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Detection of alternariol in Estonian grain samples

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Abstract. Current testing for mycotoxins in cereals and feed in Estonia includes aflatoxin, ochratoxin, zearalenone and vomitoxin. Research at the Agricultural Research Centre (ARC) has shown that ~30% of analysed Estonian grain samples contain one or more of these mycotoxins.

The Estonian Research Institute of Agriculture (ERIA) has mycological evidence that grain samples could contain other mycotoxins than the four mentioned above. Other mycotoxins have also been detected in nearby countries (Finland, Norway). We suggest that current analyses for mycotoxins in Estonian grain should be expanded.

Alternaria was the main mould in grain samples on Saku experimental fields in 2008. This mould causes several plant diseases and severe damage of cereals. The aim of the current work was to implement methods for detection of the *Alternaria* toxin alternariol (AOH). Our results show that 4 out of 10 grain samples contained alternariol.

Key words: *Alternaria* spp, alternariol, high pressure liquid chromatography (HPLC)

INTRODUCTION

Alternaria is widely spread mould genus which can be found on plants, in soil, food and indoor air. According to Macrae et al. (1993), *Alternaria* is found in grain which has been dried on the field or where harvesting has been delayed because of rain, but also in grain which has grown in high humidity and has been damaged by early frost (Azcarate, 2008). In 1992–94 on average *Alternaria* spp occurred on 72% of spring wheat seeds and on 45% of winter wheat seeds in Estonia. Most frequent were *A. alternata* and *A. tenuissima* (Lõiveke et al., 2004b).

The major problems associated with *Alternaria* mycotoxin contamination of agricultural products are illustrated by focusing on various crops and their relevant diseases, e.g. black rot of tomato, olive, and carrots; black and grey rot of citrus fruits; black point of small-grain cereals; and *Alternaria* diseases of apples (Logrieco et al., 2009).

According to Sinha & Bhatnagar (1998), *Alternaria* diseases usually appear as spots on leaves, stems, fruits, sometimes surrounded by a discoloured halo, as tuber and fruit rot, or as black heads of cereal plants; but signs of disease may also be seeding damping-off, collar rot, and blights of several kinds of crop plants.

Alternaria species, including *alternata, citrii, tenuissima*, produce toxic components like alternariol, alternariol monomethyl ether and tenuazonic acid.

Alternariol is a hazardous mycotoxin because it has cytotoxic and mutagenic effects against bacteria and mammalian cells, and also fetotoxic and teratogenic effects in mice and hamsters (Azcarate, 2008; Tiemann, 2009). Results show that ca 0.8 µg AOH stopped progesterone production in pigs and 1.6 µg stopped cell development (Tiemann, 2009).

Although *Alternaria* is one of the major fungal genera found in grain, the presence of *Alternaria* mycotoxins has been largely ignored in these products. So far there are no special regulations and limits to *Alternaria* toxins in food (Azcarate, 2008).

Since 1991 some mycotoxins have been regularly analysed in domestic and imported food in Estonia (Akk, 2004). At present, ARC is responsible for monitoring mycotoxins in Estonia. The same types of experiments have also been conducted in laboratories of the Health Protection Inspectorate in Tartu and Tallinn. ARC has worked out a quick and inexpensive method to analyse grain and feeds (toxicity + nutrient compound) (Akk & Nõges, 2004). The Food quality and safety monitoring program (1998–2005) has shown that almost half of analysed Estonian grain samples contained mycotoxins.

Lõiveke has concluded that the total concentration of mycotoxins in grain feed is unacceptably high and may be the main reason why Estonian herbal and animal food is not able to compete in foreign markets (Lõiveke et al., 2004).

MATERIALS AND METHODS

Materials and instruments

The analysed grain samples were harvested in autumn 2008 on Estonian farmers' fields, including field trials of ERIA, and were subjected to a biotest with *Stylonichia mythilus*. Ten grain samples, more or less toxic in this test, were chosen for further analysis of alternariol: 4 wheat, 4 barley and 2 oat samples. The content of various mycotoxins is shown in Table 1.

Samples were ground in a domestic coffee mill. Deionised water with electric resistance ≥ 18.2 MΩ produced with MilliQ, from Millipore Corp., Billerica, MA, USA. HPLC grade methanol, from Rathburn Chemicals Ltd, Walkenburg, Scotland, was used in all experiments. The standard of alternariol from Alternaria sp. (purity approx. 96%) was purchased from Sigma-Aldrich, USA, cat. no. A 1312. A circulate shaker type WU-4 (Premed, Poland) was used for extraction. A rotary vacuum evaporator (NE-1, Weaton, USA) was used for removal of extraction solvent and a type Sigma 113 micro centrifuge (B. Braun Biotech International GmbH, Melsungen, Germany) was used for precipitation of solid debris before chromatography. HPLC instrument Series 200 from Perkin Elmer, Norwalk, CT, USA was used for chromatography, including a low pressure gradient pump, diode array spectrophotometric detector, column Zorbax Eclipse XDB-C18 4.6 x 250 mm, 5 µm (Agilent Technologies) with guard column AJO-4286 3 x 4 mm (Phenomenex), column oven adjusted to 35 °C and Rheodyne manual injector. All were controlled by mutually linked software TotalChrom Workstation v. 6.3.1 and TurboScan 200.

