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# Rapid screening method for the determination of diethylstilbestrol in edible animal tissue by column liquid chromatography with electrochemical detection

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ABSTRACT

A rapid and sensitive screening method for the determination of residues of diethylstilbestrol in edible animal tissue is described. The analyte was extracted from the tissue with *tert.*-butyl methyl ether, reextracted with 1 *M* sodium hydroxide and further cleaned up by solid-phase extraction with $C_{18}$ cartridges. Analysis was performed by isocratic elution with a phosphate-buffered mobile phase, methanol–0.05 *M* phosphate buffer pH 3.5 (67:33), on a Nucleosil 5-$\mu$m $C_{18}$ column with electrochemical detection at +0.90 V. The average recovery of *trans*-diethylstilbestrol in spiked samples is 66%, with a standard deviation of 14% ($n=22$) in the range 0.5–2.0 $\mu$g/kg. The detection limit is 0.1–0.2 $\mu$g/kg, although at this level other compounds may interfere and give rise to false positive results.

## INTRODUCTION

The synthetic oestrogen diethylstilbestrol (DES) has been extensively used in livestock and poultry breeding to promote liveweight gain and feed conversion efficiency. However, its suspected carcinogenicity and the persistence of residues in the meat have led to regulations regarding its use. In Spain, as in other countries, the use of DES and other stilbenes has been prohibited by law since 1984, because of the potential health risks to the consumers of meat containing residues of such compounds [1].

To control the illegal use of these drugs various methods have been developed. They generally include the analysis of urine samples, which have higher concentrations of residues than meat or serum. However, urine is not always available, and muscle tissue and meat have to be analysed. The residue concentrations of veterinary drugs and, in particular, DES in meat are ten times lower than the corresponding concentrations in urine, and lie in the sub-$\mu$g/kg range. Therefore very sensitive methods have to be used to detect the illegal use of DES in meat. Various authors have used gas chromatography–mass spectrometry (GC–MS) [2–6] or radioimmunoassay (RIA) [7], but these techniques are not always available in control laboratories. High-performance liquid chromatography (HPLC)

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is often used in the clean-up procedure for residue analysis. However, the determination of DES residues by HPLC needs a very sensitive and specific detector: UV absorbance is not specific for DES analysis [8] and fluorescence measurement requires a derivatization step [9–11].

Therefore, liquid chromatography with electrochemical detection (ED) was chosen for the proposed new method described here. Low limits of DES can be detected with minimal sample preparation, as ED is a sensitive and selective technique, which has been successfully applied to the determination of other growth-promoting hormones [12]. The method permits the detection of sub-$\mu$g/kg levels of DES in meat. A possible confirmation step uses the same method applying with different potentials.

## EXPERIMENTAL

### *Apparatus and reagents*

*tert.*-Butyl methyl ether (Merck, Darmstadt, Germany) was residue-analysis grade, methanol (Romil Chemicals, Loughborough, U.K.) was HPLC grade. Sodium hydroxide (Merck) and a Sep Pak $C_{18}$ cartridge (Millipore-Waters, Milford, MA, U.S.A.) were used. Water was Milli-Q (Millipore-Waters) deionized. The HPLC system (Millipore-Waters) was composed of a dual liquid chromatographic pump (M-6000), a universal injector (U6K) and an electrochemical detector (Model 460) with glassy carbon electrodes and an Ag/AgCl reference electrode. The detector was set at sensitivity range 1 nA and filter 2. The applied potential was 0.9 V.

The detector was connected to a Digital data processor, which permits reprocessing of the obtained chromatograms by automatic and manual (scanner) integration of the selected peaks.

The chromatographic column used throughout this work, at room temperature, was packed with Nucleosil $5C_{18}$ (20 cm × 8 mm I.D.) (Macherey-Nagel, Düren, Germany), and methanol–0.05 *M* phosphate buffer pH 3.5 (67:33) was the mobile phase. The flow-rate was 1 ml/min. DES was purchased from Sigma.

### *Extraction and clean up of meat samples*

Samples of bovine muscle were minced and mixed in a Braun homogenizer. All samples were analysed in sets of eight which was the capacity of the centrifuge. A 10-g mass of the homogenate was placed into a screw-topped glass bottle (50 ml), 15 ml of *tert.*-butyl methyl ether were added, and the mixture was vigorously shaken for 15 s and centrifuged at 3000 *g* for 10 min. The supernatant was collected, and the extraction was repeated with a fresh 15-ml portion of *tert.*-butyl methyl ether. The combined organic layers were transferred to a 100-ml separatory funnel and extracted with 10 ml of 1 *M* sodium hydroxide.

