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DETERMINATION OF RESIDUES OF ANABOLIC DRUGS IN MEAT BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The residues in meat of seven estrogenic drugs used in anabolic preparations for animal production were analysed as trimethylsilyl ethers by electron-impact gas chromatography–mass spectrometry following a simple clean-up procedure. The compounds under investigation were: 17β-estradiol, diethylstilbestrol, hexestrol, dienestrol, stilbestrol, ethynylestradiol and zeranol. The method includes extractive homogenization of 10 g of meat in tetrahydrofuran, followed by liquid–liquid partition between acetonitrile and hexane and finally a chromatographic purification step on a small silica gel column. Gas chromatography was carried out on a 10-m glass capillary column coated with SE-54 using a temperature program from 100 to 250°C. The capillary column was connected to the ion source by an all-glass open-split interface with a scavenger gas-line. Detection of anabolic residues was performed with selected ion monitoring on intensive ions in the mass region above *m/e* 400, resulting in a detection limit of 1–5 ppb (10^9). Quantitative determinations were performed using dodecyl gallate as an internal standard applying the signal ratio of the drug and the standard.

INTRODUCTION

In many countries anabolic drugs are used to improve the growth rate and the feed conversion of animals in livestock breeding. The use of drugs in meat production involves a possible health risk if residues remain in the meat. Therefore, sensitive methods of residue analysis are necessary for the surveillance of food from animal produce in the market.

Biological methods based on morphological alterations in the sexual organs of test animals are now of minor importance. Chromatographic methods, including thin-layer chromatography, gas chromatography and high-performance liquid chromatography, were developed for the residue analysis of single compounds as well as for groups of anabolic drugs. Reviews covering the chromatographic methods until 1976 and 1978 were given by Ryan[1] and Günther[2].

In the last two years numerous modifications and improvements of existing

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procedures as well as the development of new methods have been published, indicating the widespread interest in this field[3–16]. A continuing urgent need for reliable methods for anabolic drug residue analysis is evident. Competitive protein binding methods are widely used because of their high sensitivity and simple handling. The most prominent type is the radioimmunoassay, which combines high specificity with high sensitivity. Detection methods are now available for most of the anabolic drugs used in animal production[17–22]. It must be considered as a drawback of the radioimmunoassay that specific antibodies and radiolabelled test compounds of high specific radioactivity are necessary for each hormone, drug and their metabolites.

A competitive protein binding method using the natural estrogen receptor protein from bovine uterus cytosol was developed as a screening procedure for estrogenic components in anabolic drugs[23,24]. This assay has to be combined with specific methods for qualitative and quantitative determination of the various compounds in question.

The most promising method to meet this goal is gas chromatography–mass spectrometry (GC–MS). GC–MS has been successfully used for many years in clinical chemistry for the analysis of steroid hormones and their metabolites in urine and blood samples, as well as for doping controls in the urine of athletes[25–30].

The application of GC–MS for residue analysis in meat has been reported only in a few cases. Day *et al.*[31] described the confirmation of diethylstibestrol (DES) residues in beef liver as the bisdichloroacetate with GC–MS using the GC method of Donoho *et al.*[32]. Höllerer and Jahr determined estrogenic anabolics in bovine liver with GC–MS using the trimethylsilyl ethers of these drugs. The detection limit for the stilbene derivatives investigated by them in meat samples was reported as 4–40 ppb[33].

In this paper we report the determination of residues of anabolic drugs in meat at the ppb level. The method includes a simple clean-up, the formation of trimethylsilyl ethers and the detection and identification of the derivatives with electron-impact GC–MS using chromatographic separation with glass capillary columns.

EXPERIMENTAL

Instrumentation

A Finnigan Model 4000 quadrupole mass spectrometer with an integrated data system Finnigan/Incos 2300 was used for all measurements. The ion source was a combined type for electron impact and chemical ionization. The mass spectrometer was connected to a Finnigan gas chromatograph Model 9610 via an all-glass open-split interface of our own design[34]. Gas chromatographic analyses were carried out on wall-coated glass capillary columns in the Finnigan Model 9610 gas chromatograph or in a Carlo Erba gas chromatograph Model 2101 equipped with a flame ionization detector.

