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Analysis of Tartaric Acid in Fermentation Broth

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Leu,

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1979

ANALYSIS OF TARTARIC ACID IN FERMENTATION BROTH

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of the Requirements for the Degree

Master of Science

by

Lih-Jen Sun Leu

August, 1979

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ANALYSIS OF TARTARIC ACID IN FERMENTATION BROTH

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Dean of the Graduate College

ANALYSIS OF TARTARIC ACID IN FERMENTATION BROTH

Lih-Jen Sun Leu August, 1979 41 pages

Directed by: Larry P. Elliott, M. R. Houston, and L. B. Lockwood

Department of Biology Western Kentucky University

Tartaric acid accumulation in fermentation broth of Gluconobacter suboxydans was investigated qualitatively with paper chromatography and thin-layer chromatography. Comparisons were made using six different solvent systems and four different chemical indicators commonly used in chromatography.

Attempts were made to establish a routine method for quantitative analysis of tartaric acid. Spectrophotometry was used to detect possible metabolites resulting from glucose oxidation by G. suboxydans in different analytical separations. A combined method of ion-exchange column chromatography and paper chromatography followed by ammonium meta-vanadate spectrophotometric analysis differentiated 5-oxogluconic acid and tartaric acid and thus gave better quantitative results in the determination of tartaric acid from fermentation broths.

ACKNOWLEDGMENTS

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Graditude is also extended to Western Kentucky University for offering the educational opportunity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

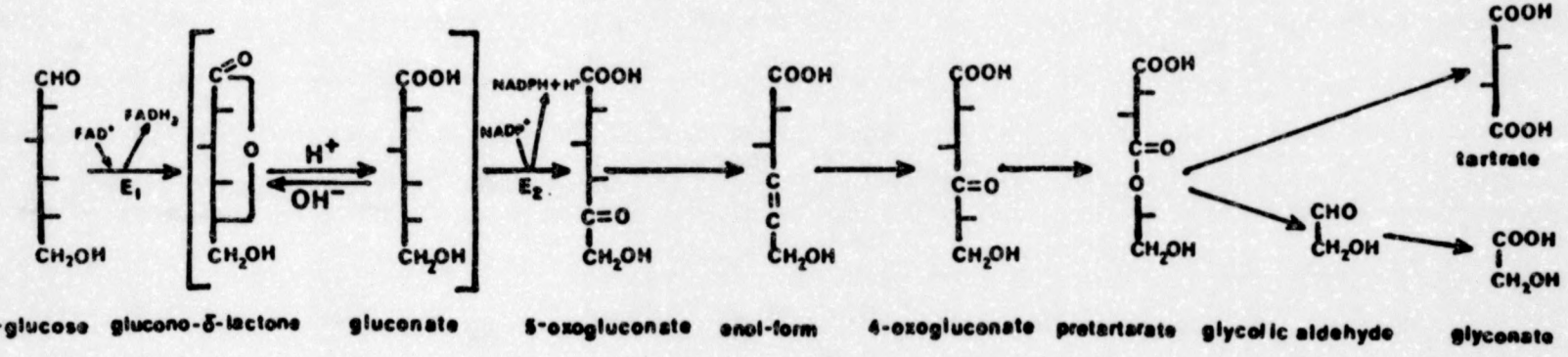
The feasibility of microbial tartaric acid fermentation has been studied for many years. Stubbs et al. (17) reported that Acetobacter suboxydans (Gluconobacter suboxydans) grown on 10% glucose at 25°C produced 89.5% 5-oxogluconic acid by 33 hours. Wood and Schwerdt (23) demonstrated by using paper chromatography that glucose was converted by G. suboxydans to an oxogluconic acid, but they did not prove whether the oxogluconic acid and 2-oxogluconic acid or 5-oxogluconic acid was produced.

Lockwood and Nelson (14) reported that 5-oxogluconic acid formed by G. suboxydans from glucose was the main intermediate which was further degraded to L-(+)-tartaric acid by Pseudomonas fluorescens. Yamada et al. (24) corroborated their work and also detected five different acids produced from glucose by G. suboxydans. Four of the five acids were purified and identified as 5-oxogluconic acid, 2-oxogluconic acid, L-(+)-tartaric acid and glycolic acid.

Kotera et al. (11) studied the mechanism of glucose oxidation to tartaric acid by G. suboxydans using chromatography. They proposed a hypothetical pathway from glucose to tartaric acid through 5-oxogluconic acid and 1,2-dihydroxyethyl hydrogen L-(+)-tartaric acid (renamed "pretartaric acid") (Figure 1).

The microbial production of tartaric acid has been under study in Western Kentucky University's laboratory for several

Figure 1. Suggested metabolic pathway from glucose to tartaric acid utilized by Gluconobacter suboxydans.



E_1= GLUCOSE OXIDASE E_2= 5-OXOGLUCONATE REDUCTASE

years. Ware (22) studied the feasibility of tartaric acid production from glucose in a broth initially inoculated with G. suboxydans followed by inoculation with P. fluorescens (renamed P. reptilovora). Daniel (4) and Dibble (5) studied the feasibility of using fungi to synthesize tartaric acid. Marootkhanian (15) demonstrated the constitutive nature of 5-ketogluconate reductase (D-glucose:NAD(P^+), oxidoreductase, 1.1.1.69) from G. suboxydans; its optimal pH in cell-free extracts was found to be 9.5. Leu (12) demonstrated a 60% yield in 5-oxogluconic acid by G. suboxydans grown in 3% glucose-yeast extract broth at a pH of 6.8.

Tartaric acid is one of the most widely distributed plant acids and has a number of food and industrial uses. Currently, all of the tartaric acid used in this country is imported and is produced mainly as a by-product from the tartar of the wine industry; thus, the amount of tartaric acid produced is limited. Development of a domestic source of tartaric acid by microbial fermentation would be beneficial to the United States. To facilitate this goal a routine method for the determination of tartaric acid in fermentation broth would be beneficial for future research in industrial tartaric acid production.

The two objectives of this research were (1) to demonstrate qualitatively tartaric acid accumulation in fermentation broth of G. suboxydans and (2) to establish a routine method for the quantitative determination of tartaric acid produced by microorganisms during fermentation.

MATERIALS AND METHODS

Chemicals

Commercial grade glucose monohydrate was obtained from CPC International Inc., Englewood Cliffs, NJ. D-gluconic acid (sodium salt), glunoco-δ-lactone, 2-oxogluconic acid (2-keto-D-gluconic acid, hemicalcium salt), 5-oxogluconic acid (5-keto-D-gluconic acid, potassium salts), glycolic aldehyde (glycoaldehyde) and Dowex - 1 (1 x 1 - 100) (dry mesh 50-100, chloride form) were purchased from Sigma Chemical Company, St. Louis, MO. Tartaric acid, ferrous sulfite, and hydrochloric acid were purchased from General Chemical Division, Allied Chemical, Morristown, NJ. Fumaric acid, citric acid, pyrogallol, sodium hydroxide, dextrose, n-butanol, isopropyl ether, formic acid, ethanol, bromophenol blue, acetic anhydride, triphynolphosphine, acetone and dimethyl yellow were purchased from Matheson Coleman and Bell Co., Inc., Rutherford, NJ. Malic acid, sulfuric acid, tannic acid, acetic acid, ammonium hydroxide, hydroxylamine hydrochloride, ammonium meta-vanadate, and pyridine were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Hydrogen peroxide and ethyl acetate were purchased from Fisher Scientific Co., Fair Lawn, NJ. Potassium thiocyanate, resorcinol, phenol, methanol, ferric chloride, silver nitrate, and Amberlite (IR-120 H C. P.) were purchased from Mallinckrodt, Inc., St. Louis, MO. Yeast extract was purchased from Difco Laboratories, Detroit, MI. Whatman #1

chromatography paper (25 cm x 25 cm x 0.01 mm) was purchased from W&R Balston, LTD, England. Uniplate, (20 cm x 20 cm, pre-coated with cellulose, 250 μm in thickness) was purchased from Analtech, Inc., Newark, DE.

Equipment

Glass jars, 18 cm x 26 cm, were used for developing the paper chromatograms. Glass tanks, 30 cm x 20 cm x 20 cm, were used for thin-layer chromatograms. A Bausch & Lomb Precision Spectrophotometer (Model 505) Rochester, NY was used to determine absorbance in the pyridine-acetic anhydride method. A Cary 14 (Varian Instrument Division, Palo Alto, CA) was used to determine the absorbancy peaks in the pyridine-acetic anhydride and ammonium meta-vanadate methods. Graduated burettes (59 cm x 0.6 cm) containing 21 cm of Amberlite or 18 cm of Dowex were used for ion-exchange chromatography. A standard curve was obtained by using a Bausch & Lomb Spectronic 20 to determine the absorbancy of tartaric acid at different concentrations after reaction with ammonium meta-vanadate.

Source of Gluconobacter suboxydans and its Maintenance

A lyophilized culture of Gluconobacter oxydans subsp. suboxydans NRRL B-755 was obtained from the Northern Regional Research Laboratory of the U. S. Department of Agriculture, Peoria, IL. The bacteria were activated in either 10% mannitol (w/v) or glucose (w/v) with 0.3% yeast extract (w/v) broth on a rotary shaker at 28°C for 36 to 48 hours. The

activated bacteria were transferred to stock slants composed of 10% mannitol or 10% glucose, 0.3% yeast extract and 2% agar (w/v), incubated for 2 - 4 days at 28°C and then stored at 4°C until used. The stock cultures were transferred every two months to insure viability.

Methods for the Qualitative Identification of Tartaric Acid

1. Ekkert's Method (8)

Tartaric acid (0.1 g) or 1 ml of 1M tartaric acid solution was mixed with 0.02 g of pyrogallol in a 20 ml test tube. Five milliliters of concentrated sulfuric acid were then added and the solution heated in a water bath at 95 - 100°C for 5 to 10 min. A violet color indicated the presence of tartaric acid. When β-naphthol was substituted for pyrogallol, a blue-green color developed.

2. Fenton's Method (9)

Ferrous sulfate (0.1 g) was added to 5 ml of a 0.5M tartaric acid solution. Then several drops of 30% hydrogen peroxide (v/v) and an excess of alkali (1 ml of 1 N NaOH) were added. A violet color denoted a positive test.

