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DETERMINATION OF ZERANOL AND ITS METABOLITES IN BOVINE MUSCLE TISSUE WITH GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

This paper describes the quantitative method of determination of chosen substances from resorcylic acid lactones group: zeranol, taleranol, α-zearalenol, β-zearalenol, and zearalanone in bovine muscle tissue. The presented method is based on double diethyl ether liquid-liquid extraction (LLE), solid phase extraction (SPE) clean up, and gas chromatography mass spectrometry (GC-MS) analysis. The residues were derivatised with a mixture of N-methyl-N-trimethylsilyltrifluoroacetamide, ammonium iodide, and DL-dithiothreitol (1,000:2:5, v/w/w). The GC-MS apparatus was operated in positive electron ionisation mode. The method was validated according to the European Union performance criteria pointed in Decision Commission 2002/657/EC. The average recoveries of all analytes at 1 μg kg^{-1} level were located between 83.7% and 94.5% values with the coefficients of variation values <25%. The decision limits (CCα) and detection capabilities (CCβ) for all analytes ranged from 0.58 to 0.82 μg kg^{-1} and from 0.64 to 0.94 μg kg^{-1}, respectively. The procedure has been accredited and is used as a screening and confirmatory method in control of hormone residues in animal tissues.

Key words: bovine muscle tissue, zeranol, GC-MS.

Zeranol (ZER, α-zearalanol, Ralgro®) is one of the non-steroidal synthetic oestrogenic growth promoters related to the oestrogenic mycotoxin zearalenone (ZON). It depresses the endogenous gonadotropins, luteinising hormone (LH), and follicle stimulating hormone (FSH) (18, 19). ZER binds to the oestrogen receptor in various species of animals with a binding affinity similar to that of diethylstilboestrol, which is much greater than that of 17β-oestradiol (2). ZER increases liveweight gain in food producing animals (17, 23). The United States Food and Drug Administration (USFDA) has allowed the use of zeranol as a growth stimulant for animal production since 1969 in accordance with good veterinary animal husbandry practice (17).

After administration, ZER is mainly metabolised to structurally related taleranol (TAL) and zearalanone (ZAN). The Codex Alimentarius Commission has established that maximum concentration of residues calculated as equivalent of ZER should not exceed 0.2 μg kg^{-1} in muscle tissue, 10 μg kg^{-1} in the liver, 2 μg kg^{-1} in the kidneys, and 0.3 μg kg^{-1} in fat. Improper use of hormones may lead to the presence of harmful residues in edible tissues, which may pose health risks to consumers from the consumption of meat products. According to the opinions of some researchers, ZER do not present any harmful effects to the health of consumer, when it is used for anabolic purposes under the appropriate conditions. Administration of ZER and TAL to food producing animals has been banned in EU from 1985 up till now, so no residue of these compounds should be present in animal tissues (8). Therefore the monitoring of the residues of these substances is necessary to ensure that they are not present at any levels, which pose a threat to public health (9). However, both compounds can be found naturally in urine from various animal species after receiving of feed containing ZON, structurally related mycotoxin, which exists in a metabolic relationship with zeranol and is produced by different species of *Fusarium* molds (3, 6, 15, 26). Therefore, it is advisable to test the samples for ZER, its metabolites TAL and ZAN, as well as for the presence of ZON and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL). Monitoring of ZER and related compounds from RAL's group requires the availability of sensitive analytical methods for screening, and even more so for confirmatory purposes.

Over the years, radioimmunoassay (RIA) and immunoassay (ELISA) techniques were mainly applied for the analysis of ZER (21). However, it was not possible to determine ZER metabolites and other substances from RAL's group, simultaneously in one analysis. Modern gas chromatography-mass spectrometry (4, 5, 20, 21, 24) and liquid chromatography-mass spectrometry (11, 13, 16, 21, 22),

have been the main techniques commonly applied for the determination of ZER, its metabolites, and related mycotoxins in food, feed, and biological matrices (21). These methods should fulfil additional criteria listed in Decision Commission 2002/657/EC for confirmatory purposes. The aim of this study was to develop a sensitive and selective GC-MS method for determination of RAL's in muscle tissue.

