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Note

Improved method for assay of formic acid by gas–liquid chromatography

KENNETH S. BRICKNELL and SYDNEY M. FINEGOLD

Infectious Disease Section, Wadsworth Hospital Center, Veterans Administration, Los Angeles, Calif. 90073, and the Department of Medicine, U.C.L.A. Medical Center, Los Angeles, Calif. 90024 (U.S.A.)

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Formic acid is one of a number of metabolic end-products produced by bacterial cultures. Determination of formic acid is necessary for the conclusive identification and classification of certain micro-organisms, particularly anaerobes. Current methods are far from efficient. The usual procedure used in the microbiology laboratory involves diethylether extraction of a portion of the culture broth. Recovery of formic acid by this method is poor[1,2]. The extract is analyzed by gas–liquid chromatography (GLC). Flame ionization detectors (FID) show no response to formic acid, so a thermal conductivity detector (TCD) is used. The TCD has a low sensitivity; in our experience it does not detect formic acid if the extract concentration is less than 5 μmole/ml. This low sensitivity, coupled with the low extraction efficiency, leaves much to be desired. Other disadvantages are that formic acid retention time varies depending upon the column condition and the concentration of formic acid; and that formic acid produces a poorly defined peak.

The purpose of this paper is to present a simple and sensitive method for the determination of formic acid as dimethylformamide. It involves a two-step reaction: (i) methylation of the formic acid and (ii) conversion of the ester into dimethylformamide (DMF).

Moore and Brown[3] reported the application of this method to the analysis of insect defense mechanisms. Extracts of the insects were treated with ethereal diazomethane and then with dimethylamine (DMA) to form DMF. In our bacterial culture analysis by GLC[4], a portion of the broth supernatant is routinely methylated with boron trifluoride–methanol reagent, followed by chloroform extraction for the determination of non-volatile fatty acids (lactic, succinic, pyruvic, etc.). Thus the first step of the reaction, *i.e.*, methylation, has already been accomplished and the diazomethane treatment used by Moore and Brown is not needed. A few microliters of the chloroform extract are treated with DMA and the mixture injected directly into the GLC instrument. Minimal detectable level on a Resoflex column connected to a TCD is 2 μmole/ml formic acid.

MATERIALS

All reagents used are analytical grade. Formic acid and chloroform were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.), boron trifluoride–methanol

14% (w/v) from Applied Science Labs. (State College, Pa., U.S.A.), N,N-dimethylformamide and dimethylamine from Eastman Organics (Rochester, N.Y., U.S.A.). Anhydrous dimethylamine (Eastman Organics Catalog No. 601) was diluted in chloroform to a concentration of 40%. The anhydrous form has a boiling point of 7.4° and the vial must be cooled in ice water before opening (see Discussion).

Reaction vials were PTFE screw-capped mini-aktors (Applied Science Labs.). Micro-syringes of 10 and 50 μl capacity were obtained from Hamilton (Reno, Nev., U.S.A.). The gas chromatograph used was a Varian Model 1420, dual-column TCD with a 6 ft. × 1/8 in. O.D. stainless-steel column packed with Resoflex (Burrell Corporation, Pittsburgh, Pa., U.S.A.). The carrier gas was helium, flow-rate 60 ml/min. Detector filament current was 200 mA, attenuation 1×. The recorder was a Varian Model A-25 at 1 mV full scale deflection.

EXPERIMENTAL

The sample was methylated as outlined in the Wadsworth Anaerobic Bacteriology Laboratory Manual[4]. Culture broth supernate (1.0 ml) was mixed with 1.0 ml of boron trifluoride–methanol reagent and left overnight at room temperature in a stoppered tube.

The mixture was extracted with 0.2 ml chloroform. A 30-μl aliquot of the extract was withdrawn, using a 50-μl capacity micro-syringe, and placed into a reaction vial. Three microlitres of DMA were added, then the tube was capped, and briefly mixed.

Upon completion of the reaction, the vial was chilled in ice water and centrifuged briefly. (It was observed that some of the reaction mixture vaporizes and condenses on the inside surface of the reaction vial. Considering the very small volumes involved, cooling and centrifuging ensures a homogeneous sample).

