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Microchemical Journal

devoted to the application of microtechniques in all branches of science

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Phosphorimetry

The Application of Phosphorescence to the Analysis of Organic Compounds

By **M. Zander**
Translated by **T. H. Goodwin**

This is the first major publication to deal exclusively with the analytical procedure known as phosphorimetry. Beginning with an introduction to the theoretical and experimental foundations of the phosphorescence of organic compounds, the book discusses the phosphorescence properties of numerous classes of compounds and of many individual substances. The techniques and instrumentation of phosphorimetry itself, as well as a comprehensive and up-to-date collection of analytical applications, are also presented in detail. The book concludes with numerous examples from many fields of scientific interest.

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Microchemical Journal, Vol. 13, No. 2

Briefs

Thin-Layer Chromatography of Isomeric Steroid Oximes and Their Homo-Aza-Lactams. III. TLC of Estra-1,3,5(10)-trien-3-ol-17-one and its Derivatives. B. MATKOVICS, AND GY. GÖNDÖS, *Institute of Organic Chemistry, University of Szeged, Szeged, Dóm-tér* 8, Hungary.

The solvent system *n*-heptane–acetone (2:1,v/v) appears to be the most favorable for separating estrone and its derivative.

Microchem. J. **13**, 171 (1968).

Thin-Layer Chromatography of Steroid Ketones and Their Derivatives. IV. TLC of Bile Acids, Keto-bile Acids, and Their Derivatives. B. MATKOVICS AND ZS. TEGYEY, *Institut of Organic Chemistry, "József Attila" University, Szeged, Hungary.*

Solvent systems are described for the thin-layer separation of bile acids and their derivatives. Mixtures of nonpolar and polar solvents containing a high percentage of the former are best suited.

Microchem. J. **13**, 174 (1968).

Titrimetric Microdetermination of Glycine. A. MASOOD AND O. C. SAXENA, *Chemical Laboratories, University of Allahabad, Allahabad, India.*

Titrimetric microdetermination of glycine by oxidizing with gold chloride in alkaline medium is described.

Microchem. J. **13**, 178 (1968).

A Micromethod for Silica-Gel Column Chromatography of Urinary Organic Acids. J. M. ORTEN, W. GAMBLE, C. B. VAUGHN, AND K. C. SHRIVASTAVA, *Department of Biochemistry, Wayne State University, School of Medicine, Detroit, Michigan 48207.*

Automatic, continuous gradient elution silica-gel column chromatography is used in the separation and quantitative estimation of different urinary organic acids under various physiological as well as pathological states.

Microchem. J. **13**, 183 (1968).

Metal Chelates of Eriochrome Black T in Aqueous Solutions. SHRIKANT B. DABHADE AND SATENDRA P. SANGAL, *Laxminarayan Institute of Technology, Nagpur University, Nagpur, India.*

The elementary observations on the chelate formation of Eriochrome black T have been described. The behavior of the reagent in aqueous solution and different conditions under which stable complexes are formed have been worked out for magnesium, zinc, cadmium, calcium, aluminium, gallium, indium, scandium, yttrium, lanthanum, praseodymium, neodymium, samarium, thorium, uranium, chromium, iron, cobalt, nickel, molybdenum, and copper metals.

Microchem. J. **13,** 193 (1968).

Azulene Procedure for Chromatographic Analysis of Aromatic and Heterocyclic Aldehydes, Carbohydrates, and Other Aldehyde Precursors. C. R. ENGEL AND E. SAWICKI, *Bureau of Disease Prevention and Environmental Control, National Center for Air Pollution Control, U. S. Department of Health, Education, and Welfare, Public Health Service, Cincinnati, Ohio 45226.*

A new location method for many types of conjugated aldehydes and their precursors is introduced. This method, with azulene as the reagent, is compared with a number of other location methods. Evidence is presented for the location of aromatic aldehydes, furfural derivatives, sugars, dinitrophenylhydrazones, and azines on paper and silica gel plates with azulene. This location procedure was applied to the identification of 5–hydroxymethylfurfural in extracts of effluents from a coffee-roasting plant. Techniques described in this paper have been developed for eventual application to analyses of airborne particulate.

Microchem. J. **13,** 202 (1968).

Titrimetric Microdetermination of Samarium and Neodymium. O. C. SAXENA, *Chemical Laboratories, University of Allahabad, Allahabad, India.*

Titrimetric microdetermination of samarium and neodymium has been described with sodium rhodizonate. Trivalent salts of samarium and neodymium form violet colored complexes in the ratio of 1:3. The complex is dissolved in dilute acid and the rhodizonic acid that is attached with the metal ion is estimated by titrating against a standard solution of ceric sulfate using *N*-phenyl anthranilic acid as indicator. Interference of cations has been observed.

Microchem. J. **13,** 222 (1968).

Determination of Formaldehyde via EDTA Titration. LÁSZLO SZEKERES AND ÁGNES KELLNER, *Chemical Institute of the Veterinary University, Budapest, Hungary.*

Formaldehyde is treated with EDTA and $Hg(NO_3)_2$. One mole of EDTA is equivalent to 1 mole of formaldehyde.

Microchem. J. **13,** 227 (1968).

The Application of Fuchsine Dyes in the Detection of Esters of *o*-Phthalic Acid in Thin-Layer Chromatography. JÓZEF SLIWIOK, *Department of Organic Chemistry, Normal School, Katowice, Poland.*

The work on the application of the use of fuchsine dyes in the detection of various compounds by means of thin-layer chromatography has been applied to esters of *o*-phthalic.

Microchem. J. **13**, 230 (1968).

Spectral Behavior of Some Cinnamic Acids. J. MÉNDEZ AND M. I. LOJO, *CSIC, Santiago de Compostela, Spain.*

Spectral changes in the presence of a complexing reagent are reported for nine cinnamic acids, naturally occurring in the plant kingdom. The advantage of the method is emphasized for some methoxy derivatives, which are difficult to distinguish in plant tissues.

Microchem. J. **13**, 232 (1968).

Automatic Catalytic Microdetermination of Copper. GEORGE COLOVOS AND CONSTANTIN PAPADOPOULOS, *Aristotelian University of Thessaloniki, Thessaloniki, Greece.*

An automatic reaction rate method is described for the microdetermination of copper. It is based on the catalytic action of copper on the reaction of 2,4–diaminophenol with hydrogen peroxide. Procedures for the determination of 0.2–12.0 μg copper per ml are given.

Microchem. J. **13**, 236 (1968).

The Application of Fuchsine Dyes in the Detection of Barbituric Acid Derivatives in Thin-Layer Chromatography. JÓZEF ŠLIWIOK, *Department of Organic Chemistry, Normal School, Katowice, Poland.*

This paper is the continuation of a study involving the use of a new fuchsine dye for the detection of organic compounds by means of thin-layer chromatography.

Microchem. J. **13**, 245 (1968).

Analysis of Multicomponent Chromium Mixtures. H. KHALIFA AND B. ATEYA, *Cairo University, Faculty of Science, Giza, Egypt, U.A.R.*

Multicomponent mixtures containing invariably chromium are successfully analyzed, by potentiometric back-titration of excess EDTA with mercury(II) using silver amalgam as indicator electrode. One titration is carried out after masking chromium with citrate and the other after boiling the mixture with EDTA. In ternary and quaternary mixtures, iron(III) and copper(II) are de-

termined iodometrically, whereas mercury(II) in quaternary mixtures is determined potentiometrically with iodide, without interference from other components.

Microchem. J. **13**, 247 (1968).

A Microspectrophotometric Method for the Determination of Bilirubin. MARTHA I. WALTERS AND HORACE W. GERARDE, *St. Francis Hospital, Bronx, New York and Fairleigh Dickinson University, Teaneck, New Jersey.*

The purpose of this report is to show that it is possible to determine bilirubin in plasma containing limited amounts of hemoglobin by measuring the absorbance at a single wavelength using a Coleman Jr. spectrophotometer and plasma dilutions obtainable with the Unopette system.

Microchem. J. **13**, 253 (1968).

Microdetermination of Perchlorate as Chloride by Oxygen Flask Combustion. G. E. SECOR, B. A. RICCI, AND L. M. WHITE, *Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, California 94710.*

Oxygen flask combustion combined with a coulometric titration finish is used for a fast, simple, and accurate determination of total chlorine in perchlorate compounds. Respective amounts of chloride–, chlorate–, and perchlorate ions present together in a solution can be determined as chloride by analyzing three separate aliquots.

Microchem. J. **13**, 273 (1968).

A New Rapid Microassay for Enzymatic Reactions Involving Glyoxylate. PHILIP FURMANSKI, HENRY C. REEVES, AND SAMUEL J. AJL, *Department of Biochemistry, Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19141.*

A rapid, microassay for enzymatic reactions involving glyoxylate is discussed. This assay is based on the quantitative determination of residual glyoxylate—^{14}C following isolation by thin-layer chromatography.

Microchem. J. **13**, 279 (1968).

The Separation of Copper and Iron Valencies by Paper Chromatography. AGNES PAUL AND P. B. JANARDHAN, *Department of Analytical and Inorganic Chemistry, Madras University, Madras, South India.*

This paper is a study of the mechanism of separation of copper and iron oxidation states leading to an explanation of the differential migration of the different valency states.

Microchem. J. **13**, 286 (1968).

Methods for the Isolation and Characterization of Constituents of Natural Products. VIII. Gas-Liquid Chromatographic Resolution of Alcohol Ester, Amide, and Thioester Derivatives of Pyruvic Acid 2,6–Dinitrophenylhydrazone. R. Bassette, C. R. Brewington, and D. P. Schwartz, *Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C. 20250.*

Gas-liquid chromatographic techniques have been employed for the resolution of homologous series of primary, secondary, and tertiary alcohol ester– primary amine amide– and thioester derivatives of pyruvic acid 2,6–dinitrophenylhydrazone. The derivatives can be separated isothermally or by temperature programming and can be detected by flame or electron capture detectors.

Microchem. J. **13,** 297 (1968).

Methods for the Isolation and Characterization of Constituents of Natural Products. IX. Separation of Alcohol, Primary Amine and Thiol Derivatives of Pyruvic Acid 2,6–Dinitrophenylhydrazone into Classes. D. P. Schwartz and C. R. Brewington, *Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C. 20250.*

Quantitative column and qualitative thin-layer chromatographic procedures are described for separating a mixture of alcohol, primary amine, and thiol derivatives of pyruvic acid 2,6-dinitrophenylhydrazone into classes. Magnesium oxide is used as the adsorbent in the column procedure and Silica Gel G in the thin-layer technique. The alcohols, amines, and thiol derivatives which are eluted in that order, show differences in color during chromatography which aid in their proper classification.

Microchem. J. **13,** 310 (1968).

Titrimetric Microdetermination of Alanine. O. C. Saxena, *Chemical Laboratories, University of Allahabad, Allahabad, India.*

Titrimetric microdetermination of alanine with gold chloride in alkaline medium is described.

Microchem. J. **13,** 316 (1968).

Microdetermination of L-Leucine and DL-Valine. O. C. Saxena, *Chemical Laboratories, University of Allahabad, Allahabad, India.*

L-leucine and DL-valine have been determined in micro quantities by oxidizing with gold chloride in alkaline medium. Sixteen and one-half and 13.5 atoms of

oxygen are required for complete decomposition of L-leucine and DL-valine, respectively. Excess of alkali inhibits the reaction. Interference by organic compounds is observed.

Microchem. J. **13,** 321 (1968).

Improved Instrumental Carbon, Hydrogen, and Nitrogen Analysis with Electronic Integration. JOHN H. GRAHAM, *Rohm and Haas Company, Bristol, Pennsylvania 19007.*

The use of the new Polypak gas chromatographic column in the F & M Model 185 Carbon, Hydrogen, and Nitrogen Analyzer has given improved resolution of component peaks making electronic integration now feasible. Electronic integration using the Infotronics Integrator has improved accuracy and precision as well as reducing total analysis time.

Microchem. J. **13,** 327 (1968).

MICROCHEMICAL JOURNAL 13, 171–173 (1968)

Thin-Layer Chromatography of Isomeric Steroid Oximes and Homo-Aza-Lactams

III. TLC of Estra-1, 3, 5(10)-trien-3-o1-17-one and Its Derivatives

B. MATKOVICS AND GY. GÖNDÖS

Institute of Organic Chemistry, "József Attila" University, Szeged, Hungary

Received June 13, 1967

As a continuation of our previous study in TLC line (*1, 6*) we shall consider the TLC separation of estrone its ethers and acetate as well as their oximes and their rearrangement products namely the D-homo-aza-lactams. Comparative examinations and structural identification of homo-aza-lactams occurring in Beckmann rearrangement and Schmidt reaction were described in some of our earlier publications (*5, 7*). The D-homo-aza-lactams derived from the esterone and estrone 3-methyl ether, were described by Regan and Hayes (*9*) and we too, carried out the completion of these examinations (*4*).

Numerous papers, summaries, and books (*2, 3, 8*) describe the TLC separation of estrone and its derivatives occurring in nature due to their biological importance. Consequently our paper can furnish a completion from a preparative organic chemical viewpoint concerning the separation of the above mentioned rearrangement products.

MATERIALS AND METHODS

Compounds

Estra-1,3,5(10-trien-3-ol-17-one, mp 269–271°C (I); estra-1,3,5(10)-trien-3-ol-17-one, mp 252–253°C (II); 17-keto-17*a*-aza-D-homo-1,3,5 (10)-estratrien-3-ol, mp 353–354°C (III); 3-methoxy-estra-1,3,5(10)-trien-17-one, mp 160–162°C (IV); 3-methoxy-estra-1,3,5(10)-trien-17-one oxime, mp 196–198° C (V); 3-methoxy-17-keto-17*a*-aza-D-homo-estra-1,3,5(10)-trien, mp 224–226°C (VI); 3-*c*.pentyloxy-estra-1,3,5 (10)-trien-17-one mp 152–153°C (VII); 3-*c*.pentyloxy-estra-1,3,5, (10)-trien-17-one oxime, mp 173–175°C (VIII); 3-*c*.pentyloxy-17-keto-17*a*-aza-D-homo-estra-1,3,5,(10)-trien mp 246–247°C (IX); estra-1,3,5 (10)-trien-3-acetate-17-one, mp 120–121°C (X); estra-1,3,5(10)-trien-

3-acetate-17-one, mp 246–248°C (XI); 17-keto-17*a*-aza-D-homo-estra-1,3,5(10)-trien-3-acetate, mp 140–141°C (XII); 3-acetoxy-13,17-seco-1,3,5(10),13(18)-estra-tetraen-17-nitril, mp 190–192°C (XIII); 13,17-seco-1,3,5(10),13(18)-estra-tetraen-3-ol-17-oic acid, mp 160°C (XIV).

Solvent Systems

(1) Cyclohexane–chloroform–acetic acid (70:20:10; v/v); (2) benzene–methanol (95:5; v/v); (3) benzene–ether (50:50; v/v); and (4) *n*-heptane–acetone (2:1; v/v).

Spraying Reagents

(A) Orthophosphoric acid (sp gr 1.70)–water (1:1; v/v); Drying: 20 minutes at 120°C and tested in ultraviolet light; (B) J_2-vapor; and (C) conc sulfuric acid. On the glass plates (mostly 20 × 20 cm) 250 mμ Kieselgel G (according to Stahl; Merck) layers were used standardized in the usual manner (*1, 6*).

The substances were applied in a chloroform solution in about 60 μg amounts.

In ascending systems the running time is about 40–90 minutes.

TABLE 1

SEPARATION OF ESTRONE AND ITS DERIVATIVES

Compounds	R_f values			
	Solvent systems			
	1	2	3	4
I	0.33	—	—	0.31
II	0.35	0.26	0.64	0.26
III	—	0.33	—	0.35
IV	0.36	—	0.94	0.49
V	0.39	0.60	—	0.36
VI	0.42	0.34	0.05	0.50
VII	0.45	1.0	0.90	0.60
VIII	0.45	0.80	0.93	0.27
IX	0.40	0.40	0.04	0.25
X	0.48	0.92	0.95	0.42
XI	0.35	—	0.61	0.26
XII	0.43	0.78	0.45	0.23
XIII	0.40	0.54	—	0.20
XIV	0.12	0.09	—	0.06

DISCUSSION

The results of TLC are seen in Table 1. It shows clearly that the solvent systems 1–4 are suitable to separate the estrone and its derivatives, however, solvent 4 seems to be the most favorable for separation.

The TLC separation on Kieselgel G is extremely adequate for the separation of the products of the Beckmann rearrangement and Schmidt reaction, namely the D-homo-aza-lactams and the starting materials, the ketones and their oximes.

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MICROCHEMICAL JOURNAL 13, 174–177 (1968)

Thin-Layer Chromatography of Steroid Ketones and Their Derivatives

IV. TLC of Bile Acids, Keto-bile Acids and Their Derivatives

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The polarity of the bile acids are similar to that of the corticoids. Concerning the thin-layer chromatographic separation of the bile acids and their peptides, only few detailed data are found in the literature (*1,3,5*). The most efficient solvents used in the separation are strong hydrophylic ones such as alcohols and water.

The paper chromatographic separation and quantitative determination of the bile acids has been initially investigated by Sjövall (*4*).

In order to separate the ketoxime isomers of the bile acids from the starting material, keto-bile acids and their hydrolysis products, experiments have been carried out with solvents which have been previously used for the separation of these compounds.

MATERIALS AND METHODS

Compounds. 3α-, 6α-dihydroxy-cholanic acid, mp 197°C (I); 3α-, 6α-dihydroxy-methyl-cholanate, mp 71–72°C (II); 3α-hydroxy-6-keto-methyl-cholanate, mp 130–31°C (III); 3α-acetoxy-6-keto-methyl-cholanate, mp 99–100°C (IV); 3α-acetoxy-6-one-oxime-methyl-cholanate, mp 160°C (V); 3α-acetoxy-cholanic-acid-6-one-oxime, mp 110–112°C (VI); 3α-acetoxy-6-one-oxime-methyl-cholanate, mp 166–167°C (VII); 3α-, 7α-, 12α-trihydroxy-cholanic acid, mp 198°C (VIII); 3α-, 7α-, 12α-trihydroxy-methyl-cholanate, mp 151–152°C (IX); 3α-, 12α-, dihydroxy-7-keto-methyl-cholanate, mp 144–145°C (X); 3α-, 12α-diacetoxy-7-keto-methyl-cholanate, mp 115°C (XI); 3α-, 12α-dibenzoyloxy-7-keto-methyl-cholanate, mp 91°C (XII); 3α-, 12α-diacetoxy-methyl-cholanate-7-one-oxime, mp 99–100°C (XIII); 3α-, 12α-dibenzoyloxy-methyl-cholanate-7-one-oxime, mp 97°C (XIV); 3α-, 7α-dihydroxy-12-keto-cholanic acid, mp 179–187°C (XV); 3α-, 7α-dihydroxy-12-keto-methyl-cholanate, mp 154–155°C (XVI); 3α-, 7α-

diacetoxy-12-keto-methyl-cholanate, mp 174–175°C (XVII); 3α-, 7α-diacetoxy-methyl-cholanate-12-one-oxime, mp 98–100°C (XVIII); 3α-, 12α-dihydroxy-cholanic acid, mp 136–137°C (XIX); 3α-, 12α-dihidroxy-methyl-cholanate, mp 80–81°C (XX); 12α-hydroxy-3-keto-cholanic acid, mp 100–110°C (XXI); 12α-hydroxy-3-keto-methyl-cholanate, mp 144–145°C (XXII); 12α-acetoxy-3-keto-methyl-cholanate, mp 118–119°C (XXIII); 12α-acetoxy-methyl-cholanate-3-one-oxime, mp 74–75°C (XXIV).

Solvent systems. (1) Cyclohexane–chloroform–acetic acid (70:20:10; v/v); (2) benzene–methanol (95:5; v/v); (3) benzene–methanol (90:10; v/v); (4) toluene–acetic acid–water (50:5; v/v); and (5) toluene acetic–acid–water (5:1:1; v/v).

Spraying reagents. (A) Orthophosphoric acid (sp gr 1.70)–water (1:1; v/v); (B) J_2-vapor; and (C) Phosphotungstic acid reagent (fresh solution of 1.5 g phosphotungstic acid in 100 ml ethanol); drying: 5 minutes at 120°C.

Method. The same technique was used as detailed earlier (2).

DISCUSSION

The results are shown in Table 1. The thin-layer chromatography (TLC) data concerning the derivatives of different bile acids are separated into groups. The series of the TLC data I–VII related to 3α-, 6α-dihydroxy-cholanic acid (hyodeoxycholic acid) and its derivatives; VIII–XVIII refer to 3α-, 7α-, 12α-trihydroxy cholanic acid (cholic acid) and its derivatives; and XIX–XXIV are connected with 3α-, 12α-dihydroxycholanic acid (deoxycholic acid) and its derivatives.

As shown in Table 1, solvent system 3 is most suitable for the separation of hyodeoxycholic acid and its derivatives. Where no figure is given, the R_4 value was zero or there was no movement of the compound on the thin-layer.

The data presented in Table 1 shows the effectiveness of the separations achieved with different solvent systems. For example, compounds XIII and XVIII are not separated in systems 2 and 4, but are separated in systems 1 and 5. For the separation of deoxycholic acid and its derivatives, system 3 was the best suited.

The simplest and best system for these separations consists of a mixture of a nonpolar and a polar solvent, e.g., benzene:methanol,

TABLE 1

SEPARATION OF BILE ACIDS, KETO-BILE ACIDS, AND THEIR DERIVATIVES

Compounds	R_f values. Solvent systems 1	2	3	4	5
I	0.05	—	0.07	0.62	—
II	0.08	0.07	0.26	0.53	—
III	0.13	0.27	0.40	0.55	0.09
IV	0.46	0.96	0.95	0.92	0.32
V	0.29	0.88	0.92	0.72	0.29
VI	0.10	—	0.08	0.60	0.07
VII	0.32	0.46	0.62	0.77	0.27
VIII	—	—	—	0.46	—
IX	—	0.07	0.21	0.52	—
X	—	0.13	0.27	0.58	—
XI	0.24	0.81	0.87	0.70	0.27
XII	0.23	0.78	0.87	0.72	0.28
XIII	0.26	0.50	0.62	0.70	0.22
XIV	0.27	0.51	0.74	0.71	0.17
XV	—	—	—	0.47	—
XVI	0.07	0.12	0.25	0.54	—
XVII	0.36	0.62	0.89	0.68	0.28
XVIII	0.38	0.50	0.75	0.69	0.27
XIX	0.08	—	—	0.59	—
XX	0.23	0.20	0.38	0.61	—
XXI	0.13	—	0.05	0.60	—
XXII	0.21	0.15	0.34	0.65	—
XXIII	0.41	0.77	0.90	0.67	0.26
XXIV	0.42	0.44	0.63	0.69	0.22

benzene:acetone, etc., where the nonpolar percentage is much higher (90–95%) than that of the polar. These systems were found to be satisfactory for the separation of homo-aza-bile acid lactams.

In summary, from our results it has been possible to compile a comprehensive table of thin-layer separations of the bile acids and their derivatives.

Solvent system 4 was found to be the most suitable for the TLC separation of most of the derivatives. At any rate, each of the enumerated systems, 1–5 may be used to separate the different groups of bile acids and their derivatives.

SUMMARY

Solvent systems are described for the thin-layer separation of bile acids and their derivatives. Mixtures of nonpolar and polar solvents, containing a high percentage of nonpolar solvent, are most suitable.

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MICROCHEMICAL JOURNAL 13, 178–182 (1968)

Titrimetric Microdetermination of Glycine

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Received August 22, 1967

INTRODUCTION

Literature concerning the determination of glycine is quite plentiful. Glycine has been determined by oxidation with potassium permanganate (*11*), by oxidation with hypochlorite (*10*), in protein hydrolysates with ceric sulfate (*8*), potentiometrically (*5*), with dichromate heat of dilution method (*9*), colorimetrically (*7*), spectrophotometrically (*1, 3*), by oxidation with chromic acid (*4*), chromatographically (*6*). Besides these there are other methods for the determination of glycine.

The present method deals with the determination of glycine in micro amounts with gold chloride in alkaline medium. The reaction mixture consists of glycine added to a known excess of gold chloride and sodium hydroxide. Remaining excess of gold chloride is titrated (12) by acidifying and adding an excess of potassium ferrocyanide, then the remaining potassium ferrocyanide is titrated against a standard solution of ceric sulfate using *N*-phenyl anthranilic acid as indicator. Probably the following reaction takes place:

$2CH_2(NH_2)COOH + 6HAuCl_4 + 24NaOH \rightarrow N_2 + 4CO_2 + 6Au + 24NaCl + 20H_2O$. From the above equation it appears that the equivalence is 9, i.e., 4.5 atoms of oxygen are required for complete oxidation. It has also been observed that excess of sodium hydroxide inhibits the reaction.