Material and Methods

Reagents and chemicals. Solvents: diethyl ether, n-hexane, chloroform, and methanol (analytical grade) were obtained from POCh (Poland); methanol (analytical, HPLC, resi grade), ethanol, ethyl acetate (HPLC grade), and acetone (resi grade) were purchased from J.T. Baker (the Netherlands); isooctane (GC grade) was obtained from Merck (Germany). Other chemicals: concentrated acetic acid, anhydrous sodium sulphate, sodium acetate, sodium bicarbonate, sodium carbonate, and hydrochloric acid (0.1 M) were obtained from POCH (Poland); β-glucuronidase (23 U mL^{-1}), aryl sulfatase (68 U mL^{-1}) Helix Pomatia (AS HP), and tris buffer substance were obtained from Merck (Germany); SPE C_{18} 100 mg/1 mL and NH_2 500 mg/3 mL columns were obtained from Mall Baker (the Netherlands); purified water was obtained with a Milli-Q apparatus (USA). Buffers: acetate buffers (0.04 M, pH 5.2) and (0.05 M, pH 4.8), carbonate buffer (mixture of 100 mL of 10% sodium hydrogen carbonate solution with 500 mL of 10% sodium carbonate solution, pH 10.25), and TRIS buffer (0.02 M, pH 8.5) were prepared in laboratory. Derivatisation reagents: N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (GC grade), ammonium iodide (NH_4I), and DL-dithiothreitol (DTT) were purchased from Sigma Aldrich (Germany). Derivatisation mixture was prepared by dissolving 2 mg of NH_4I and 5 mg of DTT in 1,000 µl of MSTFA. Standard of ZER, internal standards of zeranol-D4/taleranol-D4 (ZER-D4/TAL-D4, 50:50), α-zearalenol-D4 (α-ZOL-D4), and β-zearalenol-D4 (β-ZOL-D4) were purchased from the Netherlands National Institute for Public Health and Environment-RIVM; standard of TAL was purchased from Australian Government National Measurement Institute; α-ZOL, β-ZOL, and ZAN were obtained from Sigma-Aldrich (Germany). Structural formulas of molecules of RAL's are presented in Fig. 1.

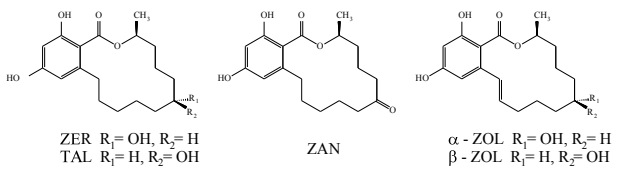


Fig. 1. Structural formulas of molecules of ZER, TAL, ZAN, α-ZOL, and β-ZOL.

All standards except ZAN were kept at 2-8°C according to the recommendations of the certificates. Primary standard stock solutions were prepared in methanol at concentration of 1 mg mL^{-1} and 10 µg mL^{-1}. Working solutions were obtained by tenfold dilution of primary standard solutions to the concentration of 1 µg mL^{-1}.

Sample preparation. The method of preparation and purification of the samples for detection of hormones published previously was investigated (25). The method was expanded to include further hormones from RAL's group and GC-MS detection was evaluated. As a result of optimisation steps the following procedure was developed.

