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QUANTITATION OF ZEARALENONE BY GAS–LIQUID CHROMATOGRAPHY ON CAPILLARY GLASS COLUMNS

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SUMMARY

A procedure is described for the quantitation of zearalenone from corn by gas–liquid chromatography. An internal standard, zearalanone, is first mixed with the finely ground corn, followed by extraction with ethyl acetate, purification by thin-layer chromatography and formation of trimethyl silyl ether (TMS) and methyl oxime (MOX) derivatives. Positive identification of zearalenone is based upon the retention times of the TMS and MOX–TMS derivatives being identical with those of the standards, comparison of chromatograms from both derivatives and characteristic twin peaks of zearalenone MOX–TMS. The detection limit of 100 parts per 10^9 zearalenone in corn could only be improved by modification of the described procedure.

INTRODUCTION

Zearalenone (F_2-toxin) is a mycotoxin produced by *Fusarium* strains[1] on crops and is often present in grain destined for both human and animal consumption[2,3]. The oestrogenic effect of zearalenone has been shown in swine[1,4] and rodents[1,5] as well as in non-human primates[6]. A survey of zearalenone in food and foodstuffs was carried out by use of several methods of analysis, *i.e.*, thin-layer chromatography (TLC)[7,8], high-performance liquid chromatography (HPLC)[9] or gas–liquid chromatography (GLC) on packed glass columns[9,10] after extraction of the samples and purification of the extracts.

The detection and measurement of zearalenone after TLC or HPLC was either based upon UV absorption[7,8,10] or fluorescence of the compound or of its derivatives[7,8,10,11]. Flame ionization[10] or electron capture[9] detection or coupled mass spectrometry and multiple ion detection[10] were employed in GLC. The best analytical results were obtained by mass spectrometry[10] which allowed the detection of 10–50 ng amounts present in the sample and the measurement of 20 ppb of zearalenone in corn. In contrast, electron capture detection after GLC enabled the determination of the 500 pg of zearalenone derivative injected, but interfering substances still present in the cleaned extract precluded analysis at levels below 1.0 ppm.

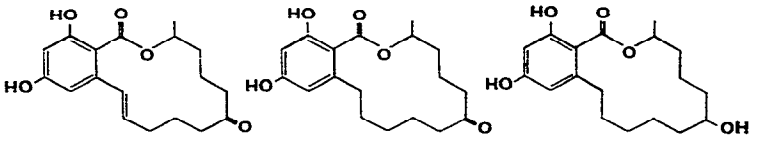
Open tubular capillary glass columns used in GLC provide a tool for the

separation of complex mixtures and have been extensively used for the study of steroid profiles[12–14]. Since zearalenone derivatives can be detected and measured after GLC, we investigated the application of GLC on capillary glass columns to the determination of zearalenone in corn extracts.

EXPERIMENTAL

Reagents

A 1 mg/ml stock solution of zearalenone (Fig. 1) in ethyl acetate was prepared and kept in the dark at 0°. Zearalanol[15] (Fig. 1) was extracted and purified from Ralgro® pellets (C.S.C. patent) obtained from Roussel-UCLAF (Paris, France). Zearalanone[15] (Fig. 1) was prepared according to Young[16] by CrO_3 oxidation of zearalanone-2,4-diacetate followed by hydrolysis in methanolic sodium hydroxide. A 1 mg/ml stock solution in ethyl acetate was prepared and kept in the dark at 0°.



ZEARALENONE ZEARALANONE ZEARALANOL

Fig. 1. Formulae of zearalanone and zearalanol derived from zearalenone.

All solvents were of Reagent grade and purchased from E. Merck (Darmstadt, G.F.R.). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), methoxyamine hydrochloride, 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and benzyltriphenylphosphonium chloride (BTPPC) were purchased from Supelco (Bellefonte, Pa., U.S.A.), Carlo Erba (Milan, Italy), Eastman-Kodak (Rochester, N.Y., U.S.A.), E. Merck and Aldrich (Beerse, Belgium), respectively.

Extraction procedure

The internal standard (25 μg of zearalanone) used was mixed with 25 g of finely ground corn to give a concentration of 1 ppm. Extraction was then carried out as described by Mirocha *et al.*[10]. Twentyfive grams of finely ground corn were mixed with 7.5 ml of water and then extracted with 60 ml of ethyl acetate for 30 min at room temperature, with occasional shaking. After filtration, the cake was extracted three times with 60 ml of ethyl acetate and the pooled filtrates dried under vacuum. The dry residue was dissolved in 5 ml of chloroform.