EXPERIMENTAL METHOD

Chemicals employed. Glycine (E. Merck grade); gold chloride (Palmston's grade); potassium ferrocyanide (ANAL-AR B.D.H. grade); ferrous ammonium sulfate (ANALAR B.D.H. grade); sodium hydroxide (E. Merck grade); sodium carbonate (ANALAR B.D.H. grade); sulfuric acid (ANALAR B.D.H. grade); ceric sulfate (Tech-

nical B.D.H. grade); and *N*-phenyl anthranilic acid (B.D.H. grade sample).

The 0.0031 *N* ceric sulfate (in 8 *N* H_2SO_4) is standardized by titrating against a standard solution of ferrous ammonium sulfate (in 1 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

The 0.0170 *N* potassium ferrocyanide (aqueous solution is standardized against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

The 0.0133 *N* gold chloride solution is standardized by adding a known excess of a standard solution of potassium ferrocyanide and then titrating the remaining potassium ferrocyanide solution against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

Procedure

The reaction mixture, comprised of a known solution of glycine, a known excess of a standard solution of gold chloride, and sodium hydroxide and distilled water, is put on a hot plate at full heat (keep in mind that the reaction mixture should not evaporate) for about 110 minutes. If the volume of the reaction mixture is reduced to about 5–8 ml (much before 110 mts.) add 20 ml of distilled water. Separate beakers containing the reaction mixture must be covered with beaker covers. After cooling at room temperature the metallic gold precipitated, corresponding to the glycine oxidized, is filtered off and thoroughly washed with distilled water. The remaining gold chloride (unused) solution in the filtrate is acidified and titrated back with a known excess of a standard solution of potassium ferrocyanide. Then the remaining excess of potassium ferrocyanide is titrated against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator. At the end point, a red brown color appears sharply.

RESULTS AND DISCUSSION

Results are shown in Table 1. The range of values estimated for glycine is from 0.0149 to 0.0377 mg/liter. The reaction between glycine and gold chloride in alkaline medium becomes complete at pH 9–11. Resulting products are N_2, CO_2, and H_2O. This method gives accurate results and glycine can be determined in micro amounts.

TABLE I

DETERMINATION OF GLYCINE

Glycine (ml)	NaOH (ml)	H_2O (ml)	$HAuCl_4$ (ml)	$K_4Fe(CN)_6$ (ml)	$Ce(SO_4)_2$ (ml)	$Ce(SO_4)_2$ used corresponds to oxidized glycine (ml)	Glycine found (mg/liter)
0.005 *N*	0.1 *N*	H_2O	0.0133 *N*	0.0170 *N*	0.0031 *N*	0.0031 *N*	
—	—	—	—	2	11.00	—	—
—	—	—	2	2	2.40	8.60	—
0.04	10	20	2	2	2.98	0.58	0.0149
0.06	10	20	2	2	3.28	0.88	0.0229
0.08	10	20	2	2	3.58	1.18	0.0304
0.10	10	20	2	2	3.86	1.46	0.0377

Interference is observed if acetone, alcohol (CH_3CH_2OH), methanol, glycols, aldehydes, antipyrine, and other aminoacids are present.

SUMMARY

Titrimetric microdetermination of glycine by oxidizing with gold chloride in alkaline medium is described. Excess of gold chloride is added to glycine in presence of excess of alkali. Remaining excess of gold chloride is titrated back by acidifying and adding a known excess of potassium ferrocyanide then the remaining excess of potassium ferrocyanide is titrated against a standard solution of ceric sulfate using *N*-phenyl anthranilic acid as indicator. Complete oxidation requires 4.5 atoms of oxygen, and at pH 9–11 the reaction is rapid. About 110 minutes are required for complete oxidation when the hot plate is at full heat. The reaction is carried out in separate beakers. The range of values estimated for glycine is from 0.0149 to 0.0377 mg/liter.

ACKNOWLEDGMENTS

Authors are grateful to Dr. Bal Krishna, Dr. M. P. Singh, and Dr. M. N. Srivastava for their kind support and encouragement; and to the C.S.I.R. (Govt. of India) for providing financial assistance.

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A Micromethod for Silica-Gel Column Chromatography of Urinary Organic Acids

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INTRODUCTION

Ever since its introduction by Martin and Synge (5), silica-gel chromatography has become an important tool for the separation and purification of chemical compounds. One of the earlier workers, Isherwood (2), successfully used it for the separation of the organic acids of fruits. Later, Donaldson, Tulane, and Marshall (*1*) modified the original method by the introduction of an automatic, continuous gradient elution technique. More recently, this method, as reported here, has been improved and simplified in the authors' laboratory and has been used successfully in the separation and quantitative estimation of the levels of different urinary organic acids under various physiological as well as pathological states.

EXPERIMENTAL METHOD

Preparation of urine samples for extraction. All urine specimens are collected during a 24-hour period and kept refrigerated. One g of sodium chloride is added to a 10-ml aliquot of rat urine, or to a 25-ml aliquot of urine from human subjects and the samples are lyophilized at a temperature of —60°C and a reduced pressure of 50 μ. The dried samples are stored in a freezer at —12°C until ready for extraction.

Preparation of acid tert-amyl alcohol. Four ml of water is pipetted into a 100-ml volumetric flask and cooled in an ice-bath. From a burette, 6 ml of concentrated sulfuric acid are added into the flask while it is still cold. The flask is kept cool, and freshly distilled *tert*-amyl alcohol is added to bring the volume to exactly the 100-ml mark. This solution is kept refrigerated until ready for use.

Extraction procedure. The residue obtained after lyophilization of the urine sample is made into a fine powder, to which 10 ml of acid *tert*-amyl alcohol is added and the mixture is shaken by an automatic wrist-action shaker (250 oscillations per minute) for 8 hours. The sample can be stored overnight in a refrigerator. Then, *tert*-amyl alcohol is added carefully to a total volume of exactly 200 ml. The container is shaken again for 4 hours, then the contents are centrifuged at 5000 rpm. The supernatant contains the organic acids which are chromatographed by the silica-gel chromatography method described below.

Assembly of the apparatus for column chromatography. The general assembly of the column chromatography apparatus is shown in Fig. 1. Into each of 180 clean Pyrex test tubes (18 × 150 mm) are placed exactly 2 ml of distilled water by means of a constant-volume automatic pipette, set to deliver 2 ml. The test tubes are then transferred to the rack of an automatic fraction collector, having an attached drop-counter unit. The purpose of the water is to retard the evaporation of the organic solvent as well as to facilitate the titration of the acids. Into Flask A are poured 450 ml of water-saturated chloroform. While the curved ends of Tubes I and H are held beneath the surface of the chloroform, 50 ml of water are added to Flask A. The stopcock at I is closed, while stopcock E is opened. Tube H is completely filled with chloroform by blowing on Tube L and immediately closing stopcock when Tube H is filled. Flask A is stoppered tightly and the stopper is held in position by fastening it with a string. To Flask B is added 750 ml. of 60% *tert*-amyl alcohol in chloroform. The *tert*-amyl alcohol–chloroform mixture is previously saturated by shaking it with 220 ml of water in a 1500-ml separatory funnel. After separation, the organic layer is removed and filtered on Whatman No. 1 filter paper to remove the excess water.

Preparation of the column. A Pyrex glass column (12 × 360 mm), fitted with a sintered-glass disk of medium porosity at the bottom, is ideal for this type of column chromatography. A disk of Whatman No. 3 filter paper, the same size as the above sintered-glass disk, is placed at the bottom of the column. The whole assembly is wetted by water-saturated chloroform. Silicic acid (100 mesh) is dried in an electric oven at 98°C for 3 days. Nine g of this dried, hot silicic acid is weighed in a 150-ml beaker, and 5.5 ml of 0.05 *M* sulfuric acid are

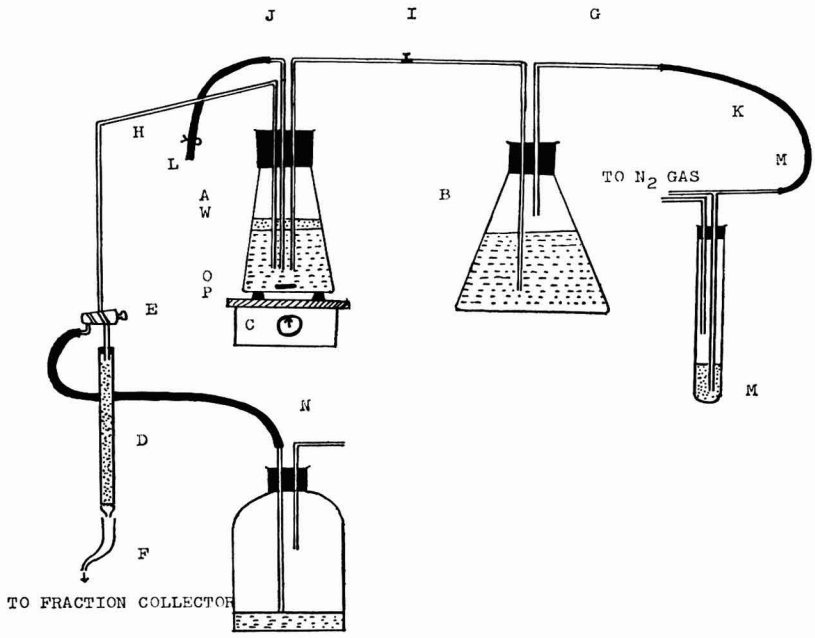

FIG. 1. Column chromatography apparatus: A. 500-ml wide-mouth Erlenmeyer flask; B. One-liter widemouthed Erlenmeyer flask; C. Magnetic stirrer; D. Silica-gel column; E. Two-way stopcock; F. Adaptor; G., H., I., J. Connection glass tubings; K., L. Rubber tubings; M. Mercury safety valve; N. Drainage tube; O. Corks; P. Asbestos plate; and W. Distilled water.

added immediately and thoroughly mixed with a glass rod to a homogenous, granular powder. To this hydrated silicic acid, a total of 60 ml of water-saturated chloroform are added slowly with continuous stirring until a uniform slurry is formed.

The slurry is poured down the column, filled up to the top each time and allowed to settle down with the aid of compressed air, until the chloroform layer almost coincides with the top of the silica gel. The previously extracted urine sample, containing the organic acids to be determined, is slowly poured down the top of the column. Again, the mixture is forced down, using gentle air pressure, until its meniscus coincides with the top of the silica gel. A filter paper disk is placed at the top of the column, which must be leveled. Next, 2 ml of water-saturated chloroform is placed on the column and forced

down, using air current, until its level almost coincides with the top of the silica gel. The column is filled to the top with water-saturated chloroform and attached to the rest of the apparatus through tubing H.

A tank of nitrogen gas is used to maintain a constant pressure of approximately 80 mm Hg in the flasks and the column. Experience has shown that a micropump (Buchler) can also be used to maintain a constant flow of solvent through the column. The solvent system coming out of Flask A is fed into the micropump, which is adjusted to maintain a flow rate of 24 drops per minute. The time needed to collect the desired 180 drops of effluent can be accurately set with an automatic "timer" unit on the fraction collector.

Titration of organic acids. After all of the 180 test tubes have been filled with 180 drops in each, the collecting tubes are removed from the fraction collector and titrated against 0.0200 *N* sodium hydroxide by an automatic titrator (Aminco), using phenol red or any other suitable indicator. A true titration value for each fraction is obtained by subtracting a baseline value from a recorded titration value. This "baseline value" is obtained when the entire procedure is carried out without the adding of acids to the column, but substituting a "blank" sample.

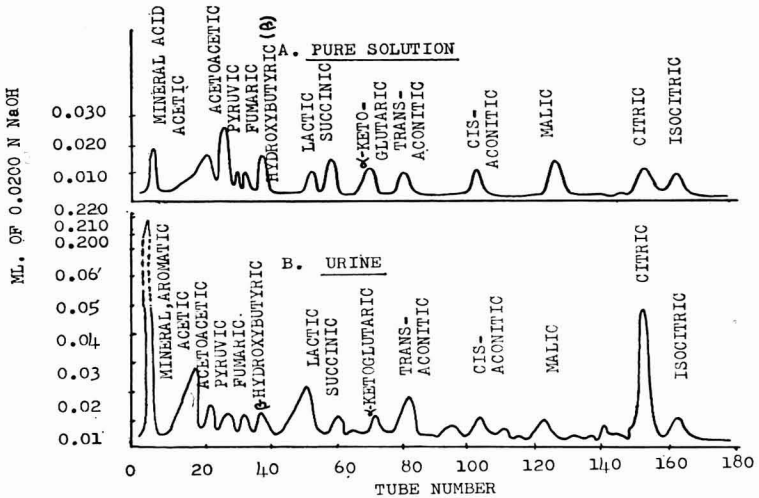

FIG. 2. Placement of acids; A. pure solution; B. urine.

Chromatography of a mixture of organic acids. Before using the above procedure for urine samples, in the authors' laboratory known mixtures of pure organic acids have also been successfully chromatographed by this method. For this purpose, a mixture of small amounts of known organic acids is prepared in *tert*-amyl alcohol. One ml of this mixture is taken and diluted with 1 ml of water-saturated chloroform, and it is placed in the column and chromatographed as described above. This procedure, of course, serves to determine the collecting tube placement of emerging peaks of each known organic acid, as well as to give the percentage "recovery" of each acid. The identity of each known organic acid isolated by the above procedure is confirmed by paper chromatography, using the method described by Ladd and Nossal (*3*).

RESULTS AND DISCUSSION

Figure 2A is a typical graphic representation of a chromatogram showing positions of the various organic acids separated from pure solution. The graph shows that separation was good for most of the organic acids. It appears that the less polar organic acids have a tendency to be eluted earlier from the column compared to the more polar ones, which come out in later fractions. It should be noted that there was a clear separation, even of *cis*- and *trans*-aconitic, and citric and isocitric acids, which are rather difficult to separate by other current methods (*4, 6*). The percentage recoveries of the acids from pure solutions are given in Table I. This table shows that there is an excellent recovery of all of the organic acids, with the exception of oxalosuccinic (data not reported) and oxaloacetic acids, which probably decompose quickly or are converted to some other intermediates.

To confirm the positions and homogeneity of the respective fractions of acids, an additional proof of their identity was needed. Although rechromatographing on the silica-gel column could serve this purpose, paper chromatography as a supplementary tool for identification and also for separation was chosen, because of its simplicity and high sensitivity. By developing chromatograms of known mixtures of acids, along with those of the extracts, and by reference to the R_f values determined for single acids, the acids in the mixtures and extract were characterized. The R_f values of the different organic acids, obtained by using Ladd and Nossal's method (3) are given in Table 2. Special

TABLE I

RECOVERIES OF ORGANIC ACIDS FROM MIXTURES OF 12 ACIDS

(μg)

Acid	Added	Recovered	Percentage recovered
	Mixture 1		
Fumaric	75	74	98.5
Succinic	135	134	99.5
Malic	140	140	100.0
Pyruvic	190	185	97.5
Lactic	20	19	95.0
cis-Aconitic	118	115	94.5
Citric	47	46	98.5
Oxaloacetic	100	71	71.0
α-Ketoglutaric	32	30	93.5
Isocitric	46	44	96.0
β-Hydroxybutyric	42	40	95.5
Acetic	146	145	99.5
	Mixture 2		
Fumaric	150	147	98.0
Succinic	70	70	100.0
Malic	75	74	98.5
Pyruvic	29	27	93.0
Lactic	60	59	98.3
cis-Aconitic	40	38	95.0
Citric	120	122	103.0
Oxaloacetic	58	40	69.0
α-Ketoglutaric	108	104	96.5
Isocitric	23	22	95.6
β-Hydroxybutyric	25	23	92.0
Acetic	70	70	100.0

notice should be taken of the fact that the R_f value of an individual organic acid was identical, whether pure acid or the mixture of pure acid, or organic acids separated from the urines were chromatographed. The data obtained by paper chromatography not only confirm the identity of the acids, but also show that separation by column chromatography, as outlined earlier, was complete. The appearance of only one spot, when a mixture of the acids and the corresponding pure acids were applied to paper, is further evidence of the identity of the acid. Likewise, it can be argued that the homogeneity

TABLE 2

R_f VALUES OF THE ACIDS

Acid	Pure acid	Separated by column chromatography from mixtures of pure acids	Separated from urine
A. Acidic solvent			
Acetoacetic	0.83	0.83	0.83
Fumaric	0.81	0.81	0.81
β-Hydroxybutyric	0.73	0.73	0.73
Lactic	0.76	0.76	0.76
Succinic	0.74	0.74	0.74
α-Ketoglutaric	0.63	0.62	0.62
trans-Aconitic	0.67	0.68	0.67
cis-Aconitic	0.70	0.70	0.71
Malic	0.46	0.47	0.47
Citric	0.41	0.41	0.41
Isocitric	0.44	0.44	0.44
B. Alkaline solvent			
Acetic	0.06	0.06	0.06
Pyruvic	0.08	0.08	0.08
Acetoacetic	0.11	0.11	0.10

of the separated acids from columns is proved by the fact that not more than one spot on the chromatogram could be observed.

The method of silica-gel chromatography was next extended to the separation and quantitative estimation of the levels of different organic acids in normal human urine and normal rat urine. The presence in the urine of stable acids has been previously well established (*4, 6*). The data obtained on the human urine samples and the rat urine samples in the present study are presented in Tables 3 and 4, respectively. A graphic representation of the pattern of the urinary organic acids in human urine is also shown in Fig. 2B.

Having established the levels of the organic acids in the normal urine samples, recovery experiments were performed as before. In brief, known amounts of the acids, as their sodium salts, were added to exactly 100 ml of the same 24-hour human urine specimen. The volume was diluted to exactly 250 ml in a volumetric flask. A 25-ml aliquot of the resulting solution was used for analysis. The results of the recovery, presented in Table 5, clearly indicate that the method

TABLE 3

ORGANIC ACIDS IN TYPICAL HUMAN URINE SAMPLES
(mg/24 hours)

Acid	I	II
A. Non-Krebs cycle acids		
Lactic	198	136
β-Hydroxybutyric	34	101
Pyruvic	30	34
Acetoacetic	90	49
Acetic	37	49
B. Krebs cycle acids		
Citric	514	550
Aconitic	36	79
Isocitric	75	66
α-Ketoglutaric	71	51
Succinic	13	35
Fumaric	32	40
Malic	21	44

TABLE 4

ORGANIC ACIDS IN TYPICAL RAT URINE SAMPLES
(mg/24 hours)

Acid	I	II
A. Non-Krebs cycle acids		
Lactic	16	15
β-Hydroxybutyric	1	1
Pyruvic	2	2
Acetoacetic	4	5
Acetic	1	1
B. Krebs cycle acids		
Critic	44	30
Aconitic	1	1
Isocitric	1	2
α-Ketoglutaric	1	1
Succinic	10	11
Fumaric	2	3
Malic	1	1

TABLE 5

RECOVERIES OF ACIDS FROM HUMAN URINE

(Av of 3 dets.)

Acid	Acid added (meq/25 ml)	Acid recovered (meq/25 ml)	Recovery %
Acetic	0.0136	0.0122	90.00
Acetoacetic	0.0057	0.0054	94.9
Pyruvic	0.0104	0.0105	100.0
Fumaric	0.0157	0.0140	89.0
β-Hydroxybutyric	0.0031	0.0028	91.0
Lactic	0.0098	0.0102	102.0
Succinic	0.0212	0.0194	91.5
α-Ketoglutaric	0.0073	0.0068	92.8
Aconitic [a]	0.0099	0.0092	92.7
Malic	0.0143	0.0141	98.5
Critic	0.0103	0.0095	92.2
Isocitric	0.0062	0.0062	96.8

[a] Includes both *cis*- and *trans*-aconitic acid.

was quite satisfactory for the extraction of the organic acids from urine.

It is evident from the data presented that silica-gel chromatographic method could be successfully used for the separation and quantitative determination of micro-quantities of the different organic acids present in the normal urine sample. This method has also been used successfully for determining the levels of the different urinary organic acids in certain physiological and pathological states (data being published elsewhere).

SUMMARY

Silica-gel column chromatographic method for the quantitative determination of micro-quantities of 12 different, physiologically most important, organic acids in mixtures, as well as in human and rat urine samples, is described. This method is simple, accurate, rapid, and has a high degree of sensitivity. It yields a high percentage of recovery and the results are reproducible.

The identity of the acids separated by this technique has been repeatedly confirmed by paper chromatography; known pure acids were compared with those eluted from the silica-gel column used in this method.

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Metal Chelates of Eriochrome Black T in Aqueous Solutions

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The use of chromogenic reagents in analytical chemistry is increasing because of their sensitivity and specificity in the microdetermination of metals. A large number of such reagents are known but to find some new chromogenic reagents for the microdeterminations of metals, a comprehensive work has been undertaken in these laboratories. Eriochrome black T also known as pentachrome black TA, Solochrome black WDFA, etc., has been extensively used for complexometric titrations of Ca and Mg. The literature on the use of this reagent in colorimetric determinations is very scarce, hence various color reactions of this reagent with different metal ions, and the conditions under which stable complexes are formed have been worked out. The present paper summarizes the results on the behavior of the reagent in aqueous solutions and also its color reactions with metals.

EXPERIMENTAL METHOD

Apparatus. A Beckman model B spectrophotometer operated on 115 V with stabilized current using 10-mm glass cells was used for the absorbance measurements. A blue sensitive photocell was used for the measurements below 600 mμ and red sensitive photocell above 600 mμ. Readings were taken against a distilled water blank.

A Leeds and Northrup direct reading pH indicator using glass calomel electrodes of the same manufacturer were used for adjusting hydrogen ion concentrations of the solutions. A Leeds and Northrup Kaulrausch slide wire with an audio frequency oscillator in the circuit, operated by 220 V/50 cycles AC mains, was used for the conductance measurements. A dip type measuring cell having a cell constant of 0.5270 was used for the purpose.

Chemicals. A freshly prepared aqueous solution of BDH sample of Eriochrome black T (abbreviated as EBT) was used for the various studies.

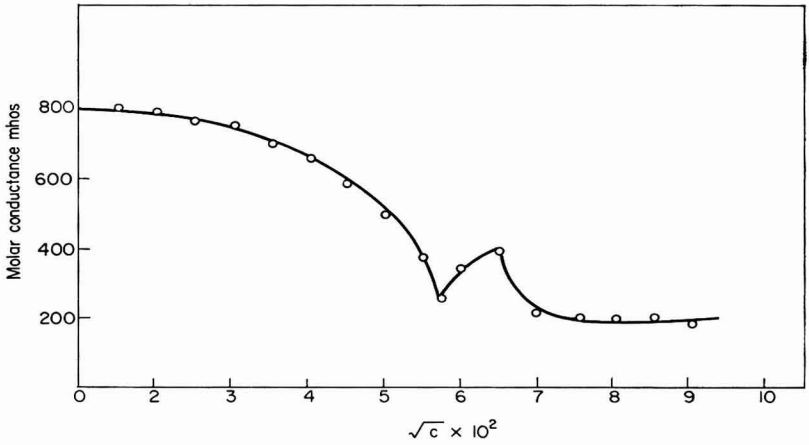

FIG. 1. Variation of molar conductance with concentration.

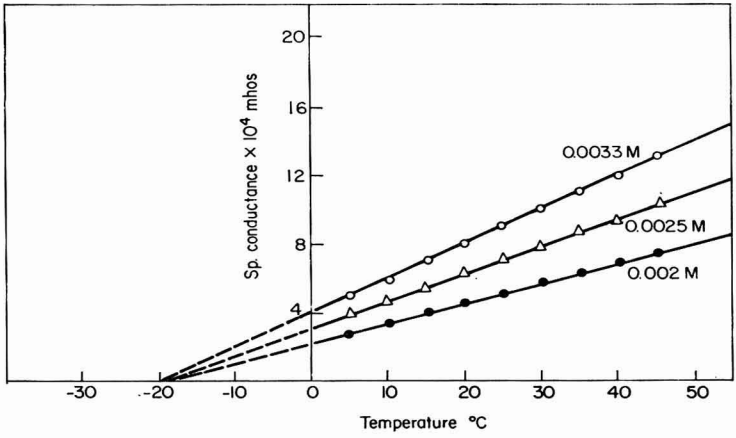

FIG. 2. Variation of specific conductance with temperature.

Standard solutions of different metal ions were prepared by dissolving the respective salts directly or by standardization. All reagents used were of AnalaR grade.

Conditions of study

All the experiments were performed at room temperature, (except where temperature is mentioned) ranging between 25 to 28°C using

freshly prepared aqueous solutions. The pH of the different solutions were adjusted using HCl or NaOH.

RESULTS AND DISCUSSION

Electrical Conductance Studies

Molar conductance of EBT. The electrical conductance of a number of solutions of EBT at various dilutions were measured at 30°C. A graph was then plotted between the square root of the concentration and the molar conductance. The curve thus obtained was not linear and resembled the curves of colloidal electrolytes as recorded by McBain (*1*) (Fig. 1).

