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Determination of α- and β-zearalenol and zearalenone in cereals by gas chromatography with ion-trap detection

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ABSTRACT

A method for determination of the estrogenic mycotoxins, α- and β-zearalenol and zearalenone, in cereals (wheat, barley, oats, corn) is described. After extraction with ethylacetate, clean-up involved a base treatment and partition with water; derivatization was by trimethylsilylation. For quantitation and confirmation a capillary gas chromatograph combined with a selective mass detector (ion trap), working in the electron impact-mode was used. The detection limit for the complete method is 1 μg/kg for each of the three mycotoxins in full scan. Recoveries from spiked cereals were 82–86%.

INTRODUCTION

trans-Zearalenone (ZON) [6-(10)-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone] is a secondary metabolite synthesized by several species of the fungal genus *Fusarium* [1]. It possesses strong estrogenic activity and has been implicated as a causative factor in hyperestrogenism, infertility and other health problems in livestock (swine, cattle, poultry) [1,2]. Numerous reports have demonstrated its worldwide occurrence in cereals and other agricultural commodities [1,3–5].

A variety of other estrogenic substances have been found along with ZON in pure cultures of *Fusarium* isolates on cereals [2,6]. *trans*-α-Zearalenol (α-ZOL) and *trans*-β-zearalenol (β-ZOL) are two of these metabolites which may occur as a diastereomeric mixture [1,6–12]. Experiments with labelled α- and β-ZOL and ZON have suggested that zearalenol is a precursor in the biosynthetic pathway leading to ZON [13]. The presence of α- and β-ZOL in feedstuffs might be of more importance than currently realized. α-ZOL is 3–4 times more estrogenic than ZON, and β-ZOL has the same or slightly less activity as ZON [8]. Both substances therefore could contribute to the total estrogenic activity of *Fusarium*-infested cereal grains, and their presence could explain why feedstuffs without detectable ZON content can be associated with hyperestrogenism in animals. This has been observed repeatedly in our laboratory.

So far the presence of zearalenols in feedstuffs has been investigated to a limited extent. α-ZOL has been detected along with ZON in three cereal samples [14], α- and β-ZOL have been extracted along with ZON from some *Fusarium*-infected stems of corn [9,10], and zearalenol (not specified whether α- or β-ZOL) has been found in one out of 248 German grain samples [15]. This scarce information creates an urgent need to determine the two zearalenols in addition to ZON in a large number of grain samples from several harvest years.

Methods used for the determination of zearalenols and ZON in cereals and animal feed include thin-layer chromatography (TLC) [9,11,12,15,16], high-performance liquid chromatography (HPLC) [8,9,17–19], gas–liquid chromatography (GC), [8,11] and/or GC-mass spectroscopy (MS) [6,8,9,20,21]. Confirmation of ZON in cereals and other commodities by GC–MS has been described in numerous other studies [1,3,4,22–24]. GC–MS has also been used for the confirmation of the two

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zearalenols and of ZON in animal tissues, biological fluids, excreta and pig and cow milk [25–29]. The advantage of GC–MS lies in the precise identification of even minute quantities without the risk of interference by substances that have identical R_f values (TLC) or retention times (HPLC, GC).

The use of large mass spectrometers in GC–MS, particularly in routine monitoring of a large number of samples, is limited by expense and problems in handling and personnel training. Instruments of the new generation of mass-selective detectors, such as the ion-trap detector which can be used as common GC detectors are easier to handle and less expensive than larger mass spectrometers. In the present study we describe the quantification and confirmation of α- and β-ZOL and ZON in cereals by GC with ion-trap detection (ITD).

EXPERIMENTAL

Chemicals and reagents

ZON, α- and β-ZOL were from Makor Chemicals (Jerusalem, Israel). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (derivatization reagent) was from Pierce (Oud-Beijerland, Netherlands). All other chemicals and solvents were of reagent grade and obtained from Merck (Darmstadt, Germany).

Cereal samples

All cereal samples (wheat, barley, oats, corn) were obtained either from farms in different districts of Baden-Württemberg (Germany) or from a feed factory, and exhibited various mycological qualities.