The syringe was flushed several times with the sample by filling and emptying it within the vial. A 10.0-μl aliquot was then injected onto the GLC column (in order to keep the DMF peak "on-scale", it may be necessary to lower the recorder baseline temporarily by adjusting the GLC balance control).

A range of standards of formic acid containing from 1.0 to 10.0 μmole/ml (0.1 to 1.0 mequiv./100 ml) was processed concurrently with the culture samples and a standard curve plotted. Values for the unknown samples were read from the standard curve.

Ratio of sample to DMA

To determine the optimum ratio of sample to DMA a set of tubes containing 20 μl of methyl formate (original formic acid concentration before methylation was 5 μmole/ml) was set up and 1, 2, 3, 4, 5, and 6 μl of DMA reagent added.

Effect of DMA reaction time

Following methylation and extraction as outlined above, 100-μl aliquots of the chloroform extract were placed in the reaction vial and 10 μl of DMA reagent added. Samples were assayed at 0, 1, 2, 4, 6 and 24 h to determine the effects of the reaction time.

Comparison of methylating reagents

Another factor examined was the difference in methylation efficiency using boron trifluoride–methanol *versus* the sulfuric acid–methanol method.

Use of the FID

We also evaluated a column packed with 6% FFAP on Porapak Q in a gas chromatograph fitted with FID using the operating parameters listed in the Wadsworth Anaerobic Bacteriology Manual[4]. The use of the FID with its increased sensitivity might permit a smaller injection volume and also lower the minimum detectable level of formic acid as DMF.

RESULTS

Ratio of sample to DMA

The resultant DMF peaks obtained from this experiment showed no significant differences (Fig. 1) and the ratio of 20-μl sample to 2-μl DMA, or multiples thereof, was adopted as standard procedure.

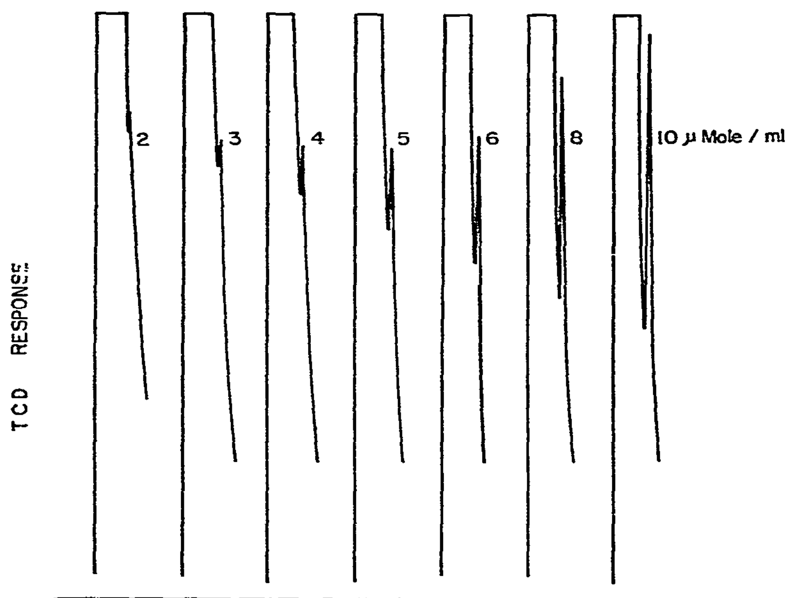

Fig. 1. GLC trace of DMF peak from formic acid at the concentrations shown.

Effect of DMA reaction time

Fig. 2 illustrates the effect of reaction time of sample/DMA; we have adopted the 2-h reaction time for our procedure. The discrepancy of the 5.0 μmole/ml sample is due to solvent evaporation from the vial causing concentration of the DMF; there was insufficient material to do the 24-h assay.