A 10 g amount of muscle tissue was fortified with the internal standards (to a concentration of 1 µg kg^{-1}). Next, 20 mL of acetate buffer (0.04 M, pH 5.2) was added and the mixture was homogenised. The pH of homogenate was adjusted to 5.2 with 0.1 M of acetic acid and 100 µL of chloroform was added. Afterwards, the hydrolysis with 200 µL of AS HP for 3 h at 60 °C (±2°C) was performed. Then, 50 mL of methanol was added and the mixture was vortexed. The sample was placed in a water bath for 10 min at 90°C (±2°C) to denature the proteins. After cooling to the room temperature, the mixture was centrifuged for 20 min at 4,000 rpm. The methanol layer was washed two times with 20 mL of n-hexane. 20 mL of water was added to methanol phase and double liquid-liquid extraction with 30 mL of diethyl ether was performed. The combined ether layers were washed with 20 mL of carbonate buffer (pH 10.25), next with 20 mL of water, and finally dried on anhydrous sodium sulphate and evaporated to dryness (60°C, N_2). The residue was dissolved in 3 mL of acetate buffer (0.05 M, pH 4.8) and passed through the C_{18} SPE column previously conditioned with 2 mL of methanol and 2 mL of TRIS buffer/methanol mixture (80:20, v/v). The column was washed with 2 mL of TRIS buffer/methanol mixture (80:20, v/v) and with 2 mL of methanol/water mixture (40:60, v/v), next was dried under vacuum for 2 min. Elution of analytes was carried out with 3 mL of acetone. The eluate was applied directly onto the NH_2 SPE column previously conditioned with 5 mL of methanol/water mixture (40:60, v/v). After that, the eluate was evaporated to dryness, transferred to derivatisation vial with ethanol, evaporated to dryness again, and derivatised with 50 µL of MSTFA/NH_4I/DTT (1,000:2:5, v/w/w) for 20 min at 60°C (±2°C). The derivative was injected into GC-MS.

GC-MS analysis. An Agilent 6890N GC with an Agilent 5973 Quadrupole MSD and Chemstation Software was used for the analysis. Chromatographic separation of RAL's was achieved on a non-polar HP-5MS capillary column (30 m, i.d. 0.25 m, 0.25 µm film thickness) using 0.9 mL min^{-1} of constant flow of helium. The samples (2 µL of injection volume) were injected in pulsed splitless mode at 250ºC. The oven temperature was kept constant at 120ºC for 2 min and then was increased by 14ºC per min to 270ºC and kept on this level for 7 min, a further rise of temperature was 15ºC to 280ºC and kept for 2 min. The injection port, MS source and Quadrupole temperatures were set as

250, 230, and 150ºC, respectively. The GC-MS apparatus was operated in positive electron impact (EI) ionisation mode at 70 eV with selected ion monitoring (SIM).

Validation study. The method was validated in accordance with Commission Decision 2002/657/EC requirements (10). The specificity, precision (repeatability and within-laboratory reproducibility), recovery, CCα, and CCβ of the method were evaluated. The calibration curves of standards solutions of RAL's with seven calibration levels (0, 0.02, 0.05, 0.1, 0.2, 0.4, and 1 μg mL^{-1}) were prepared and the parameters of linear regression were estimated. The validation process included the assays performed on spiked muscle samples. The blank bovine muscle tissue samples were fortified with the RAL's at concentrations levels of 1.0, 1.5 and 2.0 μg kg^{-1}. In addition, spiking levels of 0.5 and 5.0 μg kg^{-1} were included. Three series of analysis involving 21 fortified samples were performed. The parameters of linear regression of calibration curves for spiked samples were also determined. For checking signal specificity, 10 analyses of blank muscle tissue samples simultaneously with 10 samples of muscle fortified at a concentration of 1.0 μg kg^{-1} were prepared. For the factorial effect analysis, the software "ResVal" (v 2.0) (CRL Laboratory, the Netherlands) was used (1, 14). Additionally, during the validation process possible factors that could influence the measurement results in the ruggedness study were investigated. The minor factors, including the temperature and time of methanol extract evaporation, composition of washing solutions for C_{18} SPE column, serial numbers of C_{18} cartridges, and the temperature and time of the derivatisation reaction, were selected. For these factors ruggedness test using the approach of Youden was applied and the importance of all changes was estimated.

For proposed analytical and detection conditions, correct chromatographic separation of ZER, TAL, ZAN, α-ZOL, and β-ZOL was obtained (Fig. 2).