Extraction

A base clean-up procedure described in the literature [30] was modified. A 30-g sample of finely ground food was extracted with 200 ml of ethylacetate in a 500-ml screw-cap bottle for 2 h on a rotary shaker. The extract was filtered through two folded filters and evaporated nearly to dryness on a rotary evaporator (water bath 40°C). The residue was dissolved in 20 ml of chloroform and quantitatively transferred to a 100-ml separatory funnel (rinse flask with another 5–10 ml of chloroform followed by a brief sonication). A 10-ml aliquot of saturated sodium chloride solution was added to the separatory funnel. The chloroform solution was extracted twice with 10 ml of 1–2 *M* sodium hydroxide. Both alkali extracts were collected and the chloroform layer was discarded. The alkali phase was purified twice with 5 ml of chloroform; the chloroform layers were discarded. The pH of the alkaline phase was adjusted to 9.4–9.5 (pH meter) by 2 *M* phosphoric acid. Then the solution was extracted three times each with 15 ml of chloroform. The chloroform phases were filtered over anhydrous sodium sulphate into a round-bottomed flask (rinse with additional chloroform). The combined chloroform extracts were evaporated to dryness (up to 40°C bath temperature). Extracts which cannot be derivatized and analysed immediately should be stored in a freezer.

Derivatization

Derivatization was achieved by trimethylsilylation with BSTFA. The complete residue of the extraction was transferred quantitatively to a 1-ml autosampler vial. To the dry residue 100 μl of BSTFA reagent were added. After closing the vial with a crimping cap, the mixture was held at 60°C for 15 min. Thereafter the sample was cooled. In the splitless mode 1–4 μl and in the on-column mode 1 μl were injected into the gas chromatograph.

Apparatus

A Carlo Erba HRGC 5160 Mega Series gas chromatograph (Carlo Erba, Hofheim, Germany) equipped with the fused-silica column Permabond SE-54-DF-0.25 (25 m × 0.25 mm I.D., film thickness 0.28 μm; article no. 723056; Macherey-Nagel, Düren, Germany) was used. The carrier gas was helium (80–90 kPa). Splitless (valve 45 s closed) and on-column (autosampler; secondary cooling 45 s) modes of injection were used. The temperature of the splitless injection port was 260°C; the column temperature programme, following an initial period of 180°C for 2 min, was 4.5°C/min to 270°C, hold for 15 min, 15°C/min to 285°C and hold for 10 min. As a mass spectrometry data system, a Finnigan MAT ITD 800 ion-trap detector (Finnigan MAT, Bremen, Germany) was interfaced to the gas chromatograph. The detector was applied in the electron impact (EI) mode, being equivalent to an electron energy of 70 eV. The temperature of the transfer line was adjusted to 285°C. Only full-scan data were monitored; the selected scan range was 300/400 to 540 a.m.u.

Recovery experiments

Known amounts of ZON, α- and β-ZOL (in methanolic solution) were added to a screw-cap bottle containing 30 g of finely blended cereal samples without detectable amounts of α- and β-ZOL and ZON. After 30 min of vigorous shaking on a rotary shaker the sample was stored for 2 days in a refrigerator and then extracted.

RESULTS AND DISCUSSION

We describe for the first time the quantitation and confirmation of α- and β-ZOL and ZON in cereals by the combination of capillary GC with ITD in the EI mode.

Extracts obtained by the method of Tanaka *et al.* [31] were not pure enough for GC–MS according to our experience, even after application of Sep-Pak silica cartridges (Part No. 51900, Waters Assoc., Milford, MA, USA). This clean-up step also leads to some loss of ZON, α- and β-ZOL. For GC–MS we therefore used the base clean-up procedure described by Mirocha *et al.* [30]. This takes advantage of the solubility of ZON, α and β-ZOL in diluted alkali.

Derivatization was achieved by treating extracts with BSTFA to obtain the corresponding trimethylsilyl ethers (TMS) of ZON (di-TMS-ZON), α-ZOL (tri-TMS-α-ZOL) and β-ZOL (tri-TMS-β-ZOL). These TMS derivatives are stable in the refrigerator for several weeks.

By using other derivatization reagents, such as trifluoroacetic acid anhydride (TFAA), or by operating the ion-trap detector in the chemical ionization (CI) mode, no significant improvement, *e.g.* no intensity gain of the molecular peak, was obtained.

