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The development and characterisation of an immunoaffinity column used for the simultaneous selective extraction of *Fusarium* toxins from grain products

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RESEARCH ARTICLE

Abstract

In the present study, a multifunctional immunoaffinity column (IAC) with the binding of three antibodies against four target analytes was developed. The aim of this work was to optimise the IAC method regarding the selective and effective extraction of *Fusarium* toxins from grain products and the method used to couple to antibodies. The IAC was constructed by covalently coupling the three different highly specific antibodies (to deoxynivalenol (DON), T-2 toxin/HT-2 toxin (T-2/HT-2) and zearalenone (ZEN)) onto CNBr-activated Sepharose 4B and packing them into a solid-phase extraction cartridge. The optimal amount bound to each column was selected: 1.25 mg DON per column, 0.2 mg T-2/HT-2 per column and 0.3 mg ZEN per column. The purification conditions, including the loading, washing and eluting solutions, were optimised. The values obtained for the maximum binding capacity of the IAC for DON, T-2, HT-2 and ZEN were 261, 233, 198 and 281 ng, respectively. To obtain the quantitative results for four *Fusarium* toxins extracted from different matrices, a liquid chromatography-mass spectrometry method was utilised. The recoveries of the four *Fusarium* toxins at two different spiked concentrations were in the range of 93.3-104% with a relative standard deviation (RSD) within the range of 2.41-4.84%, indicating the high purification efficiency of the columns. After eight cycles of use, the column recovery rate still remained over 85%. The method was accurate and precise (recoveries of 89.7-105% with an RSD within 3.18-10.4%), and it was applied to analyse actual flour samples.

Keywords: immunoaffinity column, *Fusarium* toxins, grain products, liquid chromatography-mass spectrometry

1. Introduction

Fusarium toxins are secondary metabolites produced by the fungi of *Fusarium spp*, which can colonise a variety of cereals, such as maize and wheat (Krska *et al.*, 2001; Vogelgsang *et al.*, 2017; Wild and Gong, 2010). The consumption of a *Fusarium* mycotoxins-contaminated diet may induce acute and long-term chronic toxic effects resulting in erotogenic, carcinogenic, estrogenic or immunosuppressive impacts on animals and humans (Nelson *et al.*, 2014). Among these *Fusarium* toxins, zearalenone (ZEN), deoxynivalenol (DON), T-2 toxin (T-2) and HT-2 toxin (HT-2) toxins are the most common and poisonous, which generally contaminate grain products simultaneously (D'Mello *et al.*, 1999; Nelson *et al.*, 2014). It is essential to establish precise and reliable analytical methods for the detection of these four *Fusarium* toxins and eliminate the contamination of feeds and foods by *Fusarium* toxins.

The main analytical method used for the determination of *Fusarium* toxins in grain products is chromatography, such as gas chromatography (GC) (Kotal *et al.*, 1999), gas chromatography-mass spectrometry (GC-MS) (Eke *et al.*, 2004; Rodríguez-Carrasco *et al.*, 2014), high-performance liquid chromatography (HPLC) (Bartók *et al.*, 2013; Cheng *et al.*, 2017; Soleimany *et al.*, 2011) and liquid chromatography-mass spectrometry (LC-MS/MS) (Brezina *et al.*, 2014; Hamed *et al.*, 2017). Considering the complexity of the matrix and ultra-low concentration of

the mycotoxins in real samples, the samples should be pre-treated to enrich or purify the *Fusarium* toxins present. The common sample purification techniques used in the analysis of *Fusarium* toxins are liquid-liquid distribution (LLE) and solid-phase extraction (SPE) (Rodríguez-Carrasco *et al.*, 2012; Wei *et al.*, 2017). These routine techniques show low selectivity towards the particular target analytes since they capture not only the target compound, but also other components found in the matrix. It is necessary and desirable to develop more specific and efficient methods for the sample preparation and determination of multi-*Fusarium* toxins.

An immunoaffinity column (IAC) is a special SPE column based on the properties of the specific and irreversible binding between an antibody and antigen, which can extract and enrich a specific compound in one step, can be regenerated for reuse and consumes small quantities of organic solvent (Zhang *et al.*, 2013). At present, the application of the IAC technique has gradually expanded from extraction to the enrichment of target analytes from different matrices (Iha *et al.*, 2017; Kaymak *et al.*, 2018; Romagnoli *et al.*, 2010). Many IACs are specifically used for single mycotoxin clean-up (Chang *et al.*, 2017; Liu *et al.*, 2017). Some studies have reported simultaneous and selective extraction of multiple mycotoxins in food using multi-functional IACs (Irakli *et al.*, 2017; Sun *et al.*, 2017). However, there are few reports that openly discuss the method and the related parameters of IAC employed for the simultaneous and selective extraction of *Fusarium* toxins (ZEN, DON, T-2 and HT-2).