Specific conductance of EBT. The specific conductance of EBT was determined at different temperatures between 10 and 60°C with intervals of 10° C. The specific conductance was plotted against different temperatures and by extrapolation of the curves, temperature of zero conductance was determined and this was found to be —19.8°C (Fig. 2). Mushran and Prakash (2) during their studies on the colloidal systems observed that the temperature of zero conductance of true electrolytes lies near –40°C while that of the colloidal systems ranges between –15 and –35°C. On the basis of this observation it is clear that the solution of EBT behaves as colloidal when concentrated solutions are used. The experiments were done using three different concentrations viz. 0.01 *M*, 0.005 *M*, and 0.003 *M*.

Temperature coefficient of the conductance. The temperaure coefficient per 1°C per hundred of the conductance at 35°C has also been calculated with the help of the graph obtained between the specific conductance and the temperature (Fig. 2). The value of the temperature coefficient for 1°C per hundred of the conductance has been found to be from 0.8 to 1.5. These results also serve to establish the colloidal behavior of EBT in concentrated solutions, as Shivapuri and Prakash (3) reported that usually the temperature coefficient per degree centigrade per hundred of the conductance at 35°C in colloidal systems is found to be below 2.0.

Thus the above observations and results confirm that colloidal nature of EBT exists when concentrated solutions are used, therefore, it is recommended that the physicochemical studies using this reagent must be done at very dilute solutions when the reagent behaves as a

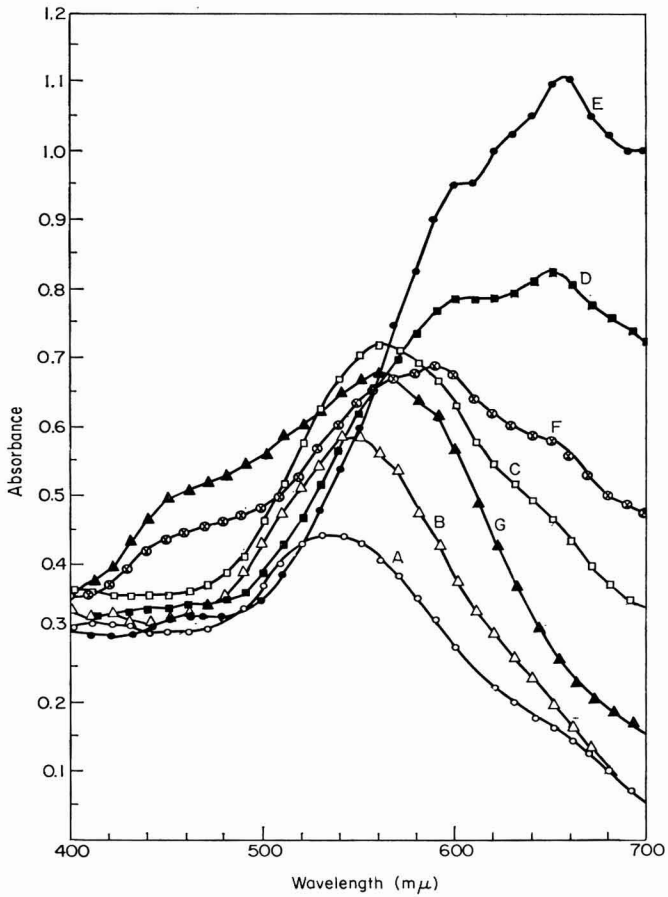

FIG. 3. Absorption spectra of EBT at different pH: A, 2.0; B, 3.9; C, 5.8; D, 6.7; E, 9.7; F, 10.4; and G, 11.0.

true electrolyte as reported by Dey (*4*) and true stoichiometry can be attained by using solutions of the order of 10^{-4} to $10^{-5}M$.

Spectrophotometric Studies

Variation of the absorption maxima of EBT with pH. Various solution of EBT to the order of 5.0×10^{-5} M were prepared at different pH's and their absorption spectra from 350 to 700 mμ were recorded. Figure 3 shows the variation of the absorption maxima of EBT with

pH. The structure of the dye indicates that the compound is ionizable and the observation on the change of λmax with pH shows that the wavelength of maximum absorption shifts as shown in Table 1.

TABLE 1

VARIATION OF λ_{max} OF EBT WITH THE CHANGE IN pH

pH range	λ_{max} (mμ)
2.0–6.0	540, 550
6.7–9.7	660
10.4–11.7	590, 560

Variation of λ_{max} of EBT Chelates with pH

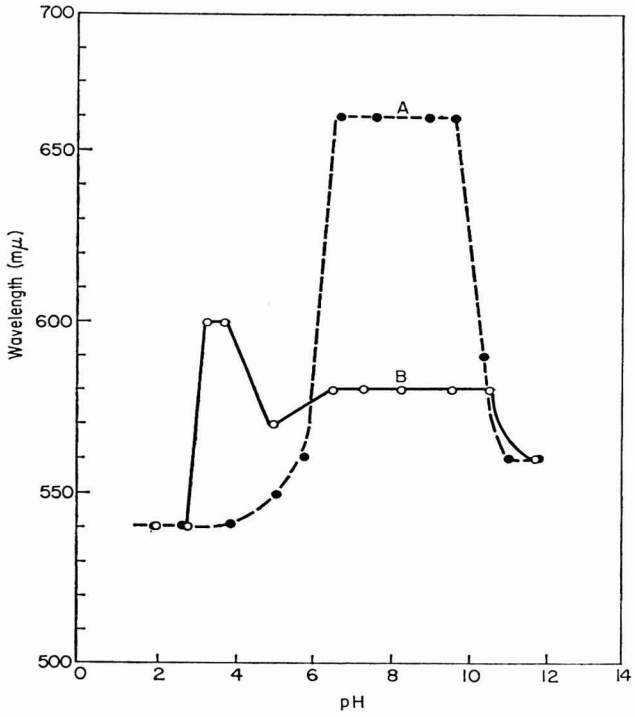

FIG. 4. Variation of absorption maxima with pH: A, EBT; and B, In—EBT Chelate.

Various solutions containing EBT (5.0×10^{-5} M) and various metal solutions (5.0×10^{-5} M) separately were prepared at different pH's ranging from 2 to 12. The change in the λ_{max} on the chelate at different pH's was noted and compared with that of the reagent for which the complete spectra in the visible range was recorded. Table 2 shows the range of pH within which a particular complex has a suitable maximum wavelength.

TABLE 2

VARIATION OF λ_{max} OF EBT CHELATE WITH pH

Cation	pH range	λ_{max} of EBT chelate (mμ)	λ_{max} of EBT (mμ)
Copper	5.8– 9.8	560	660
	1.8– 5.8	560	540, 550
Magnesium	8.4–11.0	580	660
Zinc	5.0– 6.0	570	540, 550
	6.0–11.0	570	660
Cadmium	7.8–10.2	590	660
	10.2–12.0	590	570, 560
Calcium	10.4–11.5	580	560
Aluminium	6.0–11.7	580	660
Gallium	2.0– 5.0	580	540, 550
	6.2–10.0	590	660
Indium	3.2– 4.0	630	540
	6.2–10.6	580	660
Scandium	3.8– 5.8	560	540, 550
	5.8–11.5	560	660
Yttrium	6.2–10.3	560	660
Praseodymium	7.0–10.8	550	660
Neodymium	7.0–10.8	550	660
Samarium	6.8–10.8	550	660
Thorium	6.4–10.8	570	660
Uranium	3.2– 6.0	570	540, 550
	6.0–10.2	570	660
Chromium	2.2– 5.2	570	540, 550
Iron	1.9– 3.7	560	540, 550
	6.0–10.8	540	660
Cobalt	4.8– 6.0	600	540, 550
	6.0–10.2	600	660
Nickel	4.5– 6.0	570	540, 550
	6.0–10.8	570	660
Molybdenum	1.5– 4.4	600	540, 550

The variation of λ_{max} with pH can also be studied with the help of graphs. A typical graph is given in Fig. 4. The other metals like barium, strontium, mercury, tungsten, beryllium, hafnium, and zirconium failed to give appreciable results.

Absorption spectra of the Chelates

Several mixtures containing 0:1; 1:05; 1:1; 1:2; 1:3; and 1:4 ratios of the metal and EBT were prepared at a definite pH and their absorbances were noted from 400 to 700 mμ. The λ_{max} of different

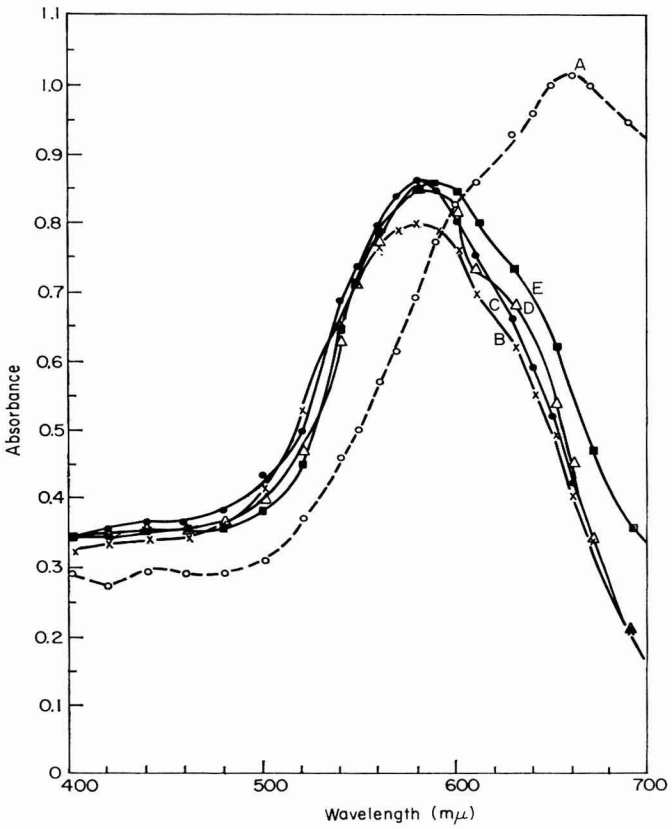

FIG. 5. Absorption spectra of In—EBT chelate at pH 7.5: A, 5.0×10^{-5} *M* EBT; B, 5.0×10^{-5} EBT; 1.0×10^{-4} *M* $InCl_3$; C, 5.0×10^{-5} *M* EBT; 5.0×10^{-5} *M* $InCl_3$; D, 5.0×10^{-5} *M* EBT; 2.5×10^{-5} *M* $InCl_3$; and E, 5.0×10^{-5} *M* EBT; 1.25×10^{-5} *M* $InCl_3$.

TABLE 3

WAVELENGTH MAXIMA OF EBT CHELATE

Cation	pH	λ_{max} of chelate (mμ)	λ_{max} of EBT (mμ)
Copper	6.8	560	660
Magnesium	9.5	580	660
Zinc	8.5	570	660
Cadmium	8.0	590	660
Calcium	10.5	580	560
Aluminium	7.5	580	660
Gallium	2.5	580	540
	7.5	590	660
Indium	3.5	630	540
	7.5	580	660
Scandium	8.0	560	660
Yttrium	8.0	560	660
Lanthanum	8.7	560	660
Praseodymium	9.5	550	660
Neodymium	8.5	550	660
Samarium	9.5	550	660
Thorium	8.0	570	660
Uranium	8.0	570	660
Chromium	3.0	570	660
Cobalt	8.0	600	660
Nickel	7.5	570	660
Molybdenum	2.5	600	540

chelates at a particular pH are recorded in Table 3. Figure 5 shows a typical graph of the In-EBT chelate at 7.5 pH.

The present work is very useful for using EBT as a chromogenic reagent for the microdetermination of different metals in solution. The detailed work on the formation constants and the analytical applications of this reagent, viz. in the spectrophotometric determinations of the metals, is in progress in these laboratories.

SUMMARY

The elementary observations on the chelate formation of Eriochrome Black T have been described. The behavior of the reagent in aqueous solution and different conditions under which stable complexes are formed have been worked

out for magnesium, zinc, cadmium, calcium, aluminium, gallium, indium, scandium, yttrium, lanthanum, praseodymium, neodymium, samarium, thorium, uranium, chromium, iron, cobolt, nickel, molybdenum, and copper metals.

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Azulene Procedure for Chromatographic Analysis of Aromatic and Heterocyclic Aldehydes, Carbohydrates, and Other Aldehyde Precursors

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INTRODUCTION

The azulene method has been used for the determination of furfural precursors in polluted air (*1*). Since these types of compounds are present in the atmosphere, methods for separating and identifying many of the individual components are needed to increase our understanding of this type of pollution. Because the compounds of interest are neither fluorescent nor colored, they are difficult to locate once they are separated.

Location methods used for furfurals and their sugar precursors include aniline-diphenylamine (*2*), anthrone (*3*), resorcinols (*4,5*), *m*-phenylenediamine (*6*), and hydrazine hydrochloride (*7*). This paper introduces a new location method for many types of conjugated aldehydes and their precursors. This method is compared with other location methods and its advantages are enumerated. Preliminary studies show that the procedure will be useful in investigating these types of compounds in polluted air.

REAGENTS AND APPARATUS [1]

Reagents and Standards

Azulene was obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin, and used without further purification. The carbo-

[1] Mention of commercial products does not constitute endorsement by the Public Health Service.

hydrates were obtained from CalBiochem and Sigma. The other chemicals used as test compounds and in sprays were obtained in the purest form possible from commercial sources; when unavailable commercially, they were synthesized in our laboratory.

The silica gel G plates (Analtech, Inc., Wilmington, Delaware) and the silica gel G F254 plates (Brinkman Instruments, Inc., Westbury, New York), both 20 × 20 cm, were coated to a thickness of 250 μ.

Whatman no. 1 paper was used in the paper chromatographic and paper electrophoretic analyses.

Apparatus

Fluorescence spectra were obtained on an Aminco-Bowman spectrophotofluorimeter equipped with a solid-state attachment, with the following settings: sensitivity 50; slit arrangement no. 2; and phototube RCA type 1P21.

The fluorescent colors were examined in a Chromato-vue cabinet (Kensington Scientific Corp., Berkeley 10, Calif.) under a 3660 Å light source.

Electrophoretic separations were done with a Gelman chamber and a 0–500-V DC power supply.

Developers for Silica Gel Thin-layer Chromatography

The main system was toluene-tetrahydrofuran (95:5, v/v); ethyl acetate–piperidine–water (8:2:1, v/v/v); *n*-propanol–water 4:1, (v/v).

Buffer for Paper Electrophoresis

The buffer contained 0.95% sodium pyrosulfite, 0.88% ammonium acetate, 0.23% acetic acid, pH 4.7.

Supplementary Reagents

When *m*-phenylenediamine was used as the reagent spray, the plates were sprayed with trifluoroacetic acid to bring out the fluorescent colors. With 4-hexylresorcinol as the reagent spray, 10 *N* NaOH was used as the final supplemental spray.

Thin-Layer Chromatographic Procedure

Except for changes in the developer and the sprays, the following technique was used in all TLC procedures, as shown by the separation of the dinitrophenylhydrazones.

The standard solutions of the dinitrophenylhydrazones and the prepared unknown dinitrophenylhydrazone extracts were spotted with a pipet 1.5 cm from the base of a glass plate coated with silica gel G. The spotted plate was placed in a glass tank containing 150 ml of toluene–tetrahydrofuran (95:5, v/v) and was developed in an ascending manner to a distance of 15 cm, in about 55 minutes. After development, the plate was removed, allowed to dry, sprayed with ethanolamine, and then examined.

Paper Electrophoresis Procedure

Whatman no. 1 paper was spotted with the samples (equivalent to about 10 μg of the compound under investigation) 2 inches from the edge of the paper. The cathode end of the paper was placed in the buffer solution so that most of the paper was wet except for the spotted end. The buffer was allowed to diffuse toward the spots from both ends. When the entire paper was wet, the separation was run at 300 V. The spots were allowed to migrate toward the cathode for about 3 hours. The pherogram was then dried and sprayed with the appropriate reagent. The visible and fluorescence colors were recorded.

Composition of Sprays

Aniline-diphenylamine spray. Solution A was 1% diphenylamine dissolved in acetone. Solution B was 85% aqueous phosphoric acid. Spray was 10 parts of A and 1 part of B.

Anthrone spray. One percent anthrone was dissolved in sulfuric acid.

Resorcinol spray. One half g of resorcinol was dissolved in 2 ml of methanol; the solution then was diluted to 25 ml with hydrochloric acid.

4-Hexylresorcinol spray. Solution A was 0.7% 4-hexylresorcinol dissolved in acetone. Solution B was 40% aqueous trichloroacetic acid. Spray was 9 parts of A and 1 part of B.

m-*Phenylenediamine spray.* One percent *m*-phenylenediamine dihydrochloride was dissolved in 76% alcohol.

9,10-Diacetoxyanthracene spray. One percent 9,10-diacetoxyanthracene was dissolved in sulfuric acid.

Azulene spray. One percent azulene was dissolved in sulfuric acid.

Guiazulene spray. One percent guiazulene was dissolved in sulfuric acid.

4,6,8-Trimethylazulene spray. One percent 4,6,8-trimethylazulene was dissolved in sulfuric acid.

Hydrazine dihydrochloride spray. One percent hydrazine dihydrochloride was dissolved in water–hydrochloric acid (4:1, v/v).

Location Procedure

After a thin-layer chromatogram was sprayed with the reagent, the immediate visible and fluorescent colors were recorded. The plate was heated in an oven for 5 minutes at 110°C, and the visible and fluorescent colors again were recorded. In addition, 10 *N* NaOH was used as the final supplemental spray with the 4-hexylresorcinol reagent, and trifluoroacetic acid was used as the final supplemental spray with the *m*-phenylenediamine reagent. When paper pherograms were sprayed with the azulene reagent, readings were made immediately. No heat was required with the use of hydrazine hydrochloride as a reagent spray on Whatman no. 1 paper, and visible and fluorescent colors were recorded directly after spraying.

Extraction of Samples of Coffee-Roast Effluent

Ether extract. Two g of a sample of coffee-roast effluent was extracted with 10 ml of acetone–water (1:1, v/v) stirred for 30 minutes, and filtered. The resulting liquid was then extracted four times with 5-ml volumes of ether. The ether layer was treated with anhydrous sodium sulfate to remove water, the ether was decanted, and the final extract was evaporated to a volume of 0.6 ml under a vacuum.

Methanol and aqueous extracts. Samples of coffee-roast effluent were extracted by Soxhlet for about 6 hours with hot methanol to prepare the methanolic extract and with hot distilled water to prepare the aqueous extract.

Preparation of 2,4-Dinitrophenylhydrazones

The 2,4-dinitrophenylhydrazone derivatives of the methanolic and ether extracts of the coffee-roast effluent sample were prepared by the following procedure. Each extract was evaporated to dryness, and

1 ml of water was added to each flask. Then, 1 ml of a 1% 2,4-dinitrophenylhydrazine solution in (1:1, v/v) water–70% perchloric acid was added to each. The precipitates that formed after 60 minutes were centrifuged, washed with 20% aqueous perchloric acid, recentrifuged, and dissolved in 0.2 ml of dimethylformamide before thin-layer chromatographic separation.

RESULTS AND DISCUSSION

Separation. Some of the thin-layer methods of separation that are useful for the various classes of compounds are described here.

For the separation of 2,4-dinitrophenylhydrazones of ketones, aldehydes, and especially furfurals, benzene-tertahydrofuran (95:5, v/v) has been used as a developer, with silica gel G as the adsorbent (8). Because of the toxicity of benzene, we substituted the less toxic toluene for our separations. Using this modified system, we could separate furfurals and their derivatives, whereas sugars and purines remained at the origin. Table 1 lists the R_f values of the compounds separated with 3-α-furylacrolein as the standard. The compounds were located by their color reactions with 1% azulene in sulfuric acid as the reagent spray. The purines were located by their development on a silica gel F254 plate, and appeared as dark nonfluorescent spots against the green fluorescent background of the plate under short-wavelength UV light. Under long-wavelength UV light, the plate shows no fluorescence. The furfurals that were separated gave the following R_f values relative to 3-α-furylacrolein (taken as 1.0): furfural, 1.07; 5-hydroxymethylfurfural, 0.18; 5-methylfurfural, 1.00; and furfuryl alcohol, 0.54. The sugars and purines were not separated, and remained at the origin.

Table 1 also lists a system used for the separation of sugars and purines. Silica gel F254 was used as the adsorbent and *n*-propanol–water (4:1, v/v) as the developer. Again 1% azulene was used as the location reagent and 3-α-furylacrolein as the standard for calculation of relative R_f values. The table shows that the sugars and purines could be separated by this system, but the furfurals could not. Thus, *n*-proponal–water (4:1, v/v) appears to be the better of the two developers for the separation of sugars and purines, and toluene–tetrahydrofuran (95:5, v/v) is better for the furfurals.

TABLE 1

COLORS WITH AZULENE AND R_f VALUES OF SOME FURFURAL AND SUGAR DERIVATIVES USING DIFFERENT SYSTEMS

Compound [a]	Si gel F254 PrOH-H_2O (4:1) Color [b]	R_f [c]	Si gel G toluene tetrahydrofuran (95:5) Color [b]	R_f [c]
Furfural		—[d]	O	1.07
Furfural barbituric acid	WP	0.70	LP	origin
Furfural MBTH [e]	LO	1.03	O	0.95
Furfural *p*-nitrophenylhydrazone	DO	1.04	O	0.66
Furfural thiobarbituric acid		—[d]	LP	origin
Furfuryl alcohol	LP	1.00	LP	0.54
3-α-Furylacrolein	DP	1.00 [f]	DP	1.00 [g]
3-α-Furylacrolein MBTH [e]	P	1.03	LP	0.95
5-Hydroxymethylfurfural	BrV	0.96	RO	0.18
5-Hydroxymethylfurfural MBTH [e]	LP	1.03	LP	0.31
5-Methylfurfural	LPk	0.98	Pk	1.00
2-Deoxyribose	Bk	0.79	LP	origin
Fructose	LPk	0.64	LPk	origin
Galactose	LPk	0.64	LPk	origin
Glucose	LPk	0.71	Pk	origin
Rhamnose	Pk	0.82	Pk	origin
Ribose	LO	0.61	LO	origin
Xylose	LO	0.79	LO	origin
Caffeine [h]	dark spot	0.71	dark spot	origin
Hypoxanthine [h]	dark spot	0.79	dark spot	origin
Theobromine [h]	dark spot	origin	dark spot	origin
Theophylline [h]	dark spot	0.84	dark spot	origin
Xanthine [h]	dark spot	origin	dark spot	origin
Purine [h]	dark spot	0.74	LBr dark spot	origin

[a] All compounds analyzed in 10-μg amounts.

[b] Colors obtained using 1% azulene in sulfuric acid as reagent spray. P=purple; O=orange; Br=brown; V=violet; Pk=pink; Bk=black; R=red; L=light; D=dark; W=weak.

[c] The R_f values are calculated as follows:

$$\frac{\text{distance compound traveled}}{\text{distance 3-α-furylacrolein traveled}} = R_f \text{ relative to 3-α-furylacrolein.}$$

[d] — = negative at 10 μg.

[e] MBTH is 3-methyl-2-benzothiazolinone hydrazone.

[f] $R_f = 0.90$.

[g] $R_f = 0.43$.

[h] Run on a silica gel F254 plate and examined under short-wavelength UV.

Separation and location methods for aromatic aldehydes were investigated. We are interested in such types of oxygenated compounds because they could be present in automotive exhausts before and even after catalytic treatment.

Aldehydes and ketones have been successfully separated by paper electrophoresis in the presence of hydrogen sulfite buffers after conversion to the hydroxysulfonic acids (9). Using Whatman no. 1 paper as the adsorbent and the sodium pyrosulfite electrophoretic buffer, we separated vanillin, p-hydroxybenzaldehyde, furfural, 5-methylfurfural, and 5-hydroxymethylfurfural and then located these compounds with the hydrazine hydrochlorine reagent.

Location. All the compounds listed in the previous section reacted with hydrazine to form azine salts, which appeared as yellow fluorescent spots on the paper. The spectra of these fluorescent spots were obtained by the use of an Aminco-Bowman spectrophotofluorimeter equipped with a solid-state attachment. Two of the fluorescence spectra thus obtained are shown in Fig. 1. The *p*-hydroxybenzaldehyde azine salt, the most sensitive, showed excitation and emission wavelengths at 388 mμ and 490 mμ, respectively, whereas the 5-hydroxymethylfurfural azine salt showed excitation and emission wavelengths at 405 mμ and 500 mμ, respectively. Spectra of the 5-methylfurfural azine salt, with $F = 405/500$, and the furfural azine salt, with $F = 400/495$, were similar to that of the salt of 5-hydroxymethylfurfural but were less sensitive.

Table 2 lists the colors and detection limits of some furfural and sugar derivatives obtained with various reagents on silica gel G plates. The results derived with azulene and its derivatives as location reagents are discussed later.

The furfurals and their derivatives reacted immediately at room temperature with all the location reagents except 4-hexylresorcinol, whereas the sugars reacted with only the anthrone and diacetoxyanthracene reagents. With 4-hexylresorcinol, only furfural barbituric acid, furfural thiobarbituric acid, and 5-hydroxymethylfurfural reacted, and the reaction was not sensitive.