A total ion chromatogram for a standard solution of the three mycotoxins is given in Fig. 1. The specific mass spectra can be seen in Fig. 2, and the GC–MS characteristics are shown in Table I. As has been shown for the TMS ether of ZON, the ratio of intensities in the higher mass region is very consistent and can be used as a diagnostic characteristic [30]. According to our experience this is true also for α- and β-ZOL (Table I). For diagnostic purpose the ions at *m/z* 536 [M^+], 446, 429, 333, 307 (α- and β-ZOL) and *m/z* 462 [M^+], 444, 429, 333, 307 (ZON) can be monitored. There is no significant difference between the mass spectra of both TMS derivatives isomers. The relative intensity of the molecular ion (536) for the β isomer appears to be higher than that for the α isomer (Fig. 2).

The recoveries of the complete method are between 82 and 86% (Table II). The limit of detection of the complete method (ion trap operated in the EI mode, full scan 400–540 a.m.u., signal-to-noise

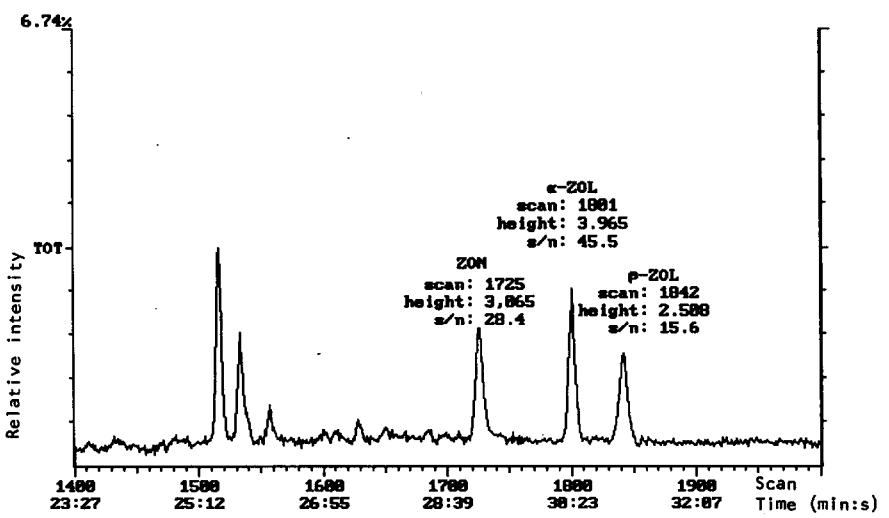

Fig. 1. Total ion chromatograms for α- and β-ZOL and ZON (standard, 2.5 ng each, trimethylsilyl derivatives, EI mode). s/n = signal-to-noise ratio.

