HPLC system 2, an ACE 3 C18, 150 mm × 2.1 mm i.d., 3 μm (Advance Chromatography Technologies, Aberdeen, Scotland) was used as analytical column and a Polar-RP column, 4 mm × 3.0 mm i.d. (Phenomenex), as guard column. The mobile phase consisted of MilliQ water–ACN–MeOH (85:5:10, v/v/v), the flow-rate was 0.2 mL/min. The injection volume was 30 μl.

2.5. External calibration

HPLC systems 1 and 2 were calibrated by injecting six standard solutions in the concentration range from 30 to 1000 ng/ml. The analysis function was obtained by linear regression of peak areas on standard concentrations.

2.6. Preparation of sol–gel immunoaffinity columns

The anti-DON antibody solution (1 mg/ml) was enriched (to 8 mg/ml) by centrifugation through an Amicon Ultra-4 ultrafiltration unit (Millipore, MA, USA). Sol–gel immunoaffinity columns were prepared by entrapping 4 mg of the monoclonal anti-DON antibody in the pores of 0.5 g silicate glass according to a previously described protocol [33]. Columns containing pure sol–gel glass were prepared without adding antibodies.

2.7. Extraction of DON

Fifty-five wheat products (26 from conventional and 29 from organic production; 40 food and 15 feed samples) were analysed in the present study (see Table 1). The 40 foodstuffs consisted of 12 pasta, 13 cookie and 15 snack samples.

Snacks, cookies, chocolate and cacao were ground in a mortar. Pasta samples were first ground in a mortar and then in a mechanical mortar for 40 min. Feed samples were ground in a kitchen blender to mill the grain into grist and afterwards minced in a coffee grinder. Wheat flour was sieved through a sieve with a pore diameter of 1 mm.

7.5 g of the ground sample was weighed and mixed with 60 ml of bidistilled water. After stirring the suspension for 10 min, a 40 ml aliquot was centrifuged for 10 min in the Sigma centrifuge at 2800 × *g* for 10 min. In the case of pasta, the supernatant was fil-

Table 1
Occurrence of DON in conventional and organic food and feed ($n = 3$). LOD: 200 ng/g, LOQ: 380 ng/g.

Conventional farming		Organic farming	
Sample	Mean ± SD (ng/g)	Sample	Mean ± SD (ng/g)
Pasta			
1	n.d.	27	n.d.
2	525 ± 12	28	n.d.
3	n.d.	29	n.d.
4	n.d.	30	n.d.
5	472 ± 7	31	n.d.
		32	n.d.
		33	n.d.
Cookie			
6	625 ± 31	34	n.d.
7	n.d.	35	n.d.
8	<LOQ	36	n.d.
9	n.d.	37	n.d.
10	595 ± 47	38	n.d.
11	n.d.	39	n.d.
		40	n.d.
Snack			
12	492 ± 37	41	<LOQ
13	540 ± 78	42	n.d.
14	n.d.	43	<LOQ
15	n.d.	44	436 ± 59
16	n.d.	45	n.d.
17	n.d.		
18	n.d.		
19	n.d.		
20	<LOQ		
21	n.d.		
Feed			
22	n.d.	46	n.d.
23	n.d.	47	<LOQ
24	n.d.	48	n.d.
25	n.d.	49	n.d.
26	498 ± 36	50	n.d.
		51	n.d.
		52	n.d.
		53	n.d.
		54	n.d.
		55	<LOQ

n.d.: below LOD.