With the aniline–diphenylamine, anthrone, diacetoxyanthracene, and resorcinol methods, all compounds gave positive results after being heated at 110°C for 5 minutes. In the *m*-phenylenediamine method,

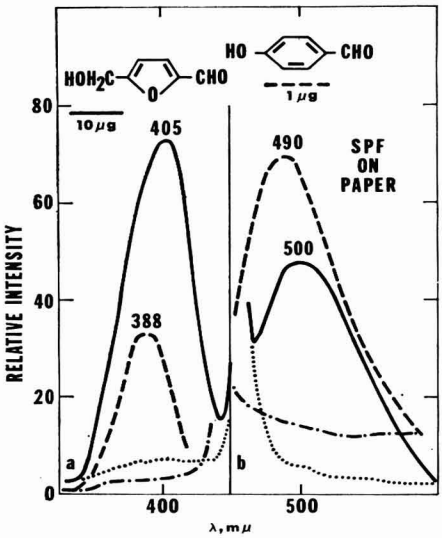

FIG. 1. Fluorescence excitation (a) and emission (b) spectra of the azine salts formed on paper after spraying with hydrazine dihydrochloride reagent. 5-Hydroxymethylfurfural (10 μg): — excitation spectrum at emiss; λ 550 mμ and MM = 0.003; — emission spectrum at exc. λ 405 mμ and MM = 0.01; ..... blank for above; F = 405/500 for 5-hydroxymethylfurfural azine salt. 4-Hydroxybenzaldehyde (1 μg): - - - excitation spectrum at emiss. λ 525 mμ and MM = 0.01; - - - emission spectrum at exc. λ 350 mμ and MM = 0.01; -.-. blank in above; F = 388/490 for 4-hydroxybenzaldehyde azine salt.

a supplementary spray of trifluoroacetic acid was needed for all the compounds to react. In the 4-hexylresorcinol method, a supplementary spray of 10 *N* NaOH was required for the furfural derivatives and sugars to react. Furfural, 5-methylfurfural, and 5-hydroxymethyl furfural 3-methyl-2-benzothiazolinone hydrazone gave negative results.

Of the methods compared here, those with anthrone and *m*-phenylenediamine appeared to be the most sensitive and that with 4-hexylresorcinol the least sensitive for the detection of these types of compounds.

All the methods were relatively simple since they required only one reagent spray (except for the 4-hexylresorcinol and *m*-phenylenediamine methods, which required a supplemental spray).

None of the methods yielded a wide range of colors; for example, in the *m*-phenylenediamine method yellow was the predominant color

TABLE 2

COLORS[a] AND DETECTION LIMITS ($\mu g/cm^2$) OF SOME FURFURAL AND SUGAR DERIVATIVES OBTAINED WITH VARIOUS REAGENTS ON SILICA GEL G

Compound	Guiazulene		4,6,8-Trimethylazulene	
	RT	110	RT	110
Furfural	—[b]	LPk-2	—	LO-3
Furfural barbituric acid	Br-2	LB-3	LY-1	0-2
Furfural MBTH[c]	—	LB-10	—	WBr-8
Furfural *p*-nitrophenylhydrazone	Y-4	WBr-10	Y-2	LBr-6
Furfural thiobarbituric acid	Y-1	LB-3	Y-1	YG-3
Furfuryl alcohol	Bk-2	LB-6	Bk-2	LB-2
3-α-Furylacrolein	LYG-2	LG-3	LYG-2	DP-1
3-α-Furylacrolein MBTH[c]	YG-2	YG-2	YG-1	YG-2
5-Hydroxymethylfurfural	WP-16	LV-4	—	LV-1
5-Hydroxymethylfurfural MBTH[c]	G-2	DP-4	G-1	DP-2
5-Methylfurfural	—	LPk-4	—	LR-4
2-Deoxyribose	Bk-2	Bk-4	Bk-2	Bk-2
Fructose	—	Br-6	—	LV-4
Galactose	—	LP-10	—	Bk-8
Glucose	—	Bk-10	—	Bk-6
Rhamnose	—	LO-10	—	O-4
Ribose	—	LP-16	—	Br-6
Xylose	—	LP-16	—	Br-6

TABLE 2 *(Continued)*

Compound	4-Hexylresorcinol			*m*-Phenylenediamine				
				RT		110		
	RT	110	10 *N* NaOH	Color	Fluor.	Color	Fluor.	TFA Fluor.
Furfural	—	—	—	LBr-13	LG-1	LV-1	LG-1	LY-1
Furfural barbituric acid	LBr-13	LBr-7	LP-7	LBr-5	WY-11	LBr-5	WY-11	WY-7
Furfural MBTH [c]	—	—	P-20	—	—	LP-3	—	LY-4
Furfural *p*-nitrophenylhydrazone	—	LY-4	Br-2	LY-4	—	LBr-2	WO-16	WO-4
Furfural thiobarbituric acid	LBr-13	LBr-7	LP-7	LY-5	WY-11	LBr-5	WY-11	WY-11
Furfuryl alcohol	—	—	LBr-16	—	LG-1	WY-13	LG-3	LG-2
3-α-Furylacrolein	—	—	LP-16	Y-2	WY-7	Y-2	Y-0.8	Y-0.6
3-α-Furylacrolein MBTH [c]	—	LBr-6	Y-4	LBr-4	—	LBr-2	WO-16	WO-4
5-Hydroxymethylfurfural	LY-36	LR-10	R-2	Y-20	LY-1	LY-20	Y-1	Y-1
5-Hydroxymethylfurfural MBTH [c]	—	—	—	LY-5	—	LBr-4	WY-10	WY-6
5-Methylfurfural	—	—	—	LY-6	—	LY-2	Y-3	WY-5
2-Deoxyribose	—	LP-7	LP-7	—	—	WY-13	G-1	G-1
Fructose	—	—	P-5	—	—	—	—	LY-6
Galactose	—	—	WP-20	—	—	LBr-20	WO-5	WY-4
Glucose	—	—	WP-15	—	—	LBr-10	WO-5	WY-4
Rhamnose	—	—	WP-20	—	—	LBr-10	WO-5	WY-4
Ribose	—	—	WP-20	—	—	LBr-10	WY-5	WY-3
Xylose	—	—	WP-20	—	—	LBr-10	WY-5	WY-2

TABLE 2 *(Continued)*

Compound	Aniline diphenylamine		Anthrone		Diacetoxyanthracene		Resorcinol	
	RT	110	RT	110	RT	110	RT	110
Furfural	LY-13	LY-6	LB-2	LG-3	LB-4	LB-4	—	LB-5
Furfural barbituric acid	LYG-6	LG-4	LG-2	LG-3	Bk-1	LG-1	LG-7	LG-5
Furfural MBTH [c]	Y-36	Y-3	—	G-3	G-4	BG-2	—	R-3
Furfural *p*-nitrophenylhydrazone	Y-6	YBr-2	Y-6	G-2	Y-2	LG-4	LY-4	RBr-1
Furfural thiobarbituric acid	LYG-5	LG-4	YG-1	LG-2	Bk-1	LBk-1	LBr-7	LP-6
Furfuryl alcohol	LY-26	Y-6	B-1	LB-1	DP-2	DP-1	LP-20	LP-2
3-α-Furylacrolein	Y-3	Y-1	LB-2	DB-1	LG-1	DG-1	P-2	B-2
3-α-Furylacrolein MBTH [c]	LY-8	YG-3	YG-4	LG-6	YG-2	LG-2	Br-4	R-2
5-Hydroxymethylfurfural	LY-26	OBr-2	LP-2	LP-2	BG-1	LG-2	R-8	R-2
5-Hydroxymethylfurfural MBTH [c]	LY-7	LG-7	Y-10	G-5	G-2	DG-2	—	LRO-4
5-Methylfurfural	LY-20	Y-5	LB-3	DB-1	BG-3	G-6	LO-36	RO-0.8
2-Deoxyribose	—	LO-8	P-2	P-2	Bk-2	DP-2	—	LO-13
Fructose	—	LO-6	B-2	LB-4	G-6	G-6	—	LPK-13
Galactose	—	LP-3	WB-13	B-2	—	G-8	—	—
Glucose	—	LP-3	—	B-2	—	G-8	—	—
Rhamnose	—	LBr-8	LB-5	DB-1	WG-16	YG-6	—	LO-10
Ribose	—	LP-6	LB-7	B-2	—	BG-2	—	LO-16
Xylose	—	LP-3	B-1	DB-1	WG-16	BG-2	—	LO-16

[a] Colors detected: O = orange; Br = brown; Y = yellow; P = purple; G = green; Pk = pink; R = red; Bk = black; V = violet; B = blue; W = weak; L = light; and D = dark.

[b] — = negative at 10 μg.

[c] MBTH is 3-methyl-2-benzothiazolinone hydrazone.

TABLE 3

LOCATION OF CONJUGATED ALDEHYDES AND THEIR PRECURSORS WITH AZULENE ON SILICA GEL AND PAPER CHROMATOGRAMS

Compound [a]	Color [c] (detection limit, $\mu g/cm^2$ spot)		
	Paper	Silica gel plate	
	after 10 min	Immediate	110°C 5 min
4-Anisaldehyde	O (4)	Pk (4)	DO (2)
Benzaldehyde	WY (13)	WY (13)	Y (5)
Cinnamaldehyde	DO (0.5)	RO (1)	RO (1)
5-Formyl-9-ethylcarbazole	YG (0.7)	OG (1)	DP (1)
5-Formylsalicylic acid	Br (7)	O (10)	LY (8)
Furfural	O (5)	LO (2)	LO (2)
4-Hydroxybenzaldehyde	O (3)	O (13)	O (0.8)
5-Hydroxymethylfurfural	O (0.7)	R (2)	DV (2)
2-Hydroxy-1-naphthaldehyde	LY (9)	Y (8)	DP (2)
2-Hydroxy-3-naphthaldehyde	LO (5)	—[b]	O (10)
Indole-3-carboxaldehyde	V (7)	—	V (10)
2-Methoxybenzaldehyde	LPk (6)	O (2)	Y (10)
5-Methylfurfural	—	Pk (10)	RO (1)
1-Naphthaldehyde	LPk (5)	—	LPk (16)
2-Naphthaldehyde	LO (2)	—	O (13)
Piperonal	Pk (0.6)	YO (6)	DO (1)
1-Pyrenealdehyde	R (7)	R (1)	DP (2)
3-Pyridinecarboxaldehyde	G (5)	—	Y (10)
Salicylaldehyde	Br (8)	O (13)	O (5)
3,4,5-Trimethoxybenzaldehyde	O (7)	O (6)	O (6)
Vanillin	Pk (0.5)	DPk (1)	DPk (1)
Veratraldehyde	R (0.7)	Pk (1)	DPk (1)
Furfural barbituric acid	Br (5)	Br (1)	LV (3)

TABLE 3 *(Continued)*

Compound [a]	Color [c] (detection limit, μg/cm² spot)		
	Paper	Silica gel plate	
	after 10 min	Immediate	110°C, 5 min
Furfural MBTH [d]	O (7)	—	Br (5)
Furfural thiobarbituric acid	LY (7)	Br (1)	LV (3)
Furfuryl alcohol	—	DP (2)	LP (2)
3-α-Furylacrolein	DP (1)	LG (1)	DP (1)
3-α-Furylacrolein MBTH [d]	Y (3)	YG (2)	YG (4)
5-Hydroxymethylfurfural MBTH [d]	Pk (4)	G (2)	DP (4)
Adenosine-5′-monophosphate	—	—	Pk (10)
Arabinose	—	—	G (2)
Deoxyadenosine	—	B (16)	B (16)
Deoxycytidine	—	—	Pk (20)
Deoxyguanosine	—	B (20)	B (20)
2-Deoxy-D-Glucose	B (7)	B (2)	B (4)
2-Deoxyribose	Bk (7)	Bk (2)	DB (3)
Deoxyribosenucleic acid	—	Br (5)	B (15)
Fructose	O (6)	RO (2)	DV (3)
Fucose	—	—	Pk (1)
Galactose	—	—	DP (8)
Glucose	—	—	DP (7)
Guanosine	—	—	O (12)
Guanylic acid (2′ and 3′)	—	—	Pk (13)
Maltose	—	—	GV (2)
Rhamnose	—	LPk (16)	RO (1)
Ribose	—	WO (16)	Br (4)

TABLE 3 *(Continued*

Compound [a]	Color [c] (detection limit, $\mu g/cm^2$ spot)		
	Paper	Silica gel plate	
	after 10 min	Immediate	110°C, 5 min
Ribosenucleic acid (Sod. salt)	—	Br (8)	Pk (10)
Thymidine	—	Bk (5)	B (12)
Trehalose	—	—	Pk (2)
Xylose	—	WO (16)	Br (5)
Acrolein 2,4-dinitrophenylhydrazone	LY (7)	—	LG (13)
9-Anthraldehyde 2,4-dinitrophenylhydrazone	LG (4)	—	LG (16)
9-Anthraldehyde 3-nitrophenylhydrazone	G (5)	—	LB (13)
Benzaldehyde 2,4-dinitrophenylhydrazone	Y (3)	—	LY (25)
Benzaldehyde 3-nitrophenylhydrazone	Y (3)	—	LY (25)
Cinnamaldehyde 4-nitrophenylhydrazone	Y (5)	—	LY (13)
Crotonaldehyde 2,4-dinitrophenylhydrazone	LY (7)	—	LY (13)
4-Diethylaminobenzaldehyde 4-nitrophenylhydrazone	YBr (7)	—	LY (18)
3,4-Dimethoxybenzaldehyde 2,4-dinitrophenylhydrazone	Y (4)	LY (4)	R (4)
3,4-Dimethoxybenzaldehyde 4-nitrophenylhydrazone	LO (4)	—	O (10)

TABLE 3 *(Continued)*

Compound [a]	Color [c] (detection limit, $\mu g/cm^2$ spot)		
	Paper after 10 min	Silica gel plate	
		Immediate	110°C, 5 min
2,4-Dinitrophenylhydrazine	LY (9)	—	LG (16)
Formaldehyde 2,4-dinitrophenylhydrazone	LO (7)	—	LB (13)
Furfural 2,4-dinitrophenylhydrazone	Y (4)	LY (13)	Bk (13)
Furfural 4-nitrophenylhydrazone	Y (4)	Y (2)	Br (4)
5-Hydroxymethylfurfural 2,4-dinitrophenylhydrazone	O (5)	LY (18)	LP (18)
4-Nitrophenylhydrazine	LY (15)	LY (13)	Bk (13)
Piperonal 4-nitrophenylhydrazone	Y (5)	—	Pk (14)
Piperonalazine	R (4)	R (4)	R (4)
Salicylaldehydeazine	O (7)	LY (15)	Y (15)

[a] Negative results were obtained with acetaldehyde 2,4-dinitrophenylhydrazone, acetone 2,4-dinitrophenylhydrazone, acetophenone, erythrose, formaldehyde, glucosamine, glucuronic acid, *m*-hydroxyacetophenone, *m*-hydroxybenzoic acid, ribitol, 1,1,3,3-tetramethoxypropane, and uridine-3′ (2′)-phosphoric acid.

[b] — = negative at 10 μg.

[c] Color abbreviations are given in footnote *a* to Table 2.

[d] MBTH is 3-methyl-2-benzothiazolinone hydrazone.

observed and in the 4-hexylresorcinol method, purple was the predominant color.

Results of the use of azulene derivatives as location reagents are listed in Table 2. Tables 2 and 3 indicate that azulene is a better location reagent than either of its derivatives and gives more satisfactory results than any location reagent listed in Table 2.

Immediate colors were obtained with the azulene spray for all the furfurals, furfural derivatives, and sugars except furfural 3-methyl-2-benzothiazolinone hydrazone, galactose, and glucose. After being heated for 5 minutes at 110°C, these compounds also reacted. The sensitivity obtained with azulene appeared to be about the same as that obtained with anthrone, the best literature method. The range of colors was much wider with azulene than with anthrone, and the colors were more brilliant. The color stability with azulene was as good as or better than that with the other location reagents. The reagent stability for azulene was about 72 hours. A disadvantage in the use of the azulene reagent is the use of sulfuric acid as the solvent. Because of this, paper pherograms could not be heated in the oven for color development after spraying. In spite of this, the azulene method detects a larger number of compounds than any of the methods tested.

A variety of compounds located by the azulene method are listed in Table 3. The rate of reaction with azulene can be used as a means of differentiating compounds. Some compounds, such as 5-hydroxymethylfurfural, 1-pyrenealdehyde, and vertraldehyde react immediately, while others like fructose and rhamnose require 2–5 minutes for complete color development. A large group of compounds, such as galactose, glucose, maltose, and the dinitrophenylhydrazones need heat for the colors to develop. Because of these various rates of reaction, this method offers greater potential for characterization than any of the other literature methods we tried. The reactivities on paper and silica gel G of the compounds located by azulene are listed in Table 3. Except for 5-methylfurfural, which did not react on paper, all the aldehydes tested could be detected on both paper and silica gel chromatograms; the reactions were more sensitive, however, on the silica gel plate. In addition, furfural derivatives, such as furfuryl alcohol and furfural barbituric acid appear to react better on silica gel than on paper. Sugars reacted poorly on paper, with only a

TABLE 4

CHARACTERIZATION OF 5-HYDROXYMETHYLFURFURAL IN COFFEE ROAST EFFLUENTS

Separation system	5-Hydroxymethylfurfural and unknown			
	Developer	Spray	Color [a]	R_f
TLC-SiGel	Ethyl acetate–piperidine–water	Anthrone	B → B[b]	0.93
	(8:2:1, v/v/v); 90 min	Azulene	O $\xrightarrow{\Delta}$ O[c]	0.90
TLC-SiGel	Propanol–water	SiGel F254	Dark spot[d]	0.73
	(4:1, v/v); 70 min	Anthrone	B → B[b]	0.73
		Aniline–diphenylamine	Y	0.90
		m–Phenylenediamine–di HCl	Y + YG fl.	0.81
PE[e]	0.95% $NaHSO_3$, 0.88% NaOAc, 0.23% AcOH; Ph 4.7	Azulene	O $\xrightarrow{\Delta}$ O[c]	11.5 cm[f]

[a] B =blue; O = orange; Y = yellow; G = green; and △ = heat.

[b] Furfural B $\xrightarrow{\Delta}$ G and 5-methylfurfural B $\xrightarrow{\Delta}$ B.

[c] Furfural O $\xrightarrow{\Delta}$ O and 5-methylfurfural R $\xrightarrow{\Delta}$ V. Colors much less intense than with 5-hydroxymethylfurfural.

[d] Furfural and its 5-methyl derivatives gave no discernable dark spot.

[e] Whatman no. 1 paper, 22.6 V/cm.

[f] Refers to distance traveled.

few giving positive results. The reactions were poor because most of the sugars required heat to react, and the paper pherograms could not be heated with sulfuric acid. When the sugars were heated on the silica gel plate, good results were obtained for all the sugars. Nucleosides, nucleotides, deoxynucleosides, deoxynucleotides, DNA, and RNA, reacted on the silica gel plate less strongly and did not react on the paper. Reactions of dinitrophenylhydrazones, nitrophenylhydrazones, and azines were good on paper. On silica gel, these same compounds required heat to react, and the detection limits were much higher than those on paper.

Application. Various samples of a polluted industrial effluent were investigated. The effluent was from a coffee-roasting plant and was extracted with water in the manner previously described. The samples were separated by thin-layer chromatography on silica gel and by electrophoresis on Whatman no. 1 paper. Since it was assumed that 5-hydroxymethylfurfural was present in the unknown, this standard was run at a concentration of 10 μg with each separation. The evidence presented in Table 4 clearly shows the presence of 5-hydroxymethylfurfural in the unknown samples. In each case when a spray was used, the same color and R_f value were found for the unknown as was found for the standard, 5-hydroxymethylfurfural.

In an attempt to identify some of the compounds present in the ether extract of the dinitrophenylhydrazones obtained from the ether extract of a coffee-roast effluent, we separated 1 μl of this sample on a silica gel plate using toluene–tetrahydrofuran (95:5, v/v) as the developer. The standards, run at the same time in 10 μg amounts, were 2,4-dinitrophenylhydrazine, furfural 2,4-dinitrophenylhydrazone, 5-hydroxymethylfurfural 2,4-dinitrophenylhydrazone, and glyoxal bis-2,4-dinitrophenylhydrazone. The resulting colors after spraying with ethanolamine are shown in Fig. 2. Although the dinitrophenylhydrazones were precipitated and washed well, a small amount of the reagent, 2,4-dinitrophenylhydrazine, was carried over, as shown in Fig. 2. Quite a few unknown dinitrophenylhydrazones were present in the extract, but only 5-hydroxymethylfurfural 2,4-dinitrophenylhydrazone, and glyoxal bis-2,4-dinitrophenylhydrazone were identified. Furfural 2,4-dinitrophenylhydrazone was not found. The remaining spots were unknowns. Although not shown in Fig. 2, a methanolic extract of the dinitrophenylhydrazones from a coffee-roast effluent was also separated by the same

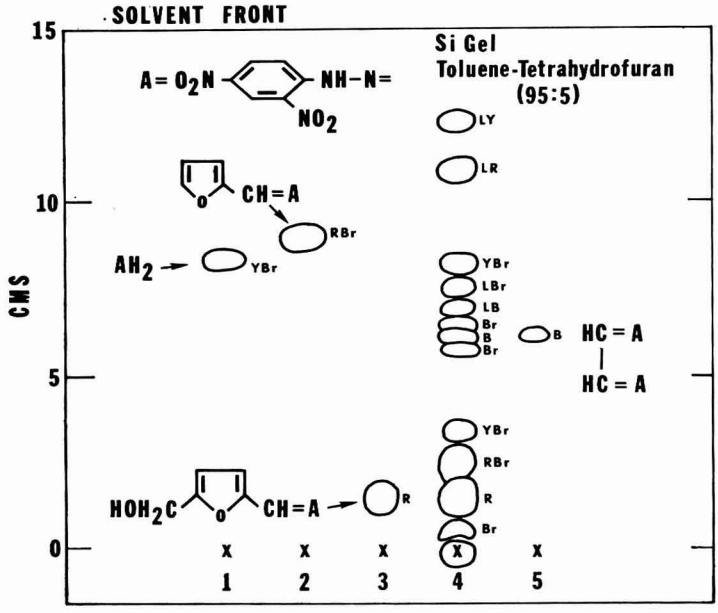

FIG. 2. Thin-layer chromatogram on silica gel with toluene–tetrahydrofuran (95:5, v/v) as developer. Standards put on in 10-μg amounts: (1) 2,4-dinitrophenylhydrazine (2) furfural 2,4-dinitrophenylhydrazone (3) 5-hydroxymethylfurfural 2,4-dinitrophenylhydrazone (4) 1 μl of ether extract of the dinitrophenylhydrazones obtained from the ether extract of a coffee-roast effluent (5) glyoxal bis-2,4-dinitrophenylhydrazone. The visible colors are shown after spraying with ethanolamine. The color abbreviations are as follows: Y = yellow; Br = brown; R = red; B = blue; and L = light.

system in an attempt to identify some of the dinitrophenylhydrazones in this sample. The only one identified was glyoxal bis-2,4-dinitrophenylhydrazone.

SUMMARY

A new location method for many types of conjugated aldehydes and their precursors is introduced. This method, with azulene as the reagent, is compared with a number of other location methods. Evidence is presented for the location of aromatic aldehydes, furfural derivatives, sugars, dinitrophenylhydrazones, and azines on paper and silica gel plates with azulene. This location procedure was applied to the identification of 5–hydroxymethylfurfural in extracts of effluents from a coffee-roasting plant. Techniques described in this paper have been developed for eventual application to analyses of airborne particulate.

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Titrimetric Microdetermination of Samarium and Neodymium

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INTRODUCTION

The literature cited concerning the determination of rare earths is ample. Workers have determined it as oxalates (*11, 19*), spectrophotometrically (*2, 3, 8, 10, 12, 17, 18, 22*), by luminescence (*20*), by absorption spectra (*6, 7*), by cupferron and neocupferron complexes (*16*), potentiometrically and conductometrically (*1, 15*), chromatographically (*9, 14*), with EDTA (*4, 21*), by radio chemical analysis (*13*) and polarographically (*5*).

The present work describes a micro method based on the formation of a violet colored complex by boiling the solution of samarium and neodymium chlorides separately with an excess of sodium salt of rhodizonic acid (RzA-Na). After filtering the precipitated complex is dissolved in an acid. Then RzA-Na, which gets attached to either samarium or neodymium complex, is oxidized with a standard solution of ceric sulfate using *N*-phenyl anthranilic acid as indicator. The results agree with those obtained by the standard method (*11*) and give concordant and precise values.

By analysis it has been found that one atom of samarium or neodymium is attached to the three molecules of RzA-Na. It is also observed than in both the cases Na, K, and Zr do not interfere but Ag, Tl, Ba, Sr, Ca, Zn, Be, Sn, Sb, Cu, Mg, Co, Ni, Bi, Ru, Rh, Ir, Te, Pt, V, Se, Cr, U, W, and Os do.