3. Tagliavini's Method (18).

One milliliter of a 1M tartaric acid solution was heated with 0.1 mg lead tetroxide and filtered (Whatman No. 1); 1 - 2 mg of potassium thiocyanate were added to the filtrate and the solution boiled for 10 min. The appearance of a black color (due to lead sulfide) indicated the presence of tartaric acid in the solution.

4. Deniges' Method (6)

Tartaric acid (crystal form, 10 mg or less) was added to 1 ml of 2% resorcinol solution; 0.5 ml concentrated sulfuric acid was then added to the solution. A red color indicated the presence of tartaric acid. If an oxidizing substance were present in the solution being analyzed, it would have to be initially reduced with zinc and hydrochloric acid.

5. Eegriwe's Method (7)

One milliliter of 1 N tartaric acid was heated with 5 ml of 95% sulfuric acid containing β, β-binaphthol. A green fluorescent color indicated the presence of tartaric acid.

Pyridine-Acetic Anhydride Method for Tartaric Acid Determination (16)

A 0.1 ml sample (containing from 25 to 200 µg of unknown metabolites or known standards) and 1.3 ml of pyridine were added to a test tube and the contents swirled briskly. Acetic anhydride (5.7 ml) was added; the solution was mixed by swirling and immediately placed in a constant temperature water bath (about 20°C). Color development was completed after 30 min. and was stable for an additional 30 min. The absorbancy was determined by either a Bausch & Lomb Precision Spectrophotometer or a Cary 14 recording spectrophotometer scanning from 200 nm to 2000 nm.

Chromatographic Solvents

The solvents used for separation in both paper and thin-layer chromatography were as follows: n-butanol/acetic acid/

water (4/1/1, v/v) (Solvent BAW) (11); aqueous 90% isopropyl ether/acetic acid (9/6, v/v) (Solvent 1A) (11); ethyl acetate/acetic acid/formic acid/water (18/3/1/4, v/v) (Solvent EAFW) (11); n-propynol/ammonium hydroxide/water (6/3/1, v/v) (Solvent PAW) (10); ethanol/ammonium hydroxide/water (20/1/4, v/v) (Solvent EAW) (2); saturated phenol in water (Solvent PW) (3).

Chromatographic Spray Reagents

1. Dimethyl yellow and Bromophenol blue (DMY/BPB) (10)

Dimethyl yellow (25 mg) and bromophenol blue (75 mg) were dissolved in 200 ml of 96% ethanol and the solution adjusted to pH 7.0 by the addition of 0.1 N sodium hydroxide (approximately 1.75 ml). After spraying the chromatogram with this solution, the acids appeared as yellow to pink spots on a bluish background. This method detected approximately 10 - 20 µg of acid.

2. Hydroxylamine-ferric chloride (1)

Chromatograms were sprayed with a freshly prepared solution of alkaline hydroxylamine prepared by mixing equal volumes of N-methanolic hydroxylamine hydrochloride and 1.1 N-methanolic potassium hydroxide. After drying in air for about 10 min., the chromatogram was sprayed with an aqueous solution containing 1 - 2% ferric chloride (w/v) and 1% hydrochloric acid (w/v).

3. Ammonium Meta-vanadate (AMV) (2)

A saturated aqueous solution of ammonium meta-vanadate was prepared by dissolving excess AMV (about 6 to 10 grams per 100 ml) into deionized distilled water. The solution was

heated to 60°C and allowed to stand for 24 hr before being filtered through Whatman No. 1 filter paper.

4. Ammoniacal Silver Nitrate (ASN) (19)

Chromatograms were passed rapidly through an ASN solution prepared by diluting 0.1 mg of a saturated aqueous solution of silver nitrate with 20 ml acetone. The low solubility of silver nitrate in acetone caused precipitation. Water was added dropwise while shaking until the silver nitrate re-dissolved. After drying, the chromatograms were sprayed with a 0.5 N aqueous ethanol solution of sodium hydroxide (2 g NaOH in 100 ml ethanol). Brown silver oxide was immediately produced. Reducing sugars formed dense black spots. Excess silver nitrate was removed from the chromatograms by immersion in 6 N ammonium hydroxide followed by washing the paper for 1 hr in running tap water and drying in an oven (approximately 100°C). Black or dark brown spots on a white background were obtained.

Ion-Exchange Chromatography (21)

A sample of fermentation broth containing about 0.15 meq of total acid was passed through the 7 cm x 0.6 cm cation exchange column of Amberlite IR-120 H C. P. The Amberlite was converted to the acid form by passage of 15 ml of 1 N hydrochloric acid solution through the column and washing with distilled water until the effluent was approximately pH 7. Before a sample containing salts was passed through the column, it was boiled with activated carbon for decolorization and then removed by centrifugation. The salt ion in the solution replaced the hydrogen ion of the resin.

The 18 cm x 0.6 cm Dowex 1 x 1 anion column was converted to the hydroxyl form by passage of 9 ml of 1 N sodium hydroxide through the column. The resin was washed with distilled water until the effluent was pH 7. A sample of 150 ml from the cation column containing not more than 0.6 meq of total acid was passed through the anion column, followed by two successive 9 - 15 ml washes with deionized distilled water. The column was eluted with 225 ml of 0.35 N acetic acid at a flow rate of 2 ml per min. in order to separate the non-volatile weak acids (the residue from the eluate). The stronger acids were eluted with 75 ml of 0.1 N hydrochloric acid. These were evaporated to dryness in a walk-in environmental room at 35^{o}C and then re-dissolved in approximately 1 ml of water for paper chromatographic separation.

RESULTS AND DISCUSSION

Methods for the Qualitative Identification of Tartaric Acid

The Ekkert's, Deniges', and Eegriwe's tests are based on the formation of a colored compound through condensation by dehydration with concentrated sulfuric acid (Table 1). Since the nature of the reactions was exergonic, a water bath at 90 - 100^{o}C was used to initiate the reaction. Due to the increase in temperature of the solution, it was difficult to obtain consistent, repeatable coloration from assay to assay. In some instances free carbon particles were formed during condensation due to over-heating of the solution. The suspended carbon particles resulted in the addition of a brown to black color to the solutions and increased the complexity of using these methods for precise qualitative analysis of tartaric acid.

Fenton's test resulted in a violet color with tartaric acid. No color reaction was apparent with glucose or 5-oxo-gluconic acid; however, the test was not sensitive when the solution contained a low concentration (0.01 g/1 ml) of tartaric acid. The lack of sensitivity of this method negated its use when analyzing fermentation broths containing trace amounts of tartaric acid.

In the Tagliavini test, a black color and precipitate indicated the presence of tartaric acid; a yellow precipitate was produced in the presence of 5-oxogluconic acid. The difficulty in collecting and qualitating the black precipitate

TABLE 1

SPECIFICITY OF SELECTED QUALITATIVE ANALYTICAL METHODS FOR TARTARIC ACID COMPARED WITH GLUCOSE AND 5-OXOGLUCONIC ACID

Method	Color Reaction		
	Tartaric acid	5-oxogluconic acid	Glucose
Ekkert	violet	dark brown	dark brown
Fenton	violet or --[1]	--	--
Tagliavini	black (ppt)[2]	yellow	yellow
Deniges	red	dark brown	reddish brown
Eegriwe	green	--	--

[1]No reaction or no coloration.

[2]Precipitate

of lead sulfide to determine the amount of tartaric acid made this a complicated and imprecise procedure.

Pyridine-Acetic Anhydride Method for Tartaric Acid Determination

Since the previous assay methods (Table 1) were not practical for qualitative analysis, the pyridine-acetic anhydride method was considered (16). The formation of a colored compound as a positive test is based upon the condensation of test compounds through dehydration by acetic anhydride instead of concentrated sulfuric acid.

The color reactions of various glucose metabolites with the pyridine-acetic anhydride method are listed in Table 2. Both 5-oxogluconic acid and tartaric acid gave a dark brown color. With triphenyl phosphine (TPP) added (personal communication, Dr. L. B. Lockwood) only tartaric acid gave a dark green color. To increase the sensitivity of this method, absorption spectra were run on each reaction mixture listed in Table 2. The addition of TPP to the solution allowed tartaric acid (green) and fumaric acid (brown) to be differentiated but did not result in a meaningful quantitative differentiation between 5-oxogluconic acid and tartaric acid (Table 2). The results from spectrophotometric analyses employing wavelengths from 340 nm to 640 nm are presented in Figure 2. Glucose, gluconic acid, glucono-δ-lactone, 2-oxogluconic acid, and glycolic acid showed little or no absorbance in the visible light region. An aqueous solution of yeast extract (0.3%, w/v) showed a slight absorbance below 400 nm. Tartaric acid