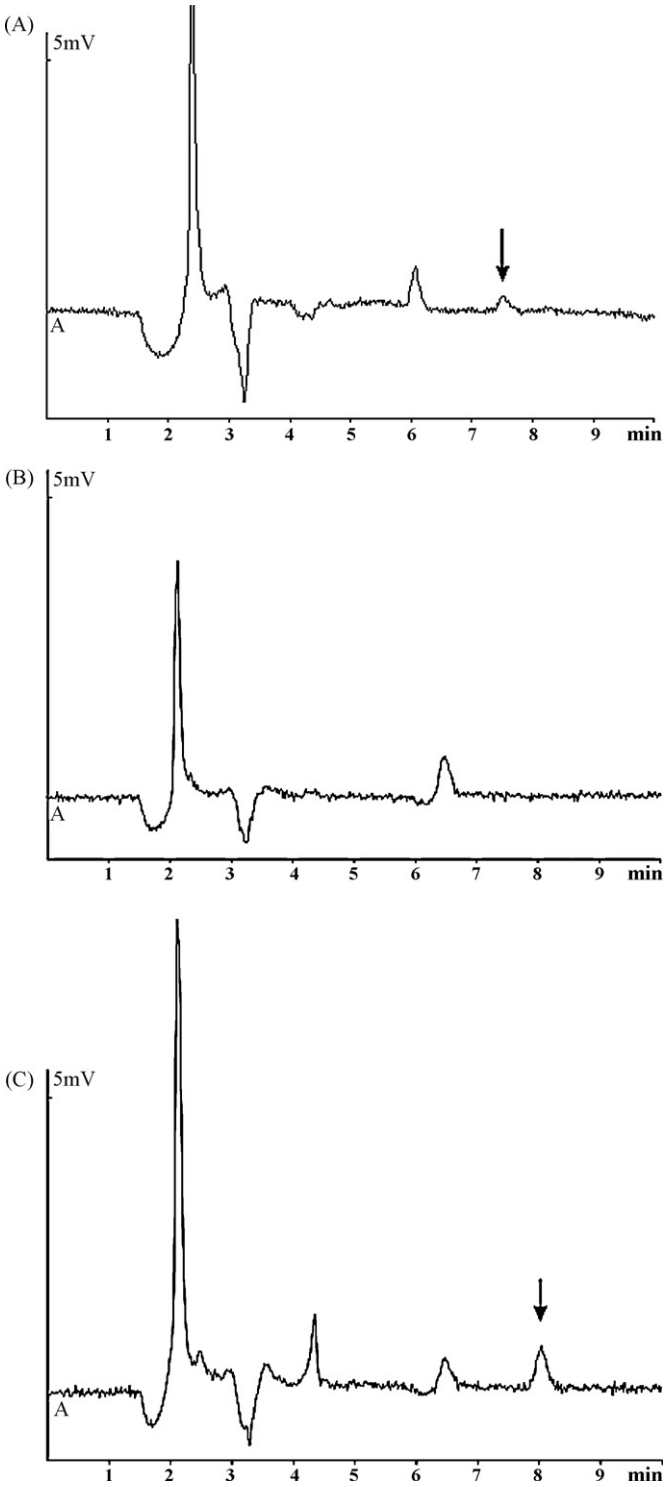

Fig. 1. Chromatograms obtained by injecting aliquots of purified extracts from food and feed samples into HPLC system 1. (A) Conventional pasta (sample 2), (B) organic feed (sample 53) and (C) organic feed (sample 53) spiked with 1004 ng DON/g sample.

tered through a borosilicate frit (porosity 5), in the case of other samples, the supernatant was first filtered through a black and then through a blue ribbon filter paper (Whatman GmbH, Dassel, Germany). In processing cookies, chocolate and cacao, the filtrates were then centrifuged in the Eppendorf centrifuge at 10,000 rpm for 10 min in order to avoid clogging of the immunoaffinity column.

2.8. Sample preparation with sol–gel immunoaffinity columns

A 5 ml aliquot of the extract was applied to the immunoaffinity column. After washing the column with 10 ml of MeOH–water (10:90, v/v) and 20 ml bidistilled water, elution was carried out with 4 ml of ACN–water (40:60, v/v). The eluate was collected in a 5 ml measuring flask. After evaporating ACN under a slight nitrogen stream the measuring flask was filled up to the ring mark with water. The column was regenerated with 20 ml of PBS. Sol–gel immunoaffinity columns were re-used up to 13 times.