EXPERIMENTAL METHOD

Chemicals Employed

Samarium trichloride (Fluka grade); neodymium trichloride (Fluka grade); sodium rhodizonate (B.D.H. grade); sulfuric acid (A.R. B.D.H. grade); ceric sulfate (technical B.D.H. grade) ferrous

ammonium sulfate (A.R. B.D.H. grade); sodium carbonate (A.R. B.D.H. grade); and *N*-phenyl anthranilic acid (B.D.H. grade).

The 0.0031 *M* ceric sulfate (in 8 *N* H_2SO_4) solution was standardized by titrating against a standard solution of ferrous ammonium sulfate (in 1 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

Procedure

Procedure for both samarium and neodymium was the same so instead of writing individual name it is mentioned as 'rare earth.'

A known solution of rare earth was boiled with an excess of sodium rhodizonate (RzA-Na) for 2–4 minutes. After cooling at room temperature the violet colored complex was filtered, thoroughly washed with distilled water, and then digested with 20 ml of 2 *N* H_2SO_4. The digested solution, containing the rhodizonic acid that was attached with the rare earth, was oxidized by titrating against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator. At the end point a red brown color was observed.

RESULTS AND DISCUSSION

The results are shown in Tables 1 and 2. The range in which samarium and neodymium are estimated vary from 0.3290 to 2.6128 mg/liter and from 0.1252 to 0.6286 mg/liter, respectively.

The reaction between samarium or neodymium and rhodizonate takes place in the following manner:

$$SmCl_3 + 3[CO{\cdot}CO{\cdot}COONa{:}NaCO{\cdot}CO{\cdot}CO] \rightarrow Sm[CO{\cdot}CO{\cdot}CO{:}NaCO{\cdot}CO{\cdot}CO]_3 + 3NaCl.$$

$$NdCl_3 + 3[CO{\cdot}CO{\cdot}CONa{:}NaCO{\cdot}CO{\cdot}CO] \rightarrow Nd[CO{\cdot}CO{\cdot}CO{:}NaCO{\cdot}CO{\cdot}CO]_3 + 3NaCl.$$

It is observed that one atom of the rare earth takes three molecules of sodium rhodizonate, which is confirmed by analysis. It is also observed by analysis that the three chloride ions get attached with the three sodium ions. Thus three sodium rhodizonate molecules are attached to one rare earth by coordinate valency.

TABLE 1

MICRODETERMINATION OF SAMARIUM

$SmCl_3$ (ml) 0.0087*M*	0.0031*M* $Ce(SO_4)_2$ used that oxidized rhodizonic attached (ml)	Amount of samarium (mg/liter)
0.25	2.12	0.3290
0.50	4.20	0.6525
0.75	6.72	0.9815
1.00	8.42	1.3078
1.50	12.60	1.9575
2.00	16.82	2.6128

TABLE 2

MICRODETERMINATION OF NEODYMIUM

$NdCl_3$ (ml) 0.0087*M*	0.0031*M* $Ce(SO_4)_2$ used that oxidized rhodizonic acid (ml)	Amount of neodymium (mg/liter)
0.1	0.84	0.1252
0.2	1.68	0.2504
0.3	2.50	0.3711
0.4	3.36	0.5008
0.5	4.22	0.6286

SUMMARY

Titrimetric microdetermination of samarium and neodymium has been described with sodium rhodizonate. Trivalent salts of samarium and neodymium form violet colored complexes in the ratio of 1:3. The complex is dissolved in dilute acid and the rhodizonic acid that is attached with the metal ion is estimated by titrating against a standard solution of ceric sulfate using *N*-phenyl anthranilic acid as indicator. Interference of cations has been observed.

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Determination of Formaldehyde via EDTA Titration[1]

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It has been pointed out by several authors that oxydizing and reducing agents can be determined volumetrically via titration with EDTA (*1–8*). This is possible in two cases. (a) If a polivalent cation is formed in a quantitative redox reaction, which cation forms a stable complex with EDTA, then the cation formed can be titrated with EDTA solution. (b) If a cation which has a stable EDTA complex is converted by a redox reaction into a noncomplex-forming cation or to a metal, then only the nonreacted amount of the cation is measured by the EDTA solution.

We have already shown previously, that the mercury(I)–mercury(II) system can be utilized for the determination of reducing or oxydizing agents, as only mercury(II) forms stable complex with EDTA (*4*). We applied these principles to the determination of formaldehyde as follows: The solution of formaldehyde was added to a mixture of known concentration of $Hg(NO_3)_2$ and EDTA, the resulting mixture was made alkaline and allowed to stand. The complex of mercury(II) is reduced and Hg_2O is formed, which disproportionately converts to mercury metal and HgO. The metallic mercury settles as a black precipitate, while the HgO forms an EDTA complex again. Thus the overall reaction is the following:

$$HgO + HCHO = Hg + HCOOH,$$

that is, 1 mole of EDTA is equivalent to 1 mole of HCHO, this is liberated during the reaction, and it can be titrated (Table 1).

Reagents. A 0.1 *M* $Hg(NO_3)_2$ solution, which also contains 63 g of HNO_3 in 1000 ml; a 0.1 *M* EDTA soln; 2 *N* NaOH soln; 2 *N* HNO_3 soln; crystalline hexamethylene tetramine; and methylthymol blue as 1:100 ground mixture with KNO_3.

[1] Part IV of the series: Chelatometric methods in the determination of oxidants and reductants. Part III of the series was published in *Chemist–Analyst* **55**, 77–78 (1966).

TABLE 1

DETERMINATION OF FORMALDEHYDE

Formaldehyde (mg)		
Taken	Found	Difference (mg)
7.50	7.58	0.08
7.50	7.62	0.12
12.75	12.60	0.15
12.75	12.36	0.39
19.12	18.60	0.52
19.12	18.72	0.40
25.50	25.95	0.45
25.50	25.86	0.36

Procedure. Add 15–25 ml of 0.1 *M* EDTA soln to 10–20 ml of 0.1 *M* $Hg(NO_3)_2$. Wait until the soln clears, then add 5–20 ml of 0.1 *N* formaldehyde soln. Adjust the pH to about 9–10 by the addition of 2 *N* NaOH, and let it stand for 2 hours. The mercury precipitates in the flask, and this precipitate does not disturb further titration. After 2 hours add the calculated amount of 2 *N* HNO_3 adjusting the pH to 4–5, then adjust to final pH of 6 by the addition of hexamethylene tetramine crystals. Titrate the excess of EDTA in the presence of methylthymol blue with 0.1 *M* $Hg(NO_3)_2$ soln. The change of color (from yellow to blue) can be observed distinctly (9).

RESULTS

As shown in Table 1 satisfactory results were obtained by the application of this simple method.

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MICROCHEMICAL JOURNAL 13, 230–231 (1968)

The Application of Fuchsine Dyes in the Detection of Esters of *o*-Phthalic Acid in Thin-Layer Chromatography.

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There are many substances used for detecting esters of *o*-phthalic acid on thin-layer chromatograms (*2*). The Kieselgel G used was producted by Merck-Darmstadt. Plates were spread at 0.7 mm thickness according to standard procedures with "Camag" apparatus. The plates were activated at 110°C for 100 min immediately before use.

Chloroform was used for the mobile phase. The chromatograms were developed to the height of 7 cm at 21°C in a period of 8 minutes. The mobile phase was dryed at 70°C for 10 minutes and afterwards the esters were detected. The plates were sprayed with water solutions of the fuchsine dyes. The conditions of the detection and the characteristics of the used dyes were the same as described in a previous publication (*1*).

Chemicals used for the chromatography analysis shown in Table 1 were pure. On the pink-red background of the chromatogram appeared violet spots of the esters. The spots were durable. The new fuchsine and basic fuchsine showed better developing abilities than acidic fuchsine. On the whole the developing abilities of the fuchsines used is higher for esters with a longer alcohol carbon chain.

TABLE 1

DEVELOPING ABILITIES OF THE APPLIED FUCHSINE DYES FOR SINGLE ESTERS OF *O*-PTHALIC ACID

Esters	New fuchsine (μg)	Basic fuchsine (μg)	Acidic fuchsine (μg)	R_f
Dibutyl phthalate	25	25	—	0.48
Dihexyl phthalate	10	10	20	0.57
Deheptyl phthalate	7	7	10	0.58
Dioctyl phthalate	5	5	8	0.61
Dinonyl phthalate	3	3	8	0.62
Didecyl phthalate	3	3	6	0.62

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Spectral Behavior of Some Cinnamic Acids

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INTRODUCTION

Cinnamic acids containing a catechol grouping at 3,4-positions have been reported (*1*) to show a characteristic shift when complexed with aluminum chloride in solution. Aluminum has also been used for detecting the presence of a hydroxyl group adjacent to a carbonyl group.

Among naturally occurring cinnamic acids, methoxy derivatives are rather difficult to detect (3). They do not present alkaline shifts and no specific chromogenic reactions are available for characterizing these compounds. Furthermore, cinnamic acids and their methoxy derivatives absorb commonly in the 230–330 mμ region and so they are not easy to distinguish from other classes (*1*). Thus, coumarins are very similar in their UV spectra to cinnamic acids. The advantage of any other additional datum is evident and with this in mind we start searching some other spectral characteristics for this group of substances and some related compounds.

EXPERIMENTAL METHOD

Cinnamic, coumaric, and methoxy-cinnamic acids were purchased from Fluka A.G., Buchs, Switzerland. For the determination of the spectra a 0.001% solution of each compound in methanol was prepared. After the neutral spectrum was recorded, three drops of a solution of 5% aluminum chloride in ethanol were added, and the spectrum was determined. The alkaline spectra were obtained after addition of two drops of 5% KOH. All spectra were determined on a manual Zeiss PMQ II spectrophotometer with 1-cm silica cells.

RESULTS AND DISCUSSION

The naturally occurring methoxy-cinnamic acids and *cis*-cinnamic acid itself do not show shifts in the ultraviolet range when their methanolic solutions are basified with alkali (Table 1), as could be

TABLE 1

INFLUENCE OF ALKALI AND ALUMINUM CHLORIDE ON THE SPECTRA OF CINNAMIC ACIDS

Compound	λ max (mμ)		
	Neutral	Alkaline	$AlCl_3$
cis–Cinnamic acid	252	252	270
trans–Cinnamic acid	271–272	265	280
o–Coumaric acid	271;320	271;316	281;332
m–Coumaric acid	221;231;273	249;280	238;284
p–Coumaric acid	291	334–335	229;318
4–Methoxy–cinnamic acid	288–289	285	230;314–315
3,4–Dimethoxy–cinnamic acid	230–232;287;315	227–229;284;309	242–244;329
3,4,5–Trimethoxy–cinnamic acid	227;293	225–226;289–292	234–235;312–313
Ferulic acid	291;314	240;306;347–349	219;243;330
Isoferulic acid	289;320	259;293;342	246–248;331–333
Sinapic acid	229;312	245;359	270
Caffeic acid	230;290;320	240;305;345	253;345–346;354
Chlorogenic acid	245;325	265;373	348

expected from substances containing no phenolic groups. Because chromogenic reactions showed ill-defined color, if any, the absorption characteristics of aluminum "complexes" have proven valuable criteria for identification.

The aluminum shift in hydroxy-cinnamic acids was ascribed to the 3,4-dihydroxyl system in the molecule (caffeic acid) but in the present paper ferulic (3-methoxy-4-hydroxy-cinnamic acid), sinapic (3,5-dimethoxy-4-hydroxy-cinnamic acid) and isoferulic (3-hydroxy-4-methoxy-cinnamic acid) acids have shown an analogous properties although the 3,4-dihydroxyl grouping is not in their structures. A phenomenon that was also showed by the three possible monohydroxy-cinnamic acids (*o*-, *m*- and *p*-coumaric acids).

Since ferulic, isoferulic, sinapic and coumaric acids give alkaline shifts (Table 1) and well defined colors with phenolic reagents, the bathochromic shifts in the presence of aluminum ion is not a relevant improvement in their characterization and the interest or importance of the method is confined to the methoxy derivatives. However, in many cases, the availability of another parameter by measurement of the absorption spectra could be a desirable time-saving technique (2).

It is rather difficult to explain the "chelating" property of aluminum ion with molecules where neither catechol nor adjacent carbonyl-hydroxyl grouping were present. It seems that another phenomenon than chelation is involved in the spectral behavior here reported and aluminum is an unspecific reagent as unpublished work had indicated for veratric aldehyde, and shikimic, *p*-hydroxy-phenylpyruvic and quinic acids.

SUMMARY

Spectral changes in the presence of a complexing reagent are reported for thirteen cinnamic acids, naturally occurring in the plant kingdom. The advantage of the method is emphasized for some methoxy derivatives, which are difficult to distinguish in plant tissues.

ACKNOWLEDGMENTS

The junior author is grateful to the CSIC of Spain and to the P.I.O. for the award of a fellowship.

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Automatic Catalytic Microdetermination of Copper

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INTRODUCTION

The analytical application of the reaction rate technique, especially for catalytic reactions, has received considerable attention in recent years (*1*). Several catalytic methods have been proposed (*2*) for the detection and determination of copper.

The catalytic action of copper, iron, and osmium on the reaction of *p*-phenylenediamine with hydrogen peroxide has been studied previously (*3*, *4*). A similar action of copper, iron, and vanadium on the oxidation of 2,4-diaminophenol (DAP) by hydrogen peroxide was observed by us. On the oxidation of DAP in aqueous solutions a red color is developed, probably due to the formation of 2-amino-*p*-quinonimine (*5*, *6*).

In the present paper are studied the catalytic effect of copper ions on the oxidation of DAP by hydrogen peroxide and the application of this reaction for the quantitative determination of copper. For the analytic applications a device, providing a precise measurement of time between two preset points on the response curve (*7*, *8*), is used.

EXPERIMENTAL METHOD

Instruments

The basic instrumental unit was a Sargent model Q concentration comparator consisting of a null-point potentiometer, as modified by Malmstadt and Pardue (*7*) for the reaction rate determination. The unit was connected to the modified Spectro-Electro Sargent titrator (*8*) and to a digital readout reaction rate adapter (Sargent model QRR).

Reagents

All reagents used were of reagent grade. The 2,4-diaminophenol solution, 0.1 *M*, was prepared by dissolving 2,4-diaminophenol dihy-

drochloride (Merck) in 0.5 *N* hydrochloric acid. All working standards, reagent solutions and samples were kept in a water bath at 30 ± 0.05°C when used.

Procedure

The Spectro-Electro titrator is switched on to the spectro-position for at least 1 hour before the measurements are started, to insure good stability from the light source. The filter wheel is dialed to the 500-mμ position and a Corning no. 3387 filter is placed in the auxiliary holder and the polarity switch is thrown to position 1. A few minutes before the measurements are started the comparator unit is turned to ON-position and the range selector switch to PNP ± 0.01 V.

Measurement step. For the 10^{-2} *M* copper stock solution curve, the comparator zero adjust control is set initially at 5.5; 0.4 ml of 3% hydrogen peroxide solution, 4.0 ml of 0.5 *N* hydrochloric acid solution and 0.02 ml of 10^{-2} *M* copper solution are injected into the reaction cell. The reagent selector is dropped to the position 2 and the ammeter's pointer is adjusted to middle by means of the auxiliary helipot resistance. The comparator adjust is set at 4.5; 1.0 ml of 0.01 *M* DAP solution is added and the start button on the model QRR reaction rate adapter is pressed at once. The analysis is completed automatically and the reading on readout dial is recorded. The procedure is repeated with 0.04, 0.06, 0.08, and 0.10 ml of copper solution. The mean value of the readings of two samples is recorded.

For the 10^{-3} *M* copper stock solution curve, the zero adjust is set first at 5.0 and then at 4.5.

Working curves are obtained for each concentration range by plotting the reciprocal time against the copper concentration.

RESULTS AND DISCUSSION

The absorption spectrum (Fig. 1) shows that the maximum absorption of oxidized DAP is at 500 mμ; copper and DAP do not absorb at the same region. Therefore, for measurements, a 500-mμ band was selected by dialing a nominal 500-mμ second order filter.

A semiquantitative appearance of catalytic action of copper on the reaction of hydrogen peroxide on DAP is presented in Fig. 2. These plottings were recorded by means of a Lumetron photometer connected with a photovolt recorder.

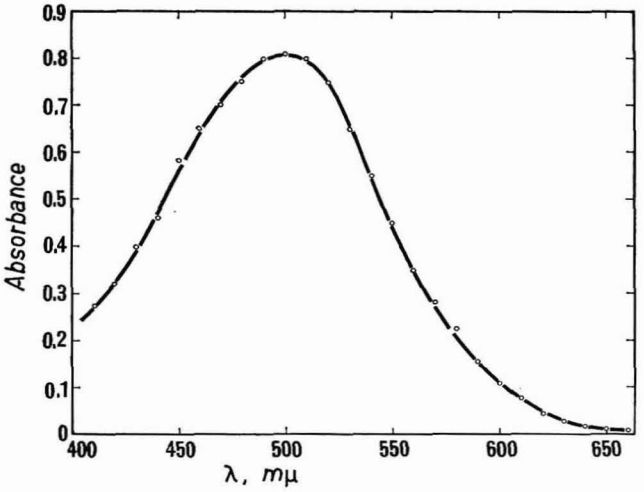

FIG. 1. Absorption spectrum of oxidized DAP.

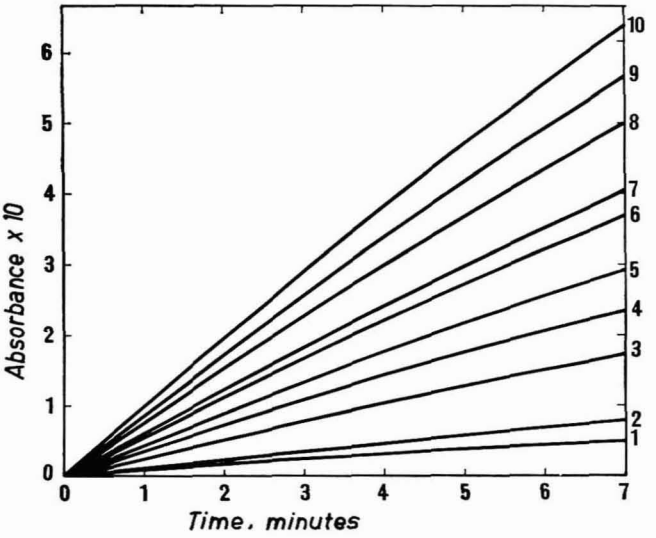

FIG. 2. Effect of copper concentration on the reaction rate of DAP oxidation with H_2O_2. (DAP 0.01 *M* in *N* HCl, 8 ml; H_2O_2 3%, 0.25 ml). (Copper concentrations: 1. zero; 2. 1.21×10^{-4} *M*; 3. 2.42×10^{-4} *M*; 4. 3.62×10^{-4} *M*; 5. 4.83×10^{-4} *M*; 6. 6.02×10^{-4} *M*; 7. 7.22×10^{-4} *M*; 8. 9.60×10^{-4} *M*; 9. 10.97×10^{-4} *M*; 10. 11.97×10^{-4} *M*).

It was observed that the rate of reaction depends on the concentrations of: DAP, Hydrogen peroxide, copper and hydrogen ions, as well as on the temperature.

The effect of each variable was studied, using the arrangement proposed by Malmstadt and Pardue (7) and modified by Malmstadt and Hadjiioannou (8).

The influence of DAP concentration is presented in Fig. 3. It is shown that, as the DAP concentration is increased the reaction rate increases up to a certain value and then becomes DAP-concentration independent. A similar curve was obtained (Fig. 4) showing the effect of hydrogen peroxide concentration on the reaction rate.

Figure 5 presents the effect of hydrogen ion concentration on the reaction rate. It is shown by increasing the hydrogen ion concentration the reaction rate decreases up to a certain value and then increases.

The irregular influence of hydrogen ion concentration on the reaction rate could be explained supposing that there is a simultaneous action of them on different catalytic reactions which are coexisting to the whole catalytic cycle.

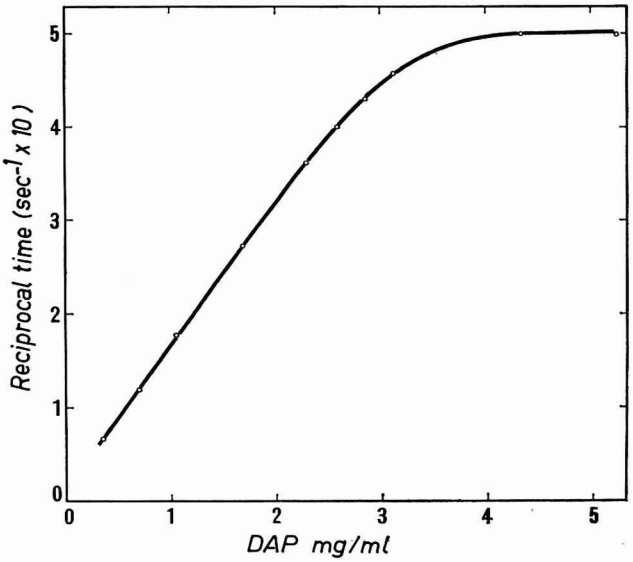

FIG. 3. Influence of DAP concentration on the reaction rate; (Cu 0.1 *M*, 0.06 ml; H_2O_2 3%, 0.25 ml; HCl 0.5 *N*, 5 ml).

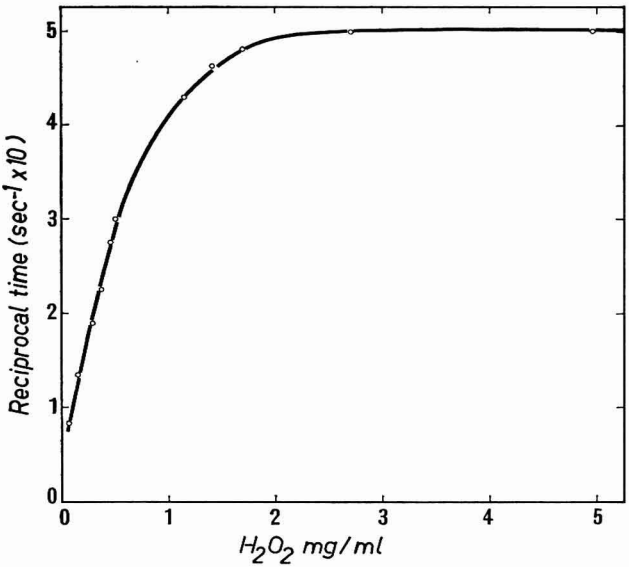

FIG. 4. Influence of hydrogen peroxide concentration on the reaction rate; (DAP 0.1, *M*, 1 ml; Cu 0.1 *M*, 0.06 ml; HCl 0.5 *N*, 4 ml).

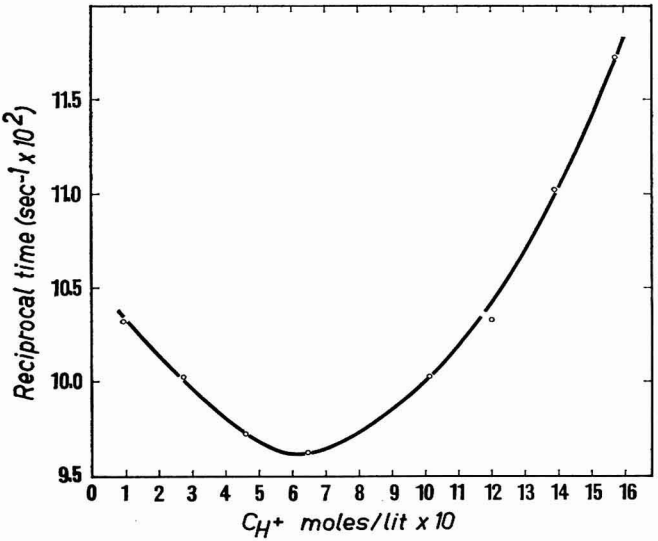

FIG. 5. Influence of hydrogen ion concentration on the reaction rate; (DAP 0.1 *M*, 1 ml; Cu 0.1 *M*, 0.01 ml; H_2O_2 3%, 0.4 ml).

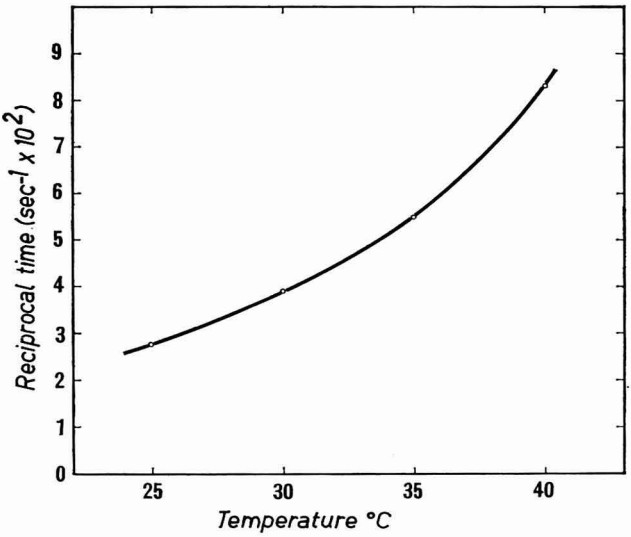

FIG. 6. Effect of temperature on the reaction rate; (DAP 0.1 *M*, 1 ml; Cu 0.01 *M*, 0.04 ml; H_2O_2 3%, 0.4 ml; HCl 0.5 *N*, 4 ml).