In our study, a multi-functional IAC with the binding of three antibodies against four target analytes (ZEN, DON, T-2 and HT-2) was utilised. The extraction conditions of the IAC were optimised, and the maximum capacity, recovery rate and stability of the IACs were evaluated. Finally, the multifunctional IACs were applied to the extraction of actual samples. The aim of this work was to select and effectively purify *Fusarium* toxins (ZEN, DON, T-2 and HT-2) from grain products using the IACs and determine the optima method to couple the antibodies to the IACs. This study provided a reference for the preparation of multifunctional IACs selective towards multi-toxins and suggested new perspectives in this area of research.

2. Materials and methods

Reagents and apparatus

Reagents: DON, ZEN, T-2 and HT-2 (100 μg/ml) were purchased from Pribolab (Beijing, China). CNBr-activated Sepharose 4B was obtained from GE Healthcare (Buckinghamshire, UK). HPLC grade methanol and acetonitrile, and the other analytical grade reagents used in this study were supplied by Dima (Buchs, Switzerland).

Buffers and solutions: (1) coupling buffer: 0.1 mol/l $NaHCO_3$, pH 8.3 containing 0.5 mol/l NaCl; (2) blocking buffer: 0.1 mol/l Tris-HCl, pH 8.0; (3) standard solutions: for DON and HT-2, 5, 10, 20, 40, 80, 100 μg/ml; for HT-2, 2, 5, 10, 20, 40, 80, 100 μg/ml; for ZEN, 2, 5, 10, 20, 40, 80, 120 μg/ml.

Apparatus: Mettler AE240 electronic balance (Mettler Toledo, Greifensee, Switzerland), UV-2300 spectrophotometer (Techcomp, Hong Kong) and Agilent 1200-6410 LC-MS/MS (Agilent, Santa Clara, CA, USA).

Preparation of the immunoaffinity column

The 0.2 g of CNBr activated Sepharose 4B was dissolved in 2 ml of 1 mol/l HCl to remove the suspended protecting groups and allowed the gel to swell in a sintered glass filter (porosity: G3). The resulting gel was washed several times with 400 ml of 1 mol/l HCl and once with 10 ml of coupling buffer. One ml of coupling buffer (containing the antibodies to: DON (0.25 mg), T2/HT-2 (0.04 mg) and ZEN (0.015 mg)) were added to the gel and reacted overnight at 4 °C. The supernatant was collected to determine the coupling efficiency using the Coomassie Brilliant Blue dye method. The remainder was loaded onto the SPE column and flushed with the coupling buffer (in 8-fold volumes of the gel) to remove the excess antibodies. The outlet of the column was sealed and the gel was immersed into double gel volumes of the blocking buffer for 2 h at room temperature to block all the remaining active groups. The column was then rinsed with 0.1 mol/l NaAc (pH 4.0) containing 0.5 mol/l NaCl and 0.1 mol/l Tris-HCl buffer (pH 8.0) containing 0.5 mol/l NaCl (in ten-fold volumes of the gel) for at least three cycles. Finally, the column was washed with 0.01 mol/l phosphate buffer saline (PBS) buffer (pH 7.4) and then stored in the same buffer containing 0.01% NaN_3 at 4 °C prior to use.

Optimisation of the purification conditions

The IAC was used to simultaneously purify four kinds of *Fusarium* toxins. In order to improve the purifying effect, the purification conditions used for the sample loading, washing and elution processes were optimised. Three kinds of loading solutions (10% methanol, ultra-pure water and 0.01 mol/l PBS), two washing solutions (ultra-pure water and 0.01 mol/l PBS) and five gradient eluting solutions (60, 70, 80, 90 and 100% methanol) were used. An aliquot of 5 ml of a mixed *Fusarium* toxin standard solution (containing 40 ng/ml DON, 40 ng/ml T-2 and HT-2 and 10 ng/ml ZEN) prepared in the different loading solutions were loaded onto the IAC. Then, 5 ml of the washing solution was applied to remove any non-specifically bound analytes. One ml of the eluting solution and 1 ml of ultra-pure water were used to elute the target analytes bound on the column. Finally, all the eluates were collected and analysed using LC-MS/MS.