Pretreatment of samples

Grain samples weighed 2.5–3 kg. After thorough mixing with a disinfected spoon, 100 g of cereals were taken for grinding. The extraction method for *Alternaria* toxins was a modified procedure based on that described by Azcarate (2008). A ground grain subsample 8.0–8.1 g was extracted with a mixture of acetonitrile and 4% KCl in water (9 : 1 v/v, 40 ml) for 30 min, followed by the addition of 1 N HCl (8 ml).

The mixture was filtered and 25 ml of the filtrate was initially clarified by addition of 50 ml of 0.05 M lead acetate and then filtered again; 40 ml of the filtrate was then extracted three times in a separation funnel with 12 ml of chloroform. The organic phases were combined, evaporated to dryness and kept at ≅+4 °C until undergoing chromatography (Azcarate, 2008). The samples were dissolved in 300 µl of chromatographic solvent before analysis, transferred into Eppendorf tubes and centrifuged at 10,000 rmp for 6 min.

Chromatography

The mobile phase for isocratic elution contained 20% (v/v) water with 300 mg l^{-1} of zinc sulphate ($ZnSO_4*H_2O$) and 80% methano; and the flow rate was 0.8 ml $min^{-1;}$ 100 µl of clarified sample solution was injected into the column. Chromatograms were recorded at 258 and 280 nm, spectra were acquired in the range of 200 to 420 nm with mean frequency 2.27 measurements per second. Gradient elution was used as the alternative method in addition to isocratic elution. The gradient was a mixture of solvent A – methanol : acetonitrile : 300 mg*l^{-1} $ZnSO_{4*}H_2O$ in water (60 : 4 : 36) and solvent B – methanol : acetonitrile : 300 mg*l^{-1} $ZnSO_{4*}H_2O$ in water (75 : 15 : 10). Initial conditions were 75% A and 25% B and after 20 min it was changed linearly to 0% A and 100% B followed by isocratic elution with solvent B for 10 minutes.

RESULTS AND DISCUSSION

Alternariol detection and verification in grain samples

Alternariol was detected in grain samples by isocratic elution. To be assured that we have found alternariol, the alternative method, gradient elution, was used (described in M&M). Using gradient elution, one of the sample peaks clearly showed the same retention time as AOH in the standard reference sample (peak retention time 10.8 min) (Fig. 1). So, the presence of AOH can be regarded as confirmed by comparison of UV-spectra of the sample peak and standard, as well as by a similar change of retention times of two compounds after modification of the composition of the eluent (Fig. 2).

Calibration of the chromatographic system was made with eight different concentrations of AOH standard for both elution systems separately. Accurate linearity between peak area and amount of standard was observed in the range of 20-500 ng of AOH injected with correlation coefficient $R^2 = 0.9997$. The results were calculated for both elution alternatives and the final results were calculated as the mean from both elution alternatives.

Analyses of chromatograms from 10 grain samples are shown in Table 1. AOH was found in three wheat samples and in one barley sample. It is possible that some of

the samples with negative results contained small amounts of AOH, but spectra of the AOH peak were not conclusive, evidently due to smaller concentration of AOH in the samples. We estimated a 0.1 mg kg^{-1} of AOH in sample grain to be the limit of reliable identification of AOH by the current method. This limit may be improved by modifying separation efficiency, e.g. by finding appropriate pre-purification methods to remove disturbing components before the final chromatography, or, with improved methods for detection, tandem mass-spectrometry has been used for this purpose (Biselli & Hummert, 2005).

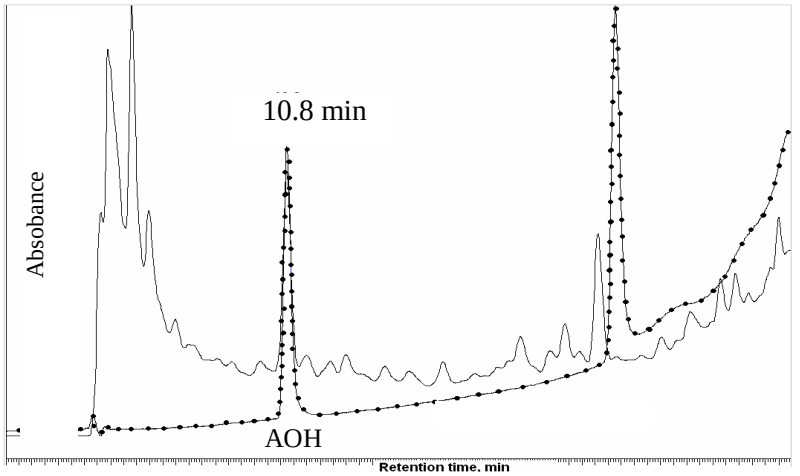

Figure 1. Wheat sample 08T-210 chromatogram (black line) compared with standard chromatogram (dotted line) in gradient elution.

