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Quantification of Diethylstilbestrol Residues in Meat Samples by Gas Chromatography–Isotope Dilution Mass Spectrometry

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A method is described for the quantitative determination of residues of the synthetic estrogen diethylstilbestrol (DES) in meat at levels as low as 0.1 ppb. Samples were enzymatically digested with subtilisin and extracted with diethyl ether. The organic phase was then subjected to a cleanup, consisting of two liquid–liquid partitionings. Residues of DES in the extract were derivatized to bis(heptafluorobutyryl)-DES and assayed by electron-impact mass spectrometry. Quantification was achieved by measuring the intensity ratio of the m/z 660 ion (molecular ion of DES) and the m/z 666 ion (molecular ion of the hexadeuteriated DES analogue). The latter not only served as the internal standard but also monitored the derivatization efficiency and stabilized the cis/trans DES isomers ratio. Analytical recovery from meat, as assayed with a ^{3}H-labeled DES, was about 90%. The method was used to confirm positive radioimmunoassay results.

Natural and synthetic hormones are used worldwide in raising and fattening of livestock and poultry as growth-promoting agents by an increased feed conversion efficiency. One of the most intensively used compounds is diethylstilbestrol (DES). Because of its carcinogenic and estrogenic properties, residues in foods are not permitted and its administration is forbidden in most countries. An increased risk of endometrial cancer in DES-treated women and cancer of the lower genital tract in daughters of these women was observed (McMartin et al., 1978). Therefore, consumer organizations support a boycott of meat imported from countries with insufficient checks or from countries that permit the administration of DES to food-producing animals. The legislation of the European Economic Community (EEC) authorizes the member states to forbid imports from countries outside the EEC of fresh meat containing residues such as estrogens and antibiotics (Directive of 12 December 1972 on the Importing of Meat from Third Countries 72/462/EEC).

Several techniques have been elaborated during the last two decades to detect and/or to measure quantitatively residues of DES in tissues, organs, and fluids of treated animals. They include bioassays, based on mouse uterus susceptibility to estrogens (Umberger et al., 1963a), colorimetry or fluorimetry (Umberger et al., 1963b), competitive protein-binding assays (Hoffmann, 1978; Hoffmann and Laschutza, 1980; Vogt, 1980; Gridley et al., 1983), and several gas chromatographic techniques with various detection systems. Among them, electron-capture (Coffin and Pilon, 1973; Donoho et al., 1973; Ryan and Pilon, 1976; Laitem et al., 1978; Tirpenou et al., 1983) and mass spectrometry (Day et al., 1975; Höllerer and Jahr, 1975; Stan and Abraham, 1980; Derks et al., 1983; Galli et al., 1983) are the most commonly employed techniques. The detection limit of the chromatographic methods is in the order of 1 ppb. Immunochemical methods are capable of greater sensitivity.

Although screening for DES is most effectively carried out on urine samples of living animals, there is still a need for reliable and accurate assays in muscle tissue, because usually this is the only sample type that is available from imported slaughtered animals.

Our aim was to develop a confirmatory method that was less laborious than existing methods and at least as sensitive. The availability of a stable isotope-labeled homologue of DES was the basis of the present work. The reliability (precision, accuracy, sensitivity) of the analytical procedure was improved by the use of an internal standard. The experience with our method covered a period of more than 1 year with samples from wholesale and retail trade.

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EXPERIMENTAL SECTION

Reagents and Materials. All reagents and solvents were analytical reagent grade and were used as supplied except methanol, which was distilled twice, and diethyl ether, which—because of the danger of a too high peroxides content—was shaken with a 25% ferrous sulfate solution, washed three times with distilled water, and subsequently distilled. The distillate was stored in brown glass bottles and renewed weekly. *trans*-Diethylstilbestrol (DES) was from Serva (Heidelberg, FRG). HPLC analysis with UV detection at 240 nm showed it to contain 5% of the cis isomer. Hexadeuteriated diethylstilbestrol [(*E*)-3,4-bis(4-hydroxyphenyl)[1,1,1,6,6,6-$^2H_6$]hex-3-ene], hereafter referred to as [$^2H_6$]DES, was synthesized as described elsewhere (De Clercq et al., 1984). The compound was found to be isotopically pure by ^{1}H NMR (360 MHz). HPLC analysis revealed it to contain 4% of the cis isomer. Subtilisin A (dialyzed and lyophilized; 29.3 Anson units/g) was obtained from Novo Industri (Copenhagen, Denmark); special care should be taken to avoid inhalation of dust from this product since allergy and irritation of the skin and mucous membranes may occur. Heptafluorobutyric acid anhydride (HFBA) was from Macherey-Nagel (Düren, FRG) and was packed in 1-mL vials with rubber septa. β-Glucuronidase/arylsulfatase was from Boehringer (Mannheim, FRG).

