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New derivatization reactions in pharmaceutical analysis*

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Abstract: The application of chemical reactions in conjunction with spectrometric and chromatographic methods is exemplified by the formation of 2-nitrophenylhydrazide derivatives for the spectrometric assay of carboxylic acids; silylation and trifluoroacetylation of drugs for gas chromatography; selective reduction of steroid ketones for their IR spectrometric identification; the use of epoxidation in discriminating between saturated and unsaturated steroids in gas chromatography; increasing the selectivity and sensitivity of the spectrometric and gas chromatographic determination of isomeric Δ^4- and Δ^5-3-ethylenedioxy steroids by treatment with hydrochloric acid; and the use of the same reagent in the difference spectrometric determination of 2,5-dimethyl-α-ethyl benzhydrol.

Keywords: *Ultraviolet-visible spectrometry; difference spectrometry; infrared spectrometry; gas chromatography; carboxylic acids; ketosteroids; 3-ethylenedioxy steroids; pyridinol carbamate; α-ethyl benzhydrol derivatives.*

Introduction

In the early days of quantitative pharmaceutical analysis, chemical reactions were involved in almost all the methods; titrimetry, gravimetry, and colorimetry. Later, however, the role of chemical reactions decreased considerably. The reason for this was the spread of UV spectrometry and other spectroscopic techniques, as well as various chromatographic methods. Using these techniques, chemical reactions can be completely omitted (e.g. in spectroscopic methods, HPLC with UV or RI monitoring, TLC densitometry based on natural absorption or fluorescence), or the reaction takes place in the measuring cell or detector only (e.g. in voltammetric methods, HPLC with electrochemical detectors, gas chromatography with a flame ionization detector).

Examining papers published in the last 1–2 decades, it is clear that the role of derivatization reactions in stationary or flowing systems is again increasing. The reasons for this seem to be the following:

(a) Using preliminary chemical reactions the field of application of spectroscopic and chromatographic methods can be greatly expanded: spectrometrically inactive materials can be determined by UV-visible spectrophotometry and by HPLC with UV detection

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(example 1 in this paper): insufficiently volatile or stable materials can be analysed by gas chromatography (example 2).

(b) The application of derivatization reactions is sometimes a useful tool in the spectroscopic (example 3) or chromatographic (example 4) identification of unknown components.

(c) The main reason for the increasing use of derivatization reactions is certainly the continually increasing demand on pharmaceutical analysts for more selective and/or sensitive methods (examples 5 and 6).

Even an outline summary of the vast number of papers in this field is beyond the scope of this review: only the most important monographs dealing with derivatization reactions in chromatography [1–4] and spectroscopy [5–7] are listed.

Derivatization of Carboxyl Groups for the Spectrometric Assays

The majority of carboxylic acids (among them drugs and biologically important derivatives) are spectrometrically inactive or absorb only in the UV region. Several derivatization methods are available for their UV-visible spectrometric determination [8–10] or for HPLC analysis with spectrophotometric [11–13] or fluorimetric [14, 15] detection. The aim of this study has been to develop a general method for the transformation of carboxyl groups to carboxamide derivatives with chromophoric or fluorophoric properties suitable for spectrometric or HPLC determination.

The key reaction of the method is between the carboxylic acid and diethyl chlorophosphite leading to a mixed anhydride [16].

