

Analysis of Acetoin and Diacetyl in Bacterial Culture Supernatants by Gas-Liquid Chromatography

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The acetoin and diacetyl contents of culture supernatants of Voges-Proskauer-positive "viridans" streptococci, *Klebsiella pneumoniae* and *Staphylococcus aureus*, were determined by a gas liquid chromatographic procedure, in which supernatants were extracted with diethyl ether and diacetyl was measured on columns of 10% (wt/wt) polyethylene glycol 400 (PEG 400) at 73 C. Acetoin was converted to diacetyl, before analysis, by a simple oxidation procedure with ferric chloride and without a distillation step. Streptococcal culture supernatants were shown by this method to contain only acetoin; supernatants of *K. pneumoniae* and *S. aureus* contained both acetoin and diacetyl.

The Voges-Proskauer (VP) test (1) indicates the presence of diacetyl or acetoin in bacterial cultures and for many years has proved useful in diagnostic bacteriology. Recently, more sophisticated techniques for detection of diacetyl or acetoin have been published (2, 3).

A sensitive method of measuring diacetyl by gas-liquid chromatography (GLC), in the presence of alcohols, was described by Doelle (3), using diethyl ether and petroleum ether extracts, injected on to a column of 30% (wt/wt) polyethylene glycol 400 (PEG 400) on a celite 545 (60/80 mesh) support, and a column temperature of 70 C.

Acetoin cannot be satisfactorily extracted from an aqueous solution with diethyl ether. Acetoin's α -diketone derivative, butane-2, 3-dione (diacetyl), however, has a much higher partition coefficient between ether and water, and can be analyzed by GLC of an ether extract. The possibility of bringing about a quantitative conversion of acetoin, in the culture supernatant, to diacetyl was therefore investigated.

Rigby (5) described a method using bismuth oxide (Bi_2O_3), which is reduced to the metal as it oxidises acetoin to diacetyl. However, Rigby states that the oxidation should be carried out in glacial acetic acid. Although it would not be impossible to evaporate the supernatant to dryness before the reaction, this method would be time consuming and tedious, and could not be used in conjunction with procedures for measuring other fermentation products from the same aliquot of supernatant. Also the risk of loss during evaporation is too great when dealing with the very small quantities of acetoin present in bacterial culture supernatants.

Brenner et al. (2) used a colorimetric method for the measurement of diacetyl, and suggested that any acetoin could be converted to diacetyl using a distillation procedure before its measurement. During the distillation step, acetoin was oxidized to diacetyl by ferric chloride.

The present work describes a highly sensitive GLC method which is complementary to the method of Drucker (4), and which permits the detection of diacetyl in the presence of acetoin, alcohol or fermentation acids in bacterial culture supernatants, and includes a very simple step for the oxidation of acetoin to diacetyl.

MATERIALS AND METHODS

Organisms. Thirty-eight VP-positive organisms were used in this study, consisting of 36 strains of streptococci, one of *Klebsiella pneumoniae*, and one of *Staphylococcus aureus*. All the organisms had been isolated from various pathological lesions during routine laboratory investigations in the Department of Bacteriology, Manchester Royal Infirmary.

Preparation of culture supernatants for GLC analysis. Cultures were grown in 100-ml volumes of Oxoid brain heart infusion broth at 37 C for 48 h in 8-ounce (ca. 240 ml) 'medical flats' bottles, and cells were removed by centrifugation at $7,500 \times g$ for 20 min at 4 C. Culture supernatants were stored at -20 C until required, to reduce loss of volatile fermentation products. All strains were grown and analyzed in triplicate.

Extraction of diacetyl. Any diacetyl present in a 10-ml aliquot of culture supernatant was extracted into 1 volume of diethyl ether containing 0.2% (vol/vol) ethyl acetate by shaking for five 30-s periods during 30 min. The extraction was carried out in a wide-necked, screw-capped, 1-ounce (ca. 30 ml) glass (universal) container. The ethyl acetate was used as an internal standard.