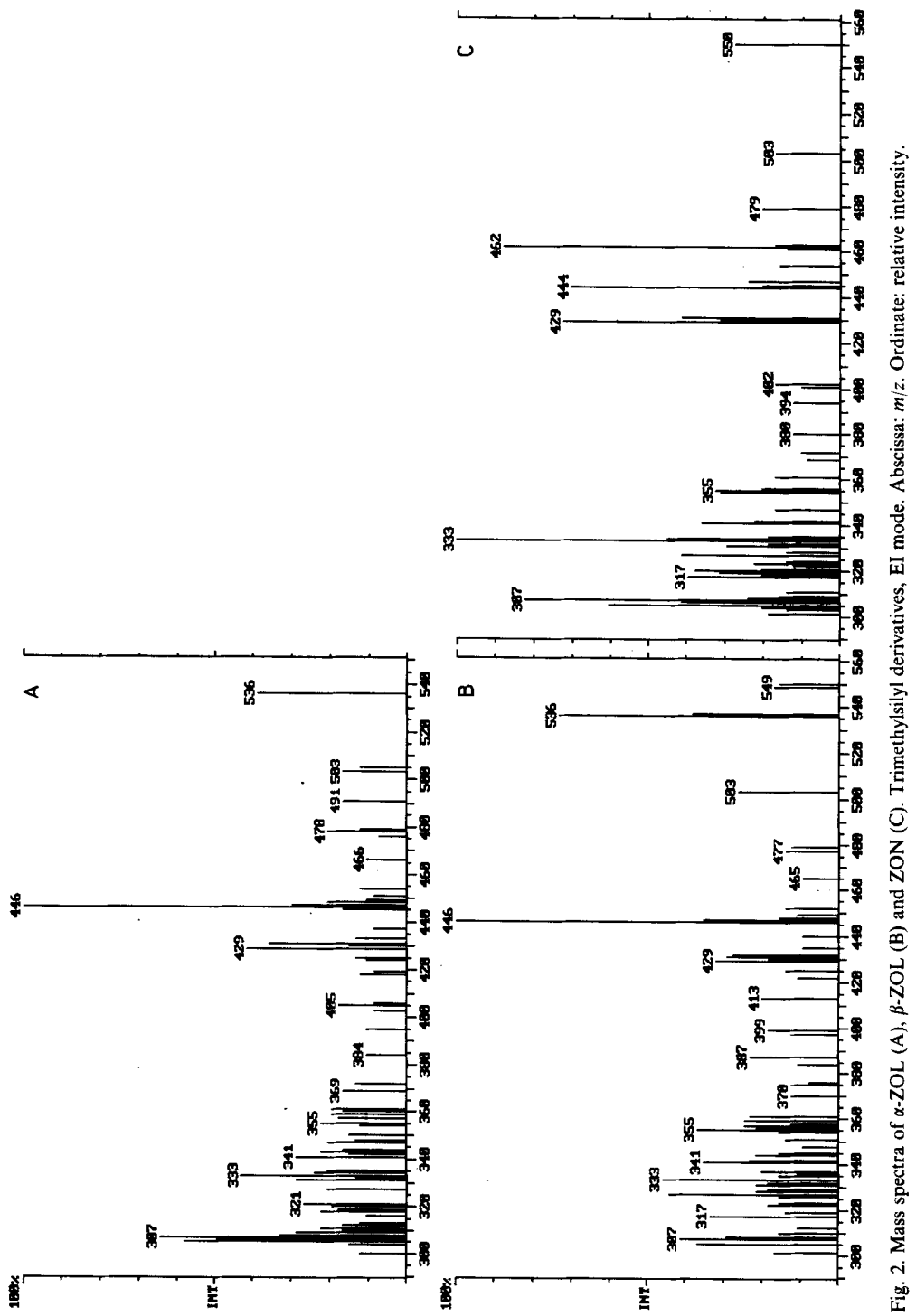

Fig. 2. Mass spectra of α-ZOL (A), β-ZOL (B) and ZON (C). Trimethylsilyl derivatives, EI mode. Abscissa: *m/z*. Ordinate: relative intensity.