For each compound at least four diagnostic ions were selected from the mass spectrum. The characteristic ions monitored for -TMS derivatives of RAL's for confirmation purposes and ion ratios were presented in Table 1.

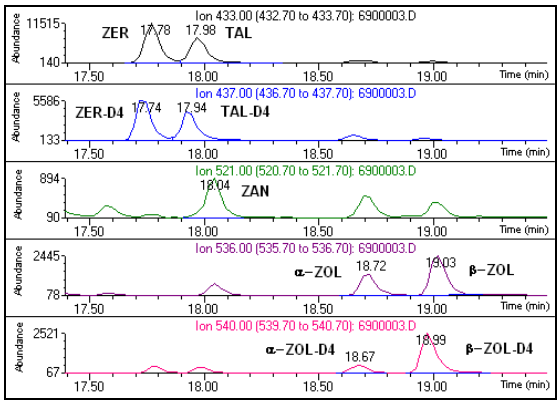


Fig. 2. GC-EI-MS chromatograms of the most intense ions of standards solution of 0.2 μg mL^{-1} (0.4 ng on-column) of RAL's.

Calculations of concentration were made based on the most intense ions underlined in the Table 1. For the calculation of the concentration of ZAN, ZER-D4 was used as internal standard. Summary of the validation results of ZER, TAL, α-ZOL, β-ZOL, and ZAN in tissue muscle samples is presented in Table 2.

Results

The linear regression parameters of standard and calibration curves were correct for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) exceeding 0.99 value for all curves. The specificity studies showed no interferences in the range of the retention times of the analytes. The apparent recovery of the tested compounds from muscle tissue ranged from 84.0% for ZAN to 100.7% for α-ZOL. The coefficients of within day and day to day variations (CV) were less than 18% and 23%, respectively, in all RAL's. The calculated values of CCα and CCβ were lower than actual recommended concentration set at 1 μg kg^{-1}. The results of the ruggedness test indicated that the minor changes of every single selected factor did not show the significant influence on the method.

Table 1
Diagnostic ions and average ion ratios of the studied RAL's hormones

Compound	Mass monitored m/z	Ion ratio average
ZER	538-523-<u>433</u>-335-307	538/433: 0.179; 523/433: 0.204; 335/433: 0.445
TAL		538/433: 0.235; 523/433: 0.217; 335/433: 0.582
ZER-D4/TAL-D4	542-527-<u>437</u>-335-307	-
ZAN	536-<u>521</u>-479-446	536/521: 0.716; 479/521: 0.454; 446/521: 0.927
α-ZOL	536-446-<u>431</u>-333-305	536/431: 0.722; 446/431: 0.790; 333/431: 0.865
β-ZOL	<u>536</u>-446-431-333-305	446/536: 0.703; 431/536: 0.577; 333/536: 0.717
α-ZOL-D4	540-450-<u>435</u>-333-305	-
β-ZOL-D4	<u>540</u>-450-435-333-305	

Table 2
Method performances for ZER, TAL, ZAN, α-ZOL, and β-ZOL

Analyte		Zeranol	Taleranol	Zearalanone	α-Zearalenol	β-Zearalenol
Calibration curve of standards	r^2	0.9968	0.9991	0.9924	0.9994	0.9991
	a	0.1414	0.1702	0.1358	0.2002	0.1086
	b	0.0174	0.0788	0.0450	0.5757	0.0639
Calibration curve of spiked samples	r^2	0.9995	0.9998	0.9989	0.9987	0.9986
	a	1.1419	2.0802	1.4673	1.3217	1.7518
	b	0.3413	0.1624	0.0559	0.5085	-0.6568
Recovery (%)	1.0[a]	95.0	93.0	84.0	93.0	84.0
	1.5	92.0	93.8	91.3	100.7	101.3
	2.0	88.7	94.9	87.4	93.7	100.6
Repeatability CV (%)	1.0	10.4	14.4	17.9	15.2	14.8
	1.5	11.0	12.3	14.2	8.8	6.5
	2.0	12.0	8.2	8.5	11.0	6.6
Reproducibility CV (%)	1.0	10.7	15.0	22.0	15.2	17.4
	1.5	11.2	13.5	15.4	12.6	6.9
	2.0	12.7	12.8	10.4	11.8	9.8
Decision limit CCα (μg kg^{-1})		0.11[b]/0.58[c]	0.06/0.60	0.16/0.58	0.17/0.77	0.18/0.82
Detection capability CCβ (μg kg^{-1})		0.19/0.65	0.10/0.64	0.27/0.69	0.29/0.89	0.30/0.94
Uncertainty U (%)		10.8	12.4	14.5	15.0	13.4