Thin-layer chromatography

Thin-layer plates (250 μm thickness) were prepared from silica gel 60 GF_{254} (E. Merck). A portion of the chloroform solution (200 μl, equivalent to 1 g of ground corn) was streaked at the bottom centre of the plate. On each side were respectively spotted 20 μl of chloroform containing a mixture of 5 μg of reference zearalenone and 5 μg of reference zearalanone. In order to eliminate non-polar lipids[17], chromatography was first carried out in benzene–hexane (3:1). After drying of the plate, a

second development in benzene–acetic acid (9:1) separated zearalenone from most contaminants[7]. No separation of zearalenone from the internal standard (zearalanone) was obtained in this system. Irradiation with UV light (254 nm) helped to locate the references zearalenone and zearalanone in the chromatographic strip. Silica gel from this strip was scraped off the plate with a razor blade. After deactivation with two drops of methanol, elution was carried out in a sintered-glass funnel with ethyl acetate. The volume of the eluate was adjusted to 10 ml, then half of it was used to prepare the trimethyl silyl ether (TMS) derivatives and the other half used for the methyl oxime–trimethyl silyl ether (MOX–TMS) derivatives.

Derivative formation

A standard solution containing 10 μg/ml of the TMS derivatives of both zearalenone and zearalanone was obtained in the following manner. Zearalenone (25 μg) and zearalanone (25 μg) were mixed, dried under nitrogen and allowed to react for 3 h at room temperature with 100 μl of BSTFA + 1% TMCS. The reaction mixture was dried under nitrogen and the residue dissolved in 2.5 ml of ethyl acetate.

The TMS derivative of the dried portion of the eluate obtained as above was prepared similarly except that 200 μl of BSTFA + 1% TMCS were used and that a portion of the reaction mixture was directly used for GLC analysis.

A standard solution containing 10 μg/ml of the MOX–TMS derivatives of both zearalenone and zearalanone were prepared as follows. A 25-μg amount of zealarenone and 25 μg of zearalanone were mixed, dried under nitrogen and allowed to react for 6 h at room temperature with 50 μl of pyridine containing 2% MOX. After drying under nitrogen, 100 μl of BSTFA + 1% TMCS were added and the reaction carried out for 3 h at room temperature. After drying under nitrogen, the residue was dissolved in 2.5 ml of ethyl acetate.

The MOX–TMS derivative of the eluate was prepared as above except that the volumes of reagents were doubled and that GLC analysis was carried out directly with a portion of the final reaction mixture.

GLC with open-tubular capillary glass columns

A Carlo Erba 2510 chromatograph equipped with a flame ionization detector (FID) was used. The setting up of capillary columns was performed according to Berthou and co-workers[13,14,18,19]. The choice of a solid injection device prepared according to Ros[20,21] was governed by the need to avoid injection of the solvent and of high-molecular-weight compounds. Capillary columns were prepared from 6 × 3 mm Pyrex tubes. Prior to coating, the capillary column was silanized [24 h at 210° with HMDS–TMCS (5:1) followed by toluene and methanol washings], deactivated with 1% BTPPC solution in dichloromethane and dried with nitrogen gas. Coating was carried out by the static method[13], the dichloromethane solution containing 0.3% of the apolar SE-52 phase being evaporated from the column under vacuum at 28°. The column used for this study was 57 m long with an internal diameter of 0.288 mm. Other conditions: thickness of the SE-52 phase, 0.21 μm; carrier gas (helium) flow-rate, 1 ml/min, v = 13.3 cm/sec; temperatures of injector and oven, 300° and 280°, respectively. Hydrogen (25 ml/min) and air (250 ml/min) were passed through the FID. The phase ratio was found at β = gas volume/phase volume = 342.

TABLE I

RETENTION TIMES AND RETENTION INDICES OF ZEARALENONE AND ZEARALANONE DERIVATIVES

See Fig. 2 for peak numbers.

