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Note

Determination of patulin and penicillic acid in unroasted cocoa beans

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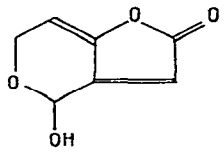
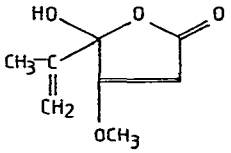
Mycotoxins consist of a large group of relatively unrelated compounds which are produced as secondary metabolites by fungi. Mycotoxicoses are a group of diseases caused by the consumption of products contaminated by these metabolites and are of considerable significance in animal and human health.

Patulin and penicillic acid are both mycotoxins which are produced by several species of *Penicillium* the former also being produced by *Aspergillus* spp. Patulin may contaminate commodities as diverse as apple juice and rice and penicillic acid has been detected in mouldy corn[1]. The chemical structures of these two mycotoxins are shown below.



PATULIN



PENICILLIC ACID

Several workers have examined foodstuffs, such as apple juice[2], grain[3] and cheese[4] for the presence of patulin and/or penicillic acid. Both toxins are usually extracted into ethyl acetate from the foodstuff. The extract is then usually submitted to a cleanup procedure, either by liquid–liquid partition and/or by column chromatography[5]. Detection and quantitation of the mycotoxins has been undertaken by thin-layer chromatography[2], gas chromatography of a derivative[6] or by high-performance liquid chromatography[7]. Owing to interference from other phenolic material in cocoa beans, a new method was needed, which would exhibit better selectivity and sensitivity. The method chosen was based on a cleanup by solvent partition and analysis by gas chromatography–mass spectrometry (GC–MS) of the silylated toxins.

EXPERIMENTAL

Reagents

The following reagents were used: glass-distilled water; ethyl acetate (AnalaR grade; hexane (glass distilled); methanol (glass distilled); dichloromethane (glass distilled); N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; Pierce, Chester, Great Britain).

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Equipment

The following equipment was used: Silverson laboratory mixer-emulsifier (heavy duty, sealed unit) fitted with a $\frac{3}{4}$-in. tubular head; Büchi rotary evaporator fitted to a vacuum water pump and used in conjunction with a heated-water bath; block heater, capable of taking 0.3 ml and 1 ml heavy-wall glass vials (Reacti-Vials; Pierce); A Hewlett-Packard 5992A coupled gas chromatograph–mass spectrometer, equipped with a glass column (1.8 m × 4 mm I.D.) packed with 3% OV-3 on Gas-Chrom Q (100–120 mesh) and operated at 180°C with a helium carrier gas flow-rate of 30 ml/min. The injection port temperature was maintained at 225°C.

Procedure

The sample (10 g; milled in coffee grinder) and ethyl acetate (40 ml) were homogenised for 2 min with a Silverson mixer. The mixture was filtered through a Whatman No. 4 filter paper (15 cm diameter) into a 250-ml round-bottomed flask. The residue in the funnel was washed with ethyl acetate and the washings were combined with the main filtrate.

The filtrate was evaporated under vacuum at 40°–45°C just to dryness, the residue was shaken with a mixture of water (5 ml) and hexane (10 ml) for 1 min. The hexane layer was discarded and the aqueous phase was further extracted twice with hexane. The aqueous layer was evaporated under vacuum at 65°–70°C and transferred to a Reacti-Vial with portions of methanol (3 × 0.25 ml). The solutions were heated at 60°C under a stream of air to remove solvent.

Silylation of this extract and of standards was effected by the addition of MSTFA (20 μl) and sufficient dichloromethane to give a volume of 100 μl. Mixtures were heated at 60°C for 20 min in the block heater. Injections of 1–2 μl of this solution and of standards were made on to the GC–MS apparatus, run initially under "peak-finder" conditions for standards in order to obtain the characteristic mass spectra (Figs. 3 and 4), followed by selected ion monitoring for the analysis of samples. The silylated penicillic acid was monitored at *m/z* 214 and 227 and the corresponding patulin derivative was measured at *m/z* 211 and 226. The sensitivity of the instrument was adjusted so that 10 ng of each derivative gave a peak which could be readily quantified.