Treatment of acetoin. Before GLC analysis, ace-

toin had to be oxidized by ferric chloride to diacetyl, the latter having a higher partition coefficient between diethyl ether and water. It was possible to avoid a distillation procedure (2) if ferric chloride (as the hexahydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) had been added to the culture supernatants and oxidation carried out in sealed vessels placed in a boiling water bath for a period of time. The reaction was carried out in new universal containers which form an adequate seal. Tests had shown that there was up to 10% loss of the diacetyl formed if old bottles were used, presumably due to slight scratches which allowed leakage of the volatile product. Preliminary investigations had been performed to determine the optimum concentration of ferric chloride and the optimum reaction time using aliquots of 0.4% (wt/vol) acetoin solutions made up in brain heart infusion broth. The diacetyl formed was extracted with diethyl ether containing ethyl acetate and analyzed by GLC. The optimum concentration of ferric chloride appeared to be 12% wt/vol and the optimum time for oxidation to be 6 min (Fig. 1 and 2). It was confirmed experimentally that 100% conversion of acetoin to diacetyl occurred under these conditions, although quantitative extraction of the diacetyl formed was not possible; hence, the use of the internal standard.

GLC analysis. Two-microliter injections were made on to a 5-foot (ca. 152.40 cm) glass column (6-mm outer diameter) packed with PEG 400 on a 100 to 120 mesh diatomite CAW (Pye Unicam), with a 5- μl Hamilton syringe. Separation was achieved at 73 C using a N_2 carrier gas flow rate of 40 ml/min. A flame ionization detector (H_2 , 40 ml/min; air, 600 ml/min) was used with a Pye Unicam Series 104 chromatograph. The peaks were recorded on a Smiths Servoscribe chart recorder; extracts were run in duplicate.

Application of method to samples. Ten-milliliter aliquots of culture supernatants were extracted with ether/ethyl acetate as described. This extract contained any diacetyl, alcohols, or fermentation acids present in the culture supernatant. After removal of the ethereal phase, the remaining broth which contained any acetoin produced by the organisms was

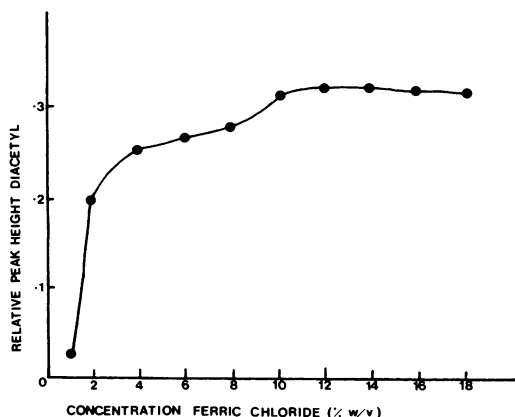


FIG. 1. Variation in diacetyl production with concentration of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ showing optimum concentration to be 12% (wt/vol).

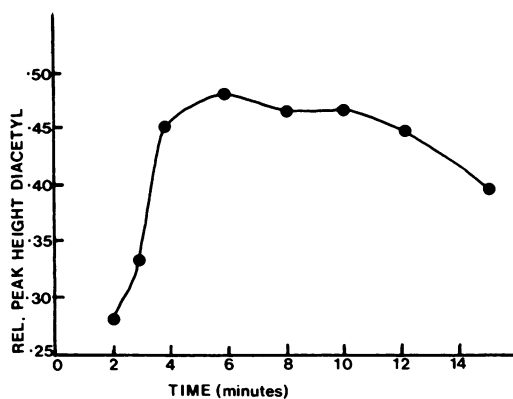


FIG. 2. Variation in diacetyl production with time of oxidation by $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ showing optimum oxidation time of 6 min.

heated after addition of ferric chloride. The treated broth was cooled to room temperature in a cold water bath and the diacetyl produced was extracted with ether/ethyl acetate by shaking as before. Fermentation acids, which are not readily eluted from the column under the conditions of use, were removed from the ether extracts before injection by shaking gently for 2 min with approximately 0.5 g of analytical reagent calcium carbonate, which had been shown to have no effect on diacetyl. The fermentation acids were removed from the diethyl ether as their ether insoluble calcium salts. Preliminary studies, using sodium hydroxide to remove acids, had shown that this resulted in destruction of diacetyl, presumably by a condensation reaction.