All nondisposable glassware was treated with chromic acid solution for 3 h and rinsed thoroughly with distilled water. Disposable Pasteur pipettes were used to transfer phases to the next tubes.

Naturally contaminated meat samples were donated by Dr. R. W. Stephany, Laboratory for Endocrinology, National Institute for Public Health and Environmental Hygiene, Bilthoven, The Netherlands.

Extraction and Cleanup. Fresh or thawed meat (1 g) was immersed in 4 mL of 0.1 M Tris solution to which 0.5 ng of [$^2H_6$]DES in 10 μL of methanol was added. This corresponded to a fortification level of 0.5 ppb. About 1 mg of Subtilisin A was dissolved in the aqueous phase, and the tube was vortexed for 30 s and then kept at 60 °C for at least 2 h.

After chilling to room temperature, the liquified contents of the tube were extracted with two 5-mL portions of diethyl ether. Due to emulsification, centrifugation was necessary after the first extraction. The combined ether layers were evaporated at 35 °C under a gentle stream of

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nitrogen. The crude extract thus obtained was taken up in 1 mL of $CHCl_3$, which was then subjected to a partition with 3 mL of 1 M NaOH (pH 14). Again, emulsification necessitated prolonged centrifugation. After the organic phase was discarded, the NaOH layer was neutralized with 3 mL of 1 M HCl and extracted twice with 5 mL of diethyl ether. The combined ether layers were dried over 2 g of anhydrous Na_2SO_4, concentrated to ca. 0.5 mL at 35 °C in a stream of nitrogen, transferred to a conical derivatization vessel with screw cap and PTFE liner, and finally taken to dryness.

When hydrolysis of DES-glucuronides was required, the chilled subtilisin digestate was neutralized with acetic acid solution, spiked with 20 μL of β-glucuronidase/arylsulfatase solution, and subsequently kept overnight at 37 °C. The procedure was then continued with the diethyl ether extractions.

Derivatization. To the dry residue were added 0.2 mL of acetonitrile and 0.05 mL of the HFBA reagent. The vessel was closed and heated at 60 °C for 30 min in a thermostated oven. Excess reagent and solvent were blown off in a stream of nitrogen, and the residue was taken up in 25 μL of hexane.

Gas Chromatography–Mass Spectrometry. The gas chromatographic–mass spectrometric analysis was done on a Hewlett-Packard 5992B instrument equipped with a 25-m length × 0.32-mm i.d., cross-linked 5% phenylmethylsilicone, fused silica capillary column (Hewlett-Packard, Palo Alto, CA) and a single-stage jet separator. Injections were done by depositing a 2–3-μL aliquot of sample extract on the tip of an all-glass moving-needle injection system (Van den Berg and Cox, 1972; Verzele et al., 1980; De Jong, 1981). Helium was used as carrier and makeup gas at flow rates of 2 and 20 mL min^{-1}, respectively. The temperature of the injection port was 290 °C, and the column was operated isothermally at a temperature of 210 °C. Under these conditions, *cis*- and *trans*-DES eluted with retention times of 1.8 and 2.3 min, respectively.

The theoretical masses of the molecular ions are 660.0995 for $(HFB)_2$-DES and 666.1365 for $(HFB)_2$-$[^2H_6]$DES. Prior to the beginning of any series of selected ion monitoring, the most intense signal of three m/z values of 0.1-amu increment around these theoretical masses was selected for optimum sensitivity and accuracy. Typically intensities at m/z 660.1 and 666.1 (window size 0.1 amu) were measured with dwell times of 30 ms and an electron multiplier voltage of 2800 V. Other ions monitored for identification were m/z 661.1 (M + 1 peak) and 631.1 (fragment ion).

RESULTS AND DISCUSSION

Enzymatic Digestion. Enzymatic digestion or deproteination of tissue samples was reported to be successful in toxicological analyses (Osselton et al., 1977). It was shown that this approach yielded far higher recoveries than conventional extraction methods and that the subsequent chromatographic analysis allowed rapid separation and detection of nanogram quantities of drugs without the need for elaborate purification procedures. They applied it to benzodiazepines, barbiturates, salicylic acid, and other acidic compounds involved in human poisoning cases.

Enzymatic digestion offers the advantage of labor-saving, as a large number of samples can be digested overnight. No differences have yet been observed in the quality of the digestate as a function of digestion time. We determined that subtilisin does not affect DES, DES-glucuronide, nor the cis–trans isomerization.