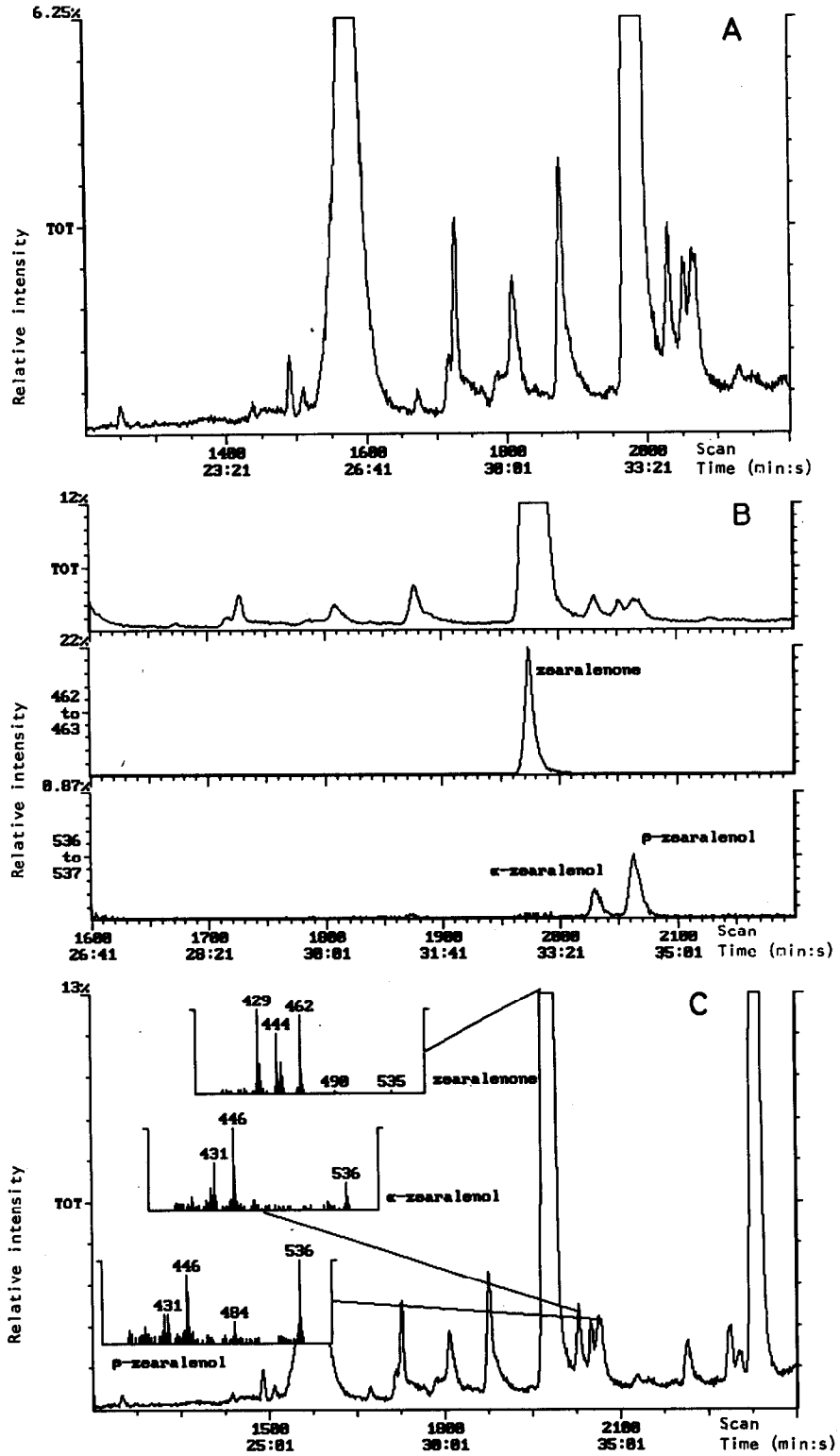

Fig. 3. Wheat sample (Bad Mergentheim district, Baden-Württemberg, 1987 harvest) containing 71 μg/kg α-ZOL, 12 μg/kg β-ZOL and 8036 μg/kg ZON. Total ion chromatogram (A), mass chromatograms of α- and β-ZOL and ZON (B), zoomed total ion chromatogram and mass spectra at retention time of α- and β-ZOL and ZON (C).