$$R{-}COOH + P(OC_2H_5)_2Cl = R{-}CO{-}O{-}P(OC_2H_5)_2 + HCl. \quad (1)$$

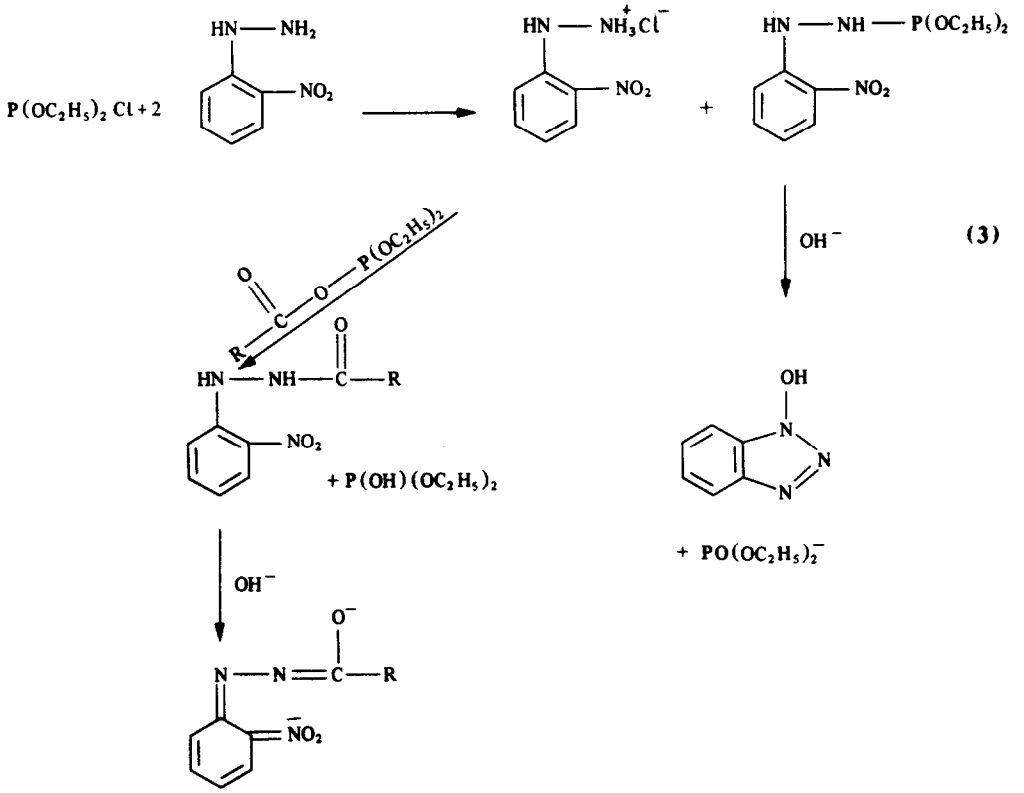
This reaction takes place very rapidly at room temperature using acetonitrile or a mixture of acetonitrile and tetrahydrofuran as solvent. Diethyl chlorophosphite should be used in large excess to avoid interference from water in the solvent (which should be under 0.05%) and to ensure complete reaction.

After completion of the reaction, any reactive and spectrophotometrically active primary or secondary amine or hydrazine can be added to form the corresponding carboxamide or hydrazide derivative:

$$R{-}CO{-}O{-}P(OC_2H_5)_2 + R'{-}NH_2 = R{-}CO{-}NH{-}R' + P(OH)(OC_2H_5)_2 \quad (2)$$

Reaction (2) is somewhat slower than reaction (1), but it can be completed at moderately elevated temperatures within 1–2 h, even with the least reactive derivatives.

At first aniline was used as the amine component in a slow, single-step process (8), the carboxanilide derivatives having a strong absorption band at 243 nm ($\epsilon \sim 14{,}500$). Here the use of 2-nitrophenylhydrazine as the amine component is described. This reagent was used by Légrádi for the detection of carboxylic acids [17] and anhydrides [18], and by Munson [19] for the quantitative determination of carboxylic anhydrides and chlorides. The use of 2-nitrophenylhydrazine as a colorimetric reagent for the determination of some water-soluble carboxylic acids was described by Horikawa and Tanimura [20]. The carboxylic acids were coupled with the reagent with the aid of water-soluble carbodiimides. The advantage of 2-nitrophenylhydrazine over aniline is that in alkaline medium (pH>12) the acyl derivatives are highly conjugated with absorption maxima around 550 nm, greatly increasing the selectivity of the method.



As is seen in reaction (3) the excess of diethyl chlorophosphite reacts with the 2-nitrophenylhydrazine reagent and the product decomposes upon addition of sodium hydroxide to form the colourless 1-hydroxy-1,2,3-benztriazol. Both reactions are almost instantaneous and as a result of this the reagent blank is negligibly small at 550 nm. By contrast isobutyl chloroformate, which is more often used to form mixed anhydrides, cannot be applied in this work because its reaction product with 2-nitrophenylhydrazine does not decompose in alkali: this would cause extremely high reagent blank absorption.