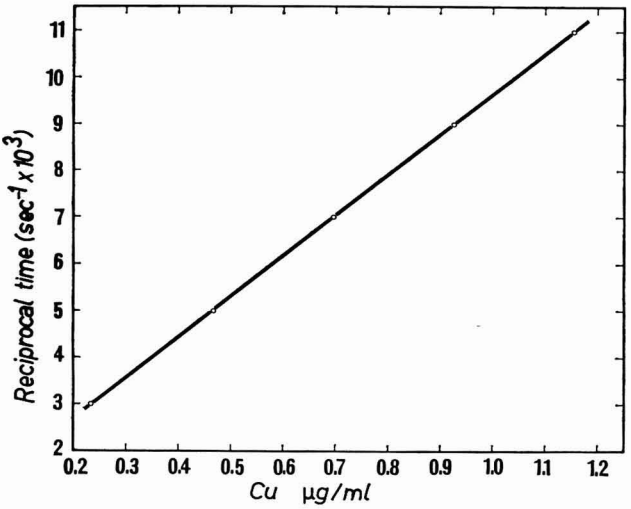

FIG. 7. Working curve for copper determination; (DAP 0.1 *M*, 1 ml; H_2O_2 3%, 0.4 ml; HCl 0.5 *N*, 4 ml).

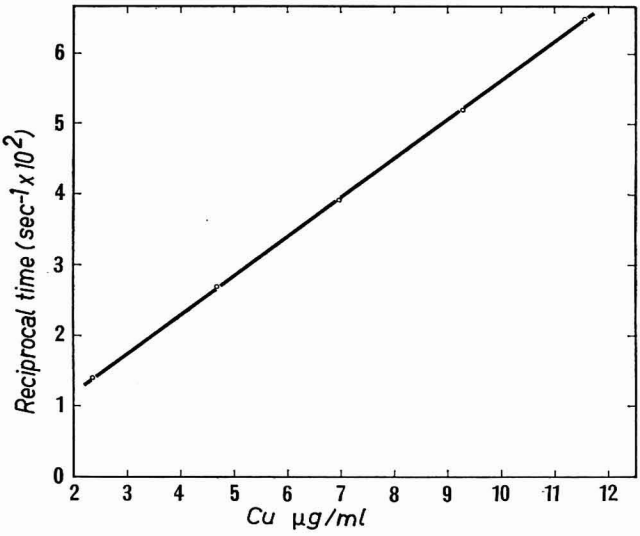

FIG. 8. Working curve for copper determination; (DAP 0.1 *M*, 1 ml; H_2O_2 3%, 0.4 ml; HCl 0.5 *N*, 4 ml).
MJ—Production No. 588

The effect of temperature is demonstrated in Fig. 6, in which the reciprocal of time is plotted against the temperature.

The relation between the reaction rate and copper concentration is presented in Figs. 7 and 8. It was found that for the given set of conditions, the reaction is first order with respect to copper concentration up to 12 µg/ml.

TABLE 1

AUTOMATIC RESULTS FOR AQUEOUS COPPER SOLUTIONS
Conditions as under procedure.

Copper (µg)		Relative error (%)
Taken	Found [a]	
2.82	2.86	+1.41
5.65	5.61	—0.88
8.47	8.64	+2.00
11.30	11.42	+1.06

[a] From straight line working curve.

TABLE 2

DETERMINED PERCENTAGES OF COPPER IN COPPER ORE SAMPLES
Conditions as under procedure.

Copper (%)		
Taken	Found	Difference
10.57	10.78	+0.21
12.36	12.66	+0.30
6.65	6.59	—0.06

Table 1 gives the results of copper determination in aqueous solutions and Table 2 the determined percentages of copper in artificial copper oxide ores (Smith and Underwood Co). In both cases the precision and accuracy was found to be within 1–3%. Generally the precision and accuracy for aqueous solutions containing 2–12 μg/ml of copper was found to be 2%.

TABLE 3

INFLUENCE OF DIVERSE IONS

Maximum concentrations of ions which cause no interference. (Solutions: DAP 0.1 *M*, 1 ml; Cu^{2+} 0.01 *M*, 0.04 ml; HCl 0.5 *N*, 4 ml; H_2O_2 3%, 0.4 ml).

Formula	Concentration (*M*)
$Alk(SO_4)_2 \cdot 12H_2O$	1.805×10^{-3}
$CdSO_4$	1.805×10^{-3}
$MgSO_4$	1.805×10^{-3}
$MnSO_4$	1.805×10^{-3}
$NiSO_4$	1.805×10^{-3}
$ZnSO_4$	1.805×10^{-3}
$Bi(NO_3)_3$	1.805×10^{-3}
$Pb(NO_3)_2$	1.805×10^{-3}
Acetic acid	1.805×10^{-3}
Tartaric acid	1.805×10^{-3}
$(NH_4)_2MoO_4$	9.025×10^{-4}
$SnCl_2$	9.025×10^{-4}
Oxalic acid	9.025×10^{-4}
$FeCl_3$	9.107×10^{-7}

Interferences

The effect of various metals that interfere in the determination of copper was also investigated. Tin, molybdenum, and iron which catalyze the reaction, interfere seriously. From the anions, only oxalates show an appreciable interfering action. Table 3 gives the minimum amount of ions which causes a deviation greater than the accuracy of the method.

SUMMARY

An automatic reaction rate method is described for the microdetermination of copper. The method is based on the catalytic action of copper on the reaction of 2,4-diaminophenol with hydrogen peroxide. The Effect of reagent concentration is studied and the maximum tolerable amounts of interfering ions are determined. Procedures for the determination of 0.2–12.0 μg copper per ml are given; 0.2–12.0 μg/ml of copper could be determined with a relative error of about 2%.

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The Application of Fuchsine Dyes in the Detection of Barbituric Acid Derivatives in Thin-Layer Chromatography

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Until now, Hg(I) nitrate (*2*) has been used in the thin-layer chromatographic detection of barbituric acid derivatives. Kieselgel G produced by Merck-Darmstadt was used. Plates were spread 0.5 mm thick according to standard procedure with the "Camag" apparatus. The plates were activated at 110°C for 100 minutes immediately before use. Mobile phase: chloroform and acetone in a volume ratio of 9:1.

The chromatograms were developed to the height of 14 cm at a temperature of 21°C in a period of 25 minutes. The mobile phase was dryed at 22°C for 24 hours and afterward the barbituric acid Derivatives were detected.

The plates were sprayed with water solutions of fuchsine dyes (*1*). The new fuchsine was the more effective developing medium than the basic fuchsine or acidic fuchsine. The spots were durable. On the red background of the chromatogram appeared pink-red spots of the barbituric acid derivatives. Chemicals used for the chromatographic analysis shown in Table 1 were of pharmaceutical purity. (See page 246 for Table 1.)

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TABLE 1

DEVELOPING ABILITIES OF FUCHSINE DYES FOR SINGLE BARBITURIC ACID DERIVATIVES

Substances	New fuchsine (μg)	Basic fuchsine (μg)	Acidic fuchsine (μg)	R_f
Prominal 5-ethyl 1-methyl 5-phenyl barbituric acid	25	100	75	0.65
Narcosan 5-methyl 5-phenyl barbituric acid	15	20	40	0.57
Dial 5,5-dialil barbituric acid	25	25	30	0.49
Luminal 5-ethyl 5-phenyl barbituric acid	15	20	40	0.42
Veronal 5,5 diethyl barbituric acid	25	35	50	0.35

MICROCHEMICAL JOURNAL 13, 247–252 (1968)

Analysis of Multicomponent Chromium Mixtures

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INTRODUCTION

The increasing use of chromium in a variety of alloys of technical importance, such as chrome steels and nickel chrome, and as an essential ingredient of many pigments, have created renewed interest in methods for analysis of its multicomponent mixtures.

In 1961 Khalifa (*5*) investigated a potentiometric–volumetric method of analysis of few binary mixtures of chromium with requisite accuracy. It was thought that back titrating excess EDTA with mercuric ions, once on cold, and once after boiling, could be made the basis underlying analysis of binary mixtures of chromium with many metal ions. However, in ammoniacal buffer, pH 8, precipitation of chromic hydroxide rendered impossible the application of such a procedure. The fact that in urotropine buffers, pH 7–8, the above phenomenon is not observed and further, the potential breaks are in average double those obtained in ammoniacal buffer, led to the conclusion that the above principle could, safely underly an accurate method of analysis of chromium mixtures. Fritz (*3*) observed interference from chromium, even on cold, and recommended tartrate and preferably citrate as masking agent.

Many of the methods cited in the literature for the analysis of chromium mixtures, including the methods recommended by A.S.T.M. (*1*), Hillebrand (*4*), and others (*2*, *6–9*), are too complicated, time consuming, and have serious interferences which limit their application. The methods of analysis presented herein, eliminate most of the above difficulties, especially because they involve no primary separations.

EXPERIMENTAL METHOD

The water used was always twice distilled from all glass equipment. The chemicals were of the highest purity available. These were nitrates of mercury(II), chromium(III), copper(II), lead, nickel, and

cadmium; citrate, hydroxide, and thiosulfate of sodium; hydroxide and chloride of ammonium, tartaric, and nitric acids; zinc oxide; potassium iodide; hexamine; pyridine; starch and criochrome black T indicators; and ethylenediaminetetraacetic acid disodium salt (EDTA).

Solutions. The mercuric nitrate solution was prepared and standardized as mentioned elsewhere. The molarities ranged between 0.0444 and 0.0507. The 0.01284 and 0.059 *M* chromium solutions were prepared essentially as mentioned before (*5*). The metal nitrate solutions were prepared and standardized according to recommended procedures. Their molarities amounted to 0.05 keeping the solutions always 0.05 *M* in respect to nitric acid. The 0.05 *M* EDTA solution was prepared and checked classically. The 0.0486 *N* thiosulfate solution was standardized against standard dichromate, cupric, and iodine solutions. The potassium iodide solutions ranged between 0.04 and 0.05 *M*. The sodium citrate solution was 0.1 *M*. The hexamine buffers, pH 7–8, and ammoniacal buffer, pH 10, were prepared as before mentioned. The 1% starch indicator solution was prepared in the usual way and properly stored.

Cell and equipment. The titration cell, pH- and potentiometer are essentially the same as those described elsewhere (*5*).

Procedures

Binary mixtures. (A) To a certain volume of the mixture is added a known excess of standard EDTA solution, citrate to mask chromium (6 ml of 0.1 *M* for each 1 mg), 20 ml of 2.5% hexamine, and the excess is back titrated with mercuric nitrate potentiometrically, using the silver amalgam as indicator electrode. This titration gives the volume of EDTA equivalent to the metal ion.

(B) To another identical volume is added a known excess of EDTA followed by boiling for 15 minutes, and cooling, the buffer is added and finally the solution is back titrated as above. This titration gives the amount of EDTA equivalent to both chromium and metal ion.

Whenever copper is a component, a pyridine buffer (1 ml of pyridine in 40 ml of water) is preferred.

Ternary mixtures. In mixtures of the type Cr + Fe(III) or Cu(II) + M, iron or copper plus metal are determined following procedure A. Iron or copper is determined iodometrically and chromium is determined following procedure (B).

Quaternary mixtures. Those containing Cr + Fe or Cu + M + Hg(II) are analyzed essentially as ternary mixtures, except for the fact that mercury is selectively titrated potentiometrically with standard iodide solution.

RESULTS AND DISCUSSION

The data in Table 1 indicate that the procedure given for such an analysis is extremely reliable. Further, it may be of great value as applied to analysis of nickel and ferro–chrome alloys. If mercury(II) is present as a third component together with any of the above mixtures, it can be selectively titrated potentiometrically with a standard iodide solution. The results shown in Table 2 indicate that, in comparison with other published procedures, the present one has the additional advantages of being simple, rapid, and extremely reliable.

TABLE 1

ANALYSIS OF BINARY MIXTURES

Chromium (mg)		Metal (mg)	
Taken	Found	Taken	Found
10.755	10.693	1.563 Fe	1.563
8.013	7,995	2.714	2.697
5.378	5.378	5.751	5.729
1.344	1.352	11.503	11.503
10.56	10.652	1.488 Ni	1.517
8.013	8.049	2.418	2.421
5.315	5.377	5.401	5.451
2.671	2.672	7.254	7.254
10.450	10.450	5.015 Hg	5.015
5.225	5.225	20.06	20.06
0.668	0.665	22.265	22.265
10.298	10.298	5.024 Pb	5.024
5.149	5.201	20.089	19.973
1.534	1.526	40.178	40.152
10.192	10.197	5.328 Cd	5.306
10.192	10.152	2.433	2.433
5.096	5.066	10.656	10.553
10,235	10.142	1.628 Cu	1.628
5.242	5.325	5.877	5.877

TABLE 2

ANALYSIS OF TERNARY MIXTURES

Chromium (mg)		Metal (mg)		Metal (mg)	
Taken	Found	Taken	Found	Taken	Found
2.580	2.582	2.714 Fe	2.714	4.784 Ni	4.836
1.935	1.936	4.071	4.071	7.086	7.086
1.289	1.289	5.428	5.428	2.392	2.392
2.580	2.592	5.428	5.428	17.814 Hg	17.894
1.935	1.936	4.071	4.071	35.628	35.308
5.160	5.217	2.714	2.714	8.907	8.947
2.705	2.663	4.071	4.071	31.144 Pb	31.092
4.057	4.059	2.714	2.714	20.762	20.928
5.409	5.409	5.428	5.428	10.381	10.310
2.671	2.658	2.714	2.714	22.257 Cd	22.302
4.007	4.031	5.428	5.428	11.129	11.308
5.342	5.375	2.714	2.714	16.693	16.726
2.671	2.709	4.830 Cu	4.830	5.986 Ni	5.998
5.342	5.349	7.245	7.245	3.591	3.514
5.342	5.409	9.660	9.660	2.394	2.456
5.342	5.342	4.830	4.830	10.331 Pb	10.187
2.671	2.656	3.622	3.622	20.662	20.546
2.671	2.616	4.830	4.830	22.257 Cd	22.548
5.342	5.270	3.622	3.670	11.128	11.263
5.342	5.246	4.830	4.830	10.171 Hg	10.141
2.671	2.694	3.622	3.622	40.684	40.523

The data in Table 3 show that by aid of the aforementioned procedure, quaternary mixtures are successfully analyzed with but slight errors in few cases.

The procedure applied for analysis of binary mixtures may be extended to the analysis of chromium plus many divalent metal ions other than those presented. The same applies to analysis of a variety of ternary and quaternary mixtures.

We failed to analyze 5-component mixtures of the type Cr + Cu + Hg + Pb + Ni, or Cd, by the procedure followed for analysis of quaternary mixtures, with the additional use of cyanide to mask all components except lead, prior to its volumetric determination with EDTA. However, the first three components were determined with

TABLE 3

ANALYSIS OF QUATERNARY MIXTURES

Chromium (mg)		Mercury (mg)		Metal (mg)		Metal (mg)	
Taken	Found	Taken	Found	Taken	Found	Taken	Found
5.342	5.384	20.462	20.462	2.714 Fe	2.714	6.045 Ni	6.162
2.671	2.627	10.231	10.231	4.071	4.071	3.627	3.697
4.006	3.976	30.693	30.693	5.428	5.428	2.418	2.418
5.342	5.278	30.693	30.621	2.714	2.714	31.230 Pb	31.290
2.671	2.643	10.231	10.303	4.071	4.071	31.230	31.317
4.006	4.028	20.462	20.414	1.357	1.357	10.410	10.422
5.342	5.274	8.907	8.907	4.071	4.071	23.527 Cd	23.500
2.671	2.636	17.814	17.731	2.714	2.714	11.763	11.904
1.335	1.350	13.360	13.360	5.428	5.428	5.881	6.166
2.671	2.709	10.171	10.171	4.830 Cu	4.830	5.986 Ni	5.998
5.342	5.349	5.085	5.085	7.245	7.245	3.591	3.514
5.342	5.342	5.085	5.085	4.830	4.830	10.331 Pb	10.187
2.671	2.656	10.171	10.171	3.622	3.622	20.662	20.546
2.671	2.646	10.171	10.171	4.830	4.830	22.257 Cd	22.548
5.342	5.270	15.256	15.256	3.622	3.670	11.128	11.263

requisite accuracy. This behavior is attributed to the probable masking of lead in the presence of large amounts of cyanide more than sufficient to mask the other four components.

The titration processes are attended with potential jumps, lying within the expected end points and ranging from 70 to 130 mV/0.1 ml of 0.05 *M* titrant. It is worthy to mention that citrate decreases potential jumps considerably owing to its tendency to decrease the concentration of free mercuric ions, just beyond the end point, by probable chelation. The literature refers to citric acid, among other hydroxy acids, as chelating with many metal ions among which mercury(II) is not mentioned.

The prime importance of the present work stems from the fact that components of the mixtures analyzed are constituents of many useful alloys such as nickel, chromium, copper, nickel–chromium or nickel–copper steels which meet numerous requirements, viz. resistance to corrosion, high tensile strength, toughness, good wearing qualities, and high shock resistance.

SUMMARY

Multicomponent mixtures containing invariably chromium are successfully analysed, by potentiometric back-titration of excess EDTA with mercury (II) using silver amalgam as indicator electrode. One titration is carried out after masking chromium with citrate and the other after boiling the mixture with EDTA. In ternary and quaternary mixtures, iron(III) and copper (II) are determined iodometrically, whereas mercury(II) in quaternary mixtures is determined potentiometrically with iodide, without interference from other components.

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A Microspectrophotometric Method for the Determination of Bilirubin

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The Unopette System[1] is widely used for various hematological tests and chemical microanalyses (*5, 6, 7, 11, 16*). The unique feature of this system is the measurement of a microvolume of specimen with a *self*-filling capillary pipette. The liquid specimen is subsequently transferred quantitatively into a premeasured volume of reagent (Fig. 1).

The spectrophotometric measurement of serum bilirubin is an ideal application for the Unopette since a simple dilution of the specimen is the only treatment required prior to measuring the absorbance of the solution. In 1954, Shinowara showed that it is possible to determine bilirubin in the presence of hemoglobin by measuring absorbance at two wavelengths (*12*). Other investigators have subsequently used differential spectrophotometry for the microdetermination of bilirubin in sera of newborn infants (*4, 10, 14, 17*). Two wavelengths were used to determine bilirubin in the presence of high concentrations of hemoglobin. However, Shinowara reported that plasma specimens, as opposed to serum specimens, contain only traces of hemoglobin. It has recently been reported (*16*) that blood specimens collected in heparinized capillary tubes were visually free of hemolysis indicating that there are low levels of plasma hemoglobin in such specimens.

The purpose of this report is to show that it is possible to determine bilirubin in plasma containing limited amounts of hemoglobin by measuring the absorbance at a single wavelength using a Coleman Jr. spectrophotometer and plasma dilutions obtainable with the Unopette system.

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[1] Becton, Dickinson and Co., Rutherford, New Jersey.

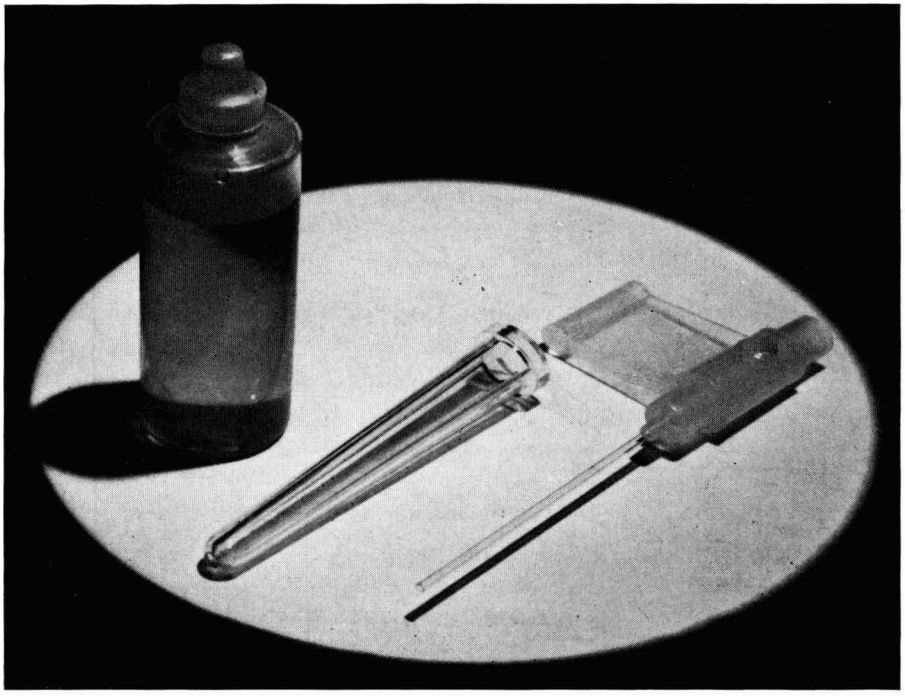

FIG. 1. Components of the Unopette System are the plastic reservoir containing diluent and the self-filling, self-measuring capillary shown with protective shield removed from the plastic capillary holder.

MATERIALS AND METHODS

1. Preparation of bilirubin standards. A stock aqueous solution of pure bilirubin (Eastman Kodak no. 2101) was prepared according to Shinowara (*12*) as follows: A weighed amount of bilirubin, usually 40 mg, was dissolved in 4 ml of phosphate buffer (pH 11.3), and 10 ml of 25% normal human albumin (Cutter) was added. This solution was transferred to a 100–ml volumetric flask and diluted to volume with Sorensen buffer (pH 7.4). An identical solution not containing bilirubin was used to prepare known mixtures of the standard solution. A similar procedure was carried out using pooled clear normal human serum containing less than 10 mg of hemoglobin/100 ml in place of human albumin. A 40-mg sample of bilirubin was dissolved in 4 ml of phosphate buffer (pH 11.3) and made up to 100

ml with the pooled serum. An identical solution not containing bilirubin was used as diluent to prepare standard solutions. The bilirubin concentration of each mixture was verified colorimetrically (9) and spectrophotometrically (see below).

2. *Blood collection and processing.* A heel puncture was made with a size 11 Bard-Parker blade (Becton, Dickinson and Co.), and 8–10 heparinized capillary tubes (1.2 mm i.d.) were filled with free-flowing blood. After centrifugation, the plasma was examined and tubes which were grossly hemolyzed were rejected. The outer surfaces of the remaining tubes were wiped free of blood, broken just above the buffy coat, and the plasma pooled in a Unopette capillary shield (Fig. 1) (which served as a micro test tube) and protected from bright light when not in use. Macro specimens were collected by venipuncture using Vacutainer tubes.[2]

3. *Dilution of plasma for the spectrophotometric determination of bilirubin.* A Unopette reservoir containing 1.0 ml of phosphate buffer, 0.067 *M* (pH 7.4) and two 25–μl capillaries [3] were used for dilution of the plasma. The technique of adding plasma to the reservoir was carried out according to standard Unopette procedure as follows: 25 μl of plasma were collected in each of two self-filling capillary tubes. Excess plasma was removed from the outside of the tube by carefully wiping with gauze. The Unopette reservoir was squeezed slightly and the capillary holder was fitted into the reservoir. By alternately relaxing and squeezing the walls of the reservoir, plasma was rinsed from the capillary into the diluent. The solution was well mixed and the second 25–μl capillary tube of plasma was similarly diluted.

4. *Spectrophotometry.* A Coleman Jr. spectrophotometer, model 6A, was used with a 7.5–mm o.d. stoppered cuvette in an adapter modified to accommodate 1–ml minimum volume. The cuvette adapter was modified by placing metal shims in the apertures so that the lower edges of the shims covered the meniscus of 1.0 ml of liquid in a 7.5–mm cuvette (Fig. 2). Distilled water was used as the optical reference. Transmittance was read to the nearest ¼ division and conversion to absorbance was made using a 4-place table. Wavelength calibration

[2] Becton, Dickinson and Co., Rutherford, New Jersey.

[3] The 50-μl Unopette capillaries were not available at the time this study was in progress.