TABLE 2

SPECIFICITY OF THE PYRIDINE-ACETIC ANHYDRIDE TEST

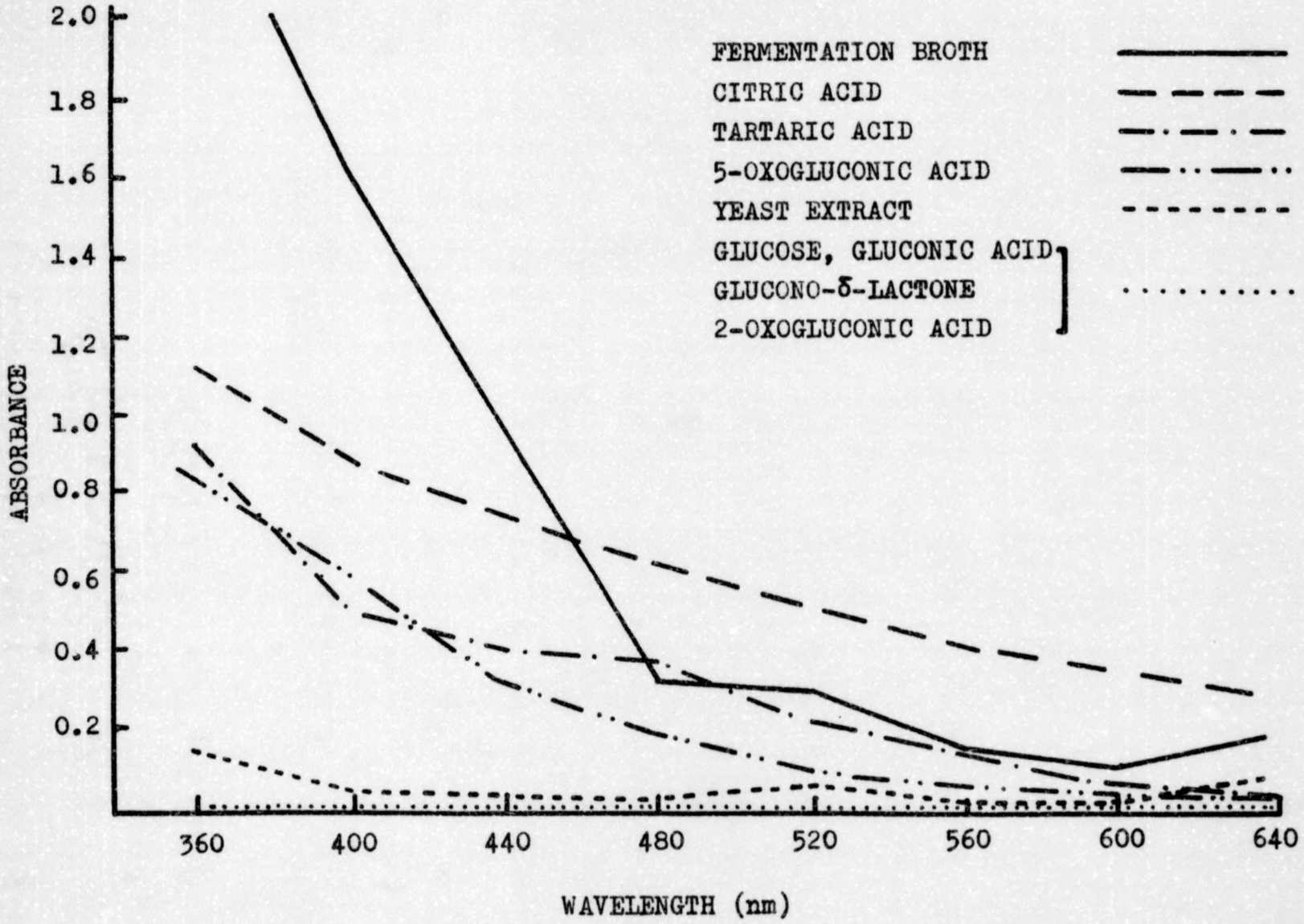
Metabolites[1]	Without TPP[2]	With TPP
Glucose	--[3]	--
Gluconic acid	light yellow	--
Glucono-δ-lactone	light yellow	--
2-Oxogluconic acid	--	--
5-Oxogluconic acid	dark brown	--
Tartaric acid	dark brown	dark green
Glycolic aldehyde	--	--
Glycolic acid	--	--
Citric acid	dark brown	dark brown
Fumaric acid	brown	brown
Malic acid	light yellow	light brown
Acetic acid	--	--

[1]Concentration of metabolites assayed (0.01 eq/l)

[2]TPP = Triphenyl phosphine

[3]No color reaction

Figure 2. Absorbancy spectra of possible fermentative metabolites using the pyridine-acetic anhydride method.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
360
400
440
480
520
560
600
640
WAVELENGTH (nm)
FERMENTATION BROTH
CITRIC ACID
TARTARIC ACID
5-OXOGLUCONIC ACID
YEAST EXTRACT
GLUCOSE, GLUCONIC ACID
GLUCONO-δ-LACTONE
2-OXOGLUCONIC ACID

and 5-oxogluconic acid gave similar absorbancy values which increased with decreasing wavelength. An absorbancy curve of citric acid was determined for comparative purposes (Figure 2). Citric acid did not accumulate in the fermentation broth. The fermentation broth showed a large and significant increase in absorbance at wavelengths below 480 nm. The increases in absorbance were probably due to a significant accumulation of metabolites, possibly 5-oxogluconic acid or tartaric acid or both, in the fermentation broth.

The similarity of absorbance in the visible region (360 - 640 nm) of 5-oxogluconic acid and tartaric acid (Figure 2) made absorbance impractical for use in the quantitative detection of tartaric acid. Subsequently, fermentation broth and known compounds were spectrophotometrically scanned in the infra-red (IR), visible and ultra-violet (UV) regions in an attempt to obtain resolvable absorbancy differences between 5-oxogluconic acid and tartaric acid. No meaningful differences in absorbancy peaks were obtained that distinguish 5-oxo-gluconic acid from tartaric acid with TPP added or without TPP (Table 3). The only metabolite having a distinguishing absorbancy peak was 2-oxogluconic acid in the IR region (peaks at 1467 nm and 1572.5 nm) without TPP added. Overlapping of absorbancy peaks in the UV region was obtained with or without TPP for all possible metabolites at 285 nm. Therefore, the pyridine-acetic anhydride method is not acceptable for quantitative analysis of microbial fermentation broths for tartaric acid.

TABLE 3

ABSORBANCE OF POSSIBLE FERMENTATION METABOLITES WITH PYRIDINE-ACETIC ANHYDRIDE METHOD

Metabolites	Without TPP[1]			With TPP			
	IR	Visible	UV	IR	Visible	UV	
Glucose	-[2]	-	285.5	-	-	283.0	276.2
Gluconic acid	-	-	285.0	-	-	280.5	276.0, 276.9
Glucono-δ-lactone	-	-	281.3	1443.8	-	291.5	279.3
2-Oxogluconic acid	1467.0, 1572.5	-	284.5	-	-	293.8, 287.6	276.5
5-Oxogluconic acid	-	-	284.3	1770.0	-	295.4	275.5
Tartaric acid	-	-	285.0	-	-	296.6, 286.0	278.8
Glycolic aldehyde	-	-	291.5	-	-	-	-
Glycolic acid	-	-	289.5	-	-	-	-
Fumaric acid	-	-	280.0	-	-	-	-
Citric acid	-	-	-	-	-	-	-
Acetic acid	-	-	-	-	-	-	-

[1] TPP = triphenyl phosphine

[2] (-) = no absorbancy peak

Ammonium Meta-vanadate Method for Tartaric Acid Determination

Ammonium meta-vanadate has been used to detect acids in solution (20) or to detect acids on chromatograms (2). Since the method is more sensitive in the detection of tartaric acid than other acids, it has commonly been used to analyze tartaric acid qualitatively and quantitatively. Ammonium meta-vanadate (0.8 ml) was reacted with each of the possible metabolites (0.1 meq/ml) which might occur during tartaric acid synthesis, and a spectrophotometrical absorbancy curve in the visible light region was determined for each compound. The absorbancy peak of each compound is recorded in Table 4. Spectrophotometric analysis revealed absorbancy differences between 5-oxogluconic acid (470.5 nm) and tartaric acid (400 nm). Except for 5-oxogluconic acid, all compounds absorbed between 396.7 - 412 nm. These absorbancy differences offer a potential means for the quantitative determination of tartaric acid spectrophotometrically.

Chromatographic Identification of Tartaric Acid in Fermentation Broth

Chromatographic determination of tartaric acid in fermentation broths or in a mixture of metabolites was investigated since this method may offer a quick, easy, accurate analytical tool for identification of tartaric acid.

Filter-paper chromatography (FPC) was initially investigated. The Rf values of different metabolites in different solvent systems were compared and are listed in Table 5.

TABLE 4

MAXIMUM ABSORBANCE OF SUSPECTED FERMENTATION METABOLITES EMPLOYING THE AMMONIUM META-VANADATE METHOD

Metabolites	Absorbance (nm)
Glucose	397
Gluconic acid	412
Glucono-δ-lactone	406.5
2-Oxogluconic acid	396.7, 352.8
5-Oxogluconic acid	470.5
Tartaric acid	400
Glycolic aldehyde	410
Glycolic acid	402.5
Fumaric acid	403.2
Citric acid	408

TABLE 5

Rf VALUES OF POSSIBLE METABOLITES FORMED DURING TARTARIC ACID FERMENTATION DEVELOPED WITH DIFFERENT SOLVENT SYSTEMS

Metabolites	Rf					
	Solvent BAW	Solvent IA	Solvent EAFW	Solvent PAW	Solvent EAW	Solvent PW
Glucose	-[1]	-	0.146	-	-	-
Gluconic acid	0.583	0.55	0.663	0.541	0.474	0.857
Glucono-δ-lactone	0.206	0.075	0.157	0.432	0.247	0.84
2-Oxogluconic acid	-	-	-	-	-	-
5-Oxogluconic acid	-	-	-	-	-	-
Tartaric acid	0.351	0.20	0.343	0.270	0.132, 0.063	0.857
Glycolic acid	0.248	0.06	0.180, 0.089	-	0.106	-
Glycolic aldehyde	-	0.425	-	-	-	-
Fumaric acid	0.811	0.825	0.955	0.405	0.253	-
Citric acid	0.461	0.26	0.489	0.216	0.053	0.886

[1]No reaction with spray reagent dimethyl yellow and bromophenol blue.

Solvent system BAW, IA, and EAFW (Table 5) were acidic solvent systems which prevented the dissociation of the acids and thus partially reduced the possibility of tailing. Solvent systems PAW and EAW were alkaline systems. The ammonium hydroxide reacted with the acids, forming ammonium salts which migrated on the filter paper in that form. Solvent system PA has a weak acid system due to the low dissociation constant of phenol in water.

Chromatograms from solvent system BAW (Figure 3) and EAFW (Figure 4) gave good separation of the compounds. In general, solvent system IA (Figure 5) showed low Rf values, and solvent system PW (Figure 6) showed high Rf values for most metabolites on the chromatograms. The faint spots on the chromatogram of the alkaline solvent systems PAW (Figure 7) and EAW (Figure 8) showed a weak color reaction between the ammonium salts and the spray reagent, dimethyl yellow and bromophenol blue. Also shown in these figures is the identity of the compounds that are found in the three fractions eluted from the anion exchange column.