Several carboxylic acids have been determined by the proposed method. The general procedure is the following.

The sample, containing up to 5 μM of carboxylic acid, is dissolved in 250 μl of anhydrous acetonitrile or (in the case of bile acids) in a 7:3 v/v mixture of acetonitrile and tetrahydrofuran. A 50 μl portion of diethyl chlorophosphite (Aldrich) is added and the mixture is allowed to stand at room temperature in a well-closed vial for 15 min. A 250 μl portion of 2-nitrophenylhydrazine reagent is then added (0.04 M in acetonitrile) and the mixture is heated at 70°C for 30 min. The contents of the vial are then transferred to a 25 ml calibrated flask with the aid of about 15 ml of ethanol. A 5 ml portion of 0.5 M aqueous sodium hydroxide is added and the flask is made up to volume with ethanol. The absorbance is read against a reagent blank at the absorption maximum (ca. 550 nm). The absorbance is stable for several hours.

The results for some characteristic examples are summarized in Table 1.

The sensitivity and the precision of the method are fairly good. Work is under way to

Table 1
Spectral data of carboxylic acids after derivatization with 2-nitrophenylhydrazine

Acid	λ_{max} (nm)	Molar absorptivity*	Regression data† a	b
Acetic acid	550	6520±1.4%	0.008	1.072
Palmitic acid	550	6850±0.8%	0.004	0.270
Cholic acid	550	7280±1.3%	−0.001	0.187
Benzoic acid	560	8030±1.2%	0.006	0.795

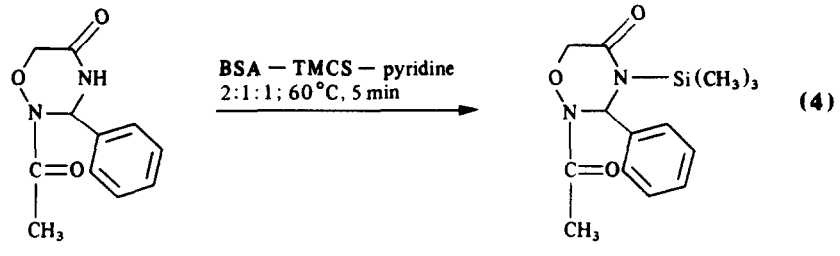
* Mean of eight determinations ± r.s.d.
† Regression equation: absorbance = $a + b \times$ concentration (mg/100 ml): $r > 0.9995$.

exploit the possibilities of this method for the determination of carboxylic acids in pharmaceutical and biological samples as well as in HPLC pre-column derivatization.

Classical Gas Chromatographic Derivatization Reactions for the Solution of New Problems

The following two examples demonstrate the usefulness of derivatization reactions in expanding the field of gas chromatography to those materials which are not volatile or stable enough to be analysed without derivatization. Two classical gas chromatographic derivatization reactions, trimethylsilylation and trifluoroacetylation, have been applied.

2-Acetyl-3-phenyl-tetrahydro-1,2,4-oxadiazin-5-one (RGH-4615), a potential anti-epileptic drug [21], was transformed to its trimethylsilyl derivative. The chromatogram can be seen in Fig. 1a.



RGH − 4615 (4)

The same reaction was not applicable to the determination of pyridinol carbamate when a method to complement the existing HPLC [22] and TLC-densitometric [23] procedures was attempted. It was found, however, that after trifluoroacetylation the determination can easily be carried out (Fig. 1b).

GC–MS revealed that the main peak in the chromatogram was the *N,N'*-bis-trifluoroacetyl derivative, while the small peak before it (the area of which does not exceed 1.5% of the total) was a by-product of the trifluoroacetylation, an asymmetrical *O,N*-bis-trifluoroacetyl derivative.

