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Analytical Methods

Sterigmatocystin presence in typical Latvian grains

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Abstract

Ninety five samples of different Latvian grains (wheat, buckwheat, barley, oats and rye) from the year 2006 and 120 samples from the year 2007 were analyzed for *Aspergillus* ssp. mycotoxin–sterigmatocystin (STC) content. 13.7% of the analyzed 2006 year samples were positive for STC with the concentration levels ranging from <0.7 to 83 μg/kg and 35% of the analyzed 2007 year samples were positive for STC with the concentration levels ranging from <1 to 47 μg/kg. A previously developed sensitive LC – Electrospray Positive Ionization – MS/MS method was applied for the analysis of STC in grains. Method includes sample extraction with acetonitrile/water solution, solid phase extraction (SPE) on Strata X SPE column, separation on reversed phase C_{18} column and STC detection by LC–MS/MS.

Keywords: Sterigmatocystin; Mycotoxin; Grains; LC–MS/MS; *Aspergillus* spp.; *Aspergillus versicolor*

1. Introduction

STC is a mycotoxin produced by fungi of many *Aspergillus species* (Atalla, Hassanein, El-Beih, & Youssef, 2003; Mucke & Lemmen, 1998). Its molecular structure is similar to those of aflatoxin B_1 (Fig. 1). It is a precursor of aflatoxin B_1 in the biological transformation (Betina, 1989).

The steps involved in the biosynthesis of STC, as well as of aflatoxins, and the molecular characterisation of genes involved in the pathway are known and described in the literature (Sweeney & Dobson, 1999). Carcinogenic properties of sterigmatocystin were already studied and published (Purchase & van der Watt, 1970). The negative impact of STC on the DNA and on the tumor suppressor gene p53, in particular, was also studied (Tong-xi, Junichi, Kazuo, Wen-Yuan, & Shu-Ying, 2000). Relatively high levels (ppm range) of STC were detected in dwellings contaminated by *Aspergillus versicolor* caused by water flooding (Nielsen et al., 1999). Contamination of cereals with *Aspergillus* fungi is a serious health risk, due to the potential of STC production by these fungi. Very little data are available concerning monitoring of foodstuffs for STC. One of the few surveys in Europe was carried out in 1983 in the United Kingdom. Out of 523 analyzed samples (about 3%) 17 were found positive (Buckle, 1983). The drawback of this survey was that the method had a relatively high limit of detection (LOD) of 20 μg/kg. This fact did not allow any conclusion concerning lower contamination levels, which are also of interest. Another larger survey was done in Brazil (Valente Soarez & Rodriguez-Amaya, 1989), in the same decade, where sterigmatocystin was not detected at all. However, again the LOD of the method applied was high (35 μg/kg). These rather high LOD values that were obtained with conventional (non-mass spectrometric) methods are rather unsatisfactoral. In order to evaluate human exposure to this mycotoxin and, more importantly, to monitor food products for existing or future legal compliance, suitable and simple analytical procedures are required that allow the precise determination of STC. Methods have been described in the literature, which

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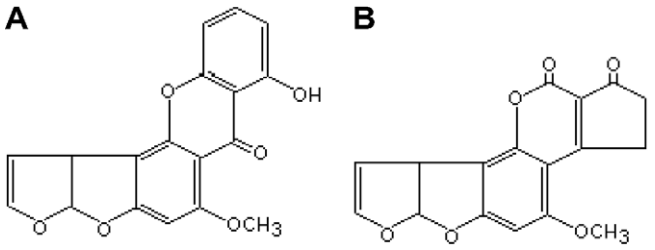

Fig. 1. Chemical structures of (A) sterigmatocystin and (B) aflatoxin B_1.