Materials

Zeranol was extracted and purified from the pharmaceutical Ralgro (TAD, Cuxhaven, G.F.R.)[35]. Hexestrol, dienestrol, and diethylstilbestrol were supplied by ICN Pharmaceuticals (Plainview, NY, U.S.A.). 17β-Estradiol and ethynylestradiol (Merck, Darmstadt, G.F.R.) were chromatographically pure. The silylating agents

trimethylchlorosilane (TMCS) and N,O-bis(trimethylsilyl)acetamide (BSA) were purchased from Fluka (Buchs, Switzerland). Silica gel 60 (70–230 mesh) for column chromatography (Art. 7734) and all other chemicals were products of Merck. WCOT-glass capillary columns with an inner diameter of 0.3 mm coated with SE-54 were used at 10-m length. The columns were supplied by Jaeggi (Trogen, Switzerland) or were prepared in our laboratory according to the barium carbonate procedure of Grob[36–38].

An Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, G.F.R.) was used to homogenize the meat and for the following solvent extraction.

METHODS

Clean-up procedure

10 g of minced meat were mixed with 25 ml of tetrahydrofuran in a 100-ml centrifuge tube and homogenized with an Ultra-Turrax homogenizer. After cleaning the homogenizer with another 25 ml of tetrahydrofuran the homogenate was centrifuged at *ca.* 1800 *g*. Then the supernatant was decanted through a plug of glass wool. The residue in the centrifuge tube was again homogenized with 25 ml of tetrahydrofuran, cleansed and centrifuged as in the first step. The supernatant solutions were combined and evaporated under a vacuum at 40°C nearly to dryness. The remaining water was vaporized by means of added ethanol. The dry residue was dissolved in 25 ml of acetonitrile and twice extracted with 25 ml of hexane. The combined hexane phases were re-extracted with 25 ml of acetonitrile, and the acetonitrile phase was again evaporated to dryness. The residue was dissolved in a small volume of benzene and fractionated on a silica gel column (200 × 15 mm I.D.). The column was prepared from a slurry of 7 g of silica gel 60 in benzene. The silica gel 60 was first deactivated by the addition of 2% (w/w) water. After the addition of the meat extract dissolved in benzene the column was washed with 50 ml of benzene. Then the estrogenic compounds were eluted with 50 ml of ethyl acetate.

Derivatization

The concentrated ethyl acetate fraction was transferred into a derivatization tube. It was evaporated to dryness with a nitrogen stream at 70°C. The residue was mixed with 0.1 ml of the silylating reagent consisting of BSA with 10% TMCS. The derivatization tube was closed with a teflon coated septum and held for 1 h at 70°C. After the tube was cooled to room temperature, 0.9 ml of hexane was added.

Gas chromatography

All gas chromatograms were run on 10-m wall-coated open tubular glass capillaries coated with the non-polar silicon phase SE-54. Helium was used as carrier gas at a flow-rate of 2.5 ml/min at room temperature. Injection port and detector were held at 230 and 260°C. A sample volume of 1 μl was splitless injected into the 'cold' column at 90°C, according to the method of Grob[39]. Just 40 sec after the injection, the split of the carrier gas was reopened and a flow-rate of *ca.* 20 ml/min was used as a septum purge. After 1 min a linear temperature program was started with a heating rate of 20°C/min to a final temperature of 250°C, which was held for 5 min.

GC–MS

The design and the operation of the all-glass open-split interface in residue analysis were described in detail elsewhere[34]. The temperature of the interface was 250°C, and the ion source was held at 180°C. Electron-impact ionization was performed at 70 eV with an ion current of 300 μA.

The GC–MS system was operated mainly in the following two modes: cyclic scan and multiple ion monitoring. In the cyclic scan mode the chromatographic run was continuously scanned with a scan time of 0.5 or 1.0 sec for the mass range from 50 to 600 *m/e*. After the run was finished, the crude data were processed using the various computer programs for data enhancement, library search or mass chromatogram reconstruction. Multiple ion monitoring was performed with a maximum of 20 selected masses in a cycling time of 0.9 sec. But mainly 6–10 selected ions were scanned with a cycling time of 0.5 sec, with longer sampling intervals on the ions with lower intensities.

When meat samples were analysed, the ion source was protected from most of the contaminating substances from the biological matrix by venting them using a scavenger gas-line installed in the open-split connection. Only the fractions from the gas chromatographic eluate of analytical interest were transferred to the ion source. Details of the procedure are given in a separate paper[34].