Standard solutions of acetoin and diacetyl were prepared and analyzed in the same way as the samples.

Semiquantitative VP test. The VP test (1) was carried out on 3 ml of culture supernatants in bijoux bottles. Equal volumes of 40% (wt/vol) potassium hydroxide and 5% (wt/vol) alcoholic α -naphthol were added. The mixture was shaken after addition of the reagents, after 20 min, and again after 40 min. The intensity of color in the bottles was compared and graded as strong, intermediate, or weak.

RESULTS

Standard curves. The relationship between the concentration of diacetyl (and acetoin measured after conversion to diacetyl), and the response (expressed as the peak height of diacetyl relative to that of ethyl acetate), was linear over the range 0.001 to 0.1% (wt/vol) diacetyl and acetoin. A typical chromatogram is shown in Fig. 3.

Diacetyl production. Diacetyl was not found in the culture supernatants of the streptococci studied before treatment. *K. pneumoniae* and *S. aureus* culture supernatants were found to be relatively low, 0.0023% (wt/vol) and 0.002% (wt/vol), respectively. The concentration of acetoin in the streptococcal supernatants ranged

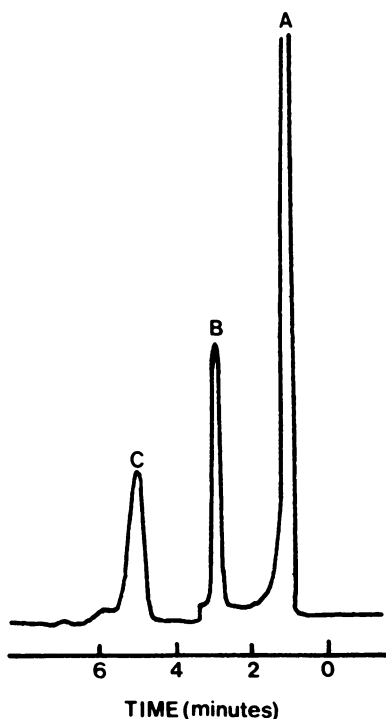


FIG. 3. Gas chromatographic separation of 0.05% (wt/vol) diacetyl (C) extracted with ether (A) containing 0.2% (vol/vol) ethyl acetate (B). Attenuation A and B, $\times 10^3$, C, $\times 10^5$. Column of PEG 400, 73 C, flow rate carrier gas, 40 ml/min.

from minimal amounts (less than 0.001% wt/vol) to 0.0312% (wt/vol); the average value was 0.008% (wt/vol).

Correlation with the VP test. Color intensity was graded as strong, intermediate, or weak 40 min after adding the reagents. A strong VP was given by supernatants containing 0.03% (wt/vol) to 0.006% (wt/vol) acetoin, intermediate color from 0.006% (wt/vol) to 0.004% (wt/vol), and weak color from 0.004% (wt/vol) to 0.001% (wt/vol). Below the latter value the VP reaction was negative or very weak. *K. pneumoniae* and *S. aureus* both gave strong color reactions, due to diacetyl rather than acetoin having been produced.

DISCUSSION

This technique provides a relatively simple but sensitive method for measuring diacetyl

together with acetoin in bacterial culture supernatants.

It is important in many cases to distinguish between acetoin and diacetyl, since Speckman and Collins (6) have shown that they can be produced by different metabolic pathways. Previous methods of differential measurement have involved separation of the two by steam distillation before estimation (7), or conversion of acetoin to diacetyl, with measurement of the total diacetyl before and after treatment (2). The former method requires specialized apparatus, the latter involves the possibility of loss of the original volatile diacetyl during conversion of acetoin. The present method requires no specialized apparatus additional to that required for GLC, and since the original diacetyl is removed before conversion is carried out, there is no risk of its loss.

Measurement of diacetyl by GLC on PEG 400 after ether extraction showed comparable sensitivity with existing methods. Doelle (3), using 30% PEG 400 as a packing material to separate C₁-C₇ acids and alcohols, noted that diacetyl had a similar retention volume of ethanol. Using only 10% PEG 400 under the present conditions (vide supra), diacetyl was well separated from ethanol. The relative retention times compared with ethyl acetate were 1.8 and 2.2, respectively.

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