[a] spiking level (μg kg^{-1}), [b] theoretical value, [c] practical value

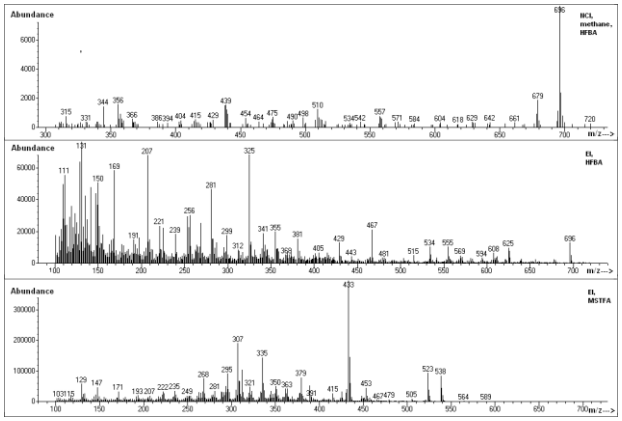


Fig. 3. The examples mass spectra of -HFB and -TMS derivatives of ZER with NCI and EI.

Furthermore, the standard deviations of the differences, calculated for all RAL's using Youden approach were lower than standard deviations of the method carried out under within-laboratory reproducibility conditions.

Discussion

The gas chromatography coupled online to mass spectrometry is a technique commonly used for the determination of volatile substances. It combines the advantages of GC: high selectivity and separation efficiency with the advantages of MS: structural information and high selectivity.

In this research, two possible ways of ionisation were checked: negative chemical ionisation mode (NCI) and electron impact ionisation mode (EI). To obtain mass spectra, standards of derivatised RAL's hormones were analysed using MS full scan mode in the range of scanning from m/z 300 to m/z 750 with NCI and from m/z 100 to m/z 750 with EI. The mass spectra recorded with negative chemical ionisation demonstrated weak fragmentation of RAL's molecules, since the mass spectra showed only intense molecular ions. Whereas, mass spectra recorded at electron impact ionisation contained both molecular ions and characteristic fragmentation ions of the derivatives. Differences in the mass spectra recorded with both types of ionisation in the example of ZER are shown in Fig. 3.

According to the guidelines of Commission Decision 2002/657/EC for confirmatory method based on GC-MS technique, four diagnostic ions (four identification points) for tested analyte should be identified. Based on the analysis of spectra it was found that negative ionisation does not give a sufficient amount of ions and for this reason can be used for screening purposes only.

Application of the electron impact ionisation yielded satisfactory fragmentation of derivatives of RAL's and the good intensity of the signals. The diagnostic ions were sufficiently characteristic for the structures of RAL's and did not originate from the same part of the molecules. The intensity of fragmentation ions was greater than 10% of the intensity of the most intense ion in the mass spectrum, which is consistent with the required criterion of intensity. For this reason, electron impact ionisation mode is suitable for confirmation, as it allows obtaining required number of characteristic ions and meets the requirements for confirmation.

The study was carried out using a single ion monitoring mode (SIM), which causes increasing of sensitivity of analyses and minimises background noise.

Two capillary columns for separation of RAL's were examined. The first one was a non polar HP-5MS column and another one was a VF-17MS column of medium polarity. Application of both columns yielded a good chromatographic separation of the RAL's compounds tested. Since the non polar columns are commonly used and recommended for analysis of hormones, the HP-5MS column was chosen for further research.

