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Note

High-performance liquid chromatographic determination of zearalenone and ochratoxin A in cereals and feed

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Cereals and mixed feed are frequently contaminated with mycotoxins produced by different fungi. Two of the more important ones are ochratoxin A and zearalenone[1–3]. In Norway ochratoxins are normally produced by *Penicillium verrucosum* and the A type is predominant. Zearalenone is like the trichothecenes a fusarium toxin. The gas chromatographic method normally used for the trichothecenes gives, however, low recovery[4].

Zearalenone and ochratoxin A can be analyzed by high-performance liquid chromatography (HPLC) under very similar conditions[5]. Howell and Taylor[6] have described a method for determination of aflatoxins, ochratoxin A and zearalenone in feed. The recoveries of ochratoxin A achieved by using this method are, however, sometimes poor.

In this paper a modified method for the determination of zearalenone and ochratoxin A is described. Aflatoxin is omitted because it is of minor importance in cereals grown in northerns countries like Norway. Modern solid phase extraction columns were used for clean up. These minicolumns are convenient to use and the solvent consumption is less than 1/10 of that of traditional columns. The procedure given was optimized for different types of cereals and feed.

One hundred samples of wheat, barley, oats and mixed feed have been analyzed by the procedure described. The detection limits were 2–5 μg/kg for zearalenone, depending on the type of feed, and 0.1–0.3 μg/kg for ochratoxin A.

EXPERIMENTAL

Reagents

Ochratoxin A and zearalenone were obtained from Sigma. The following stock solutions were made: (a) zearalenone, 20 mg/l in acetonitrile; (b) ochratoxin A, 1 mg/l in toluene–acetic acid (9:1).

Other chemicals were obtained from the following sources: Celite from Supelco; clean-up columns were Bond Elut, SI (silica), 500 mg, from Analytichem International.

All solvents used for the clean-up procedure were of p.A. grade (Merck), while the solvents used as the mobile phase for HPLC were of HPLC grade (Rathburn).

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Apparatus

The flask shaker was a universal shaking machine from Edmund Bühler, Type SM 2.5. The vacuum manifold used in connection with the clean-up columns were obtained from Supelco. The HPLC equipment was obtained from Perkin-Elmer, and consisted of a dual-pump module (Series 2) with a Rheodyne injector (Model 7125) or a Series 10 pump with an ISS-101 autoinjector. The detector was an LS-4 fluorescence spectrophotometer. The integrator system was either a LC-100 integrator or an Omega-2 data system.

The analytical column was a Nucleosil C_8, 5 μm reversed-phase column, 125 mm $\times$ 4 mm I.D. The 50-mm guard column was dry packed with LC-8 pellicular packing, 40 μm (Supelco).

Sample preparation

The samples were kept in a deep freezer at $-18°C$ until analyzed. The whole sample was ground and mixed well before an analytical sample was taken.

Extraction and clean up

A 50-g amount of ground sample was mixed with 250 ml chloroform and 25 ml 0.1 *M* phosphoric acid in a 1-l flask. Celite (10 g) was added to wheat and barley samples. The flask was shaken automatically for 45 min. Wheat and barley samples were filtered through folded filters, while the mixture of extraction solvent and solid material of oat and mixed feed samples was transferred to a 250-ml centrifuge-tube and centrifuged for 10 min at 9000 *g* before filtration. The centrifugate was then decanted and filtered through a folded filter containing 2 g Celite.

A 25-ml volume of the wheat and barley extracts, and 15 ml of the oat and mixed feed extracts, was transferred to pear-shaped flasks and evaporated almost to dryness on a rotary evaporator. Dichloromethane (10 ml) was added to the residues.

Column clean up

The column was connected to the vacuum manifold after addition of about 2 g dried Na_2SO_4 to the top of it. A 5-ml volume of hexane and 5 ml dichloromethane were washed through the column before the sample extract was transferred quantitatively to the column. The solvent was drained to the top of the layer of Na_2SO_4 and the column was washed with 10 ml dichloromethane, 10 ml hexane and 10 ml toluene. Thereafter, zearalenone was eluted with 8 ml toluene–acetone (95:5). Ochratoxin A was eluted with 6 ml toluene–acetic acid (9:1). The two fractions were collected in separate 12-ml conical centrifuge-tubes.

The eluates were evaporated to dryness under a stream of nitrogen. A 250-μl volume of acetonitrile and 250 μl 0.1 *M* phosphoric acid were added to each residue and mixed on a Whirlimixer for 1 min. The samples were then sonicated for 5 min and mixed once more on the Whirlimixer before centrifugation for 10 min at 2000 *g*. An 100-μl volume of the supernatant was transferred to an HPLC sample vial.