allow the detection of STC at levels ranging from 2 to 100 μg/kg (Scudamore, Hetmanski, Clarke, Barnes, & Startin, 1996). However, it has been recognized that the surveillance of STC below ppb level was difficult in the past due to the weak fluorescence of this mycotoxin, and that a precise determination below this level requires mass spectrometric detection (Scudamore et al., 1996). The most frequently applied analytical methods so far, are based on thin-layer chromatography (TLC) with fluorescence detection (AOAC Official Method 973.38, 1995; Athnasios & Kuhn, 1977; Gimeno, 1979; Valente Soarez & Rodriguez-Amaya, 1989). Because of its weak native fluorescence, a derivatisation procedure is generally used to visualise the STC on the developed TLC plates (Athnasios & Kuhn, 1977). The most common derivatisation approach is spraying of the TLC plate with aluminium chloride ($AlCl_3$) solution, after development and heating of the plate. With such procedures, LODs were reported to be 50 μg/kg (Athnasios & Kuhn, 1977) and 140 μg/kg (Gimeno, 1979), in cereal grains. A validated official method is also based on TLC with $AlCl_3$ spraying, and is reported to have a limit of quantification (LOQ) of 100 μg/kg. Other methods are based on high performance liquid chromatography (HPLC) with ultraviolet (UV) detection, and were applied for determination of STC in rice inoculated by *A. versicolor* at lower μg/kg levels (Neely & Emerson, 1990; Schmidt, Mondani, Ziegenhagen, & Dose, 1981) Application of HPLC with post column derivatisation with $AlCl_3$ has also been reported (Neely & Emerson, 1990), with results comparable to the official TLC method (AOAC Official Method 973.38, 1995). The lowest detection limit of 1.7 μg/kg for maize and 1.9 μg/kg for bread was reported when liquid chromatography with mass spectrometric detection (LC–MS) was applied (Scudamore et al., 1996). A method based on gas chromatography with mass spectrometric detection (GC–MS) reported an LOD of 5 μg/kg for STC in wheat (Salhab, Russel, Coughlin, & Hsieh, 1976).

In conclusion, currently the only methods with sufficient LOQs of sterigmatocystin at levels below 5 μg/kg are based on LC–MS and GC–MS.

For routine and monitoring purposes a reagent-free derivatisation procedure was also successfully applied for fluorescence induction of STC and method limit of detection was 2–11 μg/kg (Stroka, Dasko, Spangenberg, & Anklam, 2004).

Now are available LC–MS/MS based methods for routine and monitoring purposes with limit of detection 0.15 μg/kg in grains (Veršilovskis, Bartkevičs, & Miķelsone, 2007).

Neither country has legislation for STC, however some countries have set already relatively low maximum levels for sterigmatocystin (e.g., Czech Republic and Slovakia at the level 5 μg/kg for rice, vegetables, potatoes, flour, poultry, meat, milk, and 20 μg/kg for other foods) (Stroka et al., 2004) and soon after STC was recognized as a highly toxic compound, the California Department of Health Services used TD50 values from the Cancer Potency Database to produce 'no significant risk' intake levels for humans. The level resulting was 8 μg/kg body weight/day for a 70 kg adult (European Mycotoxin Awareness Network).

The aim of this study is to research typical Latvian grains (wheat, oat, rye, barley and buckwheat) from the years 2006 – 2007 for the presence and concentration of STC using sensitive LC–MS/MS method (Veršilovskis et al., 2007).

2. Materials and methods

2.1. Grain samples

Grain samples (50 wheat, 15 oats, 10 ryes, 10 barley and 10 buckwheat samples) were collected from different parts of Latvia in autumn 2006 and grain samples (20 wheat, 25 oats, 25 ryes, 25 barley and 25 buckwheat samples) were collected from different parts of Latvia in autumn 2007. After harvest, the grains were air-dried to a water content of less than 15% to avoid fungal growth during storage. Before the analysis, the samples were grounded with a laboratory-mill (Romer, Ras Mill, Union, MO, USA).

2.2. Chemicals and reagents

Methanol (HPLC-grade) and acetonitrile (HPLC-grade) was purchased from Merck (Darmstadt, Germany). Deionized water was purified with Millipore Milli-Q Plus system (Millipore, Molsheim, France). STC standard were purchased from Sigma (St. Louis, MO, USA). Argon (AGA, Latvia) was used as a collision gas in the mass spectrometry.

2.3. Preparation of standards

A stock solution of a concentration of approximately 500 μg/ml was prepared by dissolving 5 mg of STC in 10 ml of acetonitrile/methanol (50:50, v/v). An aliquot 500 μl of the stock solution was evaporated to dryness under oxygen-free nitrogen at ambient temperature and immediately redissolved in acetonitrile (5 ml). The STC concentration was established by UV spectrometry.

