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Note

Determination of sterigmatocystin in feed by high-performance liquid chromatography with column switching

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Sterigmatocystin (Fig. 1) is a toxic mould metabolite and recognized carcinogen[1–3] recently detected in feed (7.75 mg/kg) associated with acute clinical symptoms of bloody diarrhoea and death in dairy cattle[4]. It is produced by strains of *Aspergillus versicolor, Aspergillus nidulans, Bipolaris sorokiniana* and other species of the genus *Aspergillus*[5]. The main producer, *Aspergillus versicolor*, is of ubiquitous distribution and a common contaminant of a wide variety of foods and feeds[6]. Because it has been found to grow on roughages[7,8], it is possible that hay and straw intended for feeding purposes may contain sterigmatocystin. Numerous studies on the occurrence and analysis of sterigmatocystin in various foods and feeds using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) have been published[9–15]. The application of these methods to the analysis of hay and straw for sterigmatocystin yields unsatisfactory results, because some interfering compounds cannot be separated completely from sterigmatocystin by either TLC or HPLC. For this reason, we have developed an efficient and simple method for the determination of sterigmatocystin at low ppb levels in straw and hay by HPLC with column switching.

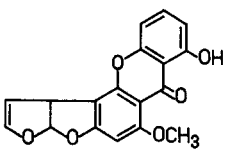

Fig. 1. Structure of sterigmatocystin.

EXPERIMENTAL

Chemicals

Sterigmatocystin was prepared in our laboratory[16] and its identity was confirmed by mass spectrometry, nuclear magnetic resonance spectroscopy and UV spectrometry. Its purity was determined by TLC and HPLC to be as high as 99%. A stock solution (5 μg/ml) was prepared in methanol. Other solutions were obtained by dilution. All solvents were distilled before use. Silica gel 60 for column chromatography (70–230 mesh) was activated for 2 h at 120°C.

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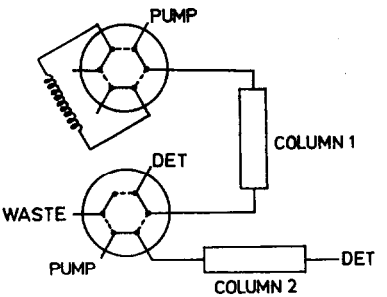


Fig. 2. Column switching system for the determination of sterigmatocystin in straw and hay (see text for details).

Apparatus

Fig. 2 is a schematic diagram of the column switching assembly employed. The chromatographic system consists of a double-headed Orlita high-pressure pump (Hewlett-Packard Model 1010 B liquid chromatograph) with two-solvent capability equipped with a sampling valve (OE-320; Labormim, Budapest, Hungary) and an additional six-port valve (Rheodyne Model 7010). A LiChrosorb RP-18 (particle size 7 μm) column (25 × 0.46 cm I.D.) for analytical work and a LiChrosorb RP-8 (particle size 10 μm) column (10 × 0.46 cm I.D.) were used. The injection and switching valve work manually, but the system can be automated easily for routine analysis. For detection a Hewlett-Packard Model 1030B variable-wavelength detector and an LCD 254 UV monitor (Laboratorní Přístroje, Prague, Czechoslovakia) were used.

Extraction and separation

The extraction and clean-up procedure is based on the AOAC method for the determination of sterigmatocystin in barley and wheat[17]. Extraction of 10 g of dried and finely ground straw or hay with acetonitrile–water containing 4% potassium chloride (9:1) was followed by partition against *n*-hexane and chloroform. After evaporation of the solvent, the residue was purified on 10 g of silica gel 60 (70–230 mesh), eluted with cyclohexane–ethyl acetate (4:1). The eluate was vacuum evaporated at 40–50°C. The residue was transferred quantitatively into a 10-ml flask with sequential portions of methanol (3 × 1 ml) and the solvent was evaporated to dryness under a gentle stream of nitrogen. The sample was redissolved in an appropriate volume (0.5–5.0 ml) of acetonitrile–$3.3 \cdot 10^{-3}$ *M* phosphoric acid (45:55) and after filtration it was injected into the HPLC system.