Derivative	*Peak number*	*Retention time (min)*	*Retention index*
Zearalenone TMS	3	27.3	2788
Zearalenone MOX–TMS*	4	30.3	2846
		31.5	2866
Zearalanone TMS	1	24.6	2727
Zearalanone MOX–TMS	2	26.7	2775

* *syn* and *anti* epimers were separated.

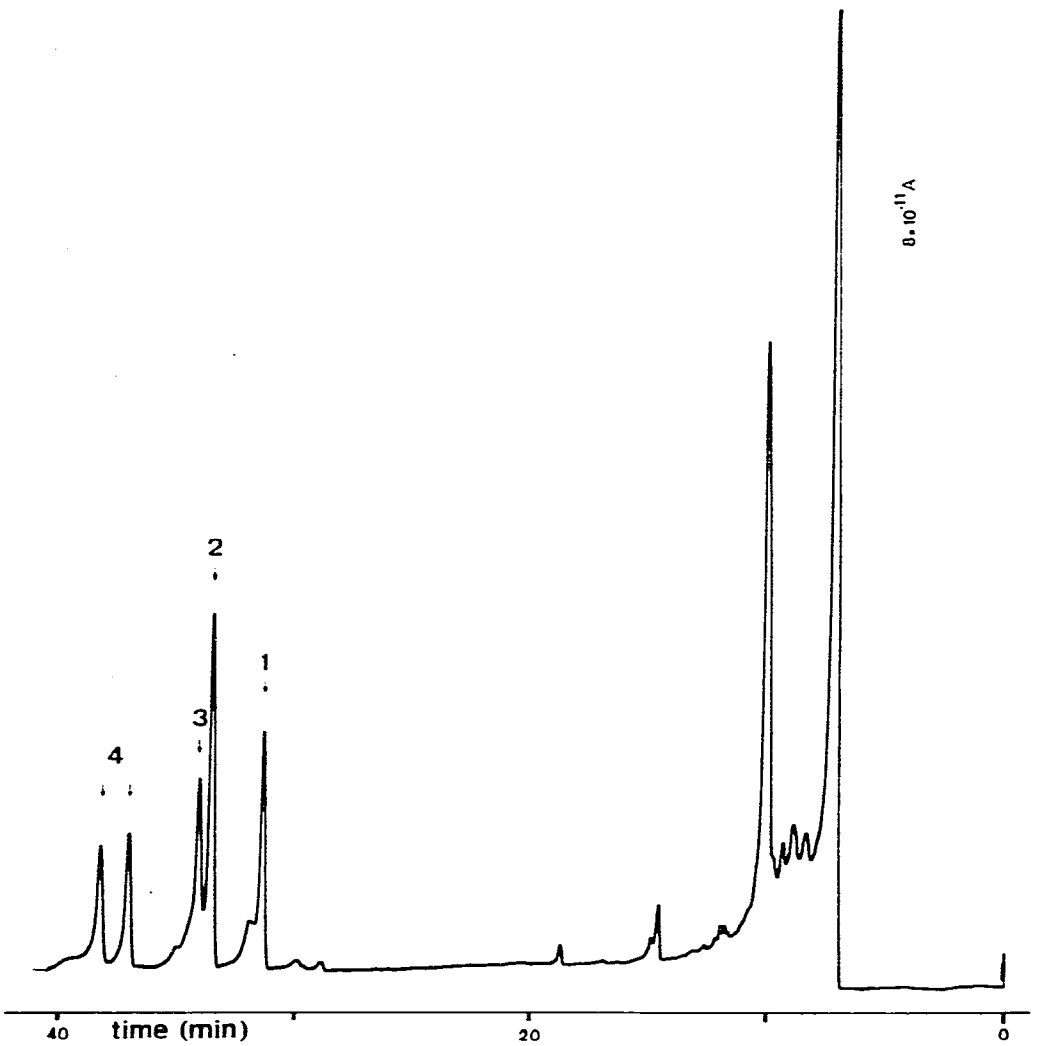


Fig. 2. Separation of TMS and MOX–TMS derivatives of zearalenone and zearalanone by GLC on a capillary glass column. Peaks: 1 = zearalanone TMS; 2 = zearalanone MOX–TMS; 3 = zearalenone TMS; 4 = zearalenone MOX–TMS (*syn* and *anti* epimers).