The calibrated stock solution (50 μg/ml) was used to prepare a standard stock solution of 5 μg/ml of STC, in acetonitrile/water (75:25, v/v). This solution was used to

spike samples for recovery experiments, and to prepare working standards 2.0 μg/ml, 1.0 μg/ml, 0.25 μg/ml, 0.05 μg/ml and 0.005 μg/ml as equivalents to 200 μg/kg, 100 μg/kg, 25 μg/kg, 5 μg/kg and 0.5 μg/kg.

2.4. Sample preparation

An amount of 25 g of homogenized sample was extracted with 84% acetonitrile in water (100 ml) for 30 min using a horizontal shaker. After filtering through a filter paper, 10 ml of the raw extract was diluted with 20 ml water and purified using Strata X (500 mg) SPE column (Phenomenex, Torrance, CA, USA). Purifying procedure: column was conditioned with 6 ml methanol, followed by 6 ml of water prior to use, then 30 ml of diluted extract was loaded in the column [using loading cartridges 100 ml and manifold (Waters, Milford, MA, USA)], column was washed with 40% acetonitrile in water, then with 40% methanol in water and STC was eluted with 4 ml of 100% acetonitrile. The resulting eluate was evaporated to dryness under nitrogen at 60 °C and redissolved in 250 μl 25% water in acetonitrile. The calibrants were prepared by spiking the blank matrix with the standard and prepared in the same way as the samples.

2.5. LC–MS/MS analysis

A Waters Alliance 2695 liquid chromatograph (Waters) was connected to a MicroMass Quattro LC triple-quadrupole mass spectrometer (Micromass, Manchester, UK). An electrospray ionization (ESI) probe in the positive mode was used in the analysis of STC. The mobile phase consisted of 0.01% formic acid in acetonitrile and 0.01% formic acid in water (75:25 v/v) used in isocratic regime. The column used was a Phenomenex Luna $C_{18}(2)$ (5 μm), 150 × 3.0 mm (Phenomenex, Torrance, CA, USA). The flow rate was 0.3 ml/min, column temperature was 30 °C and the injection volume was 50 μl. The parameters of the mass spectrometer were optimized using the STC standard. The best response was recorded with the following parameters: cone voltage 30 V, capillary voltage 3.5 kV, extractor 2 V, radio frequence (RF) lens 0.2 V, source temperature 120 °C and desolvation temperature 350 °C, cone gas flow 63 (l/h), desolvation gas flow 553 (l/h), collision energy 30 eV.

For the structural identification in multiple reaction monitoring (MRM) mode, the molecular ion $[M+H]+(m/z=325)$ was fragmented within the MS to its daughter-ions ($325>310$ and $325>281$) collision energy 30 eV, dwell 0.2 s. Argon at pressure 3.5 bar was used as a collision gas. A calibration curve constructed using external standardization in matrix. The daughter-ion ($m/z=281$) was used for the quantification of STC. The ratio between peaks of STC obtained on two MRM channels (Peak area ($325>310$)/Peak area ($325>281$)) was used for confirmation of analyte. This ratio should be 0.69 ± 0.14 for the compound to be confirmed. The MRM chromatograms of blank wheat sample, spiked wheat sample (0.5 μg/kg), and naturally contaminated oat sample are shown in Fig. 2.

2.6. Spiking for recovery studies

Spiked samples of different grains were prepared by adding 25 μl of the 0.5 μg/ml STC standard solution, 25 μl, 125 and 500 μl of the 5 μg/ml STC standard using a digital pipette to 25 g of sample in an 250 ml flask, which was left for 1 h at ambient temperature with occasional agitation to allow the acetonitrile to evaporate. These volumes of standard were equivalent to levels of 0.5 μg/kg, 5 μg/kg, 25 μg/kg and 100 μg/kg STC, respectively. Six replicates at each concentration level were prepared from each commodity for recovery experiments.

Recovery results for different grains are shown in Table 1.

2.7. Calibration and linearity

In LC–MS methods the matrix often causes the change of the response, because the matrix components disturb the ionization of the analytes (Tang & Kebarle, 1993). Because of the matrix effect, the calibrants were always prepared in blank matrix (wheat, buckwheat, barley, oat and rye). Every matrix calibrated in two linear diapasons from 0.5 to 25 μg/kg and from 25 to 200 μg/kg.

The method was linear for STC from 0.5 to 25 μg/kg and from 25 to 200 μg/kg.

The method was linear for STC up to 200 μg/kg. A tolerance of ±10% accepted for the separate calibration points for good linearity. R^2 was always greater than 0.99 on this basis, the method considered linear for the analysis of STC.