3. Results and discussion

3.1. Selectivity of the sol–gel DON immunoaffinity columns

Fig. 1 shows representative chromatograms obtained by injecting aliquots of purified extracts from food and feed samples into the HPLC–UV system (HPLC system 1). The food sample from conventional production (Fig. 1A) was found to be contaminated with DON, whereas DON could not be detected in the organic feed sample (Fig. 1B). All chromatograms demonstrate the high efficiency of the sol–gel immunoaffinity columns to remove interfering matrix compounds.

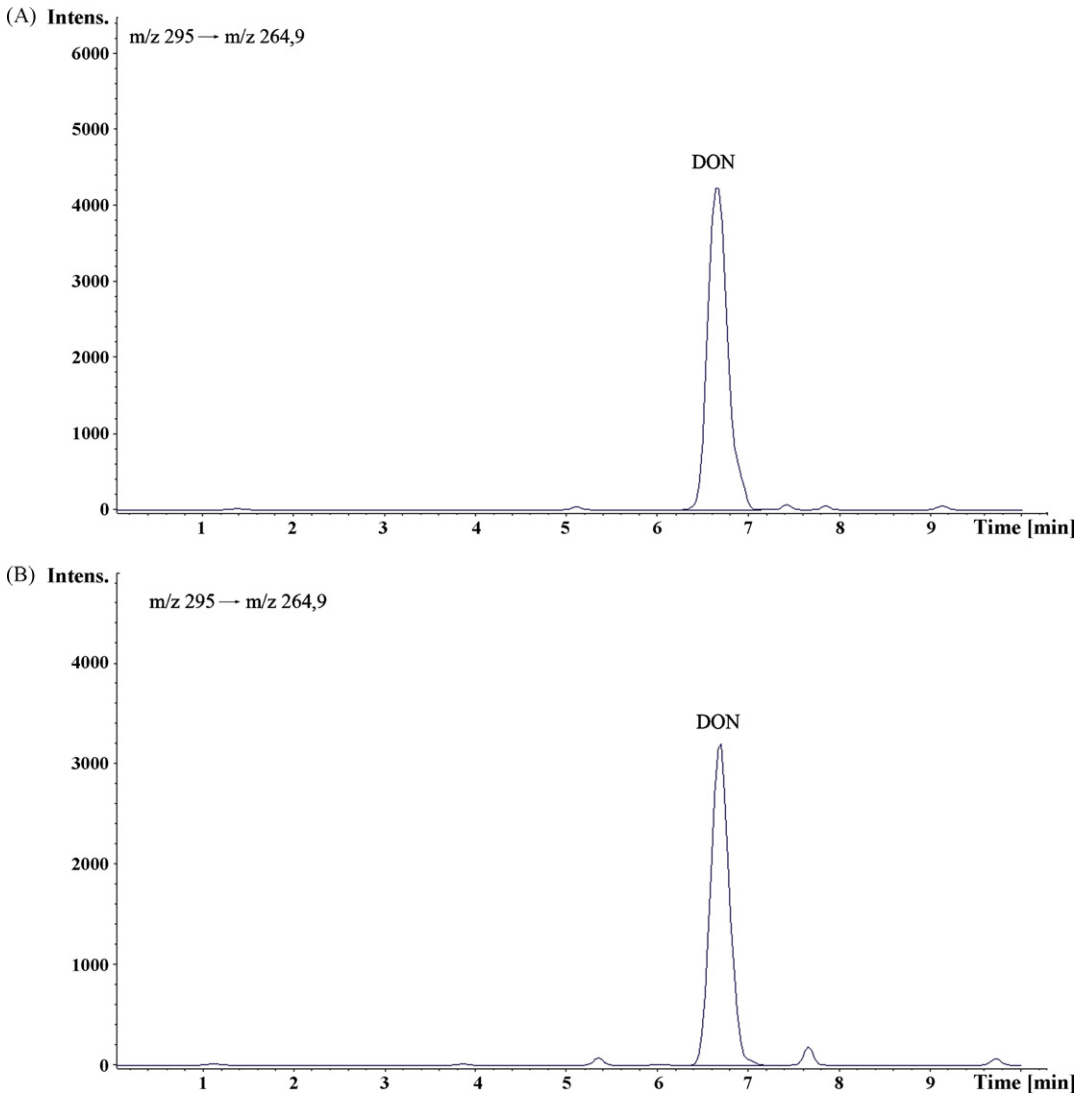

Fig. 2. Extracted ion chromatograms obtained by injecting aliquots of purified food into HPLC system 2. (A) Conventional pasta (sample 2) and (B) organic snack (sample 44).

3.2. Verification of the identity of DON

In order to confirm the identity of DON, aliquots of purified extracts from food and feed samples which were found to contain DON were analysed by LC–MS (HPLC system 2). Fig. 2 shows extracted ion chromatograms (EICs) of purified extracts from a conventional pasta (Fig. 2A) and a snack sample from organic production (Fig. 2B). In all cases, the EICs, recorded at the most abundant fragment of DON (*m*/*z* 264.9), confirm the identity of DON.

3.3. Quantitative determination of DON