Evaluation of the immunoaffinity column

The standard solution of the four mixed *Fusarium* toxins was continuously loaded onto the IAC column until all the sites on the IAC were saturated. The maximum binding capacity was then measured.

A 5 ml aliquot of the standard solution of the four mixed *Fusarium* toxins was loaded onto the column followed by washing and eluting under the optimal purification conditions. The eluting solutions were collected and analysed using LC-MS/MS. Each experiment was performed in triplicate.

To regenerate the IAC for reuse, the used IAC was washed successively with 0.1 mol/l NaAc (pH 4.0) containing 0.5 mol/ NaCl and 0.1 mol/l Tris-HCl buffer (pH 8.0) containing 0.5 mol/l NaCl in five-fold excess of the gel volume for at least three cycles. Finally, the column was washed with 200 ml of 0.01 mol/l PBS (pH 7.4) and stored in 0.01 mol/l PBS (pH 7.4) containing 0.01% NaN_3 prior to use. The stability test required the continuous measurement of the maximum capacity for the four *Fusarium* toxins eight times per month, for at least 1 month.

The application of the actual flour samples

The efficacy and precision of the method were evaluated using a recovery experiment. Ten g of the wheat flour spiked with four *Fusarium* toxins was added in a 250-ml conical flask. Forty ml of 90% methanol solution (v/v) was added and ultrasonically extracted for 40 min. Afterwards, 20 ml of the filtered extract was dried in a rotary evaporator and loaded onto the IAC, followed by washing and eluting under the optimal purification conditions. The eluant solution was analysed using LC-MS/MS. Five replicates were performed for each of the concentrations studied (25, 50, 100 μg/kg or 10, 25, 50 μg/kg).

The content of the four *Fusarium* toxins in five kinds of wheat flour and two kinds of flour samples from different areas was extracted using 90% methanol solution (v/v), purified on the IAC, and determined using LC-MS/MS. Each experiment was performed in triplicate.

LC-MS/MS parameters

Quantitative analysis of the four *Fusarium* toxins was carried out using LC-MS/MS on an Agilent 1200 HPLC series and 6410 triplequad MS. The quantitative and qualitative ion pairs of DON, HT-2, T-2 and ZEN, the parameters of the LC-MS/MS were in accordance with those reported by Zhang *et al.* (2017).

Statistical analysis

All statistical analyses were performed using SAS 9.0 for Windows software.

3. Results and discussion

The optimal amount of the binding antibodies

The IACs were separately coupled with three different antibodies (to DON, T-2/HT-2 and ZEN). Three single columns were established to acquire the optimal amount for each antibody by increasing the amount of the binding antibody. The results indicated that the optimal amount of the binding antibody for each column was 1.25 mg DON, 0.2 mg T-2/HT-2 and 0.3 mg ZEN, which were covalently coupled to 0.2 g of Sepharose 4B packed into one IAC.

Coupling dynamic curve

Three different kinds of antibodies (to DON, T-2/HT-2 and ZEN) were covalently coupled to the column and the coupling time for each antibody was determined. According to Figure 1, the amount of coupled antibody increased with time between 0-4 h. In the period between 4-6 h, the coupling efficiency reached 100%, and the three different antibodies were coupled completely. Therefore, all the antibodies could be immobilised 4 h later.

A comparison of two different coupling procedures

Two methods were chosen for the coupling process. Group A involved the preparation of three individual columns, which were then mixed. Group B involved coupling the three kinds of antibodies simultaneously. Group C involved a single column for each toxin. The three column types were prepared using the optimal amount of the individual binding antibodies to DON (1.25 mg per column), T-2/HT-2 (0.2 mg per column) and ZEN (0.3 mg per column). The recovery and RSD of the four *Fusarium* toxins in group A, B and C were 75.9-88.6, 84.1-95.4 and 78.3-88.1% and 2.61-6.03, 3.01-5.80 and 2.24-6.13%, respectively (Table 1). The recovery in group B was significantly higher than those

