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# Simple screening method for the fast determination of clenbuterol in animal feeds

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**Eine einfache Screening-Methode zur schnellen Bestimmung von Clenbuterol in tierischen Futtermitteln**

**Summary.** A simple method for the determination of clenbuterol is described. It is extracted from the sample at pH ~ 3 and then at pH ~ 11, followed by partitioning the analyte into water at pH ~ 3 and reextraction into ethyl acetate at pH ~ 9. Clenbuterol is oxidized with $KMnO_4$ to „clenbuteron" for GC-determination. Recoveries for 0.01 – 1.0 mg/kg were between 70% and 110% (standard deviation ±14%, $n = 18$).

## 1 Introduction

Recently the drug clenbuterol, 4-amino-α-[(tert-butylamino)methyl]-3,5-dichlorobenzyl alcohol, aroused public interest due to misuse in the field of calf fattening.

It was discussed that one way of application resulted from the addition of this drug to the feedstuffs.

Because our laboratory is also responsible for the official feedstuff quality control, it was therefore necessary to analyse this compound.

The necessity for the development of a fast screening method was the fact, that no methods for analysing clenbuterol in feedstuffs were available. On the other hand, methods to analyse clenbuterol in other matrix samples were available, however, with the affidavit for personal use only. Therefore a discussion on analytical methods was not possible.

The goal of this study was to develop a simple method to decide very quickly whether clenbuterol is or is not contained in a sample. An important step in this procedure is the oxidation of clenbuterol to "clenbuteron", which makes the GC-determination very easy.

## 2 Experimetal

### *2.1 Apparatus and reagents*

*Apparatus.* Mechanical shaker with time clock; Waring blender with Thyristor power regulator; centrifuge (~5000 rpm) equipped with 100 ml bottles, capable of accomodating 15 ml capacity graduated centrifuge tubes; rotary evaporator with vacuum controller; 5, 10 and 20 ml graduated quickfit test tubes; pasteur pipettes (l = 23 cm); minifunnels for filter paper (Macherey-Nagel, No. 615, $d = 7$ cm); 500 ml flasks with ISO thread GL 45 and screw cap (red) with gum septum covered with a teflon layer.

*Reagents.* Acetone for residue analysis; dichloromethane for residue analysis; ethyl acetate for residue analysis; petroleum ether for residue analysis; 5% $Na_2CO_3$ in water; 0.5% $KMnO_4$ in water; 0.1 and 0.2 mol/l $H_3PO_4$ in water; 10 mol/l KOH in water; NaCl p.a.; $Na_2SO_4$ p.a.

*Stock standard solution.* Weight 113 mg clenbuterol hydrochloride in 100ml water.

*Working and fortification standard solution.* Dilute stock solution with water to obtain concentrations for example of 1, 10, 100 and 1000 ng/ml.

### *2.2 Extraction procedure*

Weigh 40 g of a pulverized feedstuff into a high-speed blender jar and add 100 ml 0.2 mol/l $H_3PO_4$ and 100 ml acetone. Mix at low speed for 10 min and then for 1 min at high speed. An alternative technique is to mix the sample with the same solvents in a 500 ml flask over night on a mechanical shaker. Centrifuge and filter the acidic water-acetone extract. Pour an aliquot of 100 ml into a separate blender jar, add 50 ml acetone and, while slowly blending, 3 ml of 10 mol/l KOH, so that the pH is at about 11. Add 15 g NaCl and 80 ml of dichloromethane and extract on-line [1, 2] by blending at high speed for 1 min. Pour the organic extract into a 400 ml beaker and dry with solution sulphate. Take 90 ml of the organic phase (= 10 g of sample) and reduce to a volume of 1–2 ml. Add about 5 ml dichloromethane and reconcentrate. Repeat the evaporation with dichloromethane. Add about 0.5–1 g $Na_2SO_4$ to the residue. Dissolve the residue in about 1 ml of a mixture of dichloromethane and ethyl acetate (1 + 1) and transfer the sample extract into a minifunnel with filter paper ($d = 7$ cm) placed on the top of a 10 ml volumetric flask. Repeat this operation about 5 times. Rinse the filter to transfer the extract quantitatively into the 10 ml flask. Fill up with the same solvent mixture. Pipette an aliquot of 5 ml into a 20 ml graduated quickfit test tube and extract with 5 ml of 0.1 mol/l $H_3PO_4$ for 2 min to transfer clenbuterol into the aqueous phase. Centrifuge for 10 min at 5000 rpm. Pipette ~4.5 ml of the upper aqueous phase into a separate 20 ml test tube and wash twice with 5 ml petroleum ether, centrifuge for 30 s if necessary. Discard the upper petroleum ether phase. Wash with 5 ml of dichloromethane. Centrifuge for 5 min. Pipette 4 ml of the upper aqueous phase into a