RESULTS AND DISCUSSION

Samples of unroasted cocoa beans, spiked at two levels each with patulin and penicillic acid, were derivatised and analysed by GC–MS. Ion monitor chromatograms of spiked and unspiked extracts are shown in Figs. 1 and 2. Tne peak on chromatogram B in Fig. 1 at retention time 5.5 min corresponds to O-trimethylsilyl patulin and the corresponding peak in Fig. 2 at 3.6 min to O-trimethylsilyl penicillic acid. The results of analyses are summarised in Table I.

Although a peak was observed for patulin spiked at 12 μg/kg, it was too small for quantitation; the detection limit was estimated to be about 20 μg/kg. The corresponding detection limit for penicillic acid was about 10 μg/kg.

In order to determine whether the observed peaks were absent from interferences, the ratios of the peak areas on the selected ion chromatograms were compared with those from standard solutions. The results are summarised in Table II.

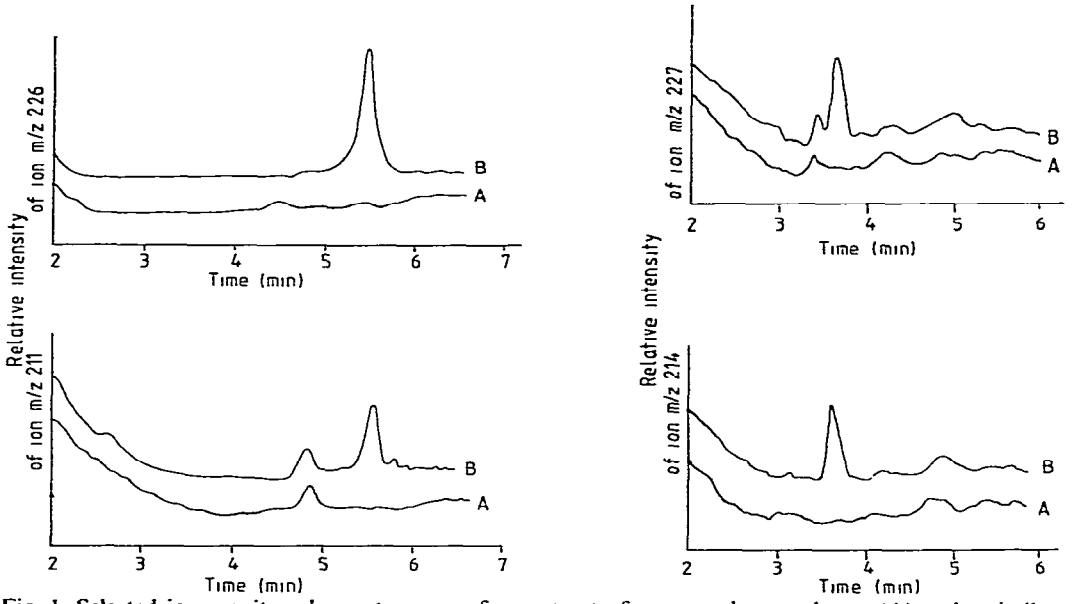

Fig. 1. Selected ion monitor chromatograms of an extract of unroasted cocoa beans (A) and a similar extract spiked with patulin at a level of 62 μg/kg (B). Analysis on a glass column (1.8 m × 4 mm I.D.) packed with 3% OV-3 on Gas-Chrom Q operated at 180 C.

Fig. 2. Selected ion monitor chromatograms of an extract of unroasted cocoa beans (A) and a similar extract spiked with penicillic acid at a level of 87 μg/kg (B). Conditions as in Fig. 1.

TABLE I

RECOVERY OF PATULIN AND PENICILLIC ACID IN UNROASTED COCOA BEANS

Mycotoxin	*Amount added (μg/kg)*	*Amount found (μg/kg)*
Patulin	0	0
	12	<10
	62	31
Penicillic acid	0	0
	17	13
	87	65

TABLE II

ANALYSIS OF PATULIN AND PENICILLIC ACID TRIMETHYLSILYL ETHERS BY MONITORING OF ION RATIOS

Mycotoxin	*Ions (m/z)*	*Ratio in standard*	*Ratio in spiked sample*	*Level in spiked sample (μg/kg)*
Patulin trimethylsilyl ether	211/226	1.31	1.34	62
Penicillic acid trimethylsilyl ether	214/227	1.01	1.05	87