The switching time can be derived from the retention time of sterigmatocystin, which was determined daily by three injections of a standard solution onto column 1 (Fig. 2), followed by elution with acetonitrile–$3.3 \cdot 10^{-3}$ *M* phosphoric acid (45:55) at a flow-rate of 1 ml/min.

Feed samples were injected onto the pre-column. After washing with acetonitrile–$3.3 \cdot 10^{-3}$ *M* phosphoric acid (45:55) for about 5 min, the eluate from column 1 containing sterigmatocystin was switched to the analytical column. When the desired volume was diverted from column 1 to column 2, the valve was placed in its original position and chromatography proceeded on column 2 with acetonitrile–$3.3 \cdot 10^{-3}$ *M* phosphoric acid (60:40) as the mobile phase at a flow-rate

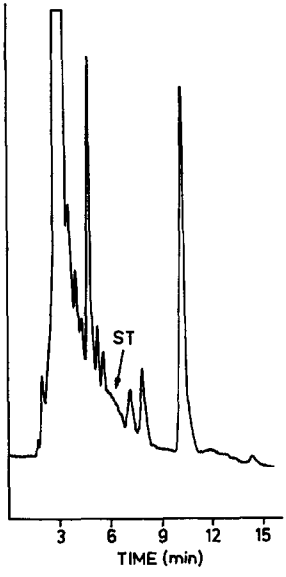
of 1.5 ml/min. Calibration graphs for sterigmatocystin in the concentration range 10–100 ng per injection were monitored in the same manner.

Detection of sterigmatocystin was performed by UV absorption measurement at 325 nm. The sensitivity was set to 0.02 a.u.f.s. The effluent from the pre-column was monitored by UV detection at 254 nm. The injection volume was 20 μl in all instances.

RESULTS AND DISCUSSION

Chromatograms of straw and hay samples extracted and purified by the AOAC method for the determination of sterigmatocystin in barley and wheat and analysed by HPLC are given in Figs. 3 and 4. The chromatograms illustrate that this procedure did not provide adequate clean-up of extracts. Compounds were detected in control samples that were not separated completely from sterigmatocystin. These peaks are variable amount and interfere with accurate quantitation. For this reason, additional purification steps are necessary, which are tedious to perform and often give rise to loss of toxin. These drawbacks can be circumvented by HPLC with column switching, reviewed by Freeman[18].

The application of two reversed-phase columns allows direct switching of an effluent segment of the pre-column to the analytical column without trapping loops or manual solvent evaporation steps. The column length, alkyl chain length of the chemically modified silica and particle size were selected in such a way that column 1 can be eluted with a markedly weaker solvent than column 2. This enables relatively



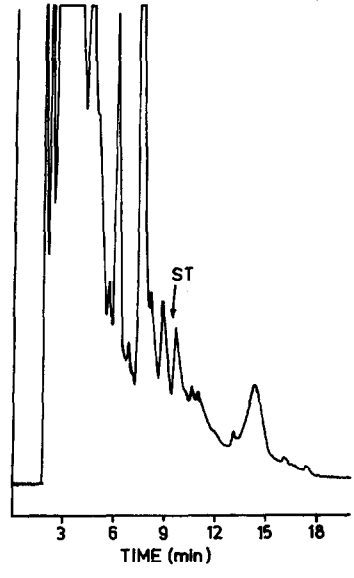
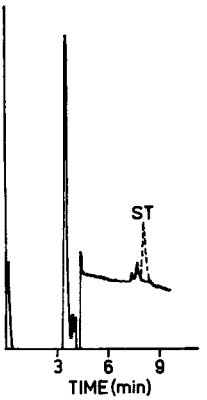


Fig. 3. HPLC separation of a control straw sample on a LiChrosorb RP-18 (particle size 7 μm) column at a flow-rate of 1.5 ml/min with acetonitrile–3.3 · 10^{-3} M phosphoric acid (60:40) as eluent and UV detection at 325 nm. ST = sterigmatocystin.