RESULTS AND DISCUSSION

Fig. 1 reveals the problems with which the analyst is confronted in GC analysis of anabolic drug residues in samples from meat. After undergoing the described clean-up procedure, an extract from meat was trimethylsilylated and separated on a 10-m SE-54 glass capillary column using a flame ionization detector. The chromatogram in Fig. 1A shows a great number of substances extracted from the biological matrix. Under the same chromatographic conditions a mixture of trimethylsilylated anabolic drugs and an internal standard was separated and recorded. The resulting chromatogram is shown in Fig. 1B. The concentration of the test substances in Fig. 1B is 10 ng in the injection volume of 1 μl. Fig. 1C shows the chromatogram of the same meat sample as in Fig. 1A, but mixed with the anabolic drugs and an internal standard at a concentration of 1 ppm. This is the same amount of substance as used for the chromatogram in Fig. 1B.

From these experiments it is evident that the combination of a sample clean-up with a non-specific gas chromatographic detector is not suitable for the determination of anabolic drug residues in meat at the ppb level. All experiments to develop a reliable multiresidue method for the estrogenic anabolics at the ppb level on the basis of gas chromatography with halogenated derivatives failed in our laboratory although various procedures for single compounds have been reported[1,2,9,32]. All these methods require laborious clean-up, which results in considerable losses of the residue compound to be detected. Therefore, we relinquished our attempts to develop a GC–ECD method as a general screening for meat samples prior to GC–MS. For this purpose we have for the past three years applied a fast biochemical assay of high sensitivity using the natural estrogen receptor from bovine uterus[23].

Highest sensitivity in GC–MS analysis can be attained with selected ion monitoring. For the successful application of this method in biological samples an im-