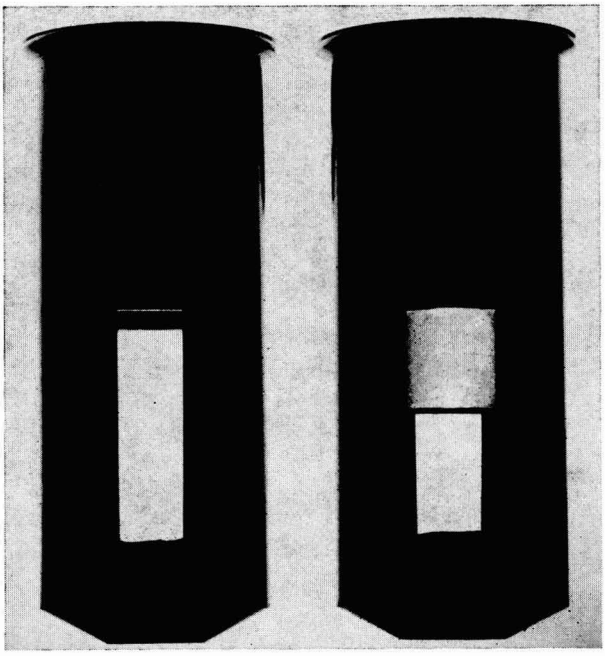

FIG. 2. Coleman Jr. spectrophotometer cuvette adapter with (right) and without metal shim inserted.

of the spectrophotometer was made with a didymium filter. A Beckman DU II spectrophotometer was used with cells having a 1–cm light path and 0.5– or 2.5–ml minimum volume.

Bilirubin concentrations were determined colorimetrically by the Malloy and Evelyn (*9*) modification of the van den Bergh reaction. Bilirubin concentrations determined with a Beckman DU II spectrophotometer were computed using Shinowara's equations (*12*):

$$Cb = 1.27\ A_{450\ m\mu} - 1.35\ A_{575\ m\mu},$$

Cb = concentration of bilirubin,

mg total bilirubin/100 ml = ($Cb \times$ dilution)—0.44.

Bilirubin, hemoglobin, and hematin concentrations were determined simultaneously by the spectrophotometric procedure of Shinowara and Walters (*13*). Unless specified, bilirubin values obtained by this procedure were designated as "expected" values.

Experimental Procedure

Precision of Dilution Using the Unopette

The dilution used with the Unopette in this study was 1:21 (50 μl of serum added to 1.0 ml of buffer). Since the dilution using the Unopette System required the addition of two 25–μl samples of serum to 1.0 ml of diluent, it was necessary to test the magnitude of error due to the double addition of sample. Six separate dilutions were made on one serum specimen. Bilirubin values obtained with a Beckman spectrophotometer using Shinowara's equations are shown in Table 1.

TABLE 1

Reproducibility of Bilirubin Concentrations Obtained at 1:21 Dilution (Beckman Spectrophotometer)

Analysis no.	Bilirubin (mg/100 ml)
1	10.27
2	10.48
3	10.27
4	10.27
5	10.27
6	10.27
mean	10.30
± SD	0.02

Comparison of Optimal Dilution (Shinowara) with Unopette Dilution

Shinowara suggested that the dilution used in the spectrophotometric bilirubin determination should have an absorbance between 0.5 and 1.2 at 450 mμ. In order to evaluate the results obtained at the high dilution a number of serum specimens were analyzed according to the procedure described by Shinowara and after dilution with the Unopette. Absorbances of all specimens were measured with a Beckman spectrophotometer and bilirubin was calculated using Shinowara's equations. Results of the parallel analyses are presented in Table 2.

Determination of the Absorptivity of Bilirubin Using a Coleman Jr. Spectrophotometer

A. Comparison of values obtained using albumin and human serum diluents. Absorbance values were obtained at 450 mμ and at 575

TABLE 2

BILIRUBIN VALUES OBTAINED WITH OPTIMAL DILUTION (SHINOWARA) AND UNOPETTE DILUTION (1:21) USING THE BECKMAN SPECTROPHOTOMETER

Specimen no.	Bilirubin (mg/100 ml)	
	Optimal dilution	Unopette dilution
1	0.7	0.8
2	1.6	1.2
3	1.6	1.6
4	1.7	1.7
5	2.3	1.7
6	5.8	5.2
7	6.3	5.7
8	7.0	6.6
9	7.4	6.9
10	7.5	7.5
11	10.6	10.3
Mean	4.8	4.5
± SD	± 3.1	± 3.1
r		0.997

mμ using bilirubin standards diluted in albumin and in serum. Absorptivities *a* were computed for each mixture, using the equations:

$a = A_{450}\text{m}\mu/C$, $\qquad a = (A_{450}\text{m}\mu - A_{575}\text{m}\mu)/C$,

$C =$ mg of bilirubin/100 ml.

Mean *a* values obtained are shown in Table 3. Absorptivities obtained with the bilirubin–albumin standard gave results 1–3 mg/100 ml higher than expected when these constants were used to compute the concentration of bilirubin in the serum standards. Similar discrepancies were observed when the constants were used to determine the concentration of bilirubin in patient specimens that were subsequently analyzed. It was concluded therefore that the absorptivities obtained from bilirubin–serum standards should be used for the determination of bilirubin in patient specimens.

B. *Determination of bilirubin using Unopette dilution.* A series of specimens containing known amounts of added bilirubin were diluted 1:21 with Unopettes and absorbances were obtained using Coleman Jr. and Beckman spectrophotometers; the same specimens were also

TABLE 3

ABSORPTIVITY FOR BILIRUBIN OBTAINED WITH COLEMAN JR. SPECTROPHOTOMETER USING ALBUMIN AND SERUM DILUENTS (UNOPETTE DILUTION)

Bilirubin concentration (mg/100 ml)	n	Protein solution	$a = A_{450\ m\mu} / C^a \times 10^{-2}$ Mean	± SD	$a = (A_{450\ m\mu} - A_{575\ m\mu}) / C^a \times 10^{-2}$ Mean	± SD
11.6–23.2	8	albumin	2.103	0.051	2.068	0.043
11.7–22.2	8	serum	2.373	0.153	2.285	0.143

C^a = mg of bilirubin/100 ml.

analyzed colorimetrically. The results obtained are presented in Table 4. Close agreement was found with two exceptions. The concentration obtained using the Coleman Jr. spectrophotometer was lower than expected in specimen A, but higher than expected in specimen F. Apparently the concentration in specimen A exceeded the limitation established for compliance with Beer's Law. When the analysis was repeated using 25 μl of plasma, the expected result was obtained. Subsequently, whenever a value of 30 mg/100 ml or greater was found the analysis was repeated using 25 μl of plasma. In order to determine the significance of the high value found in specimen F, a series of 11 specimens were analyzed according to the proposed procedure; colorimetric analyses also were done on 7 specimens. The results shown in Table 5 indicate that, although differences were found, particularly in the low concentration range, they were not clinically significant.

C. Comparison of bilirubin values calculated from a single absorbance (450) and the difference of two absorbance readings (450–575). Nineteen plasma specimens containing less than 20 mg of hemoglobin/100 ml were analyzed for bilirubin according to the proposed procedure. Bilirubin concentrations calculated from a single absorbance reading (450 mμ) and from the difference between absorbances at 450 mμ and 575 mμ are shown in Table 6. Seven grossly hemolyzed specimens were similarly analyzed for bilirubin, and the data obtained are shown in Table 7. A series of blood specimens obtained by venipuncture from an infant with hemolytic disease of the

TABLE 4

BILIRUBIN CONCENTRATIONS OBTAINED COLORIMETRICALLY, AND SPECTROPHOTOMETRICALLY USING BECKMAN AND COLEMAN JR. SPECTROPHOTOMETERS AT UNOPETTE DILUTION (1:21)

		Bilirubin (mg/100 ml)			
		Spectrophotometric			
		Coleman Jr.			
Specimen code	Known	$a = \frac{A450\ m\mu}{C}$	$a = \frac{(A450\ m\mu - A575\ m\mu)}{C}$	Beckman [a]	Colorimetric
A	38.28	30.63	30.99	38.41	40.0
B	22.97	21.28	21.44	22.66	23.4
C	19.14	18.39	18.37	18.88	19.8
D	15.31	15.68	15.65	15.73	15.4
E	11.48	12.56	12.51	12.16	10.9
F	—	1.93	1.57	0.82	0.6

[a] Computed from Shinowara's equations.

TABLE 5

BILIRUBIN CONCENTRATIONS OBTAINED COLORIMETRICALLY AND SPECTROPHOTOMETRICALLY (UNOPETTE DILUTION, AND COLEMAN JR.) FOR LOW BILIRUBIN VALUES

Specimen no.	Expected	Bilirubin (mg/100 ml) Spectrophotometric Found $a = A_{450\,m\mu}/C$	Bilirubin (mg/100 ml) Spectrophotometric Found $a = (A_{450\,m\mu} - A_{575\,m\mu})/C$	Colorimetric
1	0.9	3.6	1.9	1.1
2	1.2	3.6	3.4	—
3	1.3	2.4	1.9	1.0
4	1.6	1.3	1.3	1.2
5	1.6	2.4	2.4	1.7
6	1.6	3.7	3.0	0.9
7	2.3	1.8	1.8	2.1
8	4.3	4.5	4.3	3.9
9	5.4	5.8	6.0	—
10	5.8	6.7	5.9	—
11	6.3	6.2	6.1	—
Mean	2.9	3.8	3.4	
SD	± 2.0	± 1.7	± 1.8	
r		0.833	0.919	

TABLE 6

BILIRUBIN VALUES OBTAINED SPECTROPHOTOMETRICALLY USING SINGLE ABSORBANCE AND TWO ABSORBANCE MEASUREMENTS IN SPECIMENS CONTAINING LOW HEMOGLOBIN CONCENTRATIONS (< 20 mg/100 ml)

Specimen no.	Hemoglobin (mg/100 ml)	Bilirubin (mg/100 ml)		
		$a = \frac{A450\ m\mu}{C}$	$a = \frac{(A450\ m\mu - A575\ m\mu)}{C}$	Expected
1	18.3	1.8	1.3	0.5
2	18.7	3.6	1.9	0.9
3	0.0	2.4	2.0	1.3
4	4.2	3.7	3.0	1.6
5	6.3	4.4	4.3	4.3
6	0.0	5.8	6.0	5.4
7	12.3	8.0	7.7	7.4
8	9.0	9.5	9.3	7.7
9	12.3	10.1	9.9	9.9
10	3.1	10.7	11.0	11.1
11	0.0	11.7	11.2	11.4
12	3.1	10.5	10.8	11.9
13	7.8	15.6	15.5	14.6
14	2.5	14.3	14.5	15.8
15	0.0	18.0	18.2	20.1
16	3.0	20.3	21.0	22.7
17	0.0	21.5	21.7	24.0
18	5.4	53.8	54.7	57.8
19	5.4	55.5	55.8	57.8
Mean		14.8	14.7	15.1
SD		± 16.3	± 15.1	± 16.2
r		0.910	0.998	

TABLE 7

BILIRUBIN VALUES OBTAINED SPECTROPHOTOMETRICALLY USING A SINGLE ABSORBANCE AND TWO ABSORBANCE MEASUREMENTS IN SPECIMENS CONTAINING HIGH HEMATIN AND/OR HEMOGLOBIN CONCETRATIONS

Specimen no.	Bilirubin (mg/100 ml) $a = A_{450\,m\mu}/C$	Bilirubin (mg/100 ml) $a = (A_{450\,m\mu} - A_{575\,m\mu})/C$	Serum pigments (mg/100 ml) Bilirubin	Hemoglobin	Hematin
1	3.6	3.4	1.2	37.4	69.0
2	5.5	4.7	3.4	123.7	28.9
3	10.0	9.6	7.5	85.8	6.7
4	9.2	8.6	8.3	44.9	107.6
5	21.6	18.6	11.1	376.2	8.2
6	18.0	18.2	19.0	25.6	1.0
7	22.8	22.7	22.3	55.5	2.9
Mean	13.0	12.3	10.4		
± SD	7.20	6.97	7.13		
r	0.894	0.947			

TABLE 8

BILIRUBIN CONCENTRATIONS FOUND IN PLASMA OF ERYTHROBLASTOTIC INFANT USING SINGLE ABSORBANCE AND TWO ABSORBANCE MEASUREMENTS

Specimen (time, date)	Bilirubin (mg/100 ml)					
	$a = A_{450\,m\mu}/C$		$a = (A_{450\,m\mu} - A_{575\,m\mu})/C$		Serum pigments (mg/100 ml)	
					Bilirubin	Hemoglobin	Hematin
9 a.m. 10-18	6.7		5.9		5.8	13.1	108.5
10 a.m. 10-18 [a]	8.7		7.9		7.0	47.6	115.1
6 p.m. 10-18 [a]	14.0		13.3		13.2	47.8	105.4
6 p.m. 10-18 [b]	6.2		6.1		6.3	5.8	22.4
4 p.m. 10-19	15.3		15.0		17.1	0.0	78.8
9 p.m. 10-19 [c]	16.5		15.7		16.9	4.6	85.7

[a] Specimen taken immediately before exchange transfusion.

[b] Specimen taken immediately after exchange transfusion.

[c] Two hours before death.

TABLE 9

COMPARISON OF BILIRUBIN VALUES OBTAINED COLORIMETRICALLY AND SPECTROPHOTOMETRICALLY FROM A SINGLE ABSORBANCE MEASUREMENT AND TWO ABSORBANCE MEASUREMENTS IN PLASMA COLLECTED IN HEPARINIZED CAPILLARY TUBES

	Bilirubin (mg/100 ml)		
	Spectrophotometric		
Specimen no.	$a = / \frac{A_{450\,m\mu}}{C}$	$a = / \frac{(A_{450\,m\mu} - A_{575\,m\mu})}{C}$	Colorimetric
1	5.9	5.9	3.7
2	9.7	9.4	7.5
3	12.6	12.2	9.3
4	10.0	10.1	9.4
5	11.3	10.9	10.0
6	11.1	11.3	10.5
7	13.9	14.1	11.7
8	12.3	12.1	11.8
9	14.8	14.9	13.5
10	13.2	12.8	13.8
11	17.0	16.6	17.6
12	18.6	18.7	18.0
13	18.6	18.6	19.3
14	21.0	21.2	19.5
15	18.6	18.6	21.0
16	20.2	20.6	21.9
Mean	14.3	14.3	13.7
± SD	4.2	4.3	5.2
r	0.968	0.962	

newborn contained high concentrations of hematin and/or hemoglobin. The bilirubin concentrations obtained spectrophotometrically using a single absorbance value and the difference of two absorbance values are shown in Table 8. Blood specimens were obtained from 16 infants using the micro-collection technique described. Hemolysis was not visually evident in any of the capillary tubes. Colorimetric and spectrophotometric microanalyses for bilirubin were carried out on each specimen, and the results are presented in Table 9.

D. Studies on the feasibility of using commercially prepared solutions as bilirubin standards. Only freshly prepared standards can be used to establish or to verify the absorptivity of an instrument since bilirubin in solution is unstable even in the frozen state. Therefore, an evaluation was made to determine if the lyophilized specimens that are commercially available could be used as standards. One preparation that has high concentrations of bilirubin dissolved in normal human serum is Versatol-Pediatric.[4] Three different lots of the product were analyzed. Three ampuls of each lot were reconstituted according to the manufacturer's instructions and pooled to yield sufficient volume for spectrophotometric analysis. Visual observations revealed that all solutions were extremely turbid. Colorimetric and spectrophotometric determinations for bilirubin were done on serum standards prepared by the laboratory and on the commercial products, and a comparison of the results obtained is presented in Table 10. Despite the turbidity, values calculated from the spectrophotometric data on all three Versatol-Pediatric preparations were in close agreement with the manufacturer's assay. Using Unopette dilutions and a Coleman Jr. spectrophotometer, simultaneous analyses were then done on Versatol-Pediatric and on heparinized capillary blood specimens. Bilirubin concentrations of the infants' specimens were computed using absorptivities previously established for that instrument, and were also computed using the absorbances obtained simultaneously on Versatol-Pediatric. The results obtained on 11 infants are shown in Table 11.

DISCUSSION

The results in Table 9 show conclusively that bilirubin concentrations can be determined in plasma specimens by measuring the ab-

[4] Warner Chilcott.

TABLE 10

SPECTROPHOTOMETRIC AND COLORIMETRIC ANALYSES FOR BILIRUBIN ON STANDARDS PREPARED BY THE LABORATORY AND ON VERSATOL-PEDIATRIC

Source of standard or control specimen	Bilirubin (mg/100 ml)		
		Found	
	Expected [b]	Spectrophotometric [c]	Colorimetric
Laboratory	20.6	20.2	20.6
	16.4	16.0	16.4
	12.3	12.0	12.8
	10.3	10.2	10.4
Versatol-Pediatric (1) [a]	20.0	20.0	19.1
(2)	21.0	21.1	21.0
(3)	21.0	20.8	21.0

[a] (1), (2), (3), = Different lot numbers.

[b] From amount added or from manufacturer's assay.

[c] Shinowara (12).

sorbance at a single wavelength using a Coleman Jr. spectrophotometer and Unopette dilutions. Nearly identical results were obtained when bilirubin values were computed from single wavelength measurements or from two wavelength measurements, and both values were in close agreement with those obtained colorimetrically. Heilmeyer concluded from studies on undiluted normal serum specimens, that the measure of absorbance at a single wavelength in the blue region was inadequate for bilirubin since both hemoglobin and lipochromes absorb in this region (*8*). In specimens containing the high concentrations of bilirubin that are encountered in infants with hemolytic disease of the newborn, the absorbance at 450 mμ due to lipochromes normally present is of little significance. In this study, by using pooled serum as the protein diluent for bilirubin standards, the effect of lipochromes was eliminated optically. Although hemoglobin absorbs at 450 mμ, absorbance values for bilirubin are higher at all wavelengths significant for both bilirubin and hemoglobin (*12*). Calculations using the absorptivities reported by Meites and Hogg (*10*)

TABLE 11

A COMPARISON OF BILIRUBIN CONCENTRATIONS ON INFANTS' SPECIMENS COMPUTED FROM COMMERCIAL STANDARDS AND FROM STANDARDS PREPARED BY THE LABORATORY [a]

Specimen no.	Bilirubin concentration, Cu (mg/100 ml)			
	Single wavelength [b]		Two wavelengths [c]	
	$Cu = \frac{Au}{As} \times Cs$	$Cu = \frac{Au}{a}$	$Cu = \frac{(A450\ m\mu - A575\ m\mu)_u}{(A450\ m\mu - A575\ m\mu)_s} \times Cs$	$Cu = \frac{(A450\ m\mu - A575\ m\mu)_u}{a}$
1	4.2	4.6	3.6	3.5
2	4.9	4.8	3.7	3.6
3	7.4	7.3	6.8	6.8
4	10.6	10.3	10.1	10.1
5	12.0	11.9	11.9	12.0
6	14.3	13.8	14.1	13.7
7	14.3	13.9	13.6	13.6
8	15.5	15.3	15.6	15.4
9	16.2	16.2	15.8	15.8
10	17.3	17.3	17.4	17.4
11	20.5	20.6	20.4	20.2

[a] Abbrev.: Cs = concentration of commercial standard; As = absorbance of commercial standard; Au = absorbance of patient's specimen; and a = absorptivity of laboratory standard.

[b] $A = 450\ m\mu$.

[c] $A450\ m\mu - A575\ m\mu$.

reveal that the higher the bilirubin concentration, the less relative effect hemoglobin has at 450 mμ. In addition, a high dilution of sera containing hemoglobin greatly reduces the absorbance at 575 mμ, the hemoglobin maximum, so that the latter cannot be measured with precision. In this study the absorptivity values for bilirubin using a Coleman Jr. spectrophotometer were determined from a single measurement at 450 mμ, and from the difference in absorbances at 450 mμ and 575 mμ. Results in Tables 5 and 6 show that good correlation was obtained if hemoglobin concentrations were less than 20 mg/100 ml and if bilirubin concentrations exceeded 3 mg/100 ml. When high concentrations of hemoglobin were present, values greater than expected were obtained when either absorptivity value was used. The difference was smaller when the absorptivity derived from two wavelengths was used (see Tables 7 and 8). Although hemolysis affects the results obtained with the proposed method, this was minimized by collecting blood flowing freely from a deep skin puncture into heparinized capillary tubes. If 8–10 tubes are collected, the yield of plasma is large enough to permit the rejection of any tube that shows gross hemolysis. Correlation coefficients for bilirubin results "expected" and "found" computed for the various specimen types are presented as a tabular summary in Table 12.

TABLE 12

COMPARISON OF CORRELATION COEFFICIENTS FOR TOTAL BILIRUBIN DETERMINED USING A SINGLE ABSORBANCE AND TWO ABSORBANCE MEASUREMENTS

Specimen type	No. analyzed	Correlation coefficient $a = A_{450\ m\mu}/C$	Correlation coefficient $a = (A_{450\ m\mu} - A_{575\ m\mu})/C$
Infant plasma collected by micro-technique	16	0.968	0.962
Nonhemolyzed serum (Hb < 20 mg/100 ml)	19	0.910	0.998
Hemolyzed serum (Hb > 20 mg/100 ml)	7	0.894	0.947
Serum containing low levels of bilirubin (< 7 mg/100 ml)	11	0.833	0.919

Replicate analyses for bilirubin made on a single sample of serum showed that a microdilution using the Unopette System is a highly reproducible technique. Although Shinowara recommended that sera be diluted to give an absorbance at 450 mμ of no less than 0.5, the results presented in Table 2 show that the Beckman spectrophotometer was suitable for the determination of bilirubin in specimens where absorbance values were as low as 0.04 after dilution. On the other hand, the data in Table 5 show that with the Coleman Jr. spectrophotometer, there was not sufficient sensitivity to determine low levels of bilirubin, where concentrations were less than 3 mg/100 ml and where absorbances of the diluted specimens were less than 0.06. The upper limit of compliance with Beer's Law for this spectrophotometer was 30 mg bilirubin/100 ml.

Meites and Hogg, who also employed a Coleman Jr. spectrophotometer for a microdetermination of serum bilirubin (*10*), have used bilirubin dissolved in 5% albumin solution. Their absorptivity at 450 mμ was 1.95×10^{-2}, which is lower than the one similarly obtained in this study (Table 3). This slight, but significant, difference could be due in part to the use of different commercial preparations of serum albumin. Becker (2) recently reported that various bilirubin–albumin preparations differ markedly in their wavelength-absorption spectra. Absorptivities obtained from the bilirubin–albumin standards used in this study were not satisfactory when applied to serum specimens even though the albumin solutions were visually free of all heme pigments. On the other hand, results in Table 11 show that Versatol-Pediatric, which is a commercial bilirubin control specimen prepared in normal human serum, is entirely satisfactory for use as a standard in the spectrophotometric determination of bilirubin.

The results in Table 8 were presented to show that bilirubin concentrations obtained spectrophotometrically were not affected by the presence of other pigments. However, data on this infant also show that hematin concentrations were excessively high in all specimens. A satisfactory explanation of the significance of high hematin levels has not yet been found, although there have been reports that high values indicate poor prognosis (*1*, *3*, *15*). The observations reported here emphasize the fact that bilirubin concentrations alone are not always sufficient to reflect the clinical course of an infant with Rh isoimmunization.

SUMMARY AND CONCLUSIONS

This study shows that bilirubin in plasma can be determined spectrophotometrically with an instrument of low resolution and sensitivity (such as the Coleman Jr. spectrophotometer) at high dilutions (1:21) which give absorbance values as low as 0.06. We have also demonstrated that total bilirubin in plasma can be measured spectrophotometrically with a Coleman Jr. spectrophotometer by measuring the absorbance at a single wavelength (450 mμ) if the hemoglobin concentration is less than 20 mg/100 ml. Hemolysis can be minimized by using plasma rather than serum by collecting blood in heparinized microhematocrit tubes. Pooled human serum is to be preferred as a diluent in preparing bilirubin standards. The direct spectrophotometric determination of bilirubin incorporated into the Unopette System provides a simple, rapid, accurate micromethod for use in the clinical laboratory.

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Microdetermination of Perchlorate as Chloride by Oxygen Flask Combustion

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INTRODUCTION

Although the oxygen flask method (*8*) has been used extensively for the microdetermination of halogens in organic halogen compounds, there is only one paper (*4*), not widely available in the United States, on the application of this method for the microdetermination of total halogen in oxyhalogen compounds. We have applied the oxygen flask method to the microanalysis of some flavylium perchlorate research compounds after reexamination of the requisite conditions with other organic and inorganic perchlorates. The oxygen flask combustion technique requires less skill than other methods (Carius digestion, fusion, thermal decomposition) for reducing perchlorate-chlorine to chloride. When combined with a coulometric titration finish, it enables a fast, simple, accurate determination of total chlorine in perchlorate compounds.

EXPERIMENTAL

Apparatus. Oxygen flask combustions were carried out using the following equipment available from Arthur H. Thomas Co.: [1] oxygen flask infrared igniter (No. 6472-B); black paper precut sample wrappers (No. 6471–Q25); 500-ml Thomas-Ogg combustion flask (No. 6471–P10). The specially constructed sample carrier, overall length 35 mm, was a detachable, perforated platinum basket 10 × 10 × 5 mm with a movable bottom flap for passage of the paper fuse, welded to a 0.03-inch diameter platinum wire with an end loop.

