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

S. M. LEE and D. B. DRUCKER*
Department of Bacteriology, Medical School, Manchester, M13 9PT England
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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 (Bi₂O₃), which is reduced to the metal as it oxidizes 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 x 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 be-
tween diethyl ether and water. It was possible to
avoid a distillation procedure (2) if ferric chloride (as
the hexahydrate, FeCl₃·6H₂O) 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 concen-
tration of ferric chloride and the optimum reaction
time using aliquots of 0.4% (wt/vol) acetoine 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 acetoine to diacetyl occurred
under these conditions, although quantitative ex-
traction 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 diamonite CAW (Pye Unicam), with a 5-µl
Hamilton syringe. Separation was achieved at 73 C
using a N₂ carrier gas flow rate of 40 ml/min. A
flame ionization detector (H₂, 40 ml/min; air, 600
ml/min) was used with a Pye Unicam Series 104
chromatograph. The peaks were recorded on a
Smiths Servocribe 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 con-
tained any diacetyl, alcohols, or fermentation acids
present in the culture supernatant. After removal of
the ethereal phase, the remaining broth which con-
tained any acetoin produced by the organisms was
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. Fer-
mentation acids, which are not readily eluted from
the column under the conditions of use, were re-
moved 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 fer-
m entation acids were removed from the diethyl
ether as their ether insoluble calcium salts. Prelimi-
nary 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 sam-
ple.s.

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 re-
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 ace-
toin in the streptococcal supernatants ranged
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 C$_3$-C$_7$ 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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