Four kinds of spray reagents as indicators were tested in an attempt to increase the sensitivity of detecting the compounds on the chromatograms (Table 6). Dimethyl yellow and bromophenol blue (DMY/BPB) proved to be a general reagent for detecting acids. Hydroxylamine-ferric chloride mainly detected esters and lactones. Ammonium meta-vanadate reacted well with acids, particularly tartaric acid and fumaric acid. Ammonium silver nitrate reacted especially well with reducing

Figure 3. Chromatogram of possible fermentative metabolites separated in Solvent BAW and developed with dimethyl yellow and bromophenol blue. G, glucose; GA, gluconic acid; G-δ-L, glucono-δ-lactone; 2-Oxo, 2-oxogluconic acid; 5-Oxo, 5-oxogluconic acid; TA, tartaric acid; GlyAld, glycolic aldehyde; GlyA, glycolic acid; B, fermentation broth, E1, water eluate, WA, 0.35 N acetic acid eluate; SA, 0.1 N HCl eluate.

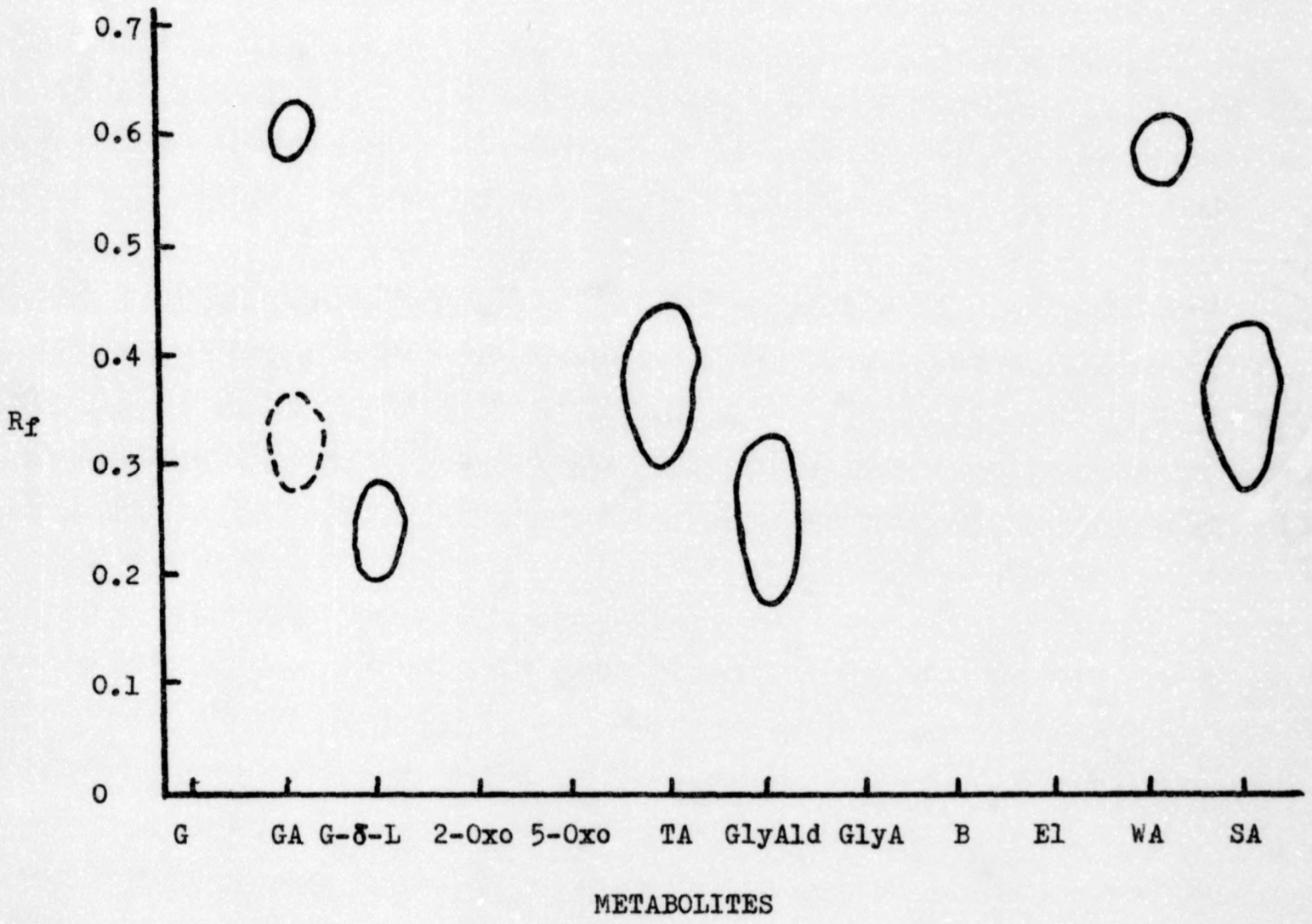

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
R_f
G
GA
G-δ-L
2-Oxo
5-Oxo
TA
GlyAld
GlyA
B
El
WA
SA
METABOLITES

Figure 4. Chromatogram of possible fermentative metabolites separated in Solvent EAFW and developed with dimethyl yellow and bromophenol blue. G, glucose; GA, gluconic acid; G-δ-L, glucono-δ-lactone; 2-Oxo, 2-oxogluconic acid; 5-Oxo, 5-oxogluconic acid; TA; tartaric acid; GlyAld, glycolic aldehyde; GlyA, glycolic acid; B, fermentation broth; El, water eluate; WA, 0.35 N acetic acid eluate; SA, 0.1 N HCl eluate.

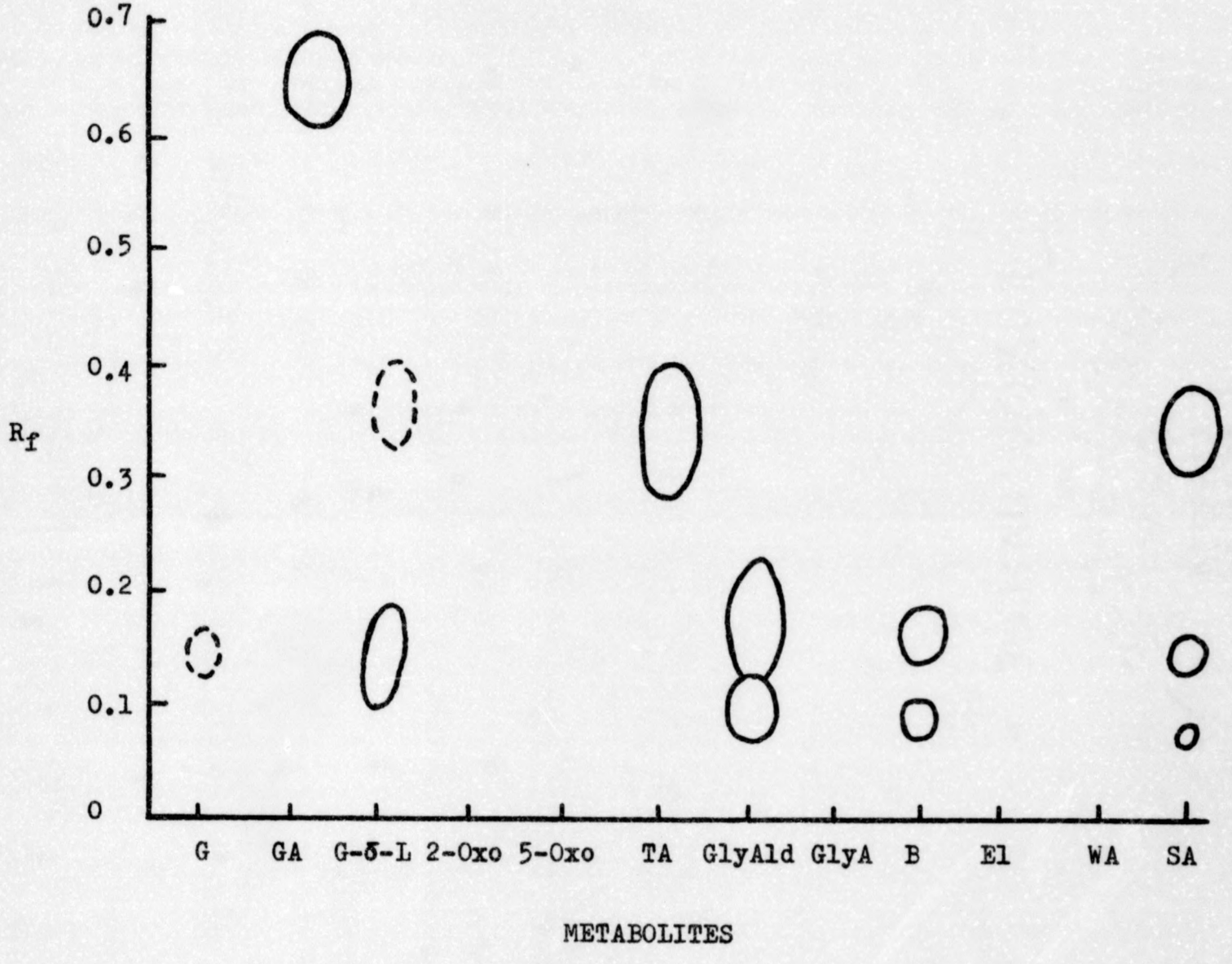

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
R_f
G
GA
G-δ-L
2-Oxo
5-Oxo
TA
GlyAld
GlyA
B
El
WA
SA
METABOLITES

Figure 5. Chromatogram of possible fermentative metabolites separated in Solvent IA and developed with dimethyl yellow and bromophenol blue. G, glucose; GA, gluconic acid; G-δ-L, glucono-δ-lactone; 2-Oxo, 2-oxogluconic acid; 5-Oxo, 5-oxogluconic acid; TA, tartaric acid; GlyAld, glycolic aldehyde; GlyA, glycolic acid; B, fermentation broth; E1, water eluate; WA, 0.35 N acetic acid eluate; SA, 0.1 N HCl eluate.