HPLC system 1 was calibrated by injecting six standard solutions in the concentration range from 30 to 1000 ng/ml in bidistilled water. A linear relationship was obtained between the DON concentrations and the peak areas over the whole concentration range, with a correlation coefficient $r = 0.9995$. The limit of detection (LOD, S/N = 3) was 25 ng DON/ml, corresponding to 200 ng DON/g sample. The limit of quantification (LOQ, S/N = 10) was found to be 48 ng DON/ml, corresponding to 380 ng/g. The method is thus suitable to detect and quantify DON within the maximum levels set by the European Commission (cookies and snacks: 500 ng/g; dry pasta: 750 ng/g; feed: 1250 ng/g).

3.4. Recovery experiments

Recovery of DON was determined by spiking wheat products in which DON could not be detected (concentration <LOD). From each sample type (pasta, cookie, snack and feed) one sample was selected. As can be seen in Table 2, recoveries were in the range from 86 to 105%. The standard deviation of the recovery was in the range from 0.9 to 8.8%, indicating the high reproducibility of the analytical method.

3.5. Occurrence of DON in food and feed

The DON concentrations of the 55 food and feed samples analysed in the present study are listed in Table 1. In general, the incidence of DON contamination was rather low. In eight samples (14.5%) the DON concentration was above the LOQ (380 ng/g), in six samples (10.9%) DON was detected but could not be quantified (>LOD (200 ng/g), but <LOQ). In seven conventional samples (two pasta, two cookie, two snack and one feed sample) but only in