HPLC analysis

A 20-μl volume of the extract was injected into the chromatograph. Methanol–0.01 *M* orthophosphoric acid (58:42) was used as the mobile phase. The flow-rate was 1 ml/min, and the excitation wavelength of the fluorescence detector was set at 270 nm

for zearalenone and 340 nm for ochatoxin A, while the emission wavelength in both cases was 465 nm.

RESULTS AND DISCUSSION

Chloroform–phosphoric acid (250:25) is an effective extraction medium for ochratoxin and zearalenone[6,7].

Oat samples and mixed feed samples did not become clear after filtration, not even after addition of Celite. A clear extract was required for the further clean-up procedure on the minicolums. Centrifugation of the extract including the feed followed by filtration gave, however, a clear extract.

Minicolumns or solid phase extraction columns were used. Up to twelve samples were easily handled at the same time. From wheat and barley samples extracts equivalent to 5 g of cereals can be placed on columns containing 500 mg of packing material. In extracts from oats and mixed feed the amount of contamination is higher, thus extracts equivalent to only 3-g samples can be placed on the columns. Otherwise a reduced recovery was observed, especially for zearalenone.

Sodium sulphate was added to the top of the silica minicolumn to reduce the water content of the dichloromethane extract, and thereby also the elution strength of the solution.

The necessity for each washing step was examined. Omission of the dichloromethane step gave a less clean chromatogram for zearalenone. Washing with toluene can in some cases be omitted, but not always. The procedure originally contained a washing step with chloroform–methanol (97:3) after the elution of zearalenone as described by Howell and Taylor[6]. A very low recovery was then occasionally obtained for ochratoxin; the toxin had been washed out with the chloroform–methanol solution.

Of the zearalenone 95% was found in the 2–6 ml fraction when eluted with 10 ml toluene–acetone (95:5), while 95% of the ochratoxin was eluted with 4 ml toluene–acetic acid (9:1). Aflatoxins may at this state be eluted with 10 ml chloroform–methanol (97:3)[6,8].

The eluate of zearalenone was normally coloured, often green. When evaporated to dryness, green drops were seen which did not dissolve in acetonitrile–phosphoric acid. Sonication and mixing with a Whirlimixer ensured the dissolution of zearalenone. By centrifugation a clear extract was obtained which can be injected directly into the HPLC system. Filtration of the extract can then be omitted.

Both a reversed-phase[6,7,9] and a normal-phase[10,11] chromatographic system have been used for the determination of zearalenone by HPLC, while only a reversed-phase has been used for ochratoxin A[6,7]. Elution of ochratoxin A, containing a carboxylic acid group, requires an acidic mobile phase. Even though zearalenone and ochratoxin A cannot be chromatographed at the same time, it is preferable to use the same mobile phase. Both acetonitrile and methanol can be used as the organic modifier of the mobile phase. Either methanol–0.01 *M* phosphoric acid (58:42) or acetonitrile–0.01 *M* phosphoric acid (40:60) was used when the Nucleosil C_8 column was used. A Supelcosil C_{18} column was also tried. This column can be used when the washing step with dichloromethane in the clean-up procedure is not omitted.

Both 275 (ref. 6) and 236 nm (refs. 9–12) have been used as excitation wavelength

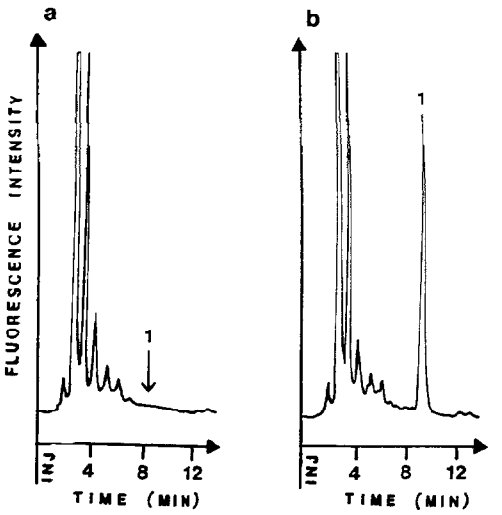

Fig. 1. Chromatogram of (a) barley sample containing no zearalenone and (b) the same sample spiked to contain 100 μg/kg zearalenone (1). Column: Nucleosil C_8, 5 μm, 50 mm × 4.6 mm + 125 mm × 4 mm I.D. Mobile phase: methanol–0.01 *M* phosphoric acid (58:42). Detector: fluorescence spectrophotometer; excitation at 270 nm, emission at 465 nm.

for zearalenone. A similar or higher signal-to-noise ratio was obtained with 275 nm as with 236 nm. The former was chosen because of the higher selectivity.