Extraction and Purification Procedure. The basic principle of the purification procedure is the partition of

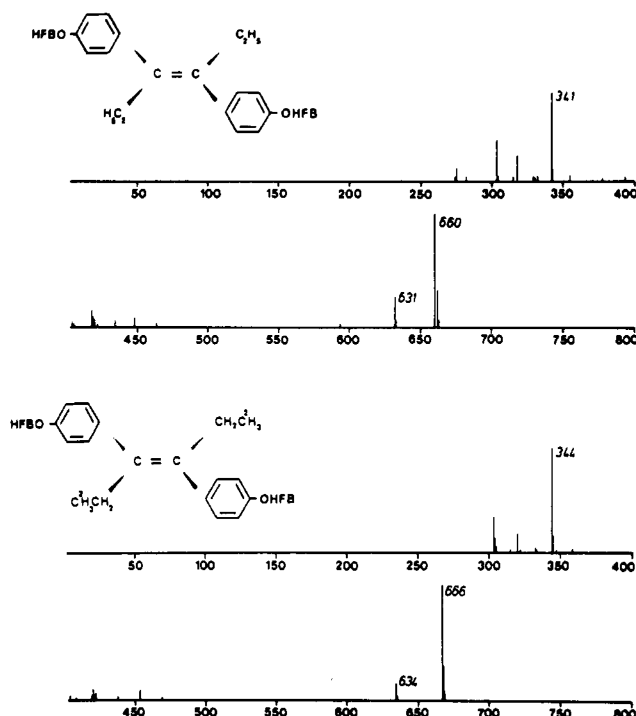

Figure 1. Mass spectra (70 eV) of *trans*-$(HFB)_2$-DES (top) and of *trans*-$(HFB)_2$-$[^2H_6]$DES (bottom).

the raw extract, which often contains considerable amounts of lipid material, between the organic phase and the NaOH solution. After neutralization and back-extraction in diethyl ether, an extract was obtained that was clean enough to be analyzed without the need of too frequent cleaning of the ion source of the mass spectrometer. The only interfering signal observed in some extracts occurred at the retention time of the *cis*-$(HFB)_2$-$[^2H_6]$DES (m/z 666). The cis fraction, however, was not counted for quantification in this method.

For immunochemical analysis the extract needs to be subjected to a more extensive cleanup. An unacceptable number of false-positive results makes further purification by column liquid chromatography indispensable. Such a procedure has been elaborated by this group (Van Peteghem and Van Haver, 1986).

Although the extraction efficiency, as determined at the 1 ppb level by means of liquid scintillation counting of tritium-labeled DES added as a tracer, was 89.6% ± 2.2% (n = 9), the addition of the deuterium-labeled internal standard before digestion of the sample compensated for all losses during sample workup in each sample analyzed. This high recovery may be explained by the presence of two phenolic OH groups, which ensures complete back-extraction from the $CHCl_3$ phase into the aqueous 1 M NaOH layer (pH 14).

Blank meat samples, spiked with DES-glucuronide, showed quantitative recovery of the free DES upon treatment of the subtilisin digestate with β-glucuronidase/arylsulfatase.

Gas Chromatography–Mass Spectrometry. The electron-impact (70 eV) mass spectra of *trans*-$(HFB)_2$-DES and of *trans*-$(HFB)_2$-$[^2H_6]$DES are shown in Figure 1. As the molecular ions are the base peaks with a high contribution to the total ion current (low fragmentation), they were selected for single-ion monitoring. The choice of a particular derivative is a compromise between good mass spectrometric characteristics and good chromatographic characteristics (volatility, stability, absence of interferences). Heptafluorobutyryl derivatives were preferred to