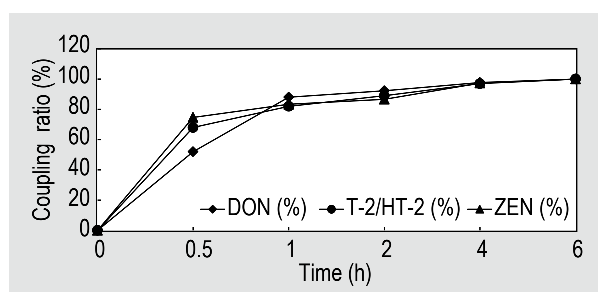

Figure 1. Coupling dynamic curve. DON = deoxynivalenol; HT-2 = HT-2 toxin; T2 = T-2 toxin; ZEN = zearalenone.

found in the other two groups (P<0.0001). Therefore, the second coupling method (B group) was selected.

Optimisation of the purification conditions

In order to improve the purification efficiency of the column, the purification conditions used in the sample, washing and elution processes were optimised. In this study, 5 ml of the mixed standard solution containing the four *Fusarium* toxins (200 ng DON, 100 ng T-2, 100 ng HT-2 and 50 ng ZEN) was loaded onto the column. A 10% methanol solution was selected as the loading solution and the recovery reached over 95% (Figure 2). The results obtained for the two washing solutions (ultra-pure water and 0.01 mol/l PBS) were similar. The recoveries of the four *Fusarium* toxins using ultra-pure water as the washing solution was 82.3-95.1%, whereas after the utilisation of 0.01 mol/l PBS, it was 72.4-104%. Thus, ultra-pure water was selected as the washing solution. The purification efficiency values obtained for the four *Fusarium* toxins increased upon increasing the methanol concentration from 60 to 90%. However, the value using 100% methanol was lower than that found using 90% methanol (Figure 3). Therefore, the 90% methanol solution was found to be the most efficient eluant. The optimal purification conditions established were: 10% methanol as the loading solution, ultra-pure water as the washing solution, and 90% methanol as the eluting solution.

Maximum capacity of the immunoaffinity column for the four *Fusarium* toxins

The maximum capacity of the IAC was measured using a breakthrough volume test. Fourteen ml aliquots of the mixed standard solution containing the four *Fusarium* toxins at a concentration of 40 ng/ml were sequentially loaded onto a single IAC. As illustrated in Figure 4, the four *Fusarium* toxins in the first three volumes (1 ml) of the standard solutions were nearly completely retained on the column. After loading the rest of the standard solution, approximately 141 ng of DON, 113 ng of T-2, 78 ng of HT-2 and 161 ng of ZEN were bound onto the column. Therefore,

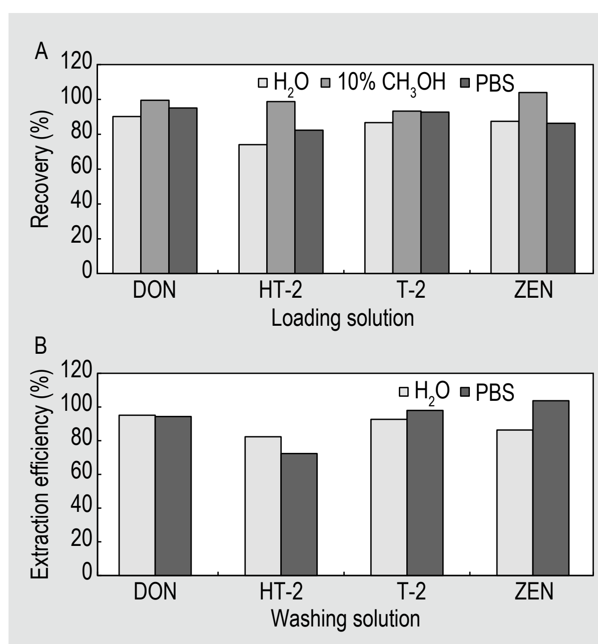

Figure 2. The effect of different loading and washing solutions on the recovery of the four *Fusarium* toxins extracted by immunoaffinity column. CH_3OH = methanol; DON = deoxynivalenol; H_2O = water; HT-2 = HT-2 toxin; PBS = phosphate buffer saline; T2 = T-2 toxin; ZEN = zearalenone.

the maximum binding capacity of the IAC for DON was calculated to be 3×40 (completely retained) + 141 (partially retained) = 261 ng per column; T-2 was calculated as 3×40 + 113 = 233 ng per column; HT-2 3×40 + 78 = 198 ng per column; and ZEN 3×40 + 161 = 281 ng per column.