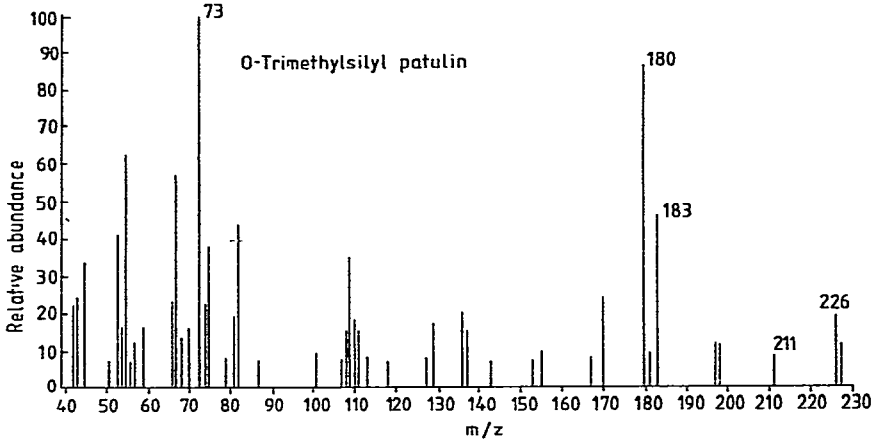

Fig. 3. Mass spectrum of O-trimethylsilyl patulin.

Comparison of the peak area ratios quoted in Table II with peak heights recorded in the mass spectra of the two silyl derivatives (Figs. 3 and 4), shows an apparent anomaly. The ratio of peak heights on ions 211/226 on Fig. 3 gives a value of 0.42, whereas the ratio quoted in Table II is 1.31. The reasons for this may be explained as follows: the peak mass recorded in Fig. 3 for the molecular ion is quoted as a whole figure number of 226, whereas accurate calculations show that the true value is 226.07. Since the spectrometer has a resolution of 2000, when set at 226.0 in the ion monitor mode, it will only measure a fraction of the total peak area. Furthermore, the mass spectrum measured in the peak-finder mode is independent of GC conditions, since the instrument is programmed to record the spectrum at the point of maximum response. However, in the selected ion mode the instrument is set to integrate the area under a chromatographic peak response of the given ion and will include the area under a falling peak. Thus, the two measurements of peak height and peak area in the two modes of operation depend on different factors and cannot be

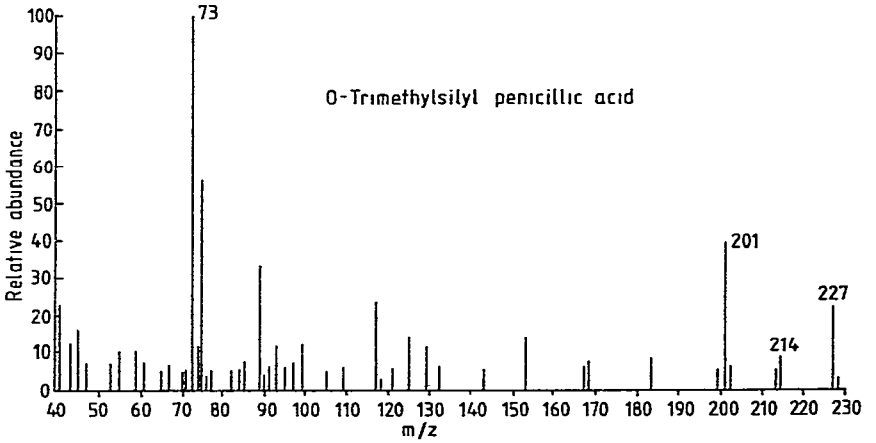

Fig. 4. Mass spectrum of O-trimethylsilyl penicillic acid.

strictly compared. When used independently, both measurements show good reproducibility and may be employed for quantitative analysis.

The analytical procedure has been used for the screening of unroasted cocoa beans for the presence of mycotoxins and has also been successfully extended to the analysis of unroasted coffee beans.

ACKNOWLEDGEMENT

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