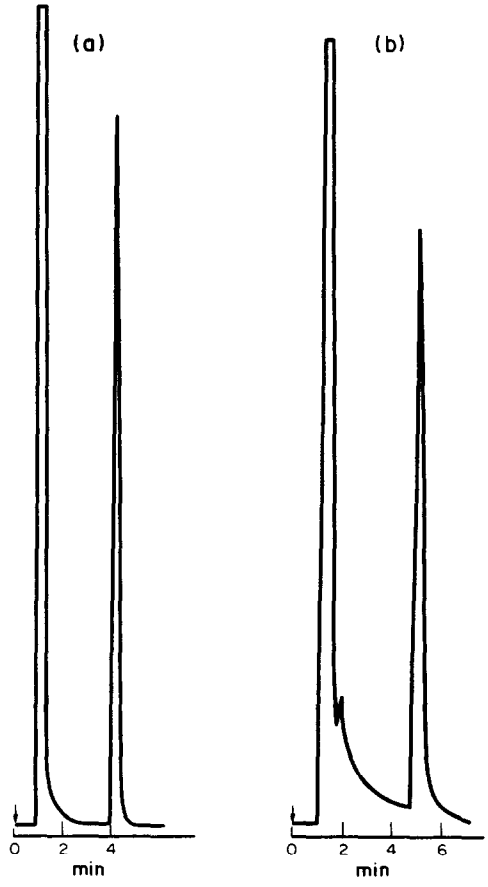
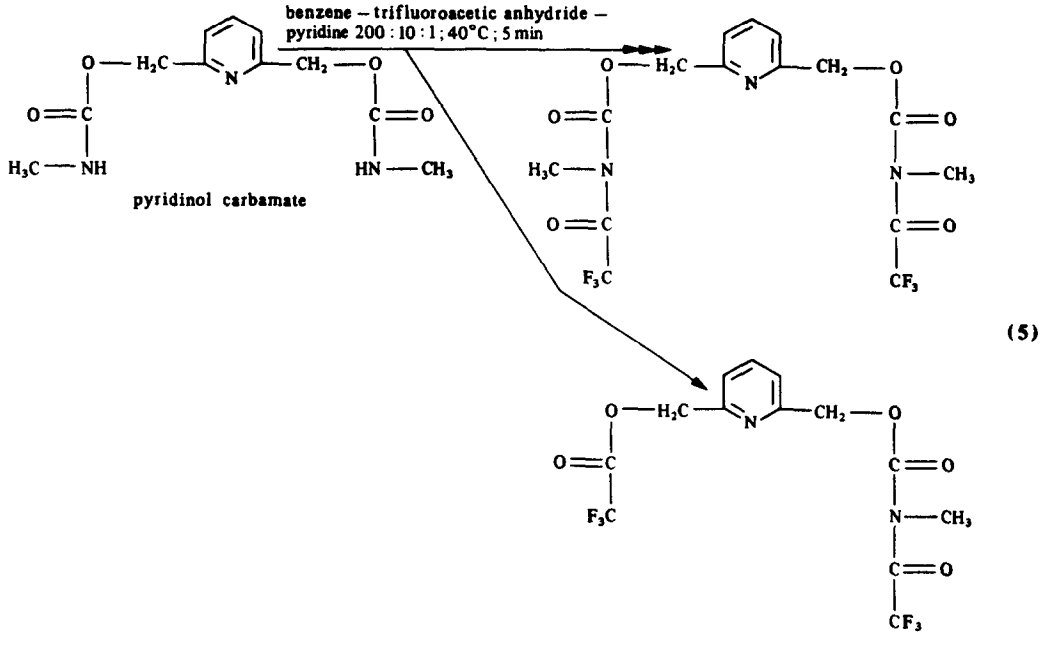


Figure 1
Gas chromatograms: 1.8 m × 2 mm glass column packed with 3% OV–101 on Gas Chrom Q: (a) 2-Acetyl-3-phenyl-tetrahydro-1,2,4-oxadiazine-5-one after trimethylsilylation (T = 230°C); (b) pyridinol carbamate after trifluoroacetylation (T = 210°C).



(5)

The Use of Calcium Borohydride Reduction in the IR Spectroscopic Identification of Ketosteroids

Although spectroscopic techniques are usually able to solve any structure elucidation problems occurring in pharmaceutical research and analysis, in some cases the application of chemical reactions prior to spectroscopy is very useful. An example is the application of the reduction of keto groups in steroids to their IR spectroscopic investigation.