3. Results and discussion

13.7% (13 samples) of the analyzed Latvian grain samples from the year 2006 were positive for STC. However, the concentration levels were quite low from 0.7 to 83 μg/kg (Table 2), but in some samples they were above maximum levels set for food products in Czech Republic and Slovakia (20 μg/kg). Only few of contaminated samples have fungi smell (one buckwheat sample has a fungi smell and it was positive for STC, one barley sample has a fungi smell, but it was not positive, and 2 wheat samples have a fungi smell, but they were not positive, however positive samples did not have a fungi smell). The weather at the year 2006 in Latvia was quite warm and dry.

Thirty five percent (42 samples) of the analyzed Latvian grain samples from the year 2007 were positive for sterigmatocystin (Table 3). The concentration levels were variable: the highest levels were detected in wheat and barley, medium in buckwheat and quite low in oats and rye samples, but again almost half of samples have STC levels above 20 μg/kg. The weather at the year 2007 in Latvia

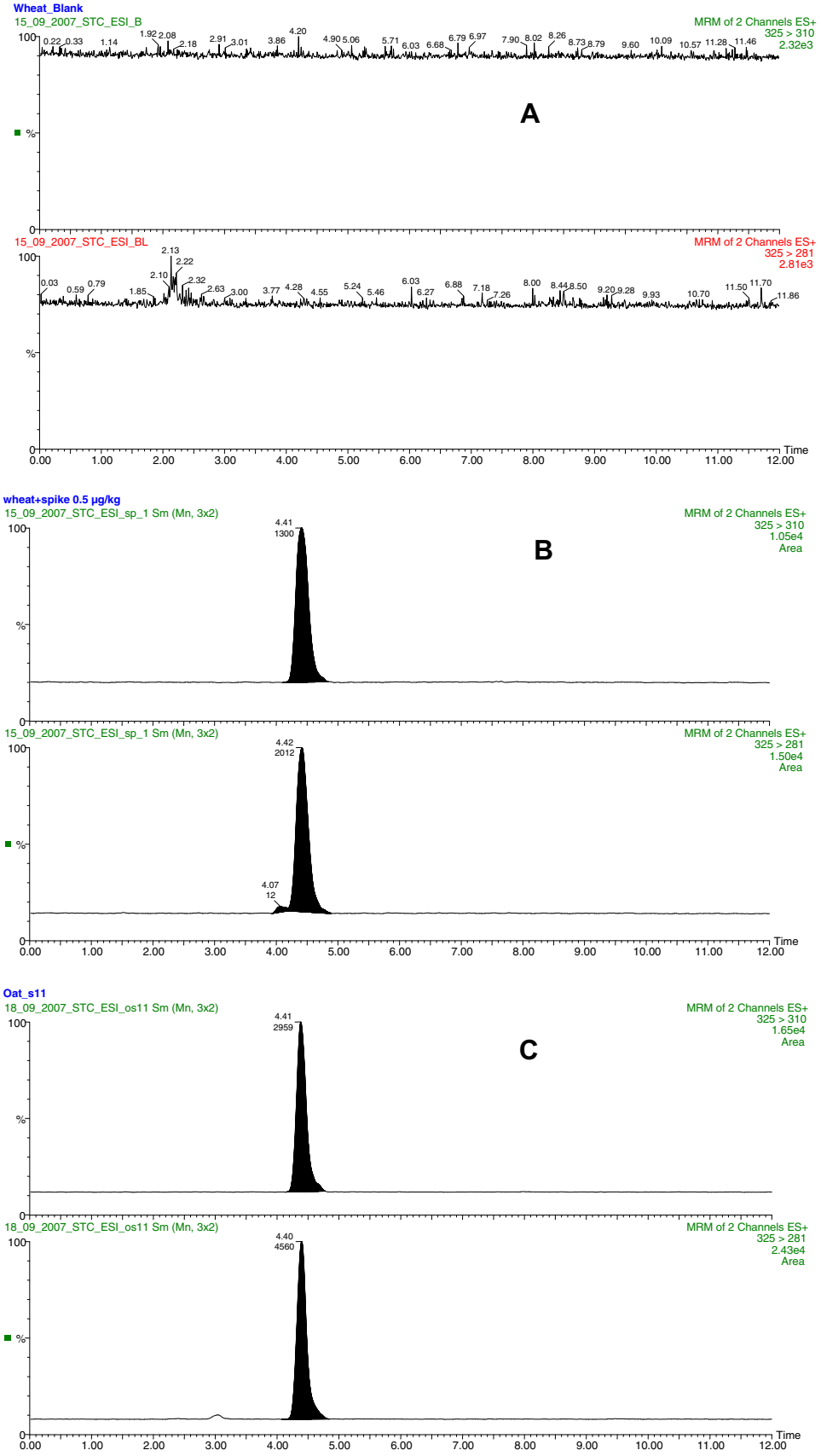

Fig. 2. MRM of blank wheat sample (A), wheat sample spiked with 0.5 µg/kg of STC (B) and naturally contaminated oat sample (C).

Table 1
Recovery of STC from spiked samples

Sample matrix	Added concentration, μg/kg	Found concentration (Mean ± SD), μg/kg	Mean recovery ($n = 6$), %	Precision RSD, %
Wheat grains	0.50	0.47 ± 0.02	94.5	4.0
	5.00	4.92 ± 0.24	98.4	4.8
	25.0	26.1 ± 1.67	104.4	6.4
	100	105 ± 6.81	105.3	6.5
Barley grains	0.50	0.45 ± 0.03	90.3	5.3
	5.00	4.89 ± 0.13	97.8	2.6
	25.0	24.3 ± 1.83	97.2	7.5
	100	98.4 ± 6.10	98.4	6.2
Oat grains	0.50	0.48 ± 0.02	96.3	4.6
	5.00	5.03 ± 0.15	100.5	2.9
	25.0	26.8 ± 1.39	107.2	5.2
	100	105 ± 7.66	105.2	7.3
Buckwheat grains	0.50	0.49 ± 0.02	97.4	4.2
	5.00	5.02 ± 0.14	100.5	2.8
	25.0	26.2 ± 0.84	104.6	3.2
	100	105 ± 4.62	105.2	4.4
Rye grains	0.50	0.44 ± 0.02	88.4	4.5
	5.00	4.02 ± 0.12	80.5	2.8
	25.0	23.2 ± 1.43	92.8	6.2
	100	95.6 ± 8.12	95.7	8.5

Table 2
STC presence and concentrations in different Latvian grains in year 2006

No.	Grains	Number of samples	Positive samples	Positive samples %	Range 0.5–25 μg/kg	Range 25–200 μg/kg
