IDENTIFICATION
OF
ACID AND ALCOHOL PRODUCTS
OF ANAEROBIC BACTERIA
BY
GAS LIQUID CHROMATOGRAPHY

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IDENTIFICATION OF ACID AND ALCOHOL PRODUCTS OF ANAEROBIC BACTERIA BY GAS LIQUID CHROMATOGRAPHY

I. IDENTIFICATION OF ALCOHOLS AND VOLATILE ACIDS

This procedure allows the identification of a number of alcohols and volatile acids which are soluble in ether (e.g., acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, heptanoic, and caprylic acids), but succinic, pyruvic, and lactic acids are not detected. These non-volatile acids are identified by the examination of methyl derivatives, Section II.

A. Cultures

1. Inoculate tubes of properly reduced Peptone-Yeast Extract (PY) medium and Peptone-Yeast Extract Glucose (PYG) medium with a few drops (0.05 – 0.10 ml) of 24- to 48-hour liquid cultures.

2. Loosen caps and incubate under anaerobic conditions for 48 hours or until adequate growth is obtained.

B. Ether Extracts

1. Acidify cultures to pH 2 or below (0.1 – 0.2 ml of 50 percent v/v aqueous H_2SO_4).

2. Transfer 4 ml of acidified culture to a 15 x 125 mm screwcap tube. (Save remainder of culture for methylation procedure if necessary.)

3. Add 1 ml ethyl ether, tighten cap, and mix by inverting tube gently about 20 times.

4. Centrifuge briefly in a clinical centrifuge (1500-2000 RPM) to break the ether-culture emulsion.

5. Place the ether-culture mixture in a freezer (minus 10°C or lower) and leave until the aqueous portion (bottom) is frozen. Pour off the ether layer into a small (13 x 100 mm) screwcap tube, add anhydrous Mg SO_4 to equal about one-half the volume of ether extract, tighten the cap, and let stand at least 10 minutes to allow removal of water from extract. (Extracts not chromatographed on the day of preparation can be held in a freezer to prevent evaporation of the ether.)

6. Inject about 14 microliters (mμ) into the column.

7. Identify alcohols and volatile acids by comparing elution times of products in extracts with those of known alcohols and acids chromatographed on the same day.

C. **Chromatograph and Operating Conditions**

1.	Chromatograph	Beckman Model GC-2A
	Type of detector	Thermal conductivity
	Type of column	6-foot x 1/4 inch stainless steel
	Packing material	Resoflex LAC-1-R-296 standard concentration (P), Burrell Corporation, Fifth Avenue, Pittsburgh, Pa.
	Operating conditions	No attenuation, 200 milliamps, oven temperature 108°C, and helium carrier gas at about 120 cc/min or 32 lbs pressure
	Recorder	Beckman 10-inch laboratory potentiometric recorder

D. **Standard Solutions**

1. Acid Standard. To 100 ml distilled water add:

acetic	0.057 ml
propionic	0.075 ml
isobutyric	0.092 ml
butyric	0.091 ml
isovaleric	0.127 ml
valeric	0.125 ml
isocaproic	0.126 ml
caproic	0.126 ml
heptanoic	0.126 ml

These quantities represent approximately 1 meq of each acid depending on the purity of the reagent. To use, acidify, extract 4 ml, and proceed as for culture.

2. Alcohol Standard. To 100 ml distilled water add:

ethanol	0.1 ml	(ca 1.7 mM)
propanol	0.035 ml	(ca 0.5 mM)
isobutanol	0.005 ml	(ca 0.05 mM)
butanol	0.01 ml	(ca 0.1 mM)
isoamylol	0.005 ml	(ca 0.05 mM)
amylol	0.005 ml	(ca 0.05 mM)

To use, acidify, extract 4 ml, and proceed as for culture.

E. **Approximate elution time, in minutes for products:**

ether 0.2	isobutyric acid 8.5
ethanol 0.5*	butyric acid 11.4
propanol 0.8	isovaleric acid 13.6
butanol 1.2	valeric acid 18.7

isoamylol 1.6	isocaproic acid 26.0
amylol 2.0	caproic acid 30.2
hexanol 2.7	heptanoic acid 40.0
acetic acid 5.0	caprylic acid 45.0**
propionic acid 7.5	

If no acids are detected or if only acetic acid is detected, analyze 1 ml of the original acidified culture for succinic, lactic, and pyruvic acids by the methylation procedure described in the following section.

* Sharp peaks representing the alcohols may be superimposed on ether and on the long, low water peak starting at about 1.1 minutes.

** It is not necessary to wait for caprylic acid before injecting the next sample unless valeric, isocaproic, heptanoic, or caproic acids are present.

II. IDENTIFICATION OF METHYL DERIVATIVES OF PYRUVIC, LACTIC, AND SUCCINIC ACIDS

A. Preparation of methyl derivatives

1. Transfer 1 ml of acidified culture to a 13 x 100 screwcap tube.

2. Add 1 ml of boron trifluoride methanol (14% v/v) and let stand at room temperature for at least 4 hours (preferably overnight) or heat in a water bath (100°C for 5 minutes or 70°C for 30 minutes).

3. Add 0.5 ml of chloroform and mix by inverting 20 times.

4. Remove the chloroform layer (bottom) to a 13 x 100 mm screwcap tube and tighten cap.

5. Inject 14 μl into the column. After testing about 15 methylated samples, recondition the column by injecting 14 μl of methanol.

B. Chromatograph and Operating Conditions

Same as employed for ether extracts, Section I.

C. Preparation of Standards

Quantities of acids required for 100 ml of standard solution

Concentration of Acid	**0.5 meq.**	**2 meq.**	**10 meq.**
lactic acid (85%)	0.042 ml	0.17 ml	0.847 ml
succinic acid	0.03 g	0.12 g	0.6 g
pyruvic acid	0.034 ml	0.135 ml	0.676 ml

Dilute each to 100 ml with distilled water. To use, acidify, methylate, and extract as for culture, Section I.

D. **Approximate elution time, in minutes, for products:**

methyl-pyruvate	2.5
methyl-lactate	3.3
methyl-succinate	12.6

III. **MEDIA**

A. **Peptone-yeast extract (PY) medium**

Peptone	20.0 g
Yeast extract	10.0 g
Salts solution*	40.0 ml
Cysteine HC1	0.5 g
Resazurin solution**	4.0 ml
Distilled water	1000.0 ml

Adjust to pH 7.2, tube in 10 ml quantities in 15 x 125 mm screwcap tubes, and autoclave at 121° C for 15 minutes.

B. **Peptone-yeast extract glucose (PYG) Medium**

Peptone	20.0 g
Yeast extract	10.0 g
Salts solution*	40.0 ml
Cysteine HC1	0.5 g
Glucose	10.0 g
Resazurin**	4.0 ml
Distilled water	1000.0 ml

Adjust to pH 7.2, tube, and autoclave as described for PY medium.

*Salts solution:

$CaC1_2$ (anhydrous)	0.2 g
$MgSO_4$	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
NaC1	2.0 g

Mix $CaC1_2$ and $MgSO_4$ in 300 ml distilled water until dissolved. Add 500 ml water and, while swirling slowly, add remaining salts. Continue swirling until all salts are dissolved. Add 200 ml distilled water, mix, and store at 4° C.

** Resazurin solution:

Dissolve one resazurin tablet (ca. 11 mg Allied Chemical Cat. #506) in 44 ml distilled water.