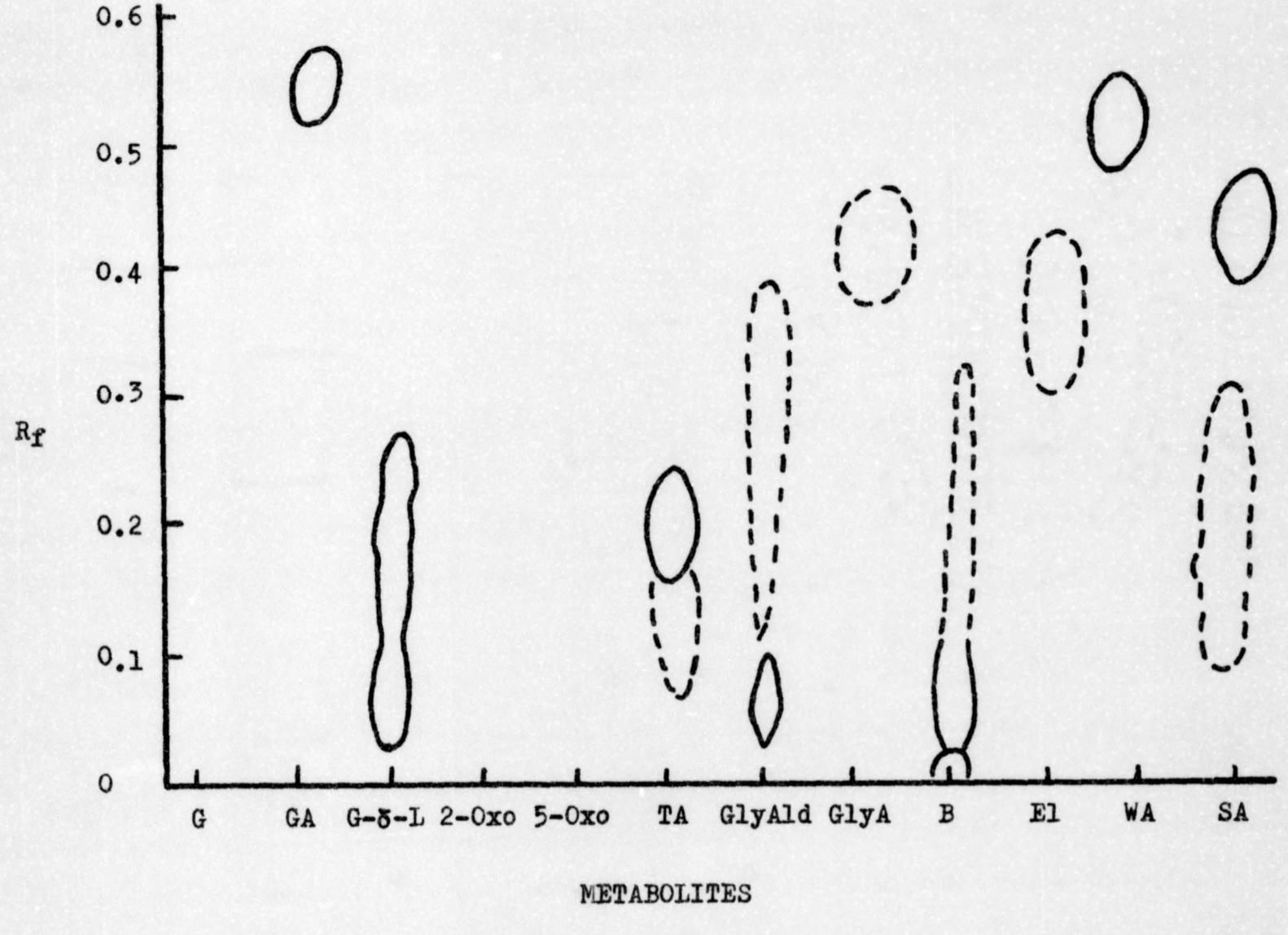

0.6
0.5
0.4
0.3
0.2
0.1
0
R_f
G
GA
G-δ-L
2-Oxo
5-Oxo
TA
GlyAld
GlyA
B
El
WA
SA
METABOLITES

Figure 6. Chromatogram of possible fermentative metabolites separated in Solvent PW and developed with dimethyl yellow and bromophenol blue. G, glucose; GA, gluconic acid; G-δ-L, glucono-δ-lactone; 2-Oxo, 2-oxogluconic acid; 5-Oxo, 5-oxogluconic acid; TA, tartaric acid; GlyAld, glycolic aldehyde; GlyA, glycolic acid; B, fermentation broth; E1, water eluate; WA, 0.35 N acetic acid eluate; SA, 0.1 N HCl eluate.

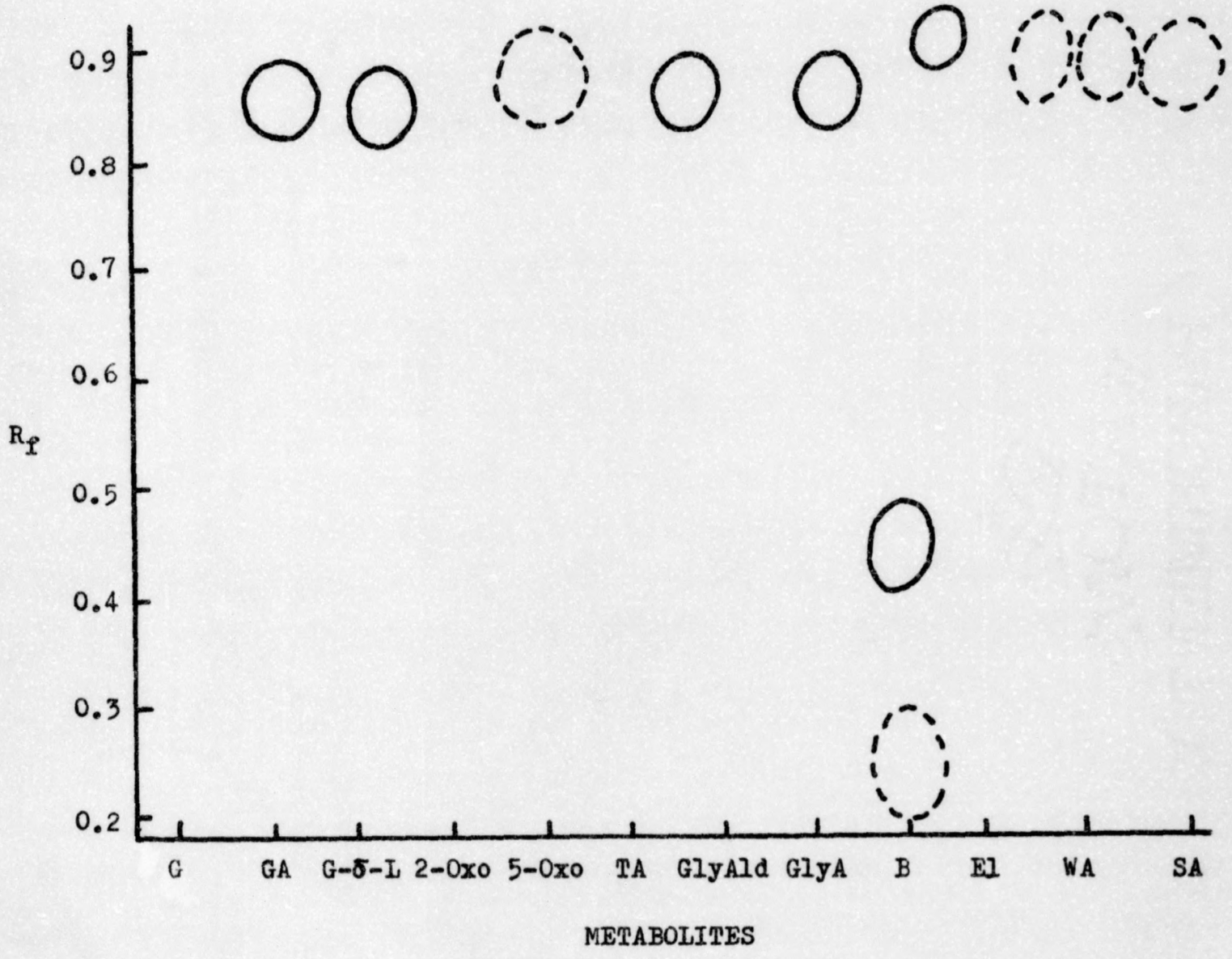

0.9
0.8
0.7
0.6
R_f
0.5
0.4
0.3
0.2
G
GA
G-δ-L
2-Oxo
5-Oxo
TA
GlyAld
GlyA
B
El
WA
SA
METABOLITES

Figure 7. Chromatogram of possible fermentative metabolites separated in Solvent PAW and developed with dimethyl yellow and bromophenol blue. G, glucose; GA, gluconic acid; G-δ-L, glucono-δ-lactone; 2-Oxo, 2-oxogluconic acid; 5-Oxo, 5-oxogluconic acid; TA, tartaric acid; GlyAld, glycolic aldehyde; GlyA, glycolic acid; B, fermentation broth; El, water eluate; WA, 0.35 N acetic acid eluate; SA, 0.1 N HCl eluate.