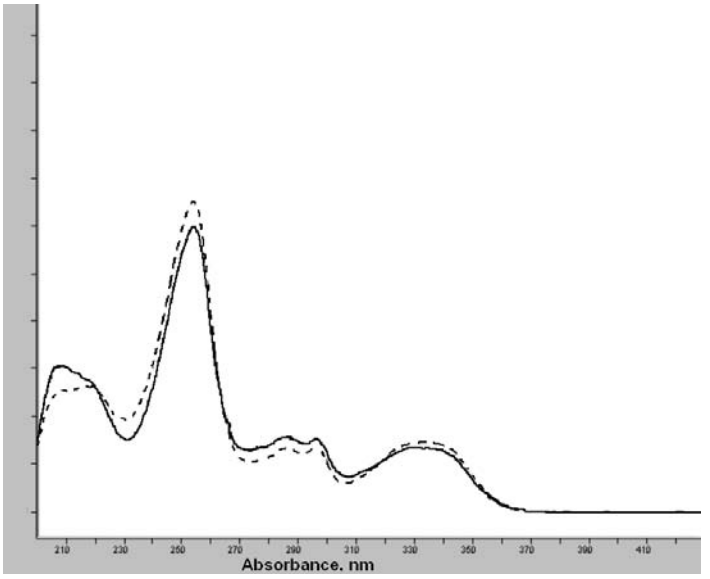

Figure 2. UV-spectrum of standard alternariol (broken line) compared with the spectrum of the corresponding peak of wheat sample 08T-210 (solid line) in the case of gradient chromatography.

Results summary

Table 1 contains the results of all 10 grain samples analysed during the study.

Table 1. AOH in grain samples

Grain	Sample sign	AOH, mg kg^{-1}	M & Y *, cfu g^{-1}	Fusarium *, cfu g^{-1}	Stylonichia mythilus survival %*, general toxicity
Oat	08T-209	-	$2.9*10^5$	$6.1*10^4$	71
Oat	08T-233	-	$6.6*10^5$	$2.2*10^4$	71
Wheat	08T-203	0.21	$5.9*10^4$	$2.6*10^3$	72
Wheat	08T-210	0.34	$6.4*10^4$	$8.5*10^3$	100
Wheat	08T-217	0.23	$5.1*10^4$	$1.8*10^3$	64
Wheat	08T-229	-	$3.3*10^3$	$1.1*10^2$	100
Barley	08T-205	-	$8.3*10^4$	$1.3*10^4$	78
Barley	08T-211	-	$4.8*10^4$	$4.3*10^3$	100
Barley	08T-215	0.13	$1.2*10^4$	$1.1*10^2$	100
Barley	08T-232	-	$4.1*10^4$	$7.6*10^3$	73

* Data were compiled at ARC. ARC analysed mould, yeast, Fusarium and general toxicity in cereal.

Of the 10 grain samples which had gone through toxicological tests at ARC, four samples contained alternariol. We believe that this is the first time alternariol has been detected in Estonian grain. The apparent high prevalence of AOH (detected in 4 out of 10 samples) clearly indicates that the analysis of mycotoxins in Estonian grain samples should be expanded.

Discussion

One research example, below, indicates the current situation in Estonian grain monitoring.

Estonian Food safety research state monitoring in 2003–2007 was carried out in the Tartu Health Protection Inspectorate laboratory. In five years, the mycotoxins have been detected in only 91 grain samples, of which 21 samples were domestic production.

Comparing these results with Tallinn University of Technology masters researches (Lapõnina, 2007; Kütt, 2009), it can be said that information about mycotoxins is extremely inadequate and research on Estonian grain products should be extended.

Therefore new mycotoxins, which have never been analysed, should be identified and proper determination methods should be used to analyse grain samples, to help improve cereal, food and feed safety and toxicological quality. Increased vigilance of toxins would have a positive effect on the economic status of the Estonian food industry and animal breeding, making it more competitive in foreign markets

CONCLUSIONS

The finding of alternariol in four out of 10 Estonian grain samples clearly shows that current testing is inadequate. The Estonian climate consists of frequent rainfall; the soil, which is excessively moist, creates an appropriate environment for several mycotoxins, including Alternaria genus moulds. We suggest that mycotoxin analysis and nomenclature should be expanded, taking into account our domestic research basis.

The current study used reverse phase HPLC with diode array detection to analyse alternariol. This method enables detection of mycotoxins with concentrations of 0.15 mg kg^{-1} or more in grain samples. To analyse for lower concentrations of alternariol, the method must be improved using either better pre-purification methods or more selective detection methods.

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