RESULTS

Chromatographic characteristics of zearalenone

To ensure a reproducible determination of zearalenone by GLC on capillary glass columns, all of the calculated parameters of the column used in this study were based upon the zearalenone TMS standard injected under conditions identical with those for the corn extract. The coating efficiency[22] was found to be 39% and the capacity factor k' was 3.84. The total number of theoretical plates found was 97,000 and the effective number of theoretical plates was 60,000. The theoretical and experimental heights equivalent to one theoretical plate (HETP)[14] were 0.23 mm and 0.58 mm, respectively. In comparison, a triacontane standard gave a coating efficiency of 46% and $k' = 5.6$. Retention indices for zearalenone TMS and other derivatives are shown in Table I.

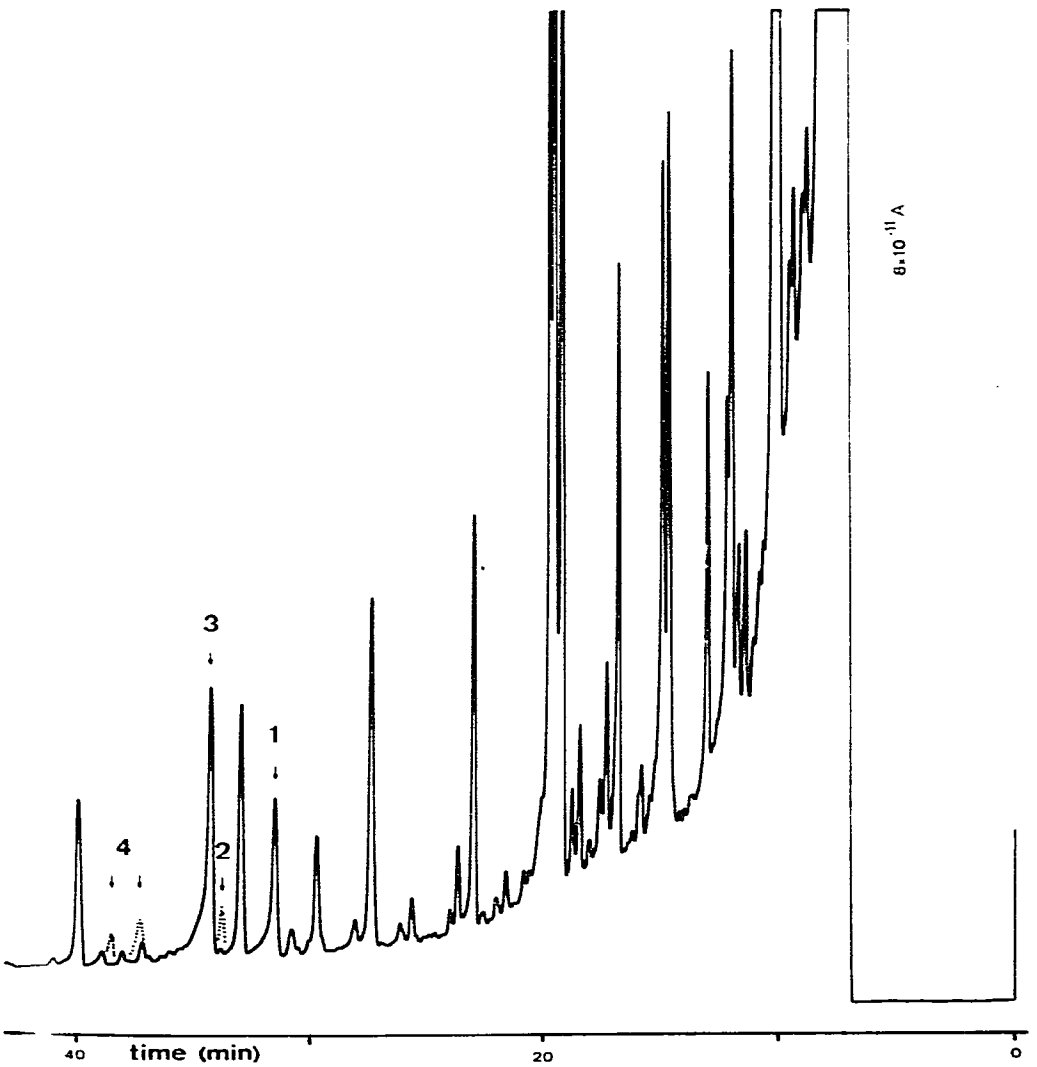


Fig. 3. TMS derivatives of a purified corn extract containing zearalenone and zearalanone as internal standard. Peaks: 1 = zearalanone TMS; 3 = zearalenone TMS. The dashed peaks were drawn after superimposition with the chromatogram of Fig. 4.