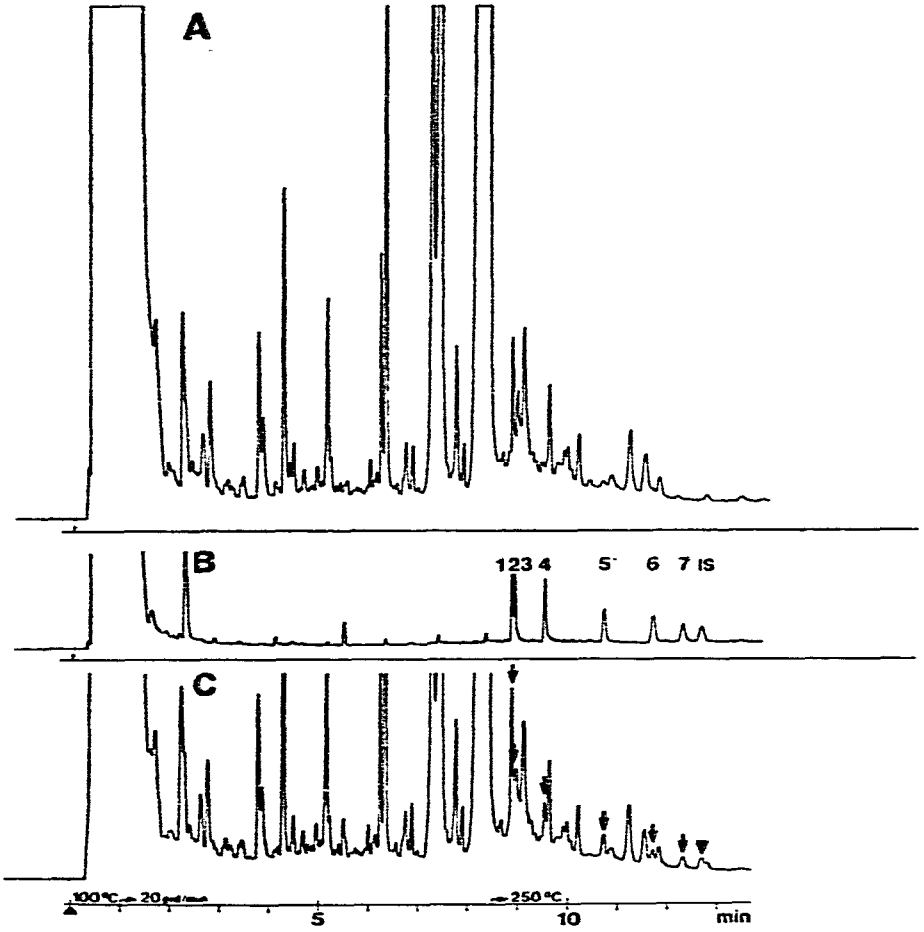

Fig. 1. Capillary gas chromatogram of a meat sample after clean-up and trimethylsilylation using a WCOT glass column of 10-m length coated with SE-54 (0.3 mm I.D.). Detector: FID. A, Meat sample free of anabolic residues. B, Standard mixture of 10 ng of hexestrol (1), diethylstilbestrol (2), dienestrol (3), stilbestrol (4), 17β-estradiol (5), ethynylestradiol (6), zeranol (7) and the internal standard dodecyl gallate (IS). C, Meat sample as in A but the standard mixture from B added. The injected amount of test substances is again 10 ng, equivalent to 1 ppm in the meat. All chromatograms at the same detector sensitivity. For experimental conditions see text.

portant prerequesite is a mass fragmentation pattern of the substances to be determined, which exhibits high ion intensities in the higher mass range. Trimethylsilyl ethers offer a good compromise of suitable mass spectral characteristics and the important gas chromatographic properties of volatility and stability. Table I lists the ions suitable for selected ion monitoring of the trimethylsilyl ethers of the analysed estrogenic anabolics and the internal standard dodecyl gallate. With the exception of the hexestrol derivative, the most intense ions from all compounds are found above *m/e* 400. The trimethylsilyl ether of hexestrol is fragmented nearly exclusively to a single stable ion, which results from cleavage of the central C—C bond and thus occurs at a very high absolute intensity. This can be seen in Fig. 2, where a plot of

TABLE I

IONS SUITABLE FOR SELECTED ION MONITORING OF THE TRIMETHYLSILYL ETHERS OF THE ANALYSED ANABOLIC DRUGS

Substance	*Symbol*	*Ions*		
		*M**	*M − 15*	*Other ions*
Ethynylestradiol	ETO	440(40)	425(100)	300(43) 285(62)
Diethylstilbestrol	DES	412(100)	397(15)	383(16)
Dienestrol	DIE	410(90)	395(51)	381(15)
Hexestrol	HEX	414(1)	399(2)	207(100)
17β-Estradiol	OST	416(100)	401(8)	285(67)
Zeranol	ZER	538(13)	523(13)	433(70) 335(27) 307(37)
Dodecyl gallate**	IS	554(100)	539(4)	281(42) 369(15)

* Molecular ion (relative abundance).

** Internal standard.

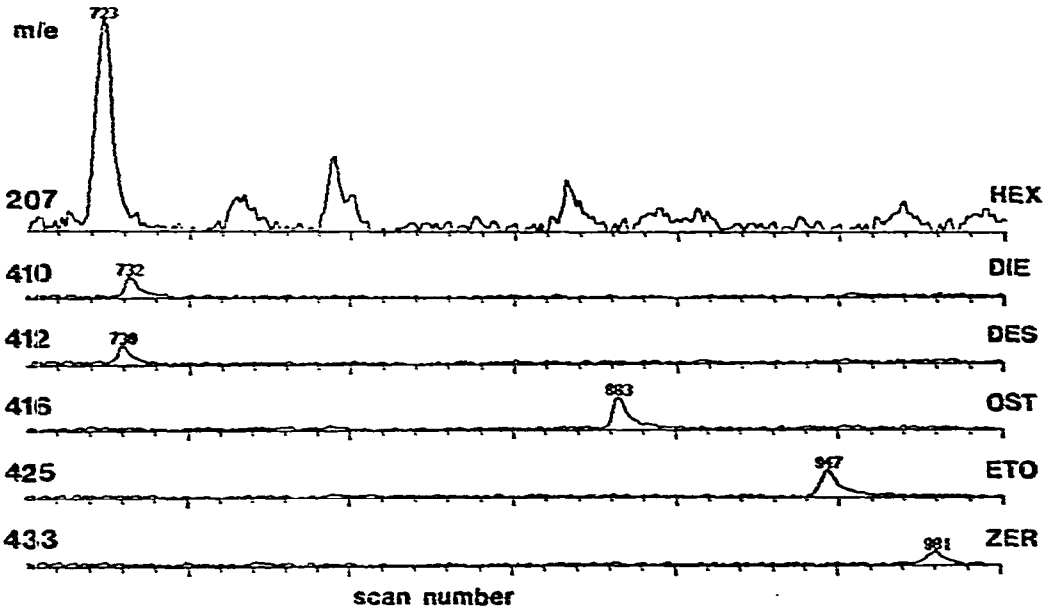

Fig. 2. Selected ion monitoring of anabolic drugs at the subnanogram level. Injected amounts: 50 pg of hexestrol (HEX), 100 pg of dienestrol (DIE), 50 pg of diethylstilbestrol (DES), 150 pg of 17β-estradiol (OST), 750 pg of ethynylestradiol (ETO) and 500 pg of zeranol (ZER), as trimethylsilyl ethers.