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Other items used were 15-ml volumetric flasks (Kontes Glass Co., special order, similar to catalog No. K-29700) and long-stemmed capillary pipettes, overall length about 23 cm (Arthur H. Thomas Co., catalog No. 8216-D).

The coulometric titrations were made with an Aminco-Cotlove automatic chloride titrator with 20 mm by 40 mm titration vials (American Instrument Co., catalog No. 4–4420B).

Reagents. The reagents were prepared according to directions (*1,2*) and include approximately 0.4 *N* aqueous KOH; 0.1 *N* NHO_3 + 10% glacial acetic acid; 0.6 *N* HNO_3 + 20% glacial acetic acid; gelatin reagent; and, standard NaCl solution (0.125 mg chloride/ml in 0.1 *N* HNO_3 + 10% glacial acetic acid).

Procedure. Weigh onto the wrapper 3–10 mg of organic perchlorate sample to give about 1.4 mg of chloride. Fold the wrapper to enclose the sample completely, insert into the platinum carrier with the fuse end protruding through the bottom slit, and hang the carrier on the stopper hook. Perform the combustion in a shielded, well-oxygenated flask containing 5 ml of 0.4 *N* KOH as absorbing solution and an encapsulated stirring bar. To ensure a gas-tight seal during the combustion, moisten the ground joint of the flask with 3 or 4 drops of absorbing solution just before seating the stopper; after the stopper is in place and held securely by hand, moisten the whole joint with several drops of distilled water and secure the stopper with a clamp without moving the parts of the joint. After the combustion, cool the flask for a few seconds by running a stream of cold water below the joint and stir magnetically 45 minutes for absorption of the combustion products. Remove the clamp and gently shake the carrier into the absorbing solution. Use a long-stemmed capillary pipette and 5 ml of 0.6 *N* HNO_3 + 20% glacial acetic acid reagent in several small portions to rinse down the stopper, ground joint, and walls of the flask and to transfer the absorbing solution to a 15-ml volumetric flask. Complete the rinse, transfer, and volume adjustment to 15 ml with small portions of about 5 ml of 0.1 *N* HNO_3 + 10% glacial acetic acid reagent. Mix well. Add 0.2 ml of gelatin reagent to each of three 4-ml aliquots and titrate at the "Medium" titration rate according to directions (*1,2*) with the chloride titrator. Average the three titration times and substract the time for a similarly determined

combusted blank. Determine the chloride factor (mg chloride/second) with standard NaCl solution by titrating under the same conditions an amount of chloride comparable to that found in the sample aliquot, correcting for a reagent blank (4 ml of the nitric–10% acetic reagent plus 0.2 ml of gelatin reagent). Optimum titration time for samples is 20–200 seconds (0.05–0.5 mg of chloride).

DISCUSSION AND RESULTS

The oxygen flask combustion technique should not be used indiscriminately to combust perchlorate compounds as some can decompose violently (*5,6,12a*). The combustion of any perchlorate should always be carried out in a well-shielded flask. When applying the method to perchlorates that might be explosive, the use of a specially designed bomb should be considered (*13*). None of the compounds reported here burned hazardously when approximately 5 mg was combusted in the usual manner. When burned as solids, most of the compounds flashed moderately, occasionally leaving a soot deposit on the wall of the flask. This did not affect the results adversely. However, low results were obtained unless the ground joint area around the stopper was wetted generously to ensure a gas-tight seal during combustion.

The results in Table 1 show that perchlorate, and chlorate, can be quantitatively reduced to chloride ion by the oxygen flask method. The conversion (*9,10,12b*) is effected probably by the high temperature (*11*) of combustion and the carbon and hydrogen (*8*) present in the wrapper. Additional organic matter was not necessary. Although the combustions were not as clean, equally good results were obtained with a quartz carrier as with a platinum carrier.

The flavylium perchlorate research compound in Table 1 was representative of several analyzed. There was no difficulty with the conversion of perchlorate to chloride with these samples or with the pyridinium perchlorate (*3*), DL-lysine · $HClO_4$ and L-arginine · $HClO_4$ that were prepared to study the method. When combusted as solids, the conversion was less reliable for $KClO_3$ and $KClO_4$, and incomplete for NH_4ClO_4. Consistently good results were obtained (cf. Table 1) for these inorganic compounds when they were completely dissolved

TABLE 1

CHLORINE RESULTS OBTAINED BY THE OXYGEN FLASK METHOD

Compound	Chlorine		
	Theor. (%)	Found (%)	Difference (%)
$C_{16}H_{13}O_6Cl$ (a flavylium perchlorate)	10.53	10.60	+0.07
		10.53	0.00
L–Arginine · $HClO_4$	12.91	13.07	+0.16
		13.16	+0.25
DL–Lysine · $HClO_4$	14.37	14.39	+0.02
		14.37	0.00
		14.33	−0.04
Pyridinium · $HClO_4$	19.74	19.80	+0.06
		19.77	+0.03
		19.74	0.00
$KClO_3$[a]	28.93	28.82	−0.11
		28.83	−0.10
		28.85	−0.08
		28.82	−0.11
$KClO_4$[a]	25.59	25.17	−0.42
		25.31	−0.28
		25.37	−0.22
NH_4ClO_4[a]	30.18	30.22	+0.04
		30.23	+0.05
		30.10	−0.08

[a] Dissolved on wrapper with hot 0.1% aq. $NaHCO_3$ and air dried before combustion.

on the wrapper with repeated applications of hot aqueous 0.1% $NaHCO_3$ solution and air dried before combustion.

Other Applications. Respective amounts of perchlorate—, chlorate—, and chloride ions present together in a solution can be determined easily as chloride by analyzing three separate aliquots. Table 2 shows the results of the analysis of a 0.1% aqueous $NaHCO_3$ solution containing weighed amounts of NaCl, $KClO_3$, and NH_4ClO_4. The amount of chloride ion was determined by direct coulometric titration of a 100-μl aliquot, and the amount of total chlorine as chloride after oxygen flask combustion (platinum carrier) of a 100-μl aliquot quantitatively transferred to a black paper wrapper and air dried. The

TABLE 2

DETERMINATION OF CHLORIDE, CHLORATE, AND PERCHLORATE AS CHLORIDE IN A SOLUTION [a]

Components determined	Chloride (mg/100 μl)		
	Theor.	Found	Difference (av.)
NaCl	0.377		
$KClO_3$	0.375		
NH_4ClO_4	0.375		
Total	1.127	(1.133, 1.133, 1.127) [b]	+0.004
NaCl	0.377		
$KClO_3$	0.375		
Total	0.752	(0.744, 0.745, 0.744) [c]	−0.008
NaCl	0.377	(0.374, 0.373, 0.374) [d]	−0.003
$KClO_3$	0.375	0.370 [e]	−0.005
NH_4ClO_4	0.375	0.387 [f]	+0.012

[a] Weighed samples were dissolved in 0.1% aq. $NaHCO_3$.
[b] By oxygen flask combustion method.
[c] By nitrite–sulfamic acid reduction method.
[d] By direct coulometric titration.
[e] By difference (c—d).
[f] By difference (b—c).

chlorate ion was reduced to chloride with nitrate as follows: A 100-μl aliquot was quantitatively transferred to a 15-ml volumetric flask. Five ml of 0.6 *N* HNO_3 + 20% glacial acetic acid reagent and 50 mg of $NaNO_2$ were added. The solution was mixed carefully and allowed to stand 10 minutes. One hundred mg of sulfamic acid was added to oxidize the excess nitrite (7) and the solution was mixed cautiously until gassing and foaming had ceased. Five ml of 0.4 *N* KOH was added and the solution made to volume with 0.1 *N* HNO_3 + 10% glacial acetic acid reagent, mixed well, and 4-ml aliquots (with 0.2 ml of gelatin reagent added to each) titrated coulometrically at the "Medium" titration rate. Appropriate blank determinations were carried out for each analysis. Perchlorate-chloride is the concentration of chloride found after the oxygen flask combustion less that found after the nitrite–sulfamic acid treatment. Chlorate–chloride is the concentration of chloride found after the nitrite–sulfamic acid treatment less that found by direct coulometric titration.

SUMMARY

Perchlorate in some organic and inorganic compounds was quantitatively reduced to chloride by the oxygen flask combustion method and determined coulometrically. Platinum and organic matter in addition to the sample wrapper were not necessary for the conversion to chloride. A method is described for determining respective amounts of chloride–, chlorate–, and perchlorate ion present together in a solution.

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A New Rapid Microassay for Enzymatic Reactions Involving Glyoxylate

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Current quantitative assay methods (*1–4*) for enzymes catalyzing glyoxylate condensation reactions (*1–5, 7–10*) are tedious, lengthy, and frequently expensive. For investigators concerned with purifying these enzymes and the separation of their isoenzymes (*5, 12, 13*), a rapid, simple, and economical assay would greatly facilitate research. This communication describes a microassay possessing these advantages. It is based on the principle that the ^{14}C-labeled gloxylate remaining at the conclusion of a reaction, or produced during the course of a reaction, can be rapidly isolated by thin-layer chromatography and quantitated by scintillation spectroscopy.

METHODS AND MATERIALS

All reagents used were the purest commercially available. Sodium glyoxylate-1-^{14}C was obtained from Nuclear Chicago, Des Plains, Illinois. Coenzyme-A (CoA) esters were prepared according to the method of Simon and Shemin (*11*) and assayed by the method of Lipmann and Tuttle (*6*).

Escherichia coli, strain E-26V (constitutive for malate synthase, E.C. 4.1.3.2) was the organism used as a source of enzymes in these studies. Cells were grown, harvested and cell-free extracts prepared as previously described (*14*).

Malate synthase was assayed at 232 mμ (*1*), in a Cary model 14 recording spectrophotometer (Applied Physics Corp., Monrovia, California).

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The microassay for determining malate synthase activity is performed in plastic disposable Microtiter trays (Cooke Engineering Company, Alexandria, Virginia). The following reaction mixture, in a total volume of 100 μl is added to a well of the tray: 4.0 μmoles of pyrophosphate buffer, pH 8.0; 0.4 μmoles of $MgCl_2$; O.1 μC of glyoxylate–1–^{14}C (specific activity 8.44 mC/mmole); 10 mμmoles of acetyl-CoA and enzyme. Two other reaction mixtures, lacking either enzyme or acetyl-CoA, are included as controls. Following incubation at 25°C, a 5–μl aliquot is removed from each well with a disposable Microcap pipette (Drummond Scientific Company, Broomal, Pennsylvania) and applied to an area on a sheet of silica gel impregnated glass microfiber (8 $\times$ 8-inch ITLC type SG, Gelman Instrument Company, Ann Arbor, Michigan) which has been previously spotted with 2 μl of glyoxylate solution (3 mg/ml). Samples may be spotted at 2–cm intervals along the origin. A chromatographic control, consisting of a 5–μl spot of glyoxylate (3 mg/ml), is included on each sheet. The spots are dried during application with a stream of hot air. The sheets are then developed in a rectangular chamber (26 $\times$ 26 $\times$ 7 cm) containing 42 ml of a solvent consisting of chloroform: methanol: formic acid (40:1:1). After 20 minutes, the sheets are removed and dried under a stream of hot air until the formic acid odor has disappeared. The location of the glyoxylate in the chromatographic control is determined by spraying that portion of the chromatogram with 0.04% bromphenol blue (ethanolic). Those experimental areas on the sheet corresponding to the glyoxylate standard are cut out, placed in 10 ml of 0.4% 2,5–bis–[(2–(5–*tert*–butyl–benzoxazolyl)]–thiophene (Packard Instrument Company, Downers Grove, Illinois) in toluene: absolute ethanol (2:1) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

In order to illustrate the validity of the microassay procedure, various parameters were examined utilizing malate synthase (Acetyl-CoA + glyoxylate $\rightarrow$ malate + CoA.SH) as the test system.

Chromatography

Table 1 gives the R_f values of glyoxylate and malate in the solvent system employed. The migration of these two compounds is sufficiently

TABLE 1

MIGRATION OF GLYOXYLATE AND ITS CONDENSATION PRODUCTS IN A CHLOROFORM: METHANOL:FORMIC ACID (40:1:1) SOLVENT SYSTEM

	R_f
Glyoxylate	0.61
Malate	0.17
α-Hydroxyglutarate	0.30, 0.86 [a]

[a] The faster migrating spot is due to the formation of α-hydroxyglutaryl-lactone.

different to enable the quantitation of residual glyoxylate without contamination from the malate formed.

It has been observed that a small amount of radioactivity always remains at the origin during chromatography of glyoxylate standards or reaction mixtures. This can be minimized by spotting small aliquots, lowering the salt concentrations in the reaction mixture and pre-spotting with glyoxylate. The assay as presented under "Methods and Materials" minimizes this difficulty and yields reproducible results.

Characteristics of the Assay

The data presented in Fig. 1 illustrate the applicability of this assay in studying the malate synthase reaction in crude undialyzed extracts. It can be seen that when the concentrations of glyoxylate and acetyl-CoA are not limiting, the disappearance of glyoxylate is proportional to protein concentration.

The data presented in Fig. 2 demonstrate the course of the reaction with time. It can be seen that under the experimental conditions employed the disappearance of glyoxylate parallels the formation of malate and is linear for approximately 7 minutes. Thus, at short time-intervals, the utilization of glyoxylate is equivalent to the formation of malate and the microassay provides a quantitative determination of the extent to which the reaction which has occurred. At later times, the formation of malate is not stoichiometric. This is to be expected since these experiments were conducted with fresh, undialyzed, crude, cell-free extracts and malate would be expected to be further metabolized.

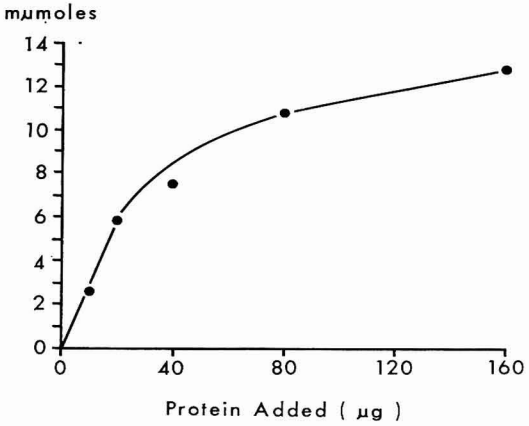

FIG. 1. Effect of protein concentration on the utilization of glyoxylate in the microassay.

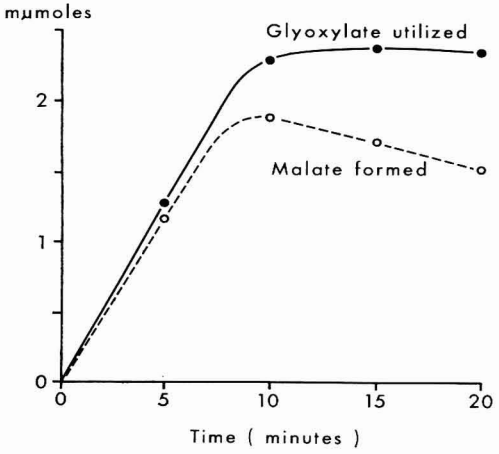

FIG. 2. Enzymatic utilization of glyoxylate as a function of time in the microassay. Malate was determined by counting the areas of the chromatograms from the microassay corresponding to a malate chromatographic standard.

Applications of the Assay

The assay described in this paper was developed especially to provide a method for the rapid quantitative determination of enzymatic reactions in which glyoxylate is a substrate. To illustrate its

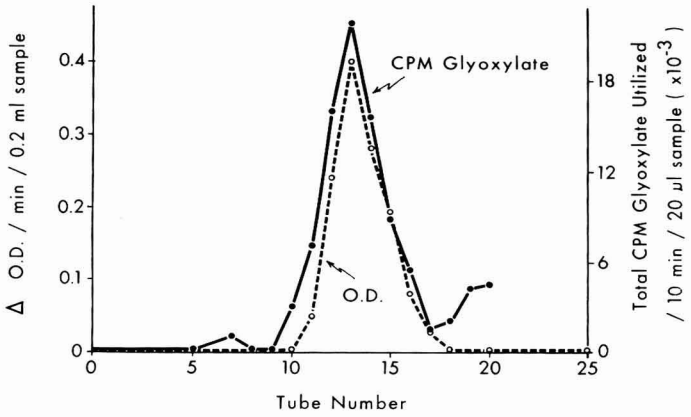

FIG. 3. Elution of malate synthase activity from a Sephadex G-100 column. —represents enzyme activity of the samples determined with the spectrophotometric assay;—represents enzyme activity of the samples determined with the microassay.

applicability, the partial purification of malate synthase was followed utilizing both the microassay and a spectrophotometric assay which is based on the cleavage of the thio–ester bond of acetyl-CoA (*1*). Six-tenths ml of a crude, cell-free extract of *E. coli* E-26V grown on propionate was placed on a 0.9 × 22–cm Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.) column, which had been equilibrated with 0.01 *M* pyrophosphate buffer, pH 8.0, containing 1 m*M* ethylenediaminetetraacetic acid. The enzyme was eluted with the same buffer, collecting 0.6–ml fractions. The results of assays of these fractions are shown in Fig. 3. It should be noted that the two curves, representing malate synthase activity as determined by the two assay procedures, are superimposable. Further, it is especially significant to mention that the microassay was completed more rapidly and with the expenditure of approximately one-tenth the amount of material employed in the spectrophotometric assay. Initial velocities can be measured with the microassay, but it is necessary to use short incubation times when the reaction rates are linear.

The microassay has also been used successfully to determine α–hydroxyglutarate synthase (E.C. 4.1.3.9) which catalyzes the condensation of glyoxylate with propionyl-CoA (*7*). Thus, if a solvent

system is available which will separate the product of a reaction involving glyoxylate from this substrate, one can employ this assay to great advantage. It can be seen from Table 1 that the solvent system utilized in assaying malate synthase is satisfactory in this respect.

The savings in time, materials, and enzyme, and the ease of handling large numbers of samples make the assay ideal for purification procedures and chromatographic enzyme separations. The sensitivity under the conditions described is in the millimicromole range and is limited only by the specific activity of the glyoxylate employed in the reaction mixture.

SUMMARY

A rapid, microassay for enzymatic reactions involving glyoxylate is discussed. This assay is based on the quantitative determination of residual glyoxylate—^{14}C following isolation by thin-layer chromatography.

ACKNOWLEDGMENTS

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The Separation of Copper and Iron Valencies by Paper Chromatography

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There is a gap in the literature explaining the separation of different oxidation states of metallic ions. There has been some work in the study of trace elements (*3, 20*) in plants and animal systems, and a few attempts in the inorganic field to resolve the oxidation states of metals, such as iron (*5, 6, 11, 12, 15, 19, 24, 25*), uranium (*7, 11, 19*), antimony (*2, 13, 16, 17*), arsenic (*13*), chromium (*2, 13, 16*), mercury (*13, 16*), plutonium (*4*), and platinum (*13*). These attempts were more or less of an empirical nature. This paper is a study of the mechanism of separation of copper and iron oxidation states leading to an explanation of the differential migration of the different valency states.

THEORETICAL CONSIDERATIONS

The migration of cations in paper chromatography is closely associated with the complexation which they undergo during elution. The cations migrate as solvo complexes or as anionic complexes. The cellulose substratum of the chromatographic paper is amphoteric, and the migration of metallic complexes on it are greatly influenced by their ionic or covalent nature. Coordination complexes can be arbitrarily divided into ionic and covalent. Ionic complexes should migrate faster in ionic solvents and covalent complexes in nonpolar solvents. This in fact is the fundamental basis by which ions in different valency states have been resolved. Metal ions in different valency states manifest varying degrees of electronegativity according to which, complexes formed by them differ in their degree of ionic or covalent nature, though this is also influenced by the nature of the ligand. During chromatographic migration, a complex can change its coordination number and hence its shape, according to the number of solvent ligands it can accommodate. Generally polar solvents are associated with ionic complexes rather than covalent ones, and nonpolar solvents with covalent

rather than ionic complexes. A transition metal which can form both covalent and ionic complexes is therefore, solvated by both types of solvents. If in one oxidation state it gives a more ionic complex than in the other with the same ligand, then the separation of the two oxidation states becomes readily possible.

EXPERIMENTAL METHOD

Preparation of Solution.

Cuprous chloride solution. A 0.2 *M* solution of Cu(I) was prepared by dissolving cuprous chloride [freshly prepared daily by the Palmer's method (*10*)] in saturated aqueous potassium chloride. This solution containing 12.72 μg of Cu/μl, was sufficiently stable for chromatographic runs of 20-minutes duration.

Cupric chloride solution. A 0.2 *M* solution was prepared by dissolving AnalaR $CuCl_2 \cdot 2H_2O$ in saturated potassium chloride. One microliter of this solution contains 12.72 μg of Cu.

Ferrous chloride solution. A 0.2 *M* solution was prepared by dissolving AnalaR iron wire in the minimum amount of A.R. HCl. One μl of this solution contains 11.17 μg of iron.

Ferric chloride solution. A 0.2 *M* solution was prepared by oxidizing the ferrous chloride solution with excess of 100 vols H_2O_2. One μl of this solution contains 11.17 μg of iron.

Chromatographic paper. Circles (15 cm diam) of Whatman no. 1 were used in all the experiments. In the case of copper, the paper was impregnated with a 2% KCl solution as this treatment was found to stabilize the Cu(I) during migration.

Spraying reagents. Spraying reagents for developing the chromatograms were chosen with a view to sensitivity and specificity. A 0.2% alcoholic solution of rubeanic acid is sensitive to 0.01μg of Cu and it gives an olive green color with Cu(II) and reddish brown with Cu(I) (*1*).

Aqueous solutions of potassium ferricyanide and ferrocyanide were (0.1%) used to detect Fe(II) and Fe(III), respectively. These reagents are sensitive to 0.1 μg of iron. Indiscriminate use of ferri and ferrocyanide reagents can interfere with the proper shade and density of the colors in the chromatograms. To get ideal shades without inter-

ference, pilot chromatograms were run to locate the spots and then the other chromatograms were carefully sprayed in the appropriate places, see Fig. 1.

Measurement of R_f values. The R_f values were evaluated by measuring the distance between the center of the spot applied and the outer boundary of the chromatographic bands, provided the bands were even and regular. When the bands were irregular, measurements were taken at several points on the band and the average was used.

Radial paper chromatography. Radial chromatography (*18*) was adopted for its well-known advantages over the other techniques, namely, (a) better band sharpness due to progressive dilution of the solute during the radial migration, (b) more than one sample can be simultaneously chromatographed by the Kawerau's modification of the technique, and (c) the facility to work with more than one detecting agent by cutting out sectors from the completed chromatograms and treating them separately. For convenience of comparison of R_f's of the ions, Kawerau's modification of the Rutter's technique was adopted in the present work. Although slight variations in R_f's even in the same system is a common experience in chromatography, there is an additional possibility of error due to variations in the physical contact between the paper and the irrigating wick. To allow for this error, the method adopted in this laboratory was to compare R_f values for fixed distance runs of the solvent front.

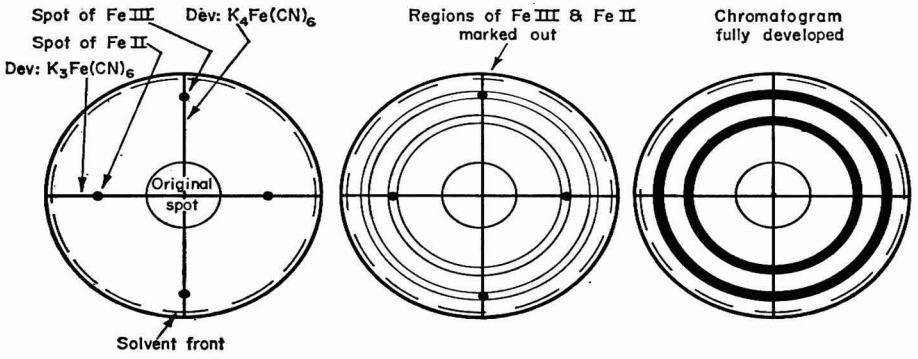

FIG. 1. Schematic representation for locating the migrated ions by two different spraying reagents.

Polarographic estimation. The chromatographically resolved ions were estimated polarographically using Radiometer Polariter PO3.

Copper usually yields a single curve in HCl media (*8*) corresponding to a reduction of Cu(II)→Cu. The presence of KCl sufficiently stabilizes the Cu(I) ion (*21*) in the supporting electrolyte (1:1 *M* HCl and KCl) so as to yield a two-step wave with $E_{1/2}$ of the first wave at —0.05 V vs S.C.E., and that of the second wave at —0.3 V. But since the region of the second wave is better-defined than the first, the second wave was adopted for the preparation of the working graph which was approximately linear. During the preliminary process of incineration and extraction, both Cu(I) and Cu(II) ions were ultimately brought into the same state of oxidation, namely Cu(II). Hence the same working graph of Cu(II) was applicable to both the oxidation states.

The regions of the cations on the chromatograms were marked and punched out individually. They were separately incinerated, the residues were extracted with conc. HCl and the solution made up to 10 ml with the supporting electrolyte, followed with 2 drops of 0.01% gelatin. The