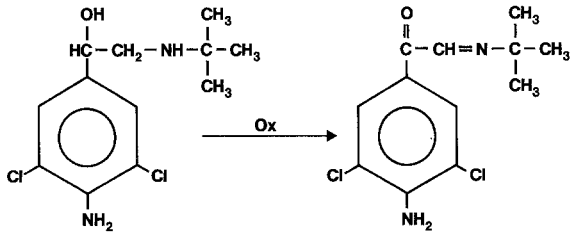


**Fig. 1.** Suggested reaction of clenbuterol to "clenbuteron" in the presence of $KMnO_4$

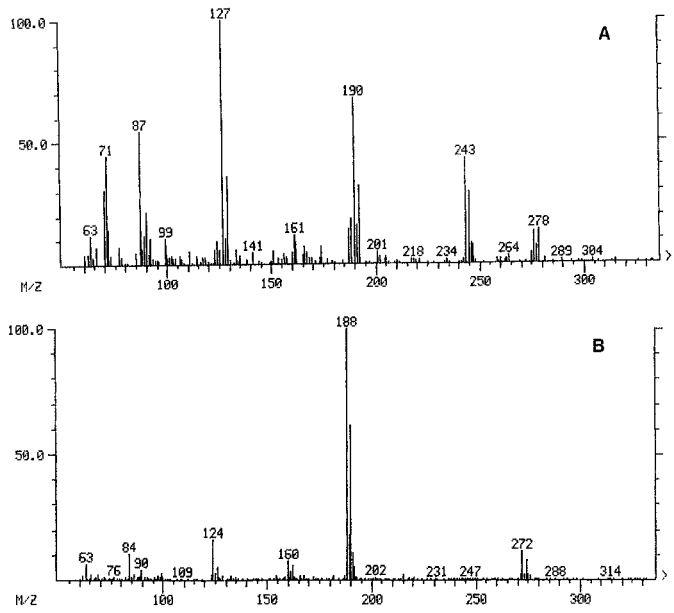


**Fig. 2A, B.** Mass spectrum of clenbuterol (**A**) and "clenbuteron" (**B**)

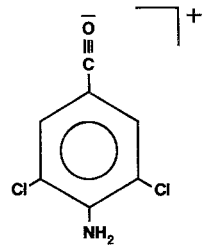

**Fig. 3.** Suggested structure of the base peak ion (m/e = 188) of "clenbuteron"

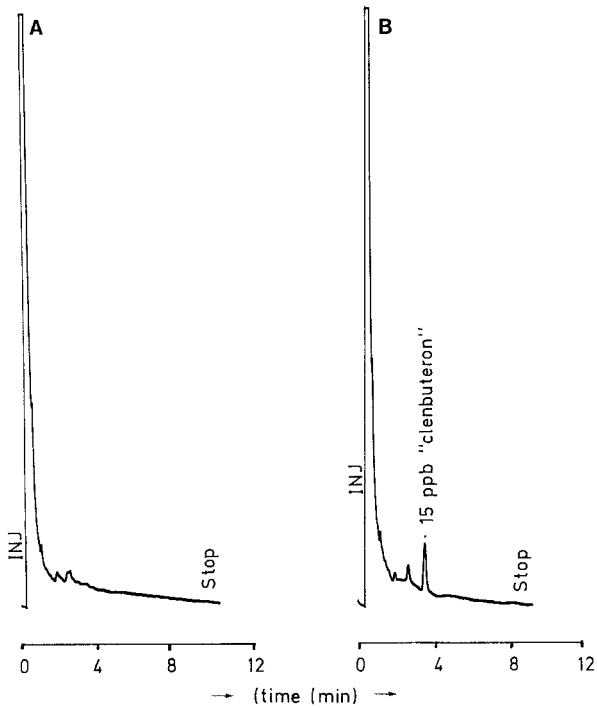


**Fig. 4A, B.** ECD-chromatogram of a sample fortified with 15 ppb clenbuterol before oxidation (**A**) and after oxidation (**B**)