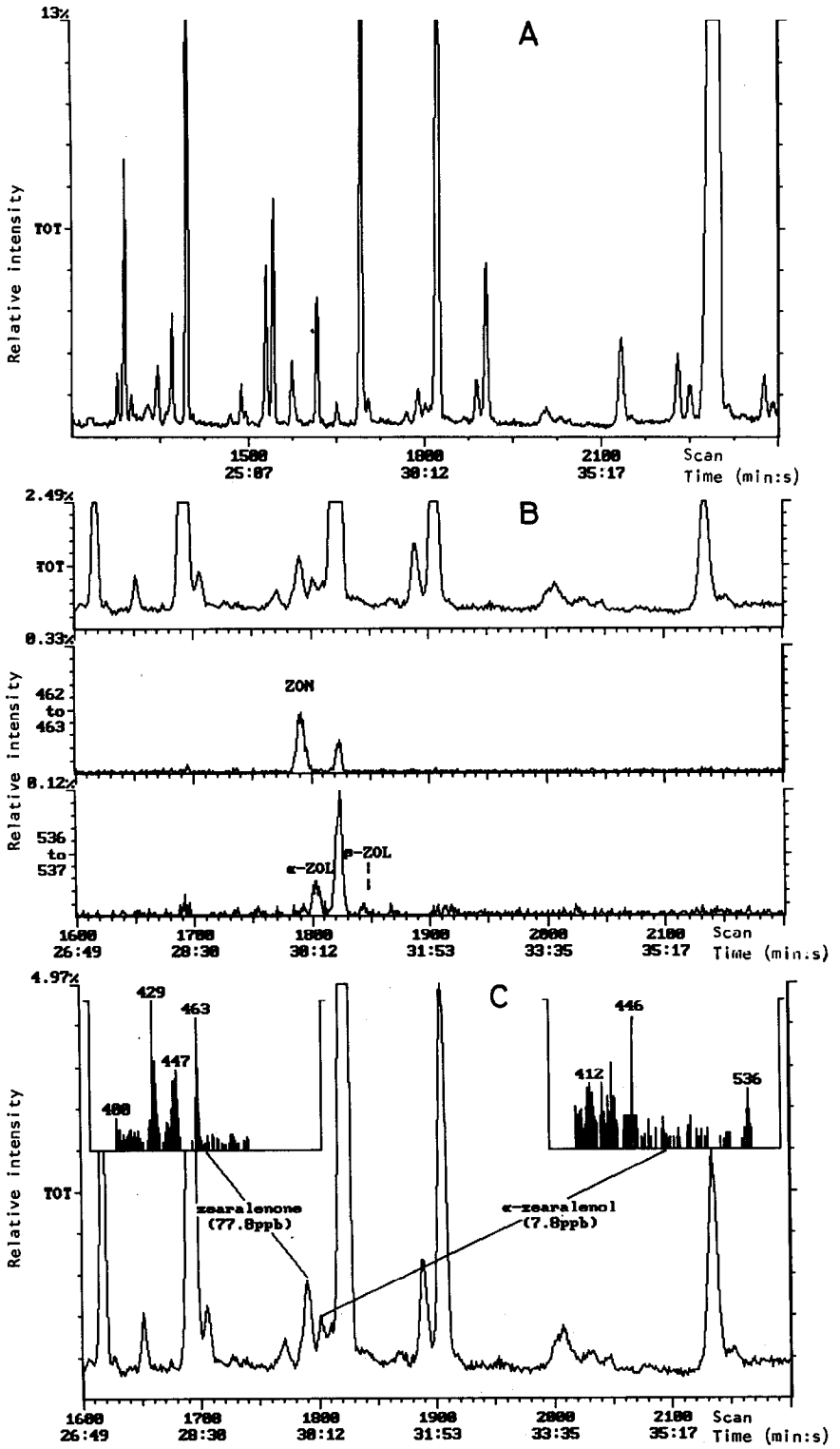

Fig. 4. Wheat sample (Göppingen district, Baden-Württemberg, 1987 harvest) containing 8 μg/kg α-ZOL and 78 μg/kg ZON, β-ZOL not detectable. Total ion chromatogram (A), mass chromatograms of ZON and α-ZOL (B), zoomed total ion chromatogram and mass spectra at retention time of α-ZOL and ZON (C). In ppb, the American billion (10^9) is meant.