the selected ions from all estrogen derivatives is shown. Hexestrol and dienestrol were in this experiment at the lowest concentration levels. It is obvious that the detection limit depends not only on the absolute ion intensity but also on the background. The chosen substance concentrations in Fig. 2 correspond with ppb levels in meat.

Fig. 3 shows the detection limit in meat samples for the three compounds of the stilbene type. These compounds are of particular importance because they exhibit the greatest health risk from the estrogenic anabolics discussed here. In the upper

plot (Fig. 3A) a meat sample free of stilbene derivatives is shown. At *m/e* 207 there is a relatively high background demonstrating a great number of compounds in the biological matrix which form this ion if electron-impact ionization is used. Fragments with *m/e* 410 are rare, whereas a number of substances yield ions with *m/e* 412. The mass fragmentogram at *m/e* 412 emphasizes the importance of a high separation power of the chromatographic system, which makes it possible to differentiate the DES residue from substances of similar chromatographic properties forming fragments of *m/e* 412. The addition of 2 ppb of the stilbene derivatives to the meat sample resulted in additional peaks in the corresponding mass chromatograms at the expected retention times (Fig. 3B). This typical experiment leads to the conclusion that the analysed meat sample is free of the three stilbene derivatives tested for at the level of 1 ppb. In this case 1 ppb can be considered as the detection limit for all three stilbenes.

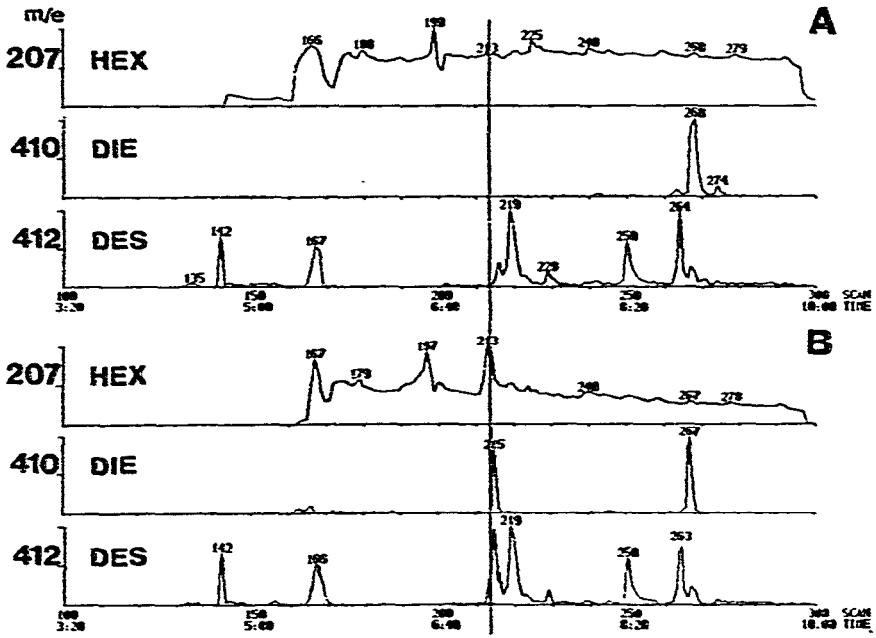

Fig. 3. Selected ion monitoring of estrogenic stilbenes in meat near the detection limit. A: Meat free of anabolic residues. B: The sample from A with an addition of 2 ppb hexestrol (HEX), dienestrol (DIE) and diethylstilbestrol (DES).