Recovery and stability tests of the immunoaffinity column

Under the optimal purification conditions, the efficiency of the IAC was investigated. A 5 ml aliquot of the mixed standard solution containing the four *Fusarium* toxins at concentrations of 20 and 40 ng/ml in 10% methanol were loaded onto the column, respectively, according to the steps described above, followed by washing and eluting. Finally, the eluting solution was collected and analysed using LC-

Table 1. Comparison of different coupling procedures.

Compound	Spiked concentration (ng/ml)	Group A		Group B		Group C	
		Recovery (%)	RSD (%)	Recovery (%)	RSD(%)	Recovery (%)	RSD (%)
DON	10	88.6	2.61	95.4	3.01	88.1	2.24
HT-2	10	75.9	3.22	84.1	4.64	79.0	3.12
T-2	10	86.0	3.81	91.6	4.92	86.4	6.13
ZEN	10	86.0	6.03	97.1	5.80	78.3	4.81

P<0.0001, the difference between the three groups was significant

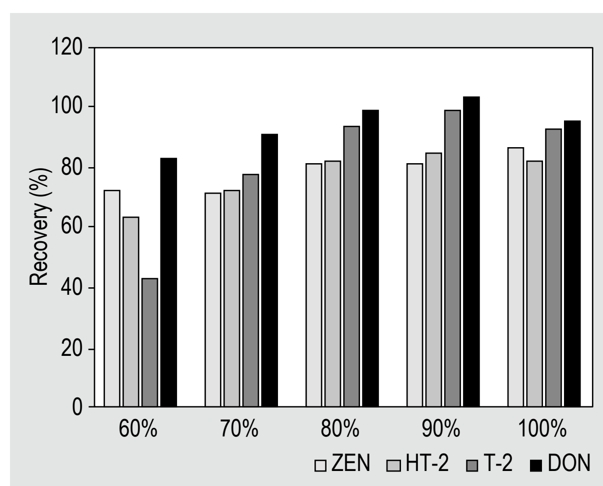

Figure 3. The effect of the percentage of methanol in eluting solution on the recovery of the four *Fusarium* toxins by immunoaffinity column extraction. DON = deoxynivalenol; HT-2 = HT-2 toxin; T2 = T-2 toxin; ZEN = zearalenone.

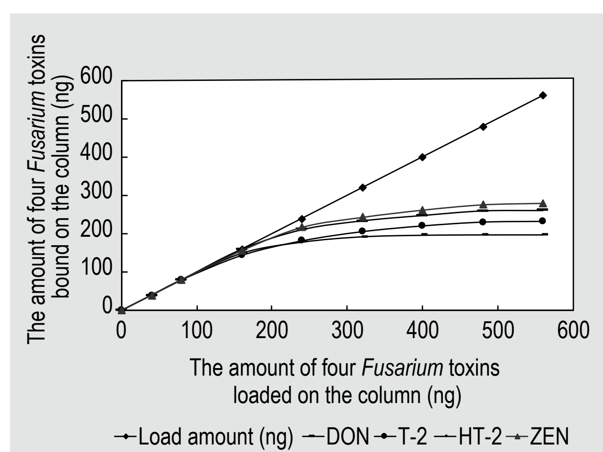

Figure 4. Maximum capacity of immunoaffinity column for the four *Fusarium* toxins. DON = deoxynivalenol; HT-2 = HT-2 toxin; T2 = T-2 toxin; ZEN = zearalenone.

MS/MS. Each experiment was performed in triplicate. The recovery of the four *Fusarium* toxins at concentrations of 20 and 40 ng/ml were in the range of 93.3-104% with an RSD within the range of 2.41-4.84%, indicating the high extraction efficiency of the column (Table 2).

Due to the high cost of the IACs, they were reused. In our study, the columns were washed with 0.1 mol/l NaAc (pH 4.0) and 0.1 mol/l Tris-HCl buffer (pH 8.0), both containing 0.5 mol/l NaCl, for at least three cycles. Then, the IACs were washed with 0.01 mol/l PBS (pH 7.4) and stored in the same PBS solution containing 0.01% NaN_3, prior to reuse. After eight cycles of usage in one month, the column recovery rate remained above 85%.