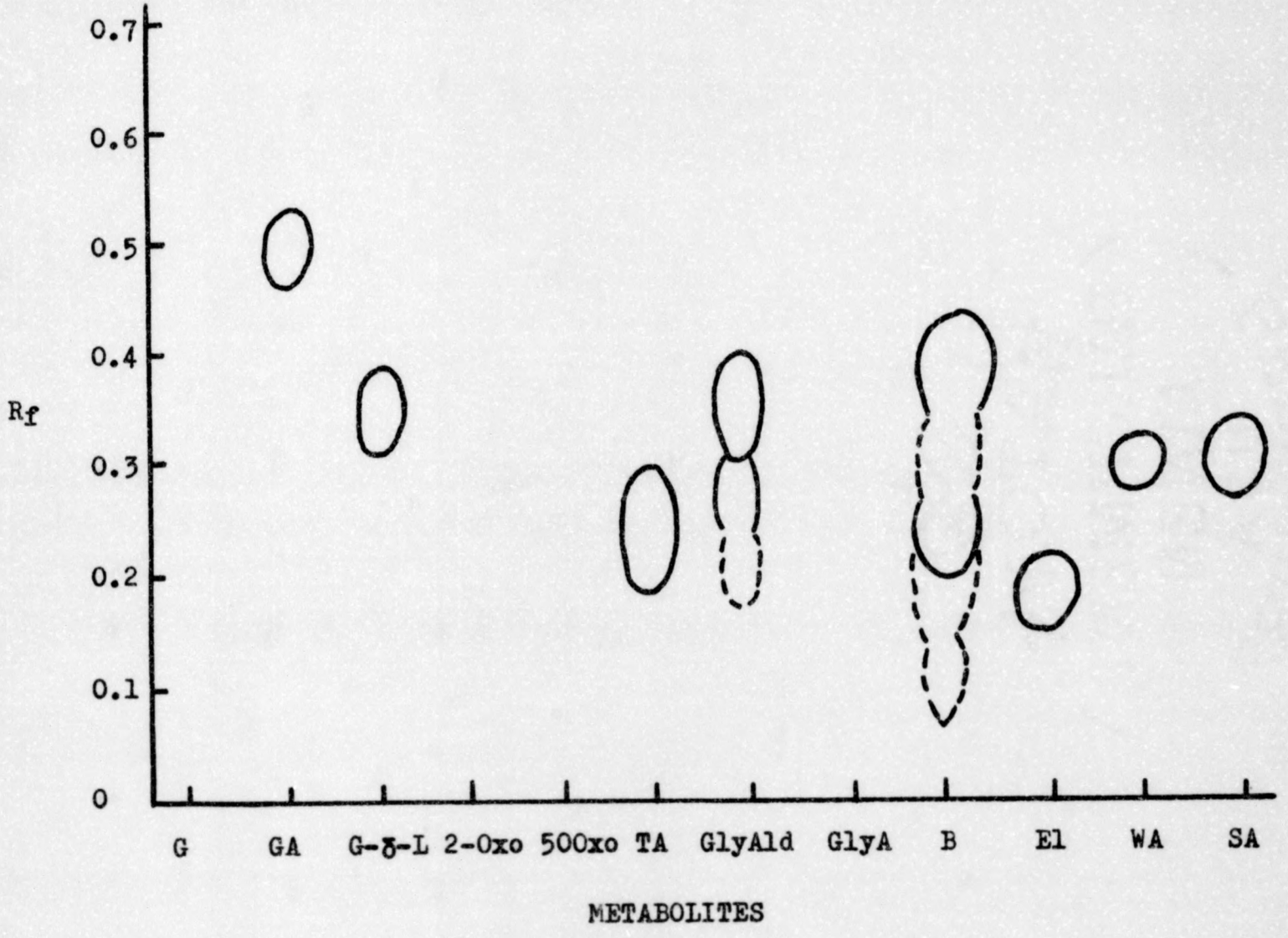
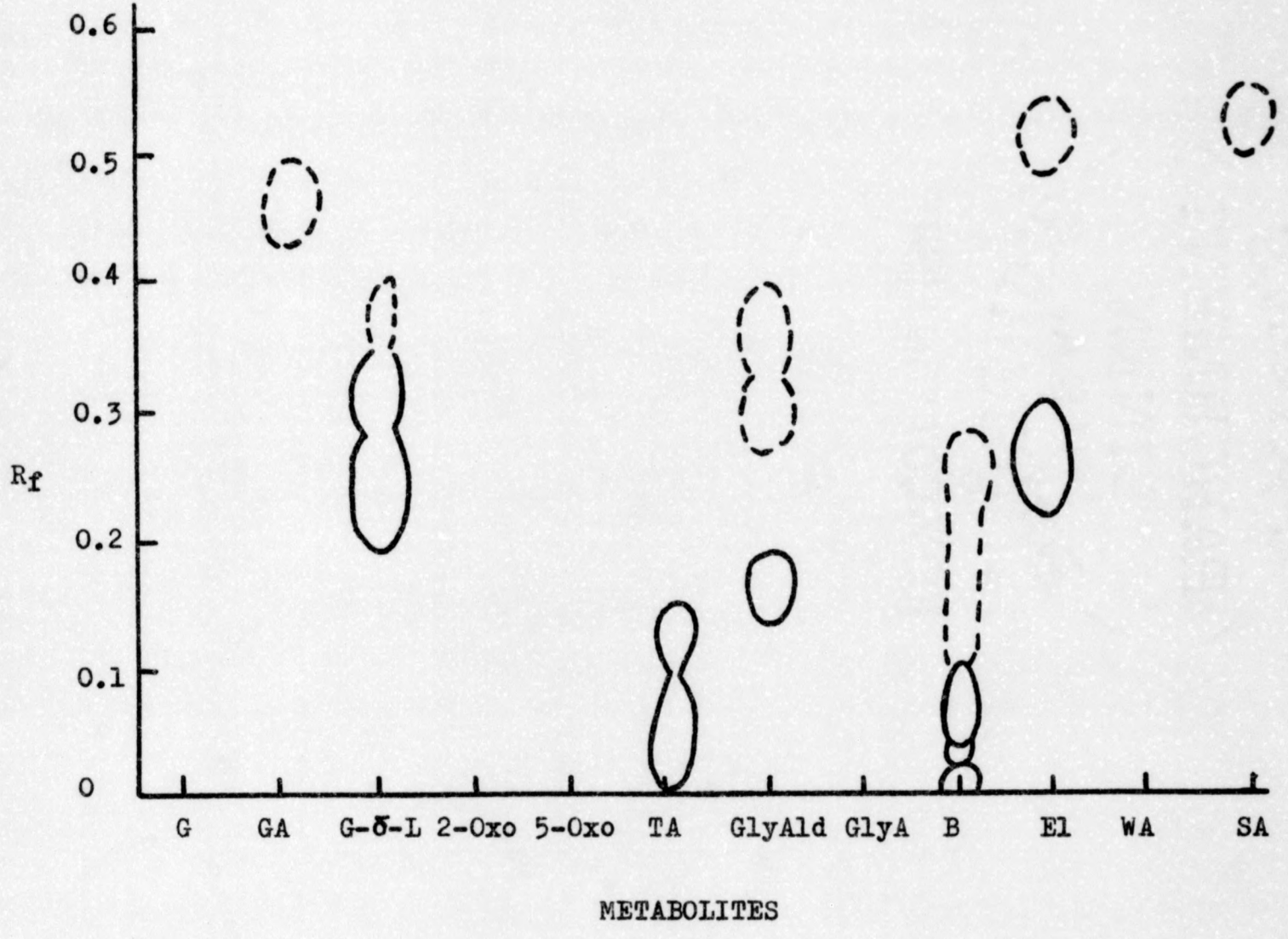

0.7
0.6
0.5
0.4
Rf
0.3
0.2
0.1
0
G
GA
G-δ-L
2-Oxo
500xo
TA
GlyAld
GlyA
B
El
WA
SA
METABOLITES

Figure 8. Chromatogram of possible fermentative metabolites separated in Solvent EAW and developed with dimethyl yellow and bromophenol blue. G, glucose; GA, gluconic acid; G-δ-L, glucono-δ-lactone; 2-Oxo, 2-oxogluconic acid; 5-Oxo, 5-oxogluconic acid; TA, tartaric acid; GlyAld, glycolic aldehyde; GlyA, glycolic acid; B, fermentation broth; E1, water eluate; WA, 0.35 N acetic acid eluate; SA, 0.1 N HCl eluate.


0.6
0.5
0.4
0.3
0.2
0.1
0
Rf
G
GA
G-δ-L
2-Oxo
5-Oxo
TA
GlyAld
GlyA
B
El
WA
SA
METABOLITES

compounds such as glucose and glycolic aldehyde. DMY and BPB gave good color spots with all the possible metabolites except glucose, 2-oxogluconic acid and glycolic aldehyde (Table 6). Hydroxylamine-ferric chloride showed a color reaction with 5-oxogluconic acid, but its color reaction with glucono-δ-lactone was too faint to be certain. Ammonium silver nitrate was sensitive to all metabolites except glycolic acid. The combined results from chromatograms sprayed with either DMY/BPB or ammonium silver nitrate gave recognizable spots and thus allowed identification of all possible metabolites. Therefore, two chromatograms must be run; one must be developed with DMY/BPB spray and the other with ammonium silver nitrate to identify all metabolites. However, using a combination of any two spray reagents on the same chromatogram was not acceptable because chemical interactions eliminated some spots.

Ion-Exchange Chromatography

Previous results indicated that the color complex formed between ammonium meta-vanadate and glucose, or possible microbial oxidative products, resulted in identifiable absorbancy peaks. However, there was some overlapping of peaks. Thus, it was postulated that improved resolution would occur if the metabolites in the fermentation broth were separated on an ion-exchange column. If a proper ion-exchange column were used, the metabolites could be eluted and collected separately for quantitative analysis. This procedure requires

TABLE 6

DIFFERENTIATION OF METABOLITES EMPLOYING VARIOUS CHROMATOGRAPHIC INDICATORS

Metabolite	Spray Reagent			
	Dimethyl yellow Bromophenol blue	Hydroxylamine ferric chloride	Ammonium metavanadate	Ammoniacal silver nitrate
Glucose	-	-	-	++
Gluconic acid	+	-	-	++
Glucono-δ-lactone	+	±	-	+
2-Oxogluconic acid	±	-	-	+
5-Oxogluconic acid	+	+	+	+
Tartaric acid	+	-	++	+
Glycolic aldehyde	-	-	-	++
Glycolic acid	+	-	+	-
Fumaric acid	+	NT	++	-
Citric acid	+	NT	+	-
Acetic acid	+	NT	NT	NT

[1] - = No color reaction; ±, weak color reaction; +, positive color reaction;

[2] ++ = Very positive and sensitive color reaction

[3] NT = Not Tested

proper anion resins and elution with appropriate acids at optimal pH values (21).

A procedure was tested as diagrammed in Figure 9. The eluates were poured into separate petri dishes or watch glasses and evaporated in a ventilated walk-in-environmental room at 35°C. The dried crystals were re-dissolved in a small amount of water (about 1 ml) for paper chromatography separation. Also the eluates were complexed with ammonium meta-vanadate and the absorption spectra determined spectrophotometrically.