Compared with the detection of the synthetic stilbene derivatives the determination of zeranol residues is more complicated. Zeranol is a commercial drug produced by hydrogenation and reduction from the naturally occurring mycotoxin zearalenone. In the animal, zeranol is partially metabolized into zearalanone. These three substances may occur together and are isolated from meat using the described clean-up procedure. After trimethylsilylation they can be separated by gas chromatography and detected with selected ion monitoring (Fig. 4). The mass spectra of the trimethylsilyl ethers are shown in Fig. 5. The fragments in the lower and middle mass

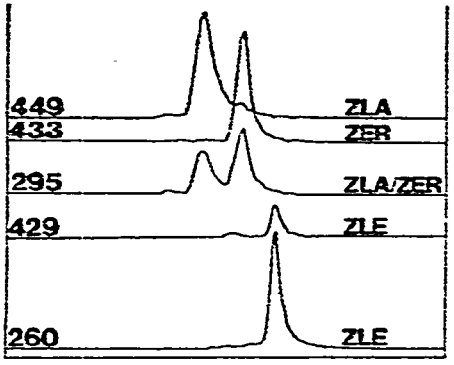

Fig. 4. Selected ion monitoring of the trimethylsilylethers of the zeranol group. Meat sample with the addition of 50 ppb of zeranol (ZER), zearalanone (ZLA) and zearalenone (ZLE).

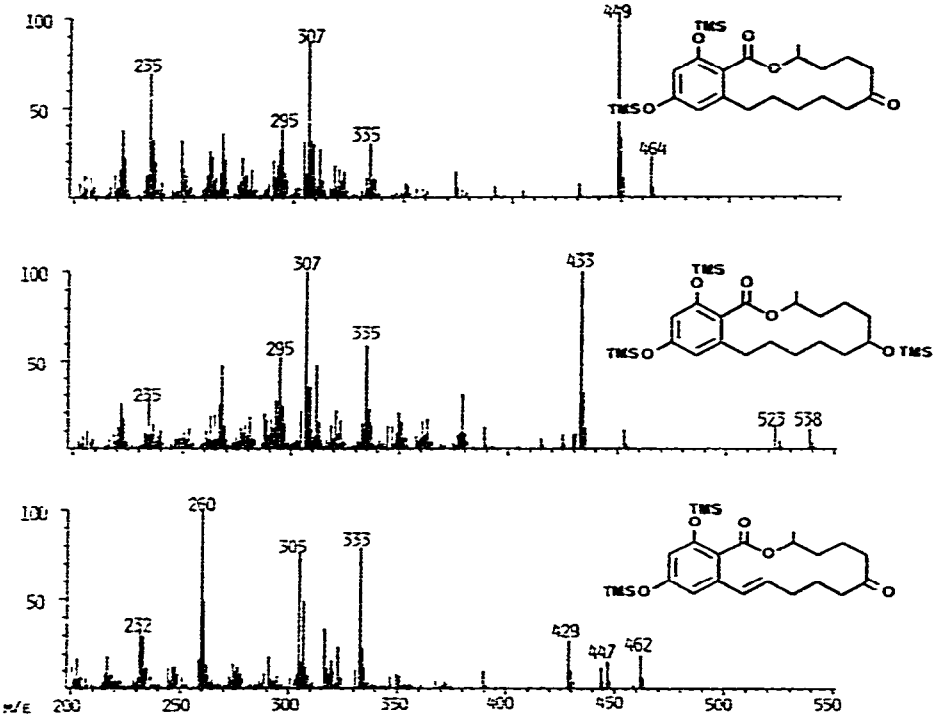

Fig. 5. Mass spectra of the trimethylsilyl ethers of the zeranol group. Electron-impact ionization under GC–MS conditions (70 eV).

regions of zeranol and zearalanone are nearly identical because of their structural similarity. Fragments from the mass spectrum of zearalenone can interfere with those from zeranol and zearalanone as isotopic peaks or by the addition of a proton. With appropriate selection of fragment ions the unequivocal detection of the three compounds can be achieved, as is demonstrated in Fig. 4.

A complex problem in residue analysis at trace level in biological samples with GC–MS is quantitation. The best method for quantitative determination of a single substance is the isotopic dilution method. At the beginning of the analysis a certain amount of the deuterated compound is added as a substance-specific internal standard. Quantitation is performed using the signal ratio of the same fragments which differ in their masses because of the incorporated deuterium[40]. This method is widely used as a reference method for steroid analysis in clinical chemistry[41,42]. The prerequisite for this method is that the deuterated drug or metabolite with high isotopic purity is at one's disposal. Deuterated anabolic drugs are not yet commercially available. On the other hand, the isotopic dilution method is obviously not of great value in multiresidue analysis. Therefore, an internal standard was chosen exhibiting good mass spectral characteristics combined with a suitable retention time in our chromatographic system. Dodecyl gallate served best for this purpose. The molecular ion at *m/e* 554 is the most intense ion in the mass spectrum (Table I) and the compound is eluted immediately after the last anabolic drug (see Fig. 1B).