The funnel was stoppered and shaken vigorously. The phases were separated (*ca.* 30 min) and the aqueous layer was collected and buffered to pH 4.5 with 20%

acetic acid. This solution was applied to a Sep Pak $C_{18}$ cartridge (Waters-Millipore), which was pretreated according to the manufacturer's instructions (5 ml of methanol and 5 ml of water). The cartridge was washed subsequently with 5 ml of water and 5 ml of methanol–water (45:55, v/v). Finally DES was eluted from the cartridge with 4 ml of methanol–water (80:20, v/v). The eluate was concentrated to *ca.* 1 ml, transferred to a 2-ml vial with the aid of methanol and evaporated to dryness under a gentle stream of nitrogen. The dry residue was dissolved in 200 μl of mobile phase.

*Detection and identification by HPLC*

The electrochemical detector had a high sensitivity, which required the use of high-quality reagents. Therefore, the mobile phase was pumped overnight through an old electrochemical detector to oxidize all the interfering compounds at the potential of +0.90 V, which was the potential used for the detection of DES. This oxidized mobile phase was used as the eluent under the chromatographic conditions described above.

A hydrodynamic voltammogram for DES was constructed in the mobile phase at pH 3.5 by successive injections of a DES standards solution (10 μl of a 40 ng/ml solution) at different electrode potentials ranging from 0.65 to 1.3 V in *ca.* 0.1 V increments.

Calibration curves were obtained by injecting different amounts of DES ranging from 0.2 to 1.8 ng per injection. The areas of the corresponding peaks were measured and plotted against the injected amounts of DES.

Recovery assays were obtained by spiking 10-g meat samples with 0.5, 1.0, 1.5 and 2.0 μg/kg DES, respectively, and comparing the areas of the *trans*-DES peaks with those of the *trans*-DES peaks of the corresponding extracted standards.

## RESULTS

Anabolics in meat are present in the free unconjugated form, and therefore the sample preparation of meat does not need the incubation step with glucuronidase that is necessary for urine samples. Some authors, however, apply an enzymatic digestion step using subtilisin to deproteinate the tissue samples, sometimes yielding far higher recoveries [6]. We did not include this enzymatic hydrolysis of the muscle protein, resulting in 'cleaner' chromatograms.

The basis principle of the first part of the sample clean-up is the partition of the raw extract, which often contains considerable amounts of lipid material, between the organic phase and the aqueous layer. The presence of the two phenolic OH groups ensures complete extraction from the ethereal phase into the aqueous NaOH solution. After neutralization, the solid phase extraction results in extracts clean enough to be analysed by HPLC with ED. The purified extract is taken up in 200 μl of mobile phase, and 10–20 μl are injected into the chromatographic system, which permits repeated injection with standard addition. Some samples,

with a suspicious peak, need a second analysis, with both DES and the sample extract being injected, to confirm the retention time.

DES is a stilbene with two phenolic groups, which can be oxidized at potentials of *ca.* 1000 mV and consequently detected electrochemically. However, other compounds present in the extract will be oxidized as well and may give rise to interference. Therefore it is important to select the lowest possible potential at which the response of DES is still considerable. If a low potential is used, less material will be deposited on the electrode.

To study the optimum detection potential, a hydrodynamic voltammogram was determined by repetitive injection of DES under the described chromatographic conditions at pH 3.5 and recording the peak current responses for each applied electrode potential. As shown in Fig. 1, the maximum response was