Identification of zearalenone in the purified extract

For the identification of zearalenone, the purified extract was divided into two equal portions and TMS and MOX–TMS derivatives were prepared. After injection of a portion of each derivative on the column, the following four criteria must be met on the chromatograms: (i) retention times of TMS and MOX–TMS derivatives are identical with those found with standard solutions (Fig. 2); (ii) there are characteristic twin peaks for zearalenone MOX–TMS derivatives (*syn* and *anti* epimers); (iii) superimposition of chromatograms from TMS and MOX–TMS, *i.e.*, contaminants from the corn extract do not interfere with zearalenone and the internal standard (Figs. 3 and 4); (iv) addition of authentic standards to the purified corn extract does not result in new peaks on the chromatograms (Fig. 5).

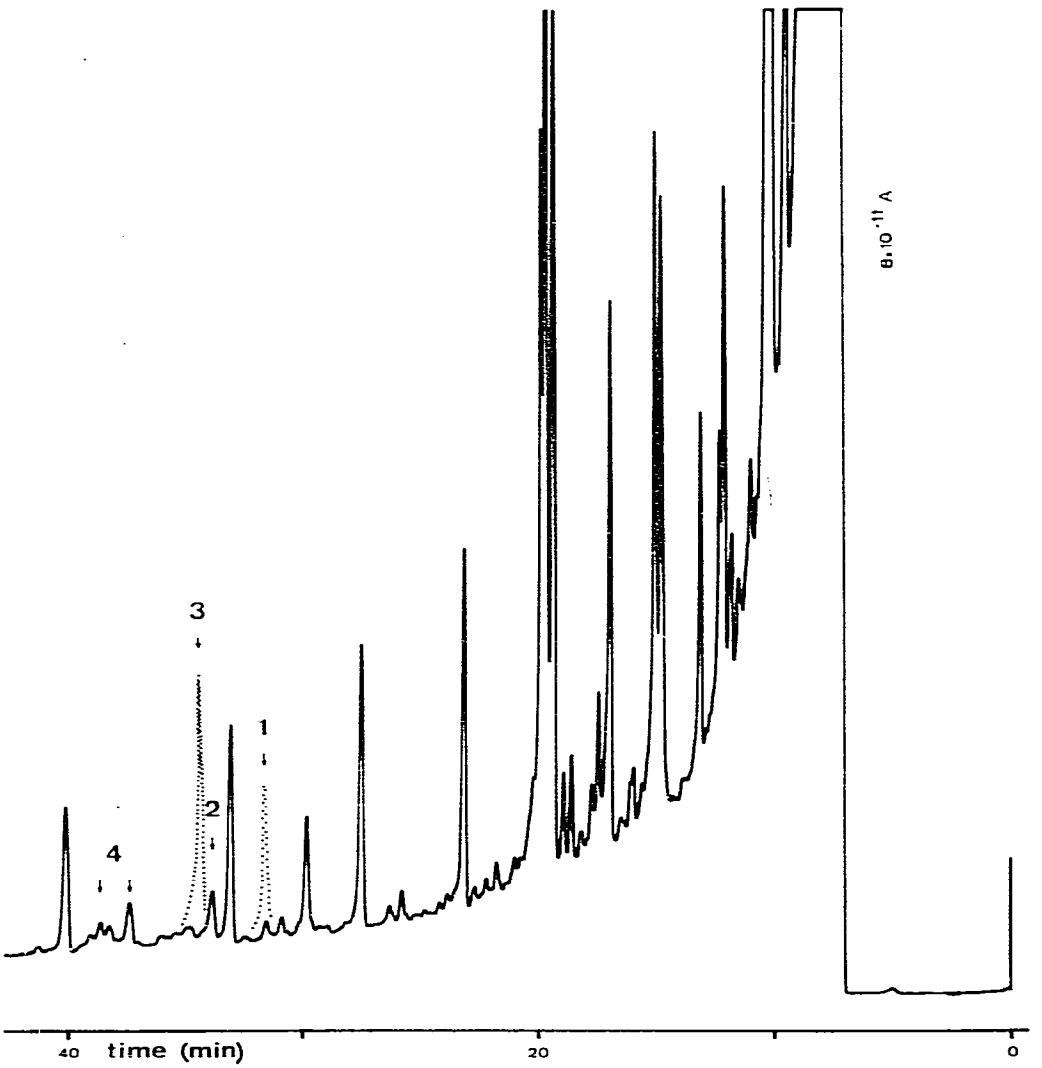


Fig. 4. MOX–TMS derivatives of a purified corn extract containing zearalenone and zearalanone as internal standard. Peaks: 2 = zearalanone MOX–TMS; 4 = zearalenone MOX–TMS (*syn* and *anti* epimers). The dashed peaks were drawn after superimposition with the chromatogram of Fig. 3

Quantitation of zearalenone

Since the extraction yields of zearalenone and zearalanone were identical, the concentration ratio for these molecules in the corn and in the chromatographed extract were equal. Thus, quantitation of zearalenone in corn was based upon the following:

$$\text{zearalenone in corn} = \frac{R_c \cdot R'_h}{R_h} \cdot C$$

where $R_c = \dfrac{\text{zearalenone concentration in standard}}{\text{zearalanone concentration in standard}}$