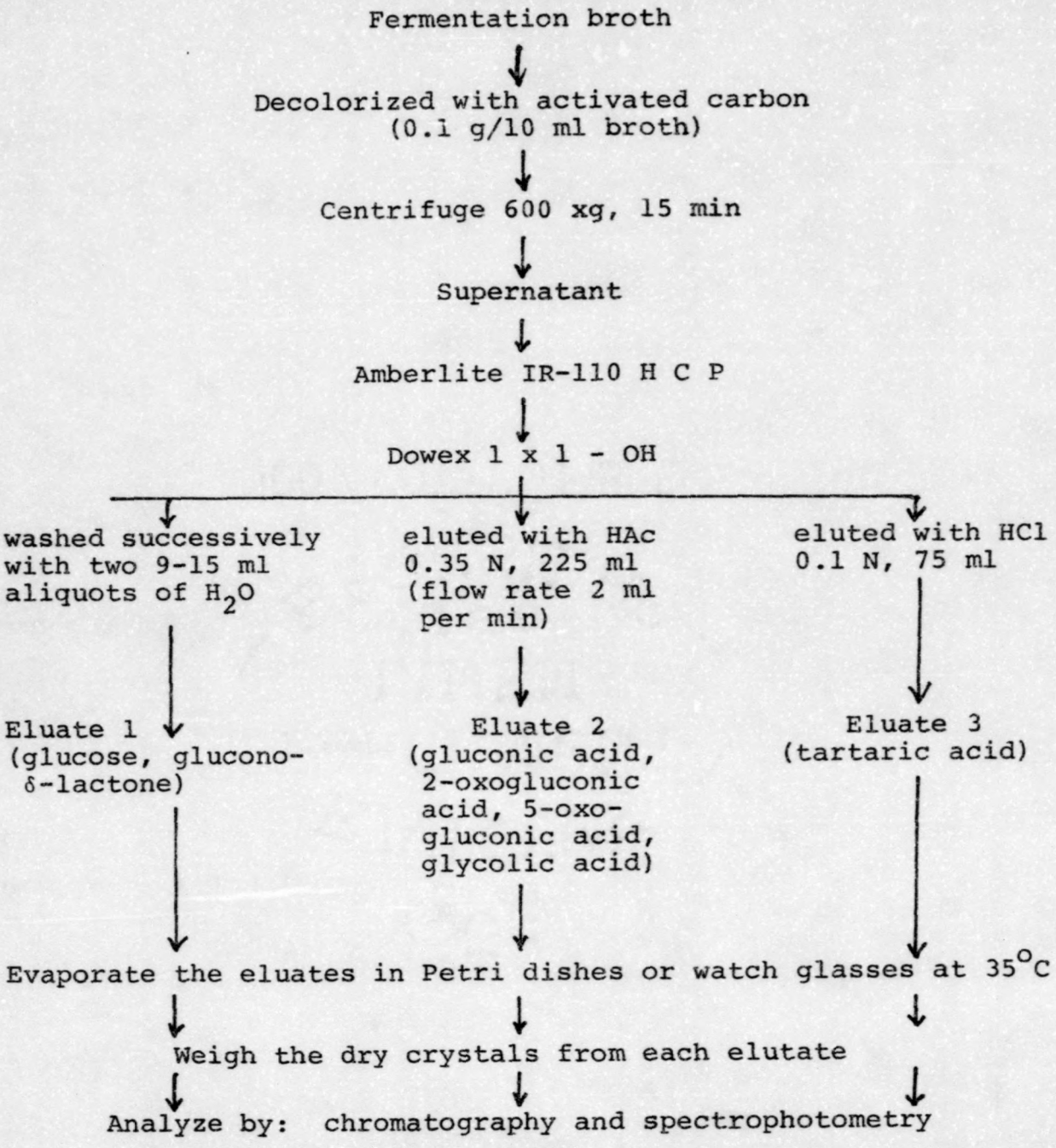
Paper chromatographic analysis of eluates from the ion-exchange column showed the presence of tartaric acid in the strong acid eluate (Figures 3, 4, 5, 6, 7, 8). The absence of spots from fermentation broth on the chromatograms were probably due to the low concentration of the metabolites. The absence of tartaric acid, particularly in the strong acid eluate in some fermentation broths, was probably due to the fact that G. suboxydans had not grown long enough for the production of tartaric acid.

Plotting the Standard Curve of Absorbance Using The Ammonium Meta-Vanadate (AMV) Method.

When de-ionized distilled water was used as a reference standard, the maximum peak of absorbancy for ammonium meta-vanadate in water occurred at 296.2 nm. Therefore, during the determination of tartaric acid concentration by using photometrical measurement this wavelength should be avoided.

Figure 9. Schematic diagram of suggested procedure for analysis of tartaric acid in fermentation broth. HAc, acetic acid; HCl, hydrochloric acid.

Ion exchange



Further reserach indicated that the sensitivity and intensity of the color reaction were related to the pH value of the solution. Abosrbancy measurements of tartaric acid (0.1 M, 1 ml) reacted with AMV (0.8 ml) at different pH values in the visible light region were made. These results indicated that the color intensity of the solutions was maximized at pH 5.8 (Table 7) and the maximum absorbancy peak was approximately 400 nm; therefore, all subsequent analyses for tartaric acid in solutions using the AMV method were adjusted to pH 5.8 and measured at 400 nm. Sodium hydroxide and hydrochloric acid, 1 N and 0.1 N, respectively, were used to adjust the pH of the solutions being analyzed. The addition of hydrochloric acid or sodium hydroxide in the adjustment of pH resulted in an insignificant modification of color.

Dimethyl Yellow and Bromophenol Blue Method for Tartaric Acid Analysis.

The reagent DMY/BPB was investigated for its possible use in forming a color complex with tartaric acid. Detection of absorbancy peaks with spectrophotometric analysis in the visible light region showed distinct absorbancy differences between 5-oxogluconic acid (588.8 nm) and tartaric acid (502.5 nm) (Table 8); however, the sensitivity of this reagent was generally dependent upon pH changes rather than upon reaction with a specific substance such as tartaric acid. Thus, this method can only be used to detect tartaric acid in fermentation broths lacking any weak acids and their salts. This method needs further refinement before it can be used for tartaric acid analysis.

TABLE 7

EFFECT OF pH ON THE DETECTION OF TARTARIC ACID

EMPLOYING THE AMMONIUM META-VANADATE METHOD

pH[1]	Absorbance at 400 nm
2.3[2]	0
2.3[3]	0
3.35	0.51
4.0	0.86
4.4	0.16
4.65	1.02
4.8	1.43
5.0	1.82
5.5	2 - ∞
5.8[4]	2 - ∞

[1]All these experimental solutions were made to 10 ml volume of the desired pH values with distilled deionized water.

[2]This experimental solution contained 0.1M tartaric acid, 1 ml only.

[3]0.1 M tartaric acid 1 ml and 0.8 ml saturated ammonium meta-vanadate solution, without pH adjusted, gave pH of 2.3 and was used as a blank.

[4]This experimental solution was used to adjust the spectrophotometer to 0 transmittance.

TABLE 8

ABSORBANCY PEAKS OF SUSPECTED METABOLITES WITH THE DMY/BPB METHOD

Metabolites	Dimethyl yellow (DMY)	Bromophenol blue (BPB)	Dimethyl yellow & Bromophenol blue (DMY/BPB)
Glucose	-[1] -	- - -	- 4950, - - 2780
Gluconic acid	5100, -	- 4270, 2880	- 5050, - 3225, 2794
Glucono-δ-lactone	5124, 3310	- 4329, 2780	- 5023, - 3110, 2800
2-Oxogluconic acid	- -	- 4344, 2815	- - 4210, - 2780
5-Oxogluconic acid	- -	5824, - 2810	- - - - 2822
Tartaric acid	5100, 3295	- 4340, 2810	- 5025, - - 2740
Glycolic aldehyde	- -	- 4210, 2810	5875, - - - 2787
Glycolic acid	- -	5864, - 2813	- - 4360, 3213, 2818
Fumaric acid	5049, 3295	- 4330, 2810	- 5018, - - 2830
Citric acid	5100, 3294	- 4330, 2813	- 5045, - 3200, 2750
Acetic acid	5058, -	- 4315, 2804	- - 4465 - -

[1]- no meaningful peaks around this area

A standard concentration absorbancy curve was prepared from readings obtained with pure tartaric acid solutions at different concentrations (from 0 to 10 mg) reacted with AMV (Table 9, Figure 10). A saturated ammonium meta-vanadate solution, 0.8 ml, was added to each test tube. Water was added to adjust the volume of the solution to 7 or 8 ml. The pH of each solution was adjusted to 5.8, and the volume of the solution was adjusted with water to 10 ml. The absorbance of the solutions was measured at 400 nm. The test tube with 0.8 ml of saturated ammonium meta-vanadate (10 ml total volume) was used to adjust the spectrophotometer to 100% transmittance, and a test tube with 10 mg tartaric acid in a total volume of 10 ml was used to adjust it to 0% transmittance. The percent transmittance was recorded first and, when converted to absorbance, gave more precise results than reading the absorbance directly.

From the data in Table 9, it is obvious that the concentrations of tartaric acid in a solution should be below 0.5 mg per 10 ml solution. A solution containing tartaric acid higher than 0.5 mg per 10 ml solution resulted in a coloration outside of the test range. Also, a solution containing tartaric acid lower than 0.05 mg per 10 ml will be more influenced by the hydrochloric acid used to adjust the pH of the solution.