The most important in the preparation of the sample for GC-MS analysis is the derivatisation step, which increases volatility of the compound and the sensitivity of determination during GC-MS action. Two derivatisation methods with different reagents, such a heptafluorobutyric anhydride (HFBA) and MSTFA, were tested. Mass spectra were recorded both for standards and internal standards of RAL's. In our experiment, only HFBA was applied and very good repeatability was obtained. For the purpose of -HFB derivatives formation, a mixture of HFBA/acetone (1:4, v/v) is commonly used. First of all, acetone was withdrawn from the use to ensure anhydrous conditions for the process. The derivatisation with HFBA reagent formed intense molecular ions and small fragmentation ions of RAL's in NCI. For this reason, the derivatisation with HFBA reagent is more appropriate for GC-MS screening than for the confirmatory methods. In the next stage, the derivatisation of RAL's with MSTFA was evaluated. The available literature on the derivatisation of RAL's indicates that MSTFA is the most commonly used reagent. Two approaches of MSTFA reagent were tested. The first one was MSTFA only and the other was the mixture of MSTFA, NH_4I, and DTT. The intensity of the signals obtained for the trimethylsilyl (-TMS) derivatives of ZER, TAL, ZAN, α-ZOL, and β-ZOL pointed out that MSTFA/NH_4I/DTT mixture showed greater potential and chemical activity than MSTFA only. The reason for this effect could be the presence of NH_4I as a catalyst, and DTT as a stabiliser, used in the mixture.

Full mass spectra of RAL's compounds recorded showed that the tri-TMS derivatives for ZER, TAL, ZAN, α-ZOL, β-ZOL, and deuterated standards were formed (Fig. 4, a-e). On the basis of the obtained mass spectra, it can be assumed that during the derivatisation reaction, a fragment of the MSTFA molecule probably substituted selectively to the cyclic structures of ZER, TAL, ZAN, α-ZOL, and β-ZOL in three chemically active centres located at positions C-7α-OH, C-14-OH, and C-16-OH of molecules. The schemes of probable fragmentation of the RAL's molecules of tri-TMS derivatives for ZER, TAL, α-ZOL, β-ZOL, and ZAN are presented in Fig. 4 (a-e).

Among the selected diagnostic ions, two were identical with standards of ZER/TAL, α-ZOL/β-ZOL, and ZER-D4/TAL-D4, α-ZOL-D4/β-ZOL-D4. Therefore, the sample suspected for ZER, TAL, α-ZOL, and β-ZOL should be analysed with and without internal standards to avoid faults in identification of RAL's hormones. The optimisation of sample pre-treatment before GC-MS analysis was not the object of this project.

In the previously published study, a method of purification of muscle sample was adapted, which was used in the procedures for the determination of hormones by high performance thin layer chromatography (HPTLC) (25).

The method performance was investigated with respect to various parameters. The method had sufficient selectivity for all RAL's tested, because no interfering peaks from endogenous compounds were found in the retention time of the target analytes. Satisfactory recoveries above 84.0% were obtained due to the use of stable isotope-labelled analogues of the analytes used as internal standards. Application of internal standards offers the advantage of compensation for analytes loss during sample preparation. The method was characterised by a good precision, since CV under within-laboratory reproducibility conditions were less than 23%. The values of obtained decision limits and detection capabilities were below the recommended concentration specified as 1 $\mu g\ kg^{-1}$.

A representative GC-EI(SIM)-MS chromatograms of bovine muscle tissue samples spiked at practical CCα level are posted in Fig 5.