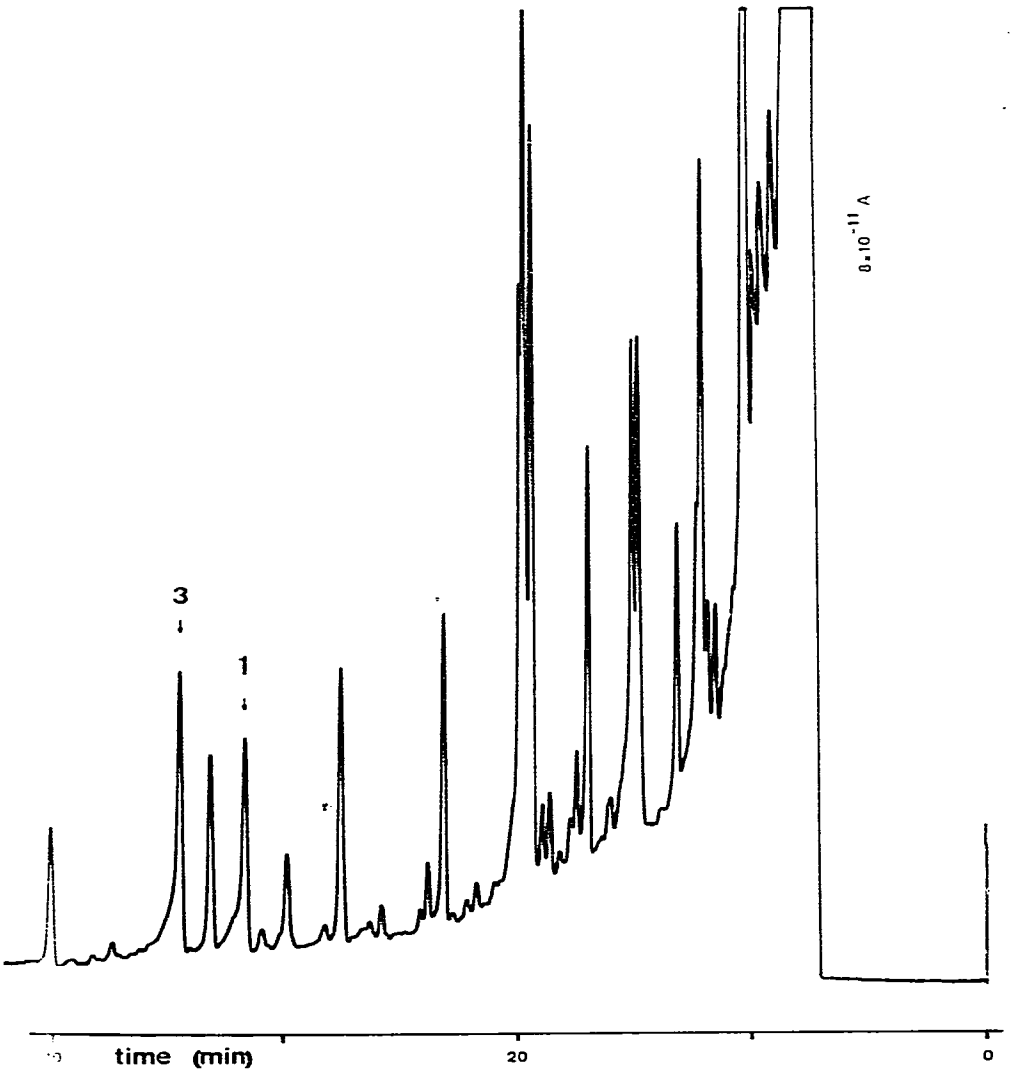


Fig. 3. TMS derivatives of a purified corn extract containing zearalenone and zearalanone as internal standard surcharged with standard zearalenone TMS. Peaks: 1 = zearalanone TMS; 3 = zearalenone TMS.

$$R_h = \frac{\text{height of peak from zearalenone TMS standard}}{\text{height of peak from zearalanone TMS standard}}$$

$$R_h' = \frac{\text{height of peak from zearalenone TMS in extract}}{\text{height of peak from zearalanone TMS in extract}}$$

C = zearalanone concentration in corn

The precision of the quantitative determinations was based upon repeated injections of the sample. The data obtained varied within 10% of the calculated mean. The accuracy of measurements increased with the quantity of zearalenone in the corn. Thus, 2 ppm of zearalenone TMS were measured with a $\pm 5\%$ accuracy from 80-mm peaks. An accuracy of $\pm 15\%$ was calculated for 1 ng of zearalenone TMS which gave a 4-mm peak at $8 \cdot 10^{-11}$ A. Since 2 μl of the purified extract were injected in this case, the detection limit for zearalenone in corn was estimated as 100 ± 15 parts per 10^9.

DISCUSSION

The use of open tubular glass capillary columns for the determination of zearalenone in corn has the advantage over other methods of high resolution. This high resolution permits the use of extracts purified only by TLC and without noticeable interference from corn contaminants. Nevertheless, the solvent system used for the development of the thin layer was chosen because of its effectiveness in removing substances which could interfere with the GLC peaks of zearalenone and zearalanone TMS and because of the identical R_F values for zearalenone and zearalanone. Solid injection of the samples according to Ros[21] avoided the solvent peak, decreased the minimal detectable quantity of zearalenone and rendered possible the introduction in the solid injector of volumes greater than 2 μl.