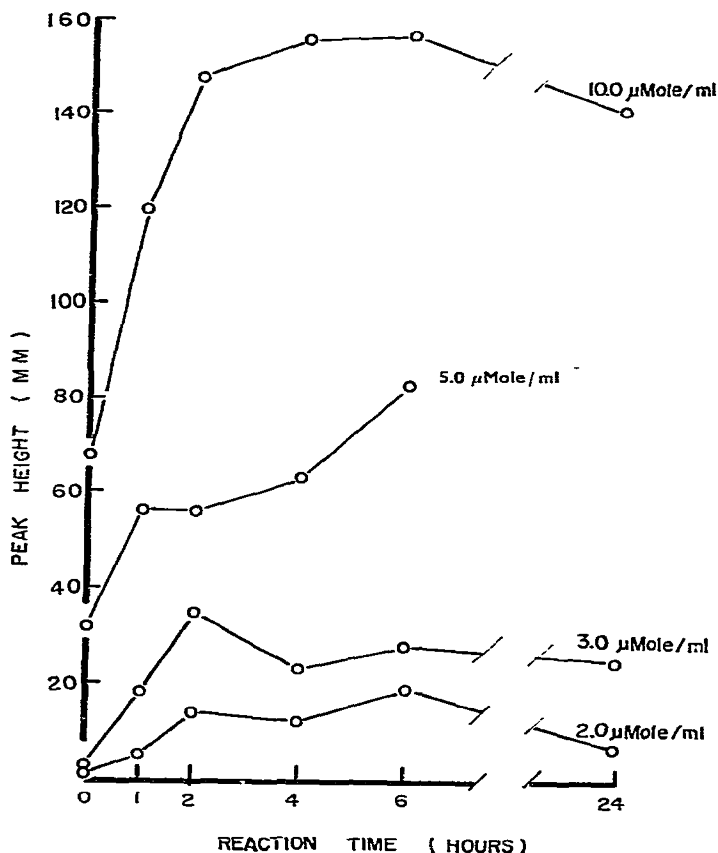

Fig. 2. Effect of reaction time for methylformate/DMA on peak height.

Comparison of methylating reagents

Comparison of the boron trifluoride–methanol reagent with sulfuric acid–methanol for methylation, using a duplicate range of aqueous standards of formic acid, showed the boron trifluoride–methanol reagent to be preferable. The resulting DMA peak using this reagent was 25% greater, confirming the more efficient methylation previously reported[5].

Use of the FID

Using the FFAP–Porapak column with the FID, DMF had a retention time of 4 min. The chloroform tended to tail, however, the DMF peak was sufficiently separated from the chloroform peak to permit use of this system. A 1.0-µl injection of the reaction mixture gave a minimum detectable level of 0.5 µmole/ml of formic acid as DMF.

DISCUSSION

In previous studies in this laboratory[6] we observed that with the combined methylation and chloroform extraction procedure, recovery of methyl ester accounts

for 50–60% of the original formic acid. The methyl formate–DMA reaction as described is *ca.* 45% complete. Even with these apparent low efficiencies the new DMF procedure allows a minimal detectable level of 2 μmole of original formic acid when assayed with a TCD.

Interference from other components in the sample is not a problem. Short-chain volatile fatty acids (C_1–C_6) are also esterified during the methylation step and their esters emerge in the solvent peak. The one component having a close retention time to DMF on the Resoflex column is "free" acetic acid. It is well known that methylation of short-chain volatile fatty acids is not 100% complete. Should the original sample concentration of acetic acid be high, the possibility of the non-methylated portion appearing on the trace is possible. However, this can be verified on the trace of the methylated sample as it is assayed for lactic, succinic, pyruvic, etc.

Due to the low boiling temperature of anhydrous DMA, caution should be used in handling it. The vial of DMA and the chloroform should both be chilled in ice water prior to opening the vial and mixing with the chloroform. The solution is stored in the refrigerator. An aqueous solution of DMA is available commercially and can be used instead of the chloroform solution. The aqueous solution was used for our preliminary experiments, but we prefer the chloroform solution to avoid any possibility of a "water peak" appearing on the GLC trace using the TCD.

Confirmation of the presence or absence of formic acid as a metabolic end-product in cultures of anaerobes is essential if positive identification of the micro-organisms is to be made. All previous methods for formic acid assay in liquid culture media have had such a low sensitivity that its presence in small quantities may easily have been missed. We are presently using the described method routinely in the laboratory, in conjunction with other criteria, for the identification and classification of micro-organisms.

The method we have described will provide a much more sensitive and reliable assay for micro-organism identification. Resolution of the DMF peak is superior and its retention time is more consistent when compared with the older solvent extraction assay method for "free" formic acid which produces a poor peak shape and variable retention time. The method could be adapted to assay other biological fluids or systems.

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