The values of CCα and CCβ were determined by the calibration curves of spiking samples according to the ISO 11843 (12). Because the theoretical values of CCα were very low, an additional experiment was conducted. Twenty samples fortified at the estimated CCα level were tested. In all samples the presence of tested analytes, except taleranol, was revealed. The average recovery ranged from 82.8% for α-ZOL to 132.2% for ZER, with CV from 2.4% for ZAN to 21.4% for α-ZOL was estimated, but in less than 50% of the samples tested at CCα level, the criteria for confirming the identity of the analytes (four identification points and relative intensities of ions) were met. Thus a new CCα of analytes were estimated by parallel extrapolation to x axis at the lowest calibration concentration in spiked samples according to requirements of SANCO/2004/2726 (7). Next the criteria for confirmation purpose were checked for new CCα values. For ZER, TAL, and ZAN the confirmation criteria were fulfilled at 90%, 85%, and 70% samples, respectively, and for α/β-ZOL at 55% samples only. Thus the developed method can be use for screening and confirmatory purposes. Lower CCα and CCβ detection limits were reported after application of GC-MS/MS (3-5) and LC-MS/MS techniques (13, 16). Application of tandem mass spectrometry allows confirmation of the analytes at low concentration levels in comparison to GC-MS.

In the evaluation of method ruggedness it was demonstrated that all selected factors did not significantly affect the analytical performance. The developed method complies with the criteria laid down by the Commission Decision 2002/657/EC for confirmatory methods of substances listed in Group A of Council Directive 96/23/EC, and therefore was applied in the official control of the residues of ZER in Poland.