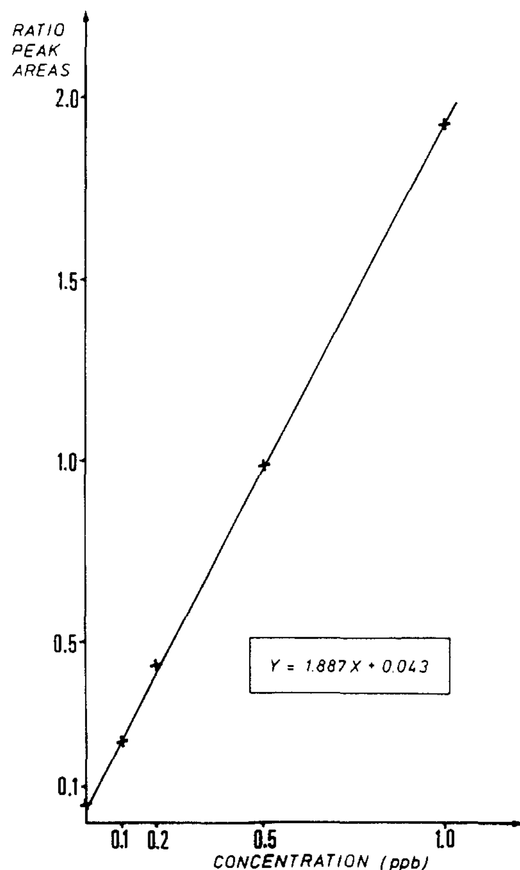

Figure 2. Example of a standard curve, established with extracts of blank meat samples to which varying amounts of DES (concentration range 0.1–1 ppb) and 0.5 ppb of internal standard were added before enzymatic digestion.

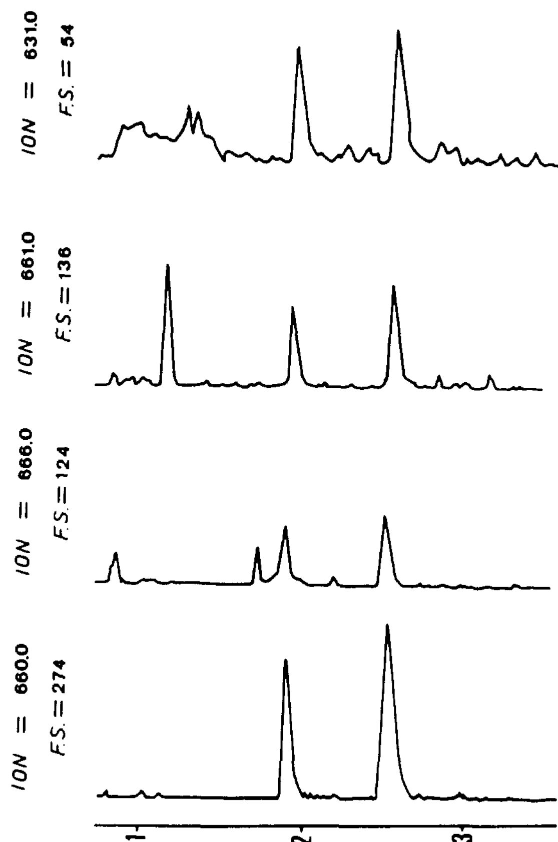

Figure 3. GC–MS data of a spiked meat sample (concentration of DES 1 ppb).

trimethylsilyl ethers for two reasons: first, the higher mass gain (m/z: $(HFB)_2$-DES, 660; $(Me_3Si)_2$-DES, 412), which made interferences less likely to occur; second, the very favorable fragmentation pattern. Moreover, HFB derivatives were easily formed, although it must be admitted that they lacked the stability of the Me_3Si derivatives. When stored at 4 °C they were stable for a maximum of 3 days.

In contrast to other methods, which use the splitless injection mode, the use of a solid (moving-needle) injection system allowed isothermal separation conditions. This considerably reduced analysis time and allowed a high sample throughput, which is of particular interest for this type of analysis. Moreover, a relatively large amount of sample extract (up to 6 μL) could be concentrated on the top of the needle without affecting the chromatographic separation. Finally, high molecular weight nonvolatile sample constituents tended to pyrolyze on the needle instead of entering the column (increased column lifetime).

Quantification. For quantitative analysis an amount of $[^2H_6]$DES, corresponding to 0.5 ppb, was added to the meat sample before enzymatic digestion. From Figure 2, established by means of spiked blank meat samples, it can be seen that the results were linear, i.e., that DES and the internal standard were recovered with the same efficiency. Although no detectable amounts of unlabeled DES could be observed from the mass spectrum of the $[^2H_6]$DES, there was a positive intercept on the y axis. The calibration curve, which was calculated by means of the method of the least squares, can be expressed as $y = 1.887x + 0.043$, where y = the ratio of peak areas of m/z 660 and 666 and x = the concentration of spiked DES in a 1-g sample of blank meat, expressed in ppb.

The deuterium-labeled DES analogue has several other functions in the analytical procedure. Besides its direct function as a reference trace, it compensates for all recovery factors during the assay and for fluctuations of the cis–trans isomerization and, last but not least, it monitors the reliability of the assay procedure. When the cis and trans peaks in the $[^2H_6]$DES channel (m/z 666) do not show up, it is clear that something has gone wrong: errors of the analyst, disturbed derivatization (trace amounts of water in the extract), decreased sensitivity of the mass spectrometer.