The internal standard chosen permitted the simultaneous separation and determination of zearalenone. Zearalanone was chosen because of its resemblance to the parent zearalenone (Fig. 1) and because it does not occur naturally in zearalenone-contaminated corn. Zearalenone and zearalanone were not separated by TLC, and their polarities and extraction yields must be of the same order. Therefore, the use of zearalanone as an internal standard considerably simplified the determination of zearalenone in corn.

The GLC conditions chosen were governed by the need to superimpose chromatograms of the TMS and MOX–TMS derivatives of the purified corn extract (Figs. 3 and 4) in order to identify zearalenone and zearalanone without possible interference. The mixture of TMS and MOX–TMS derivatives injected for identification purposes (Fig. 2) showed a poor separation of zearalenone TMS from zearalanone MOX–TMS. A minimum temperature of 270° was necessary and a resolution of 1 was sufficient for superimposition of the chromatograms. *syn* and *anti* epimers of zearalenone MOX–TMS were clearly separated on the capillary column. This is an excellent test for zearalenone since derivatives of contaminants in corn are unlikely to interfere with both of these twin peaks. In contrast, it is noticeable that zearalanone MOX–TMS epimers were not separated on the same column.

The sensitivity of the method may be improved by decreasing the minimal detectable quantity of zearalenone. Since 1 ng injected is the limit to detection by flame ionization, the volume of injection must be increased. In order to avoid column deteriorations, a more efficient purification of the extract would then have to be carried out. Thus, the cleanup procedure proposed by Mirocha *et al.*[10], involving alkaline opening of the lactone, might be successful. On the other hand, the use of an electron capture detector with appropriate halogenated derivatives[9] could lead to a better sensitivity. Finally, the measurement of peak heights could be avoided if peak areas were directly integrated. Errors due to slight dissymmetry of the peaks would then be decreased.

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