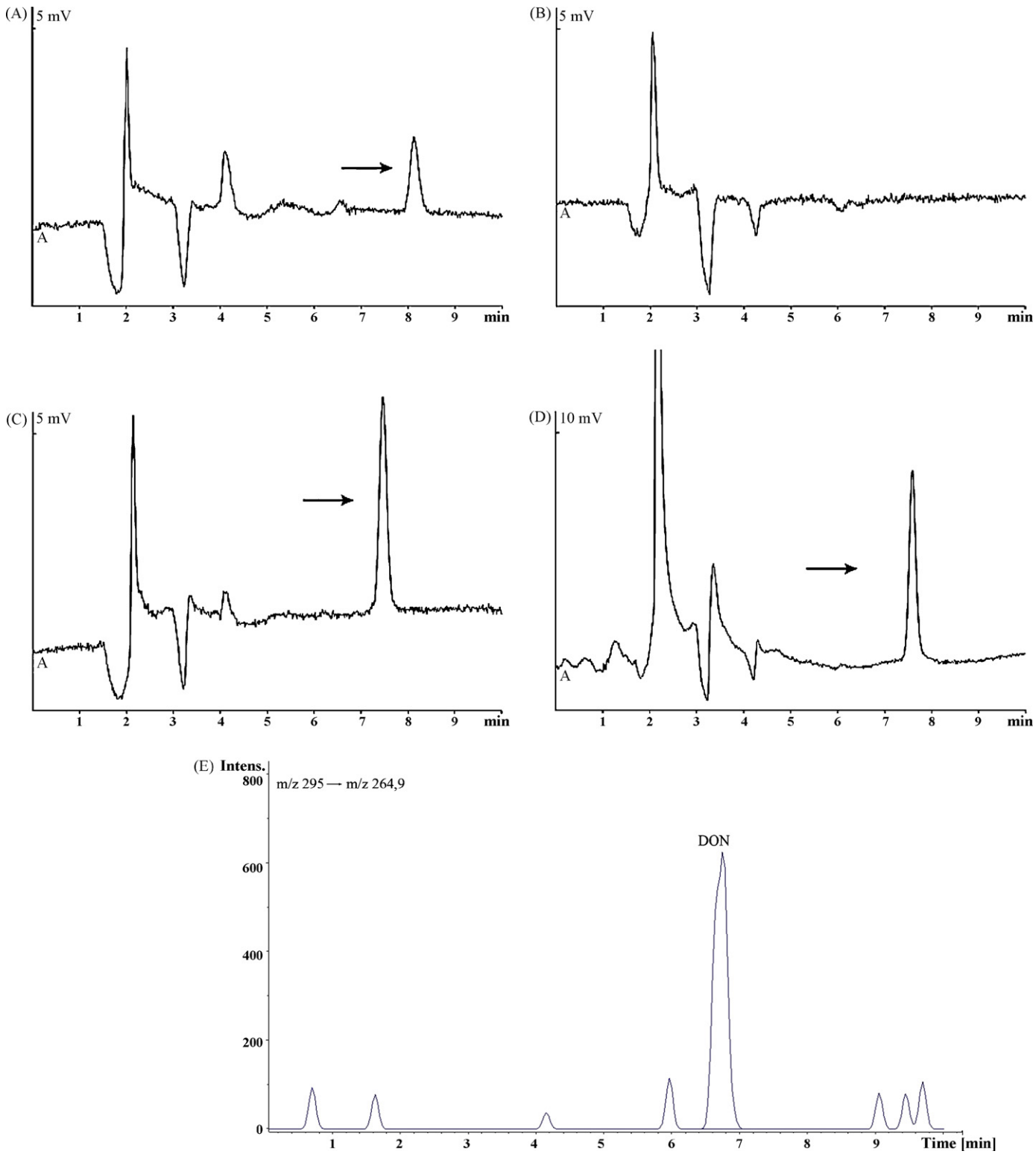

Fig. 3. Chromatograms obtained by injecting aliquots of purified extracts from chocolate containing cookies and cacao into HPLC system 1. (A) Organic chocolate cookie, (B) cookie part and (C) chocolate part from the same cookie, (D) cacao purified with a pure sol–gel column (without antibodies) and (E) EIC of the chocolate cookie.

one organic sample (snack, sample 44) the DON concentration was >LOQ.

The DON concentration in the organic sample was lower than the concentration in the conventional samples. The highest DON concentration was found in a cookie sample (sample 6). In three samples (samples 6, 10 and 13) the DON concentration exceeded the maximum levels set by the European Commission (cookies and snacks: 500 ng/g; dry pasta: 750 ng/g; feed: 1250 ng/g). In two conventional samples (cookie, sample 8, and snack, sample 20) and four organic samples (two snacks, samples 41 and 43, and two feed samples, 47 and 55) the DON concentration was between the LOD and the LOQ.

The data obtained in the present study indicate both a higher incidence of DON contamination and higher DON concentrations

Table 2
Recoveries for DON.