The estimation of the C=O stretching vibration in the 1600–1800 cm^{-1} range is a useful tool for the identification of keto and ester groups, which are among the most important functional groups in steroids. In some cases, however, this spectral range is too crowded, overlapping bands causing difficulties in the identification of the corresponding carbonyl groups. For example in Fig. 2a, a part of the spectrum of prednisone acetate is shown. Only the conjugated 3-keto group is easily identifiable at 1660 cm^{-1} (accompanied by C=C bands at 1602 and 1627 cm^{-1}). The 11 and 20-keto groups and the 21-ester group give one broad overlapping band between 1690 and 1750 cm^{-1}.

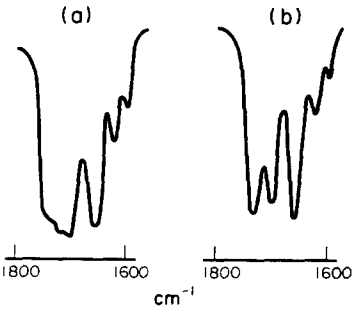
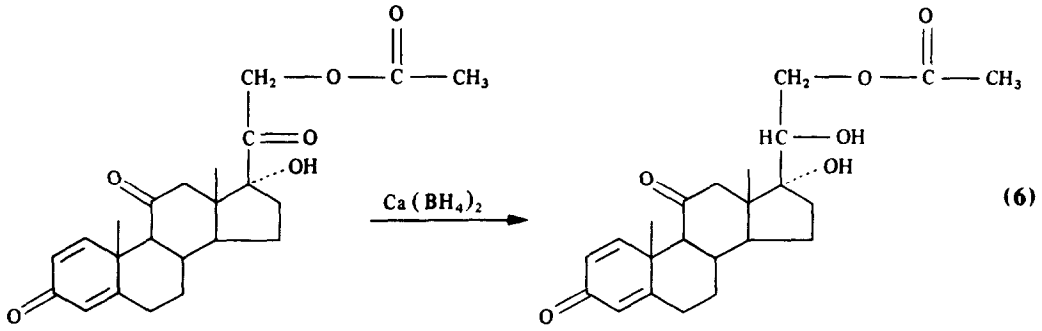


Figure 2
Infrared spectra of prednisone acetate in tetrahydrofuran; path length 0.2 mm: (a) the untreated solution; (b) after treatment with calcium borohydride.

Figure 2b shows the same spectral region after treating prednisone acetate with a large excess of calcium borohydride in tetrahydrofuran at 30°C for 60 min. Calcium borohydride was prepared *in situ* by adding equivalent quantities of sodium borohydride and calcium chloride to the solution of the steroid in tetrahydrofuran. After the reaction the unreacted reducing agent was filtered off and the spectrum recorded (tetrahydrofuran is sufficiently transparent in the range 1600–1800 cm^{-1}). From the spectrum it is



(6)

prednisone acetate

seen that the $\Delta^{1,4}$-3-keto group was not reduced, but the 20-keto group was reduced. As a consequence the bands of the 11-keto and 21-acetoxy groups at 1700 and 1733 cm^{-1} respectively were well resolved and easily identifiable. Similar results have been obtained with several other ketosteroids and ketosteroid esters. A similar technique (reduction in methanol with sodium borohydride) was introduced in the authors' laboratory for the identification and difference UV spectrometric determination of unsaturated ketosteroids [24, 25]. This method has been applied and further developed by several workers [26–30].

Identification of 3,5-Cyclo-androstan-17-one as the Impurity of 5α-Androst-2-en-17-one

In many cases, especially if reference materials are not available, derivatization reaction before chromatography greatly increases the diagnostic value of the chromatographic method in the detection and identification of impurities in drugs and intermediates. As an example the investigation of one of the intermediates of the synthesis of pipecuronium bromide, a new short acting neuromuscular blocking agent [31], is demonstrated. 5α-Androst-2-ene-17-one is prepared from 3β-hydroxy-5α-androstan-17-one mesylate by the acid-catalysed elimination of methanesulphonic acid. On the basis of analogous reactions the formation of the isomeric 3,5-cyclo-androstan-17-one as a by-product of the elimination reaction was highly probable. Using polar stationary phases such as Silar 10C (2% on Gas Chrom Q) an impurity was actually detected. Using a 1.8 m long column at 210°C the retention time of the main peak was 2.9 min while that of the impurity was 2.4 min.