1	Wheat	50	9	18	5	4
2	Barley	10	2	20	2	0
3	Oat	15	0	0	0	0
4	Buckwheat	10	2	20	2	0
5	Rye	10	0	0	0	0
Total		95	13	14	9	4

Table 3
STC presence and concentrations in different Latvian grains in year 2007

No.	Grains	Number of samples	Positive samples	Positive samples %	Range 0.5–25 μg/kg	Range 25–200 μg/kg
1	Wheat	20	8	40	2	6
2	Barley	25	11	44	2	9
3	Oat	25	6	24	6	0
4	Buckwheat	25	9	36	4	5
5	Rye	25	8	32	8	0
Total		120	42	35	22	20

was variable from quite cold and dry to quite warm and rainy.

So, it is possible that warm and rainy weather affected contamination of grains at year 2007.

Only 25.6% of totally 215 analyzed samples from the years 2006 – 2007 were positive for STC.

There are no comparable results in literature, because of high detection limits of methods described in literature and a very little data about presence of STC in grains. However, obtained results indicates possible health risk for consumers due usage of contaminated grain products.

Because a STC was found in wheat grains there is a possibility that it can be transferred to wheat grain products such us bread, crackers, cookies, etc. From barley it can be transferred to beer, etc. From oat grains it can be transferred to oat flakes and other oat food products. Buckwheat is one of the main food products in Latvia; it is widely used in different foods. Rye in Latvia is the main ingredient for rye bread production which is very important part of Latvian people daily food intake.

So, all mentioned above indicates importance of determination of STC in typical Latvian grains, because grains are basic products (raw materials) for other typical food.

4. Conclusions

This is the first two-year long study which indicates the occurrence of sterigmatocystin in typical Latvian grains and importance of the investigation of this toxin in other food products where grains are used as ingredients or as basic substances.

So, in the aspect of all mentioned above, monitoring of the presence of STC in typical Latvian grains and other food products in Latvia is clearly necessary.

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