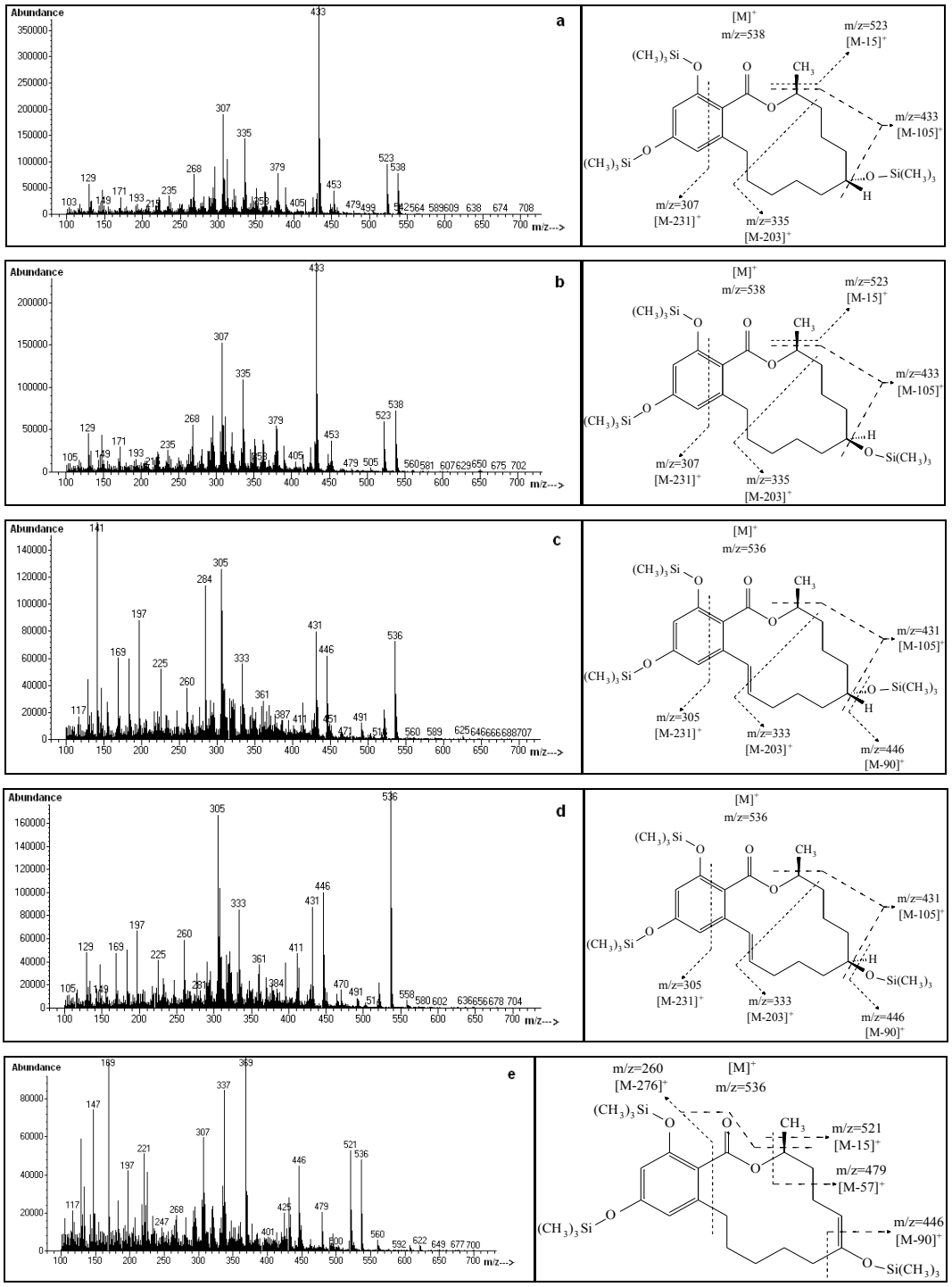


Fig. 4. Full mass spectra and the proposed schemes of (-TMS) derivatives fragmentation of ZER (a), TAL (b), α-ZOL (c), β-ZOL (d), and ZAN (e).

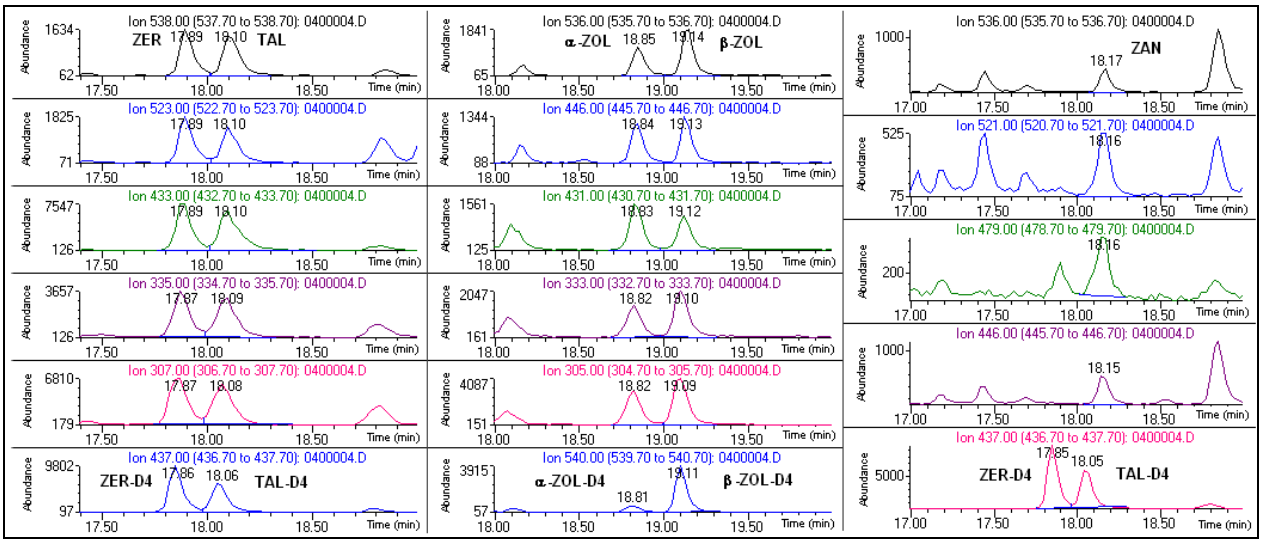


Fig. 5. GC-EI-MS chromatograms of spiked tissue sample at practical CCα level with ZER (0.58 μg kg^{-1}), TAL (0.60 μg kg^{-1}), ZAN (0.58 μg kg^{-1}), α-ZOL (0.77 μg kg^{-1}), and β-ZOL (0.82 μg kg^{-1}).

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