Sample	Spike level (ng/g)	Measured concentration (ng/g)	Recovery (%)	Mean recovery (%)	SD (%)
Pasta (sample 1)	972	859	88	87	0.9
		848	87		
		843	87		
Cookie (sample 37)	972	1025	105	105	4.0
		1050	108		
		974	100		
Snack (sample 14)	497	497	100	98	1.4
		495	100		
		484	97		
Feed (sample 53)	1004	1017	101	103	8.8
		955	95		
		1129	113		

in food and feed samples from conventional than in those from organic production. The use of synthetic nitrogen fertilizers in conventional production may result in thinner cell walls by increased growth speed, thus facilitating mould infection [35]. On the other hand, crop rotation routinely carried out in organic agriculture has been assumed to prevent the transmission of plant diseases [36].

Our data are in agreement with results published by Schneweis et al. [10]. They determined DON concentrations in three wheat varieties from conventional and organic production which had been cultivated under comparable soil and weather conditions. Higher frequency of DON contamination and higher DON levels were found in conventionally cultivated wheat.

Similar results were obtained by Schollenberger et al. [12] who found a higher incidence of DON contamination and higher DON levels in conventional samples when analysing 101 bread samples from German bakeries and food stores.

Pussemier et al. [13] analysed 93 wheat grain and 80 wholemeal wheat flour samples. In conventional samples, both the frequency of contamination and the DON concentration were higher than in organic samples.

Malmauret et al. [8] who compared DON levels in 11 organic and 11 conventional wheat samples also report a higher frequency of DON contamination in conventional samples. However, in contrast to our results and those from the papers mentioned above, a higher median DON level was found in samples from organic production.

3.6. Analysis of chocolate cookies

In addition to the 55 samples mentioned above, four chocolate containing cookies, two from conventional and two from organic production, were analysed. Fig. 3A shows the chromatogram obtained by injecting an aliquot of the purified extract of one of these cookies into HPLC system 1. The peak at the retention time of about 8 min was significantly higher than the peaks obtained for all other food and feed samples, corresponding to a DON concentration of 2225 ng DON/g sample. For the other three chocolate containing cookies, similar results were obtained. In the following experiments, we removed the chocolate part from the cookie and extracted and purified both parts (chocolate and cookie) separately. The chromatograms are shown in Fig. 3B and C. It can be seen that only in the case of the chocolate part the peak at a retention time of 8 min was obtained. Next, we extracted commercially available cacao and purified the extract by sol–gel immunoaffinity chromatography. Again, the chromatogram contained the peak at 8 min. In order to investigate if the substance eluted from the analytical column after 8 min was retained in the immunoaffinity column due to interactions with the anti-DON antibodies, the cacao extract was also loaded onto a column containing pure sol–gel glass. As can be seen in Fig. 3D, the same peak was obtained, indicating that the substance was non-specifically bound to the sol–gel glass. However, experiments carried out in our previous study [32] showed that DON is not non-specifically bound to the sol–gel glass matrix. The results indicate, that chocolate contains a compound which is non-specifically bound to the sol–gel glass matrix and co-elutes with DON in the HPLC system 1, feigning a too high DON concentration.

In order to investigate if the chocolate containing cookie was actually contaminated with DON, an aliquot of the extract (of the whole cookie) was purified by sol–gel immunoaffinity chromatography and injected into HPLC system 2. The EIC shown in Fig. 3E indicates that the cookie sample did contain DON, however, the DON concentration was lower than the LOQ of HPLC system 1.

4. Conclusions

The present paper provides data on the concentration of deoxynivalenol (DON) in 55 wheat food and feed samples, 26 from conventional and 29 from organic production. Only in eight samples, seven conventional and one organic sample, the DON concentration was above the LOQ (380 ng/g). The DON concentration in the organic sample was lower than the concentration in the conventional samples. Our results are in agreement with data previously published from other research groups.

In the case of pasta, cookie, snack and feed samples, the sol–gel immunoaffinity columns proved to be very efficient in removing interfering matrix compounds. However, in analysing chocolate containing cookies it is necessary to remove the chocolate part in order to avoid systematic errors.

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