Fig. 6 shows a plot of the signal ratio of the selected ions of the drugs and the internal standard as a function of drug concentration. With these two stilbene derivatives a linear calibration curve was found. Fig. 6 again indicates the high intensity of the *m/e* 207 ion from TMS-hexestrol in comparison with the intensity of typical fragments from the other drugs. Depending on the signal to noise ratio of the individual compounds in biological samples, quantitative determinations can be achieved starting at the level of 10–20 ppb.

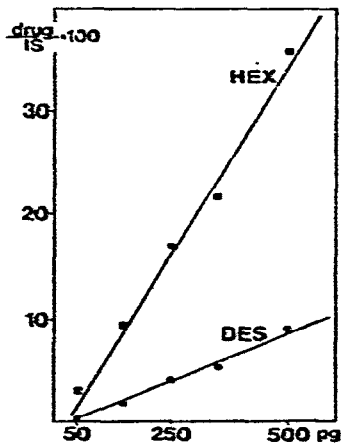

Fig. 6. Quantitative determination of estrogenic anabolics using selected ion monitoring and internal standard. Calibration plot of the signal ratio of drug to internal standard. Internal standard: 10 ng of dodecyl gallate. Amounts of hexestrol (HEX) and diethylstilbestrol (DES) are indicated on the abscissa.

The GC–MS method using the trimethylsilyl ethers of estrogenic anabolic compounds is suitable for the detection and identification of residues in meat at the lower ppb level. The proof of absence of the stilbene derivatives, which are considered as a risk to human health, can be achieved down to 1 ppb or even less, if single ion

monitoring is used. For unequivocal identification, in addition to reproducible retention times on a capillary column and a signal at a substance-specific ion, the simultaneous registration of two or three masses is necessary.

Trimethylsilyl ethers are the derivatives mostly used in GC–MS analysis of steroids in biological samples with electron-impact ionization. These derivatives were found to be suitable for some estrogenic anabolics by Höllerer and Jahr[33]. Our attempts to develop a screening procedure based on ECD–GC with halogenated acyl derivatives included a systematic study of the gas chromatographic properties of a series of derivatives. We found that heptafluorobutyrates were the most suitable. However, zeranol formed a critical derivative, as was the case with other acylating agents. It is very sensitive during chromatography and is easily destroyed if any active spots arise in the system. Heptafluorobutyrates do not exhibit better mass spectral characteristics than the trimethylsilyl ethers, so that the better chromatographic stability of the latter makes them the derivatives of choice.

In recent years other alkylsilyl ethers have been proposed for the GC–MS analysis of steroids and phenolic compounds. The most promising derivatives are the *tert.*-butyldimethylsilyl ethers, which demonstrate superior mass spectral fragmentation characteristics with electron-impact ionization[43–46]. A disadvantage of these derivatives is their low volatility, which means that higher temperatures are required. This is of particular significance with compounds containing three hydroxyl groups, such as zeranol. Although the *tert.*-butyldimethylsilyl ethers can be of importance for the mass spectral identification of some individual estrogens, the trimethylsilyl ethers are more suitable for routine trace analysis in meat.

Quantitation with GC–MS is frequently better achieved using chemical ionization (CI). Our investigations of various derivatives, including the trimethylsilyl ethers and heptafluorobutyrates, were performed with methane and isobutane as reactant gases. Under various experimental conditions we always found that with CI the total ionization of the compounds was less compared with electron-impact ionization. This means a higher detection limit for the anabolics and therefore CI is less suitable for trace analysis.

In our laboratory we start the routine analysis of anabolics in meat with a competitive protein binding method in which the estrogen receptor isolated from the cytosol of bovine uterus cells is used as a highly specific and sensitive reagent for estrogenic compounds[23]. The combination of this time-saving biochemical screening procedure with the GC–MS method enables us to focus our attention on estrogen positive samples. Moreover, the agreement of biochemical and MS data makes the trace analysis of estrogenic anabolic compounds in meat highly reliable at the ppb level.

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