Method validation

Good linearity was observed for DON (in the range of 5-100 μg/ml), HT-2 (in the range of 5-100 μg/ml), T-2 (in the range of 2-100 μg/ml) and ZEN (in the range of 2-120 μg/ml) with an r^2 value >0.9970 for each. The values of the limit of detection obtained using LC-MS/MS for DON, HT-2, T-2 and ZEN were 0.13, 0.13, 0.39 and 0.39 μg/kg, respectively. In addition, the limit of quantification values were 0.39, 0.39, 1.17 and 1.17 μg/kg, respectively. The results from the recovery experiment are listed in Table 3. The recovery of the four *Fusarium* toxins in the flour samples was in the range of 89.74-105% with an RSD within the range of 3.18-10.4%. These results showed that the method was accurate and precise.

The purification and application of the flour samples

The contents of the four *Fusarium* toxins present in seven flour samples are shown in Table 4. The flour samples all contained DON, HT-2, T-2 and ZEN. The content of ZEN in the No. 3 and No. 4 samples were beyond the limits according to GB 2761-2017 National Food Safety Standard Maximum Levels of Mycotoxins in Foods (DON≤1000 μg/kg and ZEN≤60 μg/kg in wheat, corn and barley).

Table 2. The recoveries of the four *Fusarium* toxins in the IAC.

Compound	Spiked concentration (ng/ml)	Spiked amount (ng)	Recovery (%)	RSD(%)
DON	20	100	94.2	2.41
	40	200	99.6	3.14
HT-2	20	100	98.7	2.65
	40	200	95.1	4.84
T-2	20	100	93.3	3.53
	40	200	94.2	3.34
ZEN	20	100	104	4.60
	40	200	96.4	3.71

Table 3. The recoveries of wheat flour samples fortified with the four *Fusarium* toxins.

Compound	Spiked concentration (μg/kg)	Recovery (%)	RSD (%)
DON	25	105	3.26
	50	99.6	5.71
	100	95.4	5.86
HT-2	25	92.5	7.33
	50	92.5	5.24
	100	89.7	3.18
T-2	10	101	8.12
	25	104	6.49
	50	98.3	9.55
ZEN	10	98.7	6.32
	25	93.1	10.4
	50	91.2	3.71

4. Conclusions

In this study, an IAC used for the simultaneous and selective extraction of four *Fusarium* toxins (ZEN, DON, T-2 and HT-2) from grain products was established. An IAC was constructed by covalently coupling the three different kinds of highly specific antibodies (to DON, T-2/HT-2 and ZEN) onto CNBr-activated Sepharose 4B and packing them into a SPE cartridge. The optimal amount of antibody bound onto each column was found to be DON (1.25 mg per column), T-2/HT-2 (0.2 mg per column) and ZEN (0.3 mg per column), and each was covalently coupled to 0.2 g of Sepharose 4B and packed into one multi-toxin IAC. The extraction conditions, including the loading, washing and eluting solutions, were optimised. The 10% methanol, ultra-pure water and 90% methanol were selected as loading solution, washing solution and eluting solution respectively. The maximum binding capacity of the IAC for DON, T-2, HT-2 and ZEN was 261, 233, 198 and 281 ng, respectively. The recoveries of the four *Fusarium* toxins at two different spiked concentrations were in the range of 93.3-104%. After eight cycles of use, the column recovery rate still remained over 85%. The IAC was used for extracting the four *Fusarium* toxins simultaneously. The method was accurate and precise (recoveries of 89.7-105% with an RSD within 3.18-10.4%) and it was applied to analyse actual flour samples. Therefore, the prepared IAC provided an alternative, effective and fast tool for the simultaneous extraction of the four *Fusarium* toxins from grain products.

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Table 4. The content of the four *Fusarium* toxins in seven flour samples.

Number	DON		HT-2		T-2		ZEN	
	Content μg/kg	RSD %	Content μg/kg	RSD %	Content μg/kg	RSD %	Content μg/kg	RSD %
1	32.8	4.81	17.2	5.61	8.27	1.77	15.1	5.35
2	49.4	2.09	14.6	3.27	12.6	2.20	15.0	2.04
3	135	6.57	15.9	2.69	34.7	12.4	68.7	3.54
4	187	12.3	16.6	4.34	7.02	1.89	87.6	14.1
5	51.5	9.22	14.0	0.991	8.39	1.62	16.4	6.72
6	64.0	1.55	12.1	7.02	8.27	4.35	16.7	3.16
7	46.3	4.66	15.9	3.56	20.8	3.11	16.1	4.50

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