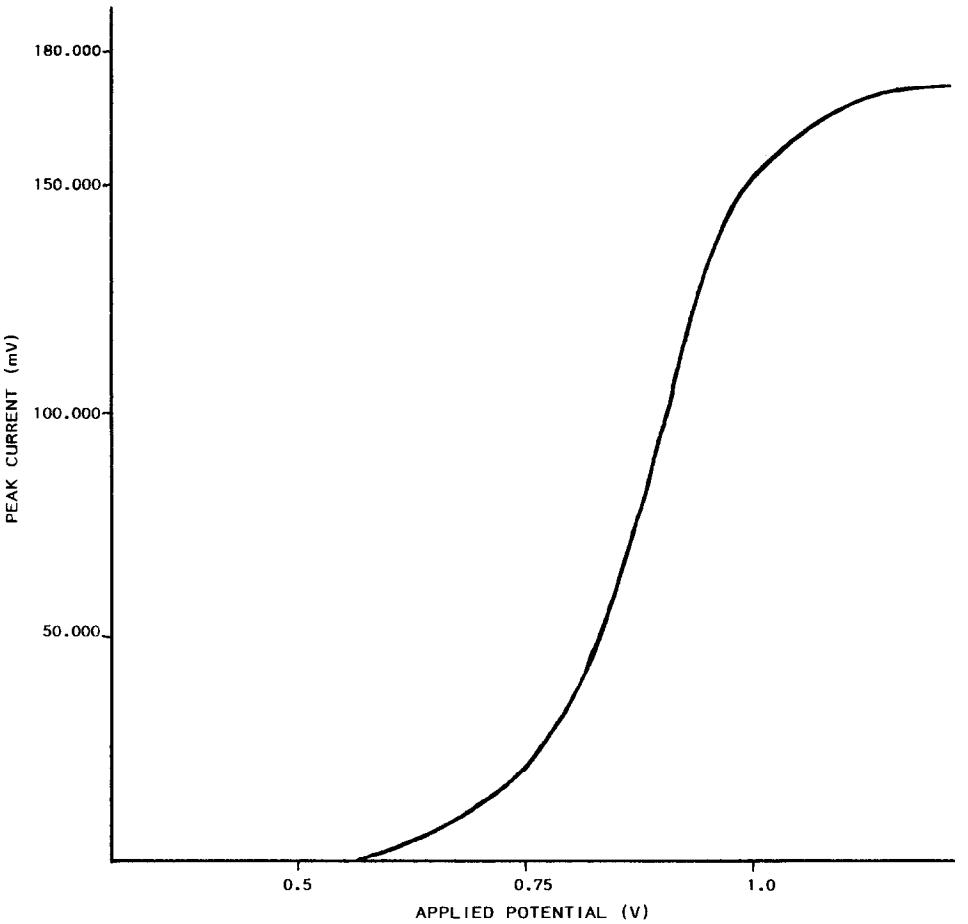


Fig. 1. Hydrodynamic voltammogram for DES under the described conditions: the peak area (mV) for *trans*-DES, as measured by the data processor, is plotted against the applied electrode potential.

reached at *ca.* 1.2 V, but 0.9 V was chosen as the working potential, because the signal-to-noise ratio was better at this lower potential and the response was still good.

The detection procedure shows a good reproducibility; however, some care must be taken. The sensitivity of the working electrode gradually declines, and we cleaned this electrode every day. Also it is essential to stabilize the electrode until the response is constant.

Estradiol, a natural estrogenic anabolic, and zeranol, a synthetic, non-steroidal anabolic, have similar retention times to DES under the condition of these experiments, and therefore their responses at the electrode potential of 0.9 V were also determined. Table I shows the relation between the injected amounts of DES, estradiol and zeranol and the corresponding responses under the same conditions.

The response for DES under these chromatographic conditions is much more sensitive than those for zeranol and estradiol, and the retention times are also slightly different, 6.6, 7.0 and 6.4 min for DES, estradiol and zeranol, respectively. To achieve better separation between these anabolics we tested some other columns: Radial Pak $C_{18}$, Nova Pak $C_{18}$ and Spherisorb ODS. However, there was no improvement so we continued to use the Nucleosil column.

Based on these results, the final conditions were selected as described in Experimental.

The linearity of the response, on the basis of peak area *versus* amount injected, was studies for five standard solutions in the range of 190–2000 pg DES injected. The regression line passed close to the origin, with a slight negative intercept: $y = 375.4x - 14.1$, where $x$ = ng DES injected and $y$ = obtained area (mV) by the data processor. The correlation coefficient was 0.9917, and the detection limit of 0.1–0.2 ng DES injected.

The mean recoveries of DES added to 10 ml of water in the range 0.5–2 μg/kg DES, amounted to 65.8% ($\sigma$ = 10.3%, $n$ = 15).