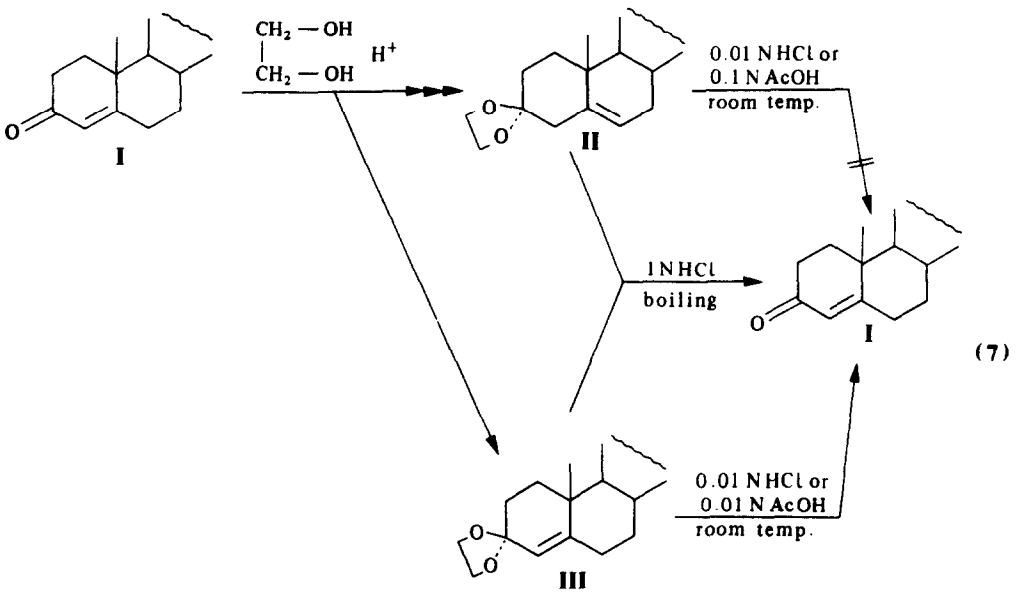
In order to prove that the impurity did not contain a double bond the sample was treated with 3-chloroperbenzoic acid in chloroform at room temperature for 30 min [32]. Under these conditions 5α-androst-2-ene-17-one was transformed to 2α,3α-epoxy-5α-androstane-17-one, its retention time being shifted to 11.5 min. The retention time of the impurity remained unchanged, further evidence that it was the 3,5-cyclo derivative.

Simultaneous Determination of Δ^4- and Δ^5-3-Ethylenedioxy Steroids

The simultaneous determination of the isomeric Δ^4- and Δ^5-3-ethylenedioxide steroids (formed upon protecting the Δ^4-3-keto group by acid-catalysed ketalization) was based on a selective hydrolytic reaction. Under very mild conditions (treatment at room temperature with 0.01 N hydrochloric acid or 0.1 N acetic acid) the Δ^4-isomer was quantitatively transformed to the Δ^4-3-keto derivative while the Δ^5-isomer remained unchanged.

Subsequent gas chromatographic separation was thus easy. For example the retention time of 17α-ethynyl-17-hydroxy-4-androsten-3-one (I), formed from 3-ethylenedioxy-17α-ethynyl-4-androsten-17-ol (II) by hydrolysis with 0.1 N acetic acid in a 3:2 v/v mixture of tetrahydrofuran and ethanol for 30 min, was 6.0 min. The unchanged 3-ethylenedioxy-17α-ethynyl-5-androsten-17-ol (II) was eluted after 8.7 min, thus enabling their simultaneous determination on a 1.8 m × 2 mm column packed with 3% SP-2100 on Supelcoport; T = 230°C.

A highly selective UV spectrometric method has also been based on the same reaction. The unreacted I, present as a contaminant in the ethylenedioxy derivative, can be determined using its UV absorption at 240 nm. After treatment of the sample with 0.01 N hydrochloric acid in an 8:2 v/v mixture of ethanol and water at room temperature for



(7)

30 min the sum of I and III can be determined at the same wavelength using the same molar absorptivity value, while after total hydrolysis (boiling with 1 N hydrochloric acid for 5 min) the sum of I, II and III can be measured. The agreement between the results obtained by the two methods is excellent.