The precision of the procedure, expressed as the coefficient of variation, was 8.1% at the spiked 0.2 ppb level (n = 6) and 2.1% at the spiked 2 ppb level (n = 6).

Cis–Trans Isomerization. *trans*-DES is the biologically active form, and commercial preparations of this substance usually contain only a few percent of the cis isomer. However, due to biological metabolization and mainly as an artifact during sample extraction, cleanup, and derivatization, a considerable extra amount of the cis isomer is formed. In the chromatographic step of the present method both isomers were well-resolved but showed identical mass spectra.

The normal cis/trans ion intensities ratio of a standard solution containing 1 ng of DES was, after transformation into $(HFB)_2$ derivatives, about 75/25. However, if DES was taken through the entire extraction, purification, and derivatization procedure, the ratio changed to about 40/60, as is demonstrated in Figure 3 (GC–MS data of a meat sample spiked with 1 ppb DES and 0.5 ppb internal standard) and Figure 4 (GC–MS data of a blank meat sample spiked with 0.5 ppb internal standard). It was a general feature that this ratio was not constant and therefore certainly could not be used as an identification criterion. It must however be emphasized that the ratios, although they might vary from sample extract to sample

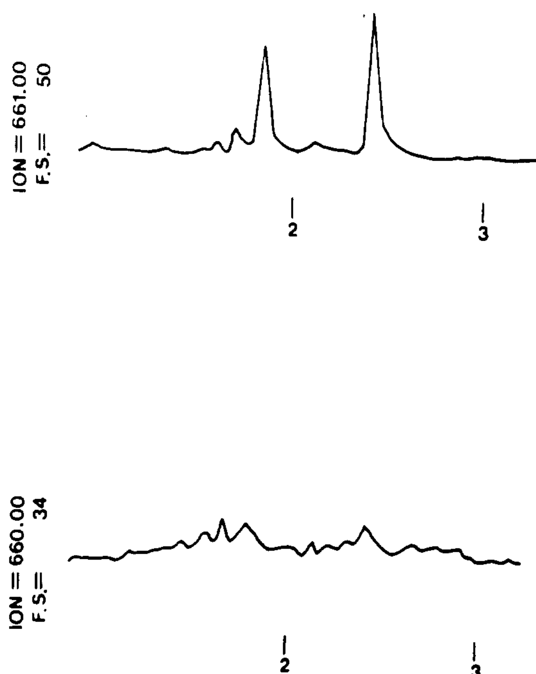

Figure 4. GC–MS data of a blank meat sample (concentration of internal standard 0.5 ppb).

extract, were comparable within the same analytical run for both $(HFB)_2$-DES and $(HFB)_2$-$[^2H_6]$DES. This is consistent with the observations of other authors (Covey et al., 1985). An additional advantage is that the metabolism of the parent drug molecule, which is the trans configuration, is ruled out by the analytical procedure. Analyte and internal standard are brought to the same equilibrium state between cis and trans. Very probably the cis–trans isomerization is both a matrix- and a concentration-dependent phenomenon. The latter assumption is based on the observation that it occurred to a larger extent in standards than in biological samples with comparable concentrations and that it occurred to a lesser extent when standards of high concentrations, corresponding to the ppm level, were derivatized.

It is clear that the isomerization in trace amounts in a biological matrix deserves further investigation. In this method only the trans peak was taken into account, although other authors sum both peaks. Anyhow, meat found to contain DES at any level or in any form is unfit for human consumption according to most food legislations.

Identification Criteria. DES is positively identified in meat samples, which were found positive upon screening by radioimmunoassay (Van Peteghem and Van Haver, 1986), if the following conditions are met simultaneously:

1. Cis and trans peaks of both analyte (DES) and internal standard ($[^2H_6]$DES) appear at the correct retention times. Under the gas chromatographic conditions described, there was a slight separation of the labeled and unlabeled compound.

2. Cis and trans peaks in the analyte and the internal standard show the same pattern, i.e., the same relative heights. Accurate area measurements were often impeded by the small peak at the leading edge of the cis peak in the internal standard channel, as can be seen from Figure 3. The absolute value of the cis/trans ratio was found to be of no practical use. The same experience is pertinent to the cis/trans ratios of other ions of the fragmentation pattern such as m/z 661 and 631.

3. The ion intensities ratios of m/z 660 to 661 and m/z 660 to 631 in the analyte are comparable with those of a standard. They were found to be 3.83 ± 0.52 ($n = 8$) for m/z 660 to 661 and 5.40 ± 0.29 for m/z 660 to 631.

Registry No. DES, 56-53-1.

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