*trans*-DES is the biologically active form, and commercial preparations of DES contain principally this isomer. However, sample extraction and clean-up

TABLE I

RESPONSE OF VARIOUS ANABOLIC DRUGS UNDER THE DESCRIBED CHROMATOGRAPHIC CONDITIONS

| Anabolic drug | Injected amount (ng) | Retention time (min) | Measured peak area (mV) |
|---|---|---|---|
| DES | 0.40 | 6.6 | 85.000 |
| 17B-Estradiol | 0.48 | 7.0 | 40.500 |
| Zeranol | 0.36 | 6.4 | 3.300 |

produce a considerable amount of the *cis*-isomer, which usually is not considered. The *cis/trans* ratio may vary during and after the entire extraction and purification. Therefore, in each series of spiked samples, the recoveries were expressed as *trans*-DES corrected for the recovery percentage obtained for *trans*-DES in the corresponding standard solution extracted under the same conditions and the same day.

In GC–MS assays, a deuterium-labelled internal standard of DES can be added, which makes the correction for *cis–trans* isomerization much easier [6]. However, in the HPLC method, we cannot use this internal standard because of its identical chemical behaviour with DES. Therefore, in our assays, we related the recoveries in spiked samples with an external standard, extracted and purified under the same conditions as the spiked and unspiked samples. Recoveries of spiked muscle samples in the range 0.5–2.0 μg/kg DES are shown in Table II. Although the standard deviation is rather high, caused by uncontrolled *cis–trans* isomerization in both standard DES and spiked muscle during the extraction procedure, the mean recovery values for the four observed levels are *ca.* 65%. Typical chromatograms of (a) an unspiked muscle sample, (b) a spiked muscle sample (1.6 μg/kg) and (c) spiked water (1.6 μg/kg) are shown in Fig. 2. The unknown peaks with retention times of 2–4 min are caused by impurities and substances that were introduced into the extracts during the purification procedure, as directly injected DES standards do not yield these peaks.

The presence of DES in suspicious samples may be confirmed using the information provided by the hydrodynamic voltammogram, and applying different electrode potentials. The ratio of the responses of the peak current at 0.9 and 0.8 V must be the same for both the sample peak and the DES standard. Under our conditions this ratio was *ca.* 3; samples exhibiting other values, were discarded as being negative samples. Estradiol had a ratio value of *ca.* 13, and zeranol was nearly undetectable at these potentials.

The proposed method has a high sensitivity and may reach values as low as *ca.*

TABLE II

RECOVERIES OF DES FROM SPIKED BOVINE MUSCLE TISSUE

| Amount added (μg/kg) | Amount found (μg/kg) | Recovery[a] (%) |
|---|---|---|
| 0.5 | 0.34 | 68 |
| 1.0 | 0.67 | 67 |
| 1.5 | 0.95 | 63 |
| 2.0 | 1.32 | 66 |
| Mean = 65.8%; $n$ = 22; S.D. = 14.1% | | |

[a] $n \geq 5$.