Difference Spectrometric Determination of 2,5-Dimethyl-α-ethyl Benzhydrol

This example demonstrates that the selectivity of a spectrometric assay can be further increased by the application of the difference technique. The cholagogue drug, 2,5-dimethyl-α-ethyl benzhydrol [33] (RGH-3395), is a weak UV absorber. Its UV spectrophotometric determination [34], based on dehydration with hydrochloric acid to form the styrene-like 1-(2,5-dimethylphenyl)-1-phenyl-1-propene (λ_{max} 247 nm, ϵ = 13,970), is fairly sensitive and selective. In the determination of this material in complex matrices (e.g. animal feedstuffs) the selectivity is not sufficient; background absorption interferes with the assay method (Fig. 3). To avoid this interference, a difference spectrometric method was introduced.

The feedstuff was extracted with ethanol. A 20 ml sample of the extract containing about 5 mg of 2,5-dimethyl-α-ethyl-benzhydrol was treated with 3 ml of 10 N hydrochloric acid at room temperature for 3 h. These reaction conditions, milder than the original ones [34], minimized the effect of hydrochloric acid on the background absorbance. After a 20-fold dilution with ethanol the absorbance at 247 nm was determined: the blank contained the same constituents at the same concentrations, the only difference being that hydrochloric acid was added to the diluted solution before the measurement. As can be seen in Fig. 3c and d, the difference between the spectra is negligible indicating that the background could be cancelled out by the difference technique.

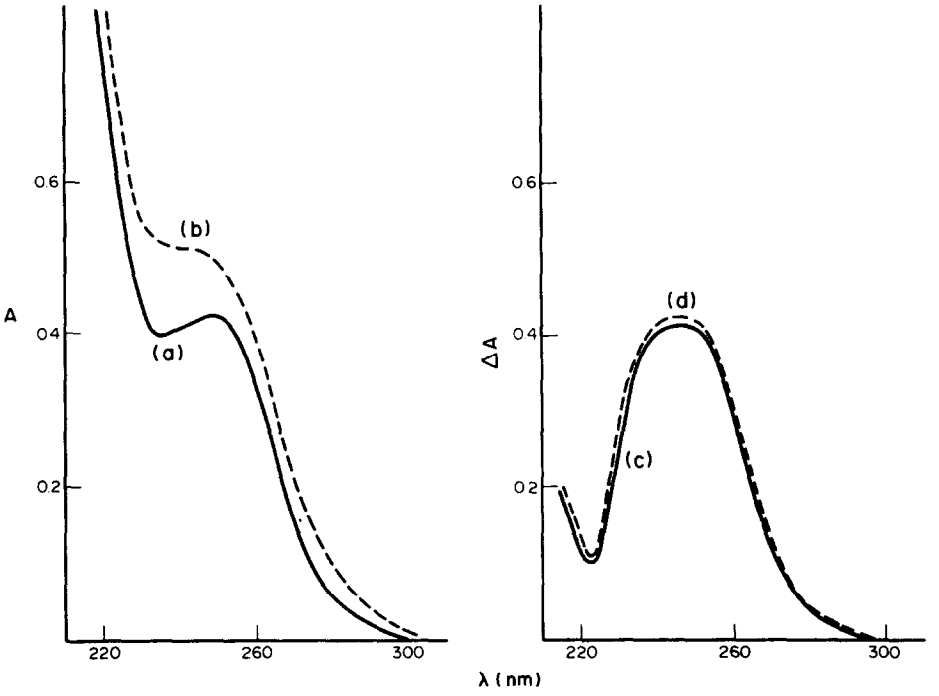


Figure 3
Ultraviolet spectra of 2,5-dimethyl-α-ethyl-benzhydrol and an animal feedstuff containing it (concentration 7.40 mg/l): (a) the active ingredient after treatment with hydrochloric acid; (b) the extract of the feedstuff after the same treatment; (c) difference spectrum of the active ingredient; (d) difference spectrum of the extract.

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