Fig. 4. HPLC separation of a control hay sample. Flow-rate, 1.0 ml/min; other conditions as in Fig. 3. ST = sterigmatocystin.



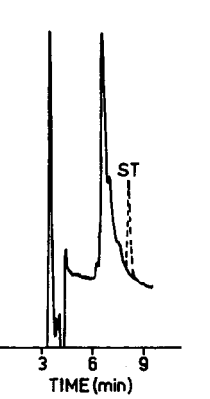


Fig. 5. Chromatogram of a straw sample spiked with 50 ppb of sterigmatocystin (ST) obtained by HPLC with column switching. The solid line refers to the response of a blank and the broken line to the spiked sample.

Fig. 6. Chromatogram of a hay sample spiked with 100 ppb of sterigmatocystin (ST) obtained by HPLC with column switching. The solid line refers to the response of a blank and the broken line to the spiked sample.

large volume segments of the pre-column to be switched directly to the analytical column without undesirable band spreading of the compound of interest.

The HPLC traces of unspiked and artificially contaminated straw and hay samples shown in Figs. 5 and 6 show the high purity of the sample extracts transferred to the analytical column by the column switching technique. No interfering peaks were detected at the retention time of sterigmatocystin and accurate quantitation was possible. The total HPLC analysis time is about 15 min. At the end of the analytical run the pre-column does not need flushing with stronger solvents in most instances. A time of 15 min is sufficient for elution of strongly retained compounds and reconditioning of the column. Another advantage of the method is the prolonged lifetime of the analytical column. It has been used in our laboratory for 3 months with daily injections without any decrease in column efficiency. Column 1 has to be repacked after about 200 injections, depending on the type of samples injected. The detection limit for sterigmatocystin in hay and straw was 25 ppb*.

TABLE I

REPRODUCIBILITY OF RETENTION TIME FOR STERIGMATOCYSTIN

Standard solutions and spiked straw samples were injected.

Parameter	*Value*
No. of injections	30
Retention time:	
Range (s)	457–481
Mean (s)	473
Standard deviation (s)	6.6
Coefficient of variation (%)	1.4

* Throughout the article, the American billion (10^9) is meant.

TABLE II

REPRODUCIBILITY OF PEAK HEIGHT FOR STERIGMATOCYSTIN

20-μl aliquots of an 11.6 ng/μl sterigmatocystin standard solution in methanol were injected: the detector sensitivity was set to 0.04 a.u.f.s.

Parameter	*Value*
No. of injections	10
Peak height:	
Range (mm)	132.5–136
Mean (mm)	134.8
Standard deviation (mm)	1.26
Coefficient of variation (%)	0.93

The retention times and peak heights ascertained for a sterigmatocystin standard solution and for contaminated straw samples provide evidence of the high reproducibility of the HPLC column switching technique employed (Tables I and II). The relationship between peak height and amount of sterigmatocystin injected was linear over the range 10–250 ng. Average recoveries of sterigmatocystin added to straw and hay in amounts from 50–200 ppb were 71.5 and 59.4%, respectively.

The column switching technique was successfully employed for the analysis of various naturally sterigmatocystin-contaminated hay samples (Fig. 7). Preliminary studies have shown that the procedure described may also be applied to the determination of sterigmatocystin in cereals, maize and silage.

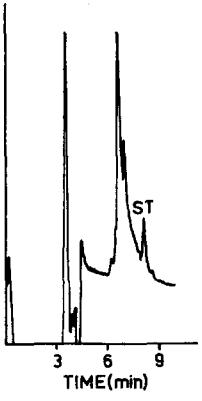


Fig. 7. Chromatogram of a hay sample naturally contaminated with 72 ppb of sterigmatocystin (ST) obtained by HPLC with column switching.

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