About 100 samples have been analyzed by the method described: 36 samples contained detectable amounts of zearalenone and 54 samples contained detectable amounts of ochratoxin. The highest amounts were found to be 137 and 1300 μg/kg,

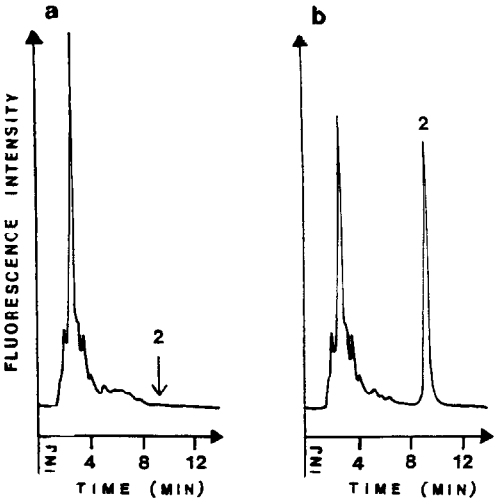

Fig. 2. Chromatogram of (a) barley sample containing no ochratoxin and (b) the same sample spiked to contain 5 μg/kg ochratoxin (2). Chromatographic conditions as in Fig. 1, except that 340 nm was used as the excitation wavelength.

TABLE I

RECOVERY TESTS DONE ON DIFFERENT TYPES OF SAMPLES

Wheat and barley samples were spiked to contain 100 μg/kg zearalenone and 5 μg/kg ochratoxin A in addition to the natural contamination by these mycotoxins. The figures were 167 and 8.3 μg/kg, respectively, for oat and mixed feed samples. See text for further information.

Recovery (%)	*Zearalenone*				*Ochratoxin*			
	Wheat	*Barley*	*Oat*	*Feed*[a]	*Wheat*	*Barley*	*Oat*	*Feed*
Mean	83	89	78	77	96	92	81	77
S.D.	8	6	6	10	8	15	15	21
n[b]	9	10	15	9	11	9	14	6

[a] Mixed feed.
[b] Number of samples.

respectively. The identity of the HPLC peaks was verified by fluorescence spectra when samples contained high amounts of the mycotoxins. These results will be published elsewhere.

Typical chromatograms of samples containing no zearalenone and ochratoxin and samples spiked to contain the same mycotoxins are shown in Figs. 1 and 2.

At least one recovery test was done for each set of samples analyzed. A 500-ng amount of zearalenone and 25 ng ochratoxin A were added to an extra aliquot of a chloroform extract of the sample. The same clean up and HPLC method was used for these extracts as for the other ones. The standard solution was added to the chloroform extract instead of to the cereal sample itself in routine analysis to save time and solvent. We found no significant difference in recovery between the two methods, the average being 8% in favour of addition to the cereals. The standard deviation was 9% for eight samples. The average of the recovery tests obtained for zearalenone and ochratoxin in different types of samples are shown in Table I.

The reproducibility of the method was checked for different types of samples.

TABLE II

REPRODUCIBILITY TEST ON FOUR DIFFERENT SAMPLES

Mean relative standard deviation: 18% (15%[a]).

Sample	*Zearalenone*				*Ochratoxin*			
	Wheat	*Barley*	*Oat*	*Feed*[b]	*Wheat*	*Barley*	*Oat*	*Feed*
1 (μg/kg)	34	4	<5	15	<0.1	86	4.5	0.8
2 (μg/kg)	41	6	<5	20	<0.1	119	4.8	0.4
3 (μg/kg)	42	6	<5	16	<0.1	105	4.0	0.5
4 (μg/kg)	29	5	<5	13	<0.1	123	4.0	0.5
Mean (μg/kg)	37	5	–	16	–	108	4.3	0.6
S.D. (μg/kg)	6	1	–	3	–	17	0.4	0.2
R.S.D. (%)	17	18	–	18	–	15	9	31

[a] Ochratoxin in feed is not included, because of its low content.
[b] Mixed feed.

Four samples were analyzed four times each for zearalenone and ochratoxin A. The results are given in Table II. The samples were ground especially well before the analysis, to remove the inhomogeneities that always exist in mycotoxin samples. Some variations in the results may nevertheless reflect inhomogeneities in the samples.

The detection limit depended on the type of sample, being highest for mixed feed and oat samples. It was found to vary between 2 and 5 μg/kg for zearalenone and between 0.1 and 0.3 μg/kg for ochratoxin.

CONCLUSIONS

A rapid, sensitive and selective method for the determination of the important mycotoxins zearalenone and ochratoxin A is presented.

The clean-up procedure given is optimized for modern solid phase extraction columns, which are much easier to handle than conventional columns. The method involves no liquid–liquid extraction or other time-consuming steps. The identification and quantification is by HPLC. Clean chromatograms are obtained in most cases, making it easy to identify the zearalenone and ochratoxin peaks. Even with complicated mixed feed samples, the mycotoxins can be determined without further purification of the eluate from the minicolumn, which is recommended by other workers[5,6].

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