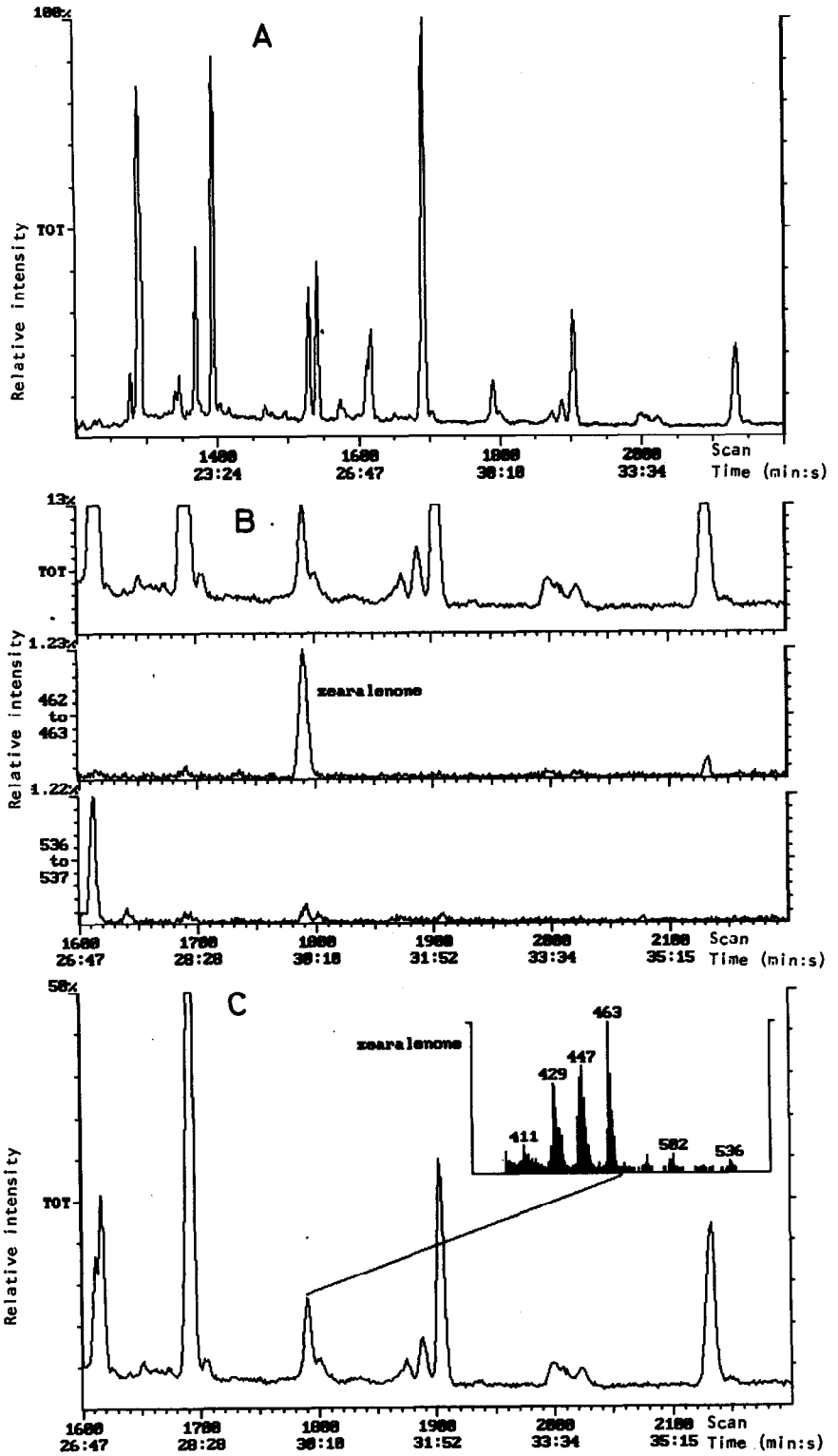

Fig. 5. Barley sample (Künzelsau district, Baden-Württemberg, 1987 harvest) containing 310 μg/kg ZON, α and β-ZOL not detectable). Total ion chromatogram (A), mass chromatogram of ZON (B), zoomed total ion chromatogram and mass spectrum at retention time of ZON (C).

TABLE I

GC–MS OF α- AND β-ZOL AND ZON (AS TRIMETHYLSILYL DERIVATIVES, BSTFA DERIVATIZATION, EI MODE)

$[M^+]$: molecular ion

Toxin	Retention time[a] (min)	Mass fragments (a.m.u.)	Ratio of intensities	Quantitation mass window (a.m.u.)
α-ZOL	About 31	536 $[M^+]$	1	536–537
		446	2	
		429	1	
		(333/307)		
β-ZOL	About 32	536 $[M^+]$	2	536–537
		446	2	
		429	1	
		(333/307)		
ZON	About 30	426 $[M^+]$	1	461–463
		444	1	
		429	1	
		(333/307)		

[a] Depending on the pressure of carrier gas helium.

ratio 3:1) is 0.5 ng per injection, *i.e.* about 1 μg/kg for α- and β-ZOL and ZON.

Using the described method cereal samples from farms were investigated. The identities of α- and β-ZOL and ZON were determined by comparing the retention times, mass spectra, and ratio of intensities of various mass fragments and ions with those of standards. In a wheat sample both α- and β-ZOL were detected along with ZON (Fig. 3); in another wheat sample α-ZOL and ZON were determined (Fig. 4). The simultaneous occurrence of all three mycotoxins as well as the occurrence of only α-ZOL together with ZON has been reported in literature [6,8,9,14].

By the method described α- and β-ZOL can be quantified and identified together with low and high concentrations of ZON (Figs. 3 and 4). The absence of α- and β-ZOL (detection limit: 1 μg/kg) has been demonstrated in a barley sample containing 310 μg/kg ZON (Fig. 5). There was no interference by other ingredients of wheat and bearley.

TABLE II

RECOVERY OF α- AND β-ZOL AND ZON FROM CEREALS (WHEAT, BARLEY, OATS, CORN)

Samples spiked with 100, 300 and 500 ng of each toxin.

Toxin	Recovery (%)
α-ZOL	82.9 ± 1.2
β-ZOL	86.1 ± 1.3
ZON	85.2 ± 1.5

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