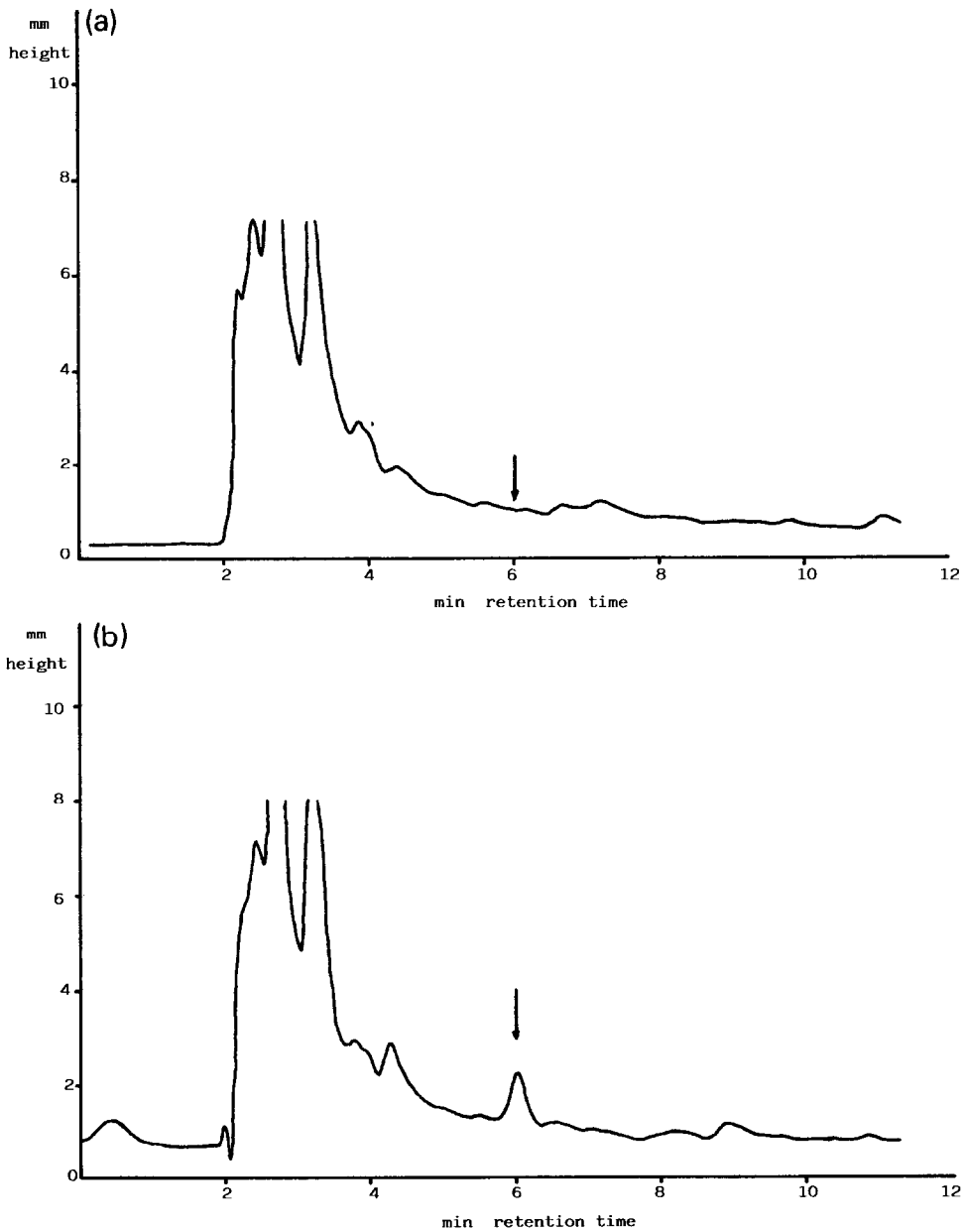


Fig. 2.

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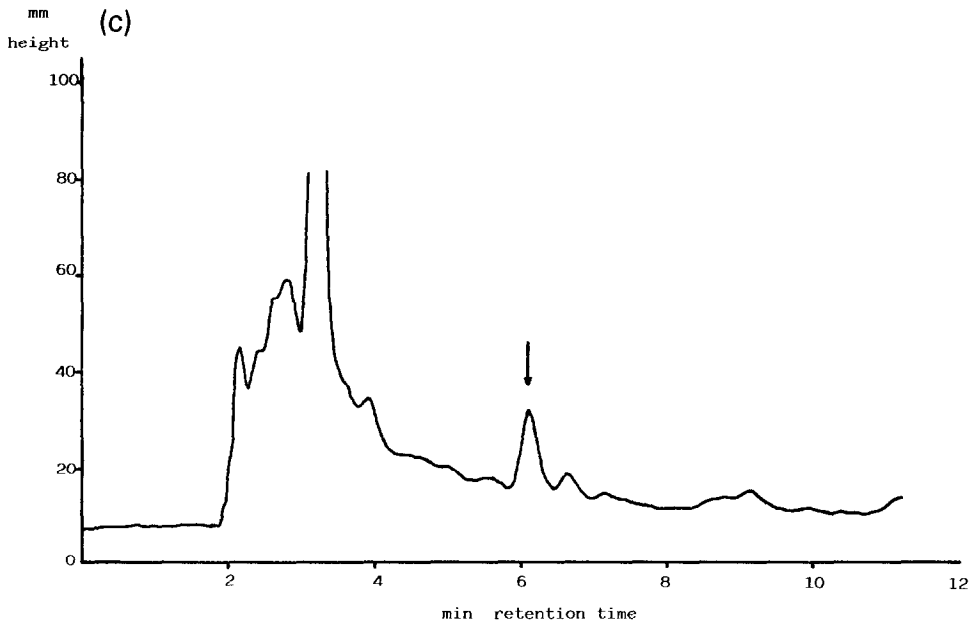


Fig. 2. Typical chromatograms of (a) an unspiked muscle tissue sample, (b) a spiked muscle tissue sample (1.6 μg/kg DES), and (c) spiked water (1.6 μg/kg DES).

0.1–0.2 μg/kg in samples in which a signal-to-noise of 2 or 3 permits the detection of very low amounts of DES.

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