A suggested routine procedure for tartaric acid analysis is as follows. Each analyst should plot a standard curve. Add 0.8 ml of a saturated ammonium meta-vanadate solution

TABLE 9

STANDARD CURVE OF TARTARIC ACID USING THE AMMONIUM META-VANADATE METHOD

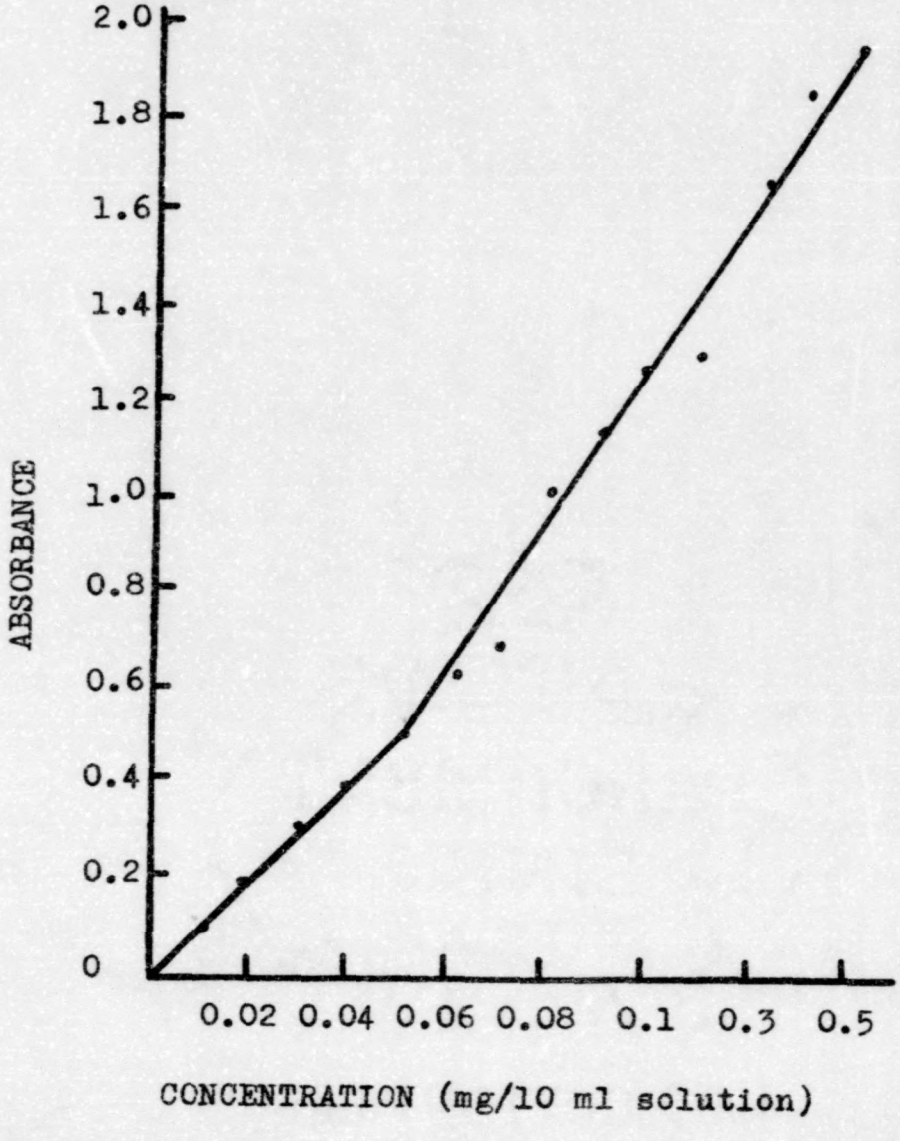
Tartaric acid Concentration (mg)	Absorbance[1]	
	Standard[2]	Mixture[3]
0.00	0	
0.01	0.21	
0.02	0.32	
0.03	0.42	0.42
0.04	0.50	
0.05	0.65	
0.06	0.68	0.71
0.07	1.02	
0.08	1.13	
0.09	1.25	1.13
0.10	1.28	
0.20	1.60	
0.30	1.83	
0.40	1.92	
0.50	2	
0.75	2	
1.00	2 - ∞	
2.50	2 - ∞	
5.00	2 - ∞	
7.50	2 - ∞	
10.0	2 - ∞	

[1]Absorbances were determined at 400 nm, pH 5.8.

[2]The standard contained tartaric at different concentrations.

[3]The mixture contained equal concentrations of glucose, gluconic acid, tartaric acid, glycolic aldehyde, and glycolic acid.

Figure 10. Standard absorbancy curve of varying tartaric acid concentrations reacted with ammonium metavanadate.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0.02 0.04 0.06 0.08 0.1 0.3 0.5
CONCENTRATION (mg/10 ml solution)

to each test tube containing tartaric acid at different concentrations (0.02 - 10 mg/10 ml); add water to make the volume of the solution to 7 - 8 ml. Very precisely, adjust the pH of the solution to pH 5.8 then add distilled water until a 10-ml volume is reached. Recheck the pH value. A test tube containing 0.8 ml AMV in 10 ml of solution adjusted to pH 5.8 should be used as a blank for standardization. Another test tube containing 0.5 mg tartaric acid and 0.8 ml AMV in a 10 ml solution (pH 5.8) should be used to adjust the spectrophotometer to 0% transmittance. The percent transmittance of each solution then should be converted to absorbance. If a test tube containing 0.1 mg of tartaric acid is used as a blank and another test tube containing 0.5 mg tartaric acid is used to adjust the spectrophotometer to 0% transmittance, the absorbance should be more precise for plotting the standard curve.

The fermentation broth should be first decolorized by adding 0.05 - 0.1 g of activated charcoal per 10 ml broth and then heating the suspension to boiling. The solution is then passed through a cation-exchange resin column to convert the tartrate to its free acid form. The free acid is separated from 5-oxogluconic acid by passing through an anion-exchange column and eluting with 0.35 N acetic acid and subsequently 0.1 N hydrochloric acid as previously described. If the concentration of tartaric acid is above 0.5 mg per 10 ml of solution, dilutions are necessary. One to eight milliliters of the strong-acid eluate should be added to 0.8 ml of a

saturated solution of ammonium meta-vanadate and the procedure continued as described previously. Employing the standard curve, the absorbancy value obtained from this reading would be used to determine the tartaric acid concentration. All dilution factors must be taken into consideration to determine the original concentration of tartaric acid in the fermentation broth.

A test run of sample mixtures containing glucose, gluconic acid, glucono-δ-lactone, glycolic acid and tartaric acid of the same concentration was carried out to check the validity of this method (Table 9). The similarity of absorbancy determinations between the standard solution of pure tartaric acid and tested mixtures indicated the practicality of the method.

LITERATURE CITED

1. Abdel Akher, M. and F. Smith. 1951. The detection of carbohydrate esters and lactones after separation by paper chromatography. J. Am. Chem. Soc. 73:58-59.

2. Buch, M. L., E. C. Dryden and C. H. Hills. 1955. Chromatographic comparison of nonvolatile acids of fresh and stored apple juice concentrate. Agr. Food Chem. 3:960-964.

3. Ho, F. H. 1971. Experimental Biochemistry 2nd Ed. The National Book Co. Taiwan, R. O. C. 541 pages.

4. Daniel, L. W. 1975. The metabolism of tartaric acid by Aspergillus ficuum. Thesis: Western Kentucky University, Bowling Green, KY.

5. Dibble, J. T. 1974. The production of epoxysuccinic acid by fungi. Thesis: Western Kentucky University, Bowling Green, KY.

6. Deniges, G. 1936. Application of the sulfo-resorcinol reaction to the succinic ion. Bull. Soc. Pharm. Bordeaux. 74:12-17.

7. Eegriwe, E. 1932. Reactions and reagents for the detection of organic compounds. I. Z. Anal. Chem. 89:121-125.

8. Ekkert, L. 1925. New color test for tartaric acid. Pharm. Zentralhalle. 66:765.

9. Fenton, H. J. H. 1922. Notes on qualitative analysis, concise and explanatory. MacMillan Co., N.Y. 47 pages.

10. Hulme, A. C. 1961. Method for the determination of organic acids. Adv. Appl. Microbiol. 3:343-393.

11. Kotera, U., T. Kodama, Y. Minoda and K. Yamada. 1972. Isolation and chemical structure of new oxidative product of 5-oxogluconic acid, and a hypothetical pathway from glucose to tartaric acid through this new compound. Agr. Biol. Chem. 36:1313-1325.

12. Leu, N. C. 1979. The effect of pH on 5-oxogluconate production by Gluconobacter suboxydans. Thesis: Western Kentucky University, Bowling Green, KY.

13. Lockwood, L. B. 1960. *Acetobacter suboxydans* fermentations. McGraw-Hill Encyclopedia of Technology, Vol. 1, pages 39-41.

14. Lockwood, L. B. and G. N. Nelson. 1951. D-Tartaric acid. U.S. Patent 2,559,650.

15. Marootkhanian, V. 1976. Constitutive nature of 5-keto-gluconate reductase of *Gluconobacter suboxydans*. Thesis: Western Kentucky University, Bowling Green, KY.

16. Marier, J. R. and M. Boulet. 1958. Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method. J. Dairy Sci. 41:1683-1693.

17. Stubbs, J. J., L. B. Lockwood, E. T. Roe, B. Tabenkin, and G. E. Ward. 1940. Keto-gluconic acids from glucose: Bacteria production. Ind. Eng. Chem. 32:1626-1631.

18. Tagliavini, G. 1922. Identification of small quantities of cream of tartar by microchemical methods. Bull. Soc. Pharm. Bordeaux. 60:169-172.

19. Trevelyan, W. E., D. P. Procter and J. S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature. 166:444-445.

20. Underhill, F. P., F. I. Peterman and A. C. Krause. 1931. The metabolism of tartrates (I). A colorimetric method for the determination of tartaric acid. J. Pharmacol. 43:351-358.

21. VanEtten, C. H. and C. E. McCrew. 1957. Ion exchange micromethods for separation of fermentation acids--determination of fumaric acid in fermentation broth. Anal. Chem. 29:1506-1509.

22. Ware, K. L. 1976. The metabolism of glucose by the combined use of *Gluconobacter suboxydans* and *Pseudomonas fluorescens*. Thesis: Western Kentucky University, Bowling Green, KY.

23. Wood, W. A. and R. F. Schwerdt. 1953. Carbohydrate oxidation by *Pseudomonas fluorescens*. I. The mechanism of glucose and gluconate oxidation. J. Biol. Chem. 201:501-511.

24. Yamada, K., T. Kodama, T. Obata and N. Takahashi. 1971. Microbial fermentation of tartaric acid from glucose: (I) Isolation and identification of tartaric acid producing microorganism. J. Ferment. Technol. 49: 85-92.