separate 20 ml test tube. Add 2 ml of 5% $Na_2CO_3$, so that the pH of this solution is now at about 9. Add 4 ml of ethyl acetate to this solution and mix for 2 min. If, for example, 20 g sample are extracted, add 2 ml instead of 4 ml ethyl acetate. Now 1 g sample is in 1 ml of extract.

### *2.3 Oxidation of clenbuterol*

The oxidation of clenbuterol to "clenbuteron" in the sample extracts and standard solutions should be prepared at the same time.

*Preparation of "clenbuteron" in the sample extract.* Pipette about 2 ml of the clear upper ethyl acetate extract – derived from the procedure of chapter 2.2 – into a 5 ml test tube. Add 2 ml of 0.5% $KMnO_4$ solution and mix well for 2–3 min. Centrifuge for 2 min at 5000 rpm. Transfer the organic phase into a 5 ml test tube, add 1 ml 5% $Na_2CO_3$ and mix for 10 s to extract the $KMnO_4$ from the organic phase. Transfer the organic phase into a 2 ml flask. The solution is now ready for GC-determination. Store the solution in a refrigerator, because "clenbuteron" decomposes slowly at room temperature.

*Preparation of "clenbuteron" in the standard solution.* Pipette 4 ml of clenbuterol standard in water into a 20 ml test tube. Add 2 ml of 5% $Na_2CO_3$, so that the pH of this solution is now at about 9. Add 4 ml of ethyl acetate to this solution and mix for 2 min to transfer clenbuterol into the organic phase. Continue as described in chapter 2.3.

### *2.4 Gas chromatography*

"Clenbuteron" is determined by GC-ECD. The separation column is a 2 m × 2 mm glass column filled with 1.5% OV-17 + 2% OV-210 on Gas Chrom Q (80–100 mesh). The oven temperature is 200° C and the gas flow 25 ml/min.

## 3 Discussion

The suggested structure of the oxidation product "clenbuteron" is shown in Fig. 1. The mass spectra of clenbuterol and "clenbuteron" are shown in Fig. 2A and B, respectively. From Fig. 2B it is seen that the molecular ion of "clenbuteron" has a mass of m/e 272, which indicates that four hydrogen atoms are eliminated from clenbuterol with

the molecular ion of the mass of m/e 276 (see Fig. 2A) by the reaction with $KMnO_4$. It is also suggested that the base peak of "clenbuteron" with the mass of m/e 188 (see Fig. 2B) is contributed by the ion with the chemical structure shown in Fig. 3. To prove this ideas, further studies are necessary (e.g. NMR, IR). This, however, was not the original question of this study. Our main interest in this study was to prove, that "clenbuteron" is oxidatively produced each time reproducibly and in addition is very easily eluted from GC-columns as shown in Fig. 4B. Contrary to this, clenbuterol cannot be determined under these conditions (see Fig. 4A).

Recoveries from untreated control samples fortified with clenbuterol at levels of 0.01 to 1.0 mg/kg are between 70% and 110% and averaged 86% with an absolute standard deviation of $\pm 14\%$ ($n = 18$).

The blank values of "clenbuteron" in untreated control samples were always lower than 0.002 mg/kg, so that the only compound, which can be chromatographed after the addition of clenbuterol is "clenbuteron" as shown in Fig. 4. Because "clenbuteron" is produced reproducibly and no other oxidation product is yielded which is chromatographed under the given conditions, the presented method is suitable for the fast determination of clenbuterol in feedstuffs.

The calibration curve of "clenbuteron" is linear, if untreated control samples were spiked with clenbuterol in the range of $0.01 - 0.4$ mg/kg.

This studies are continued to improve the reported procedure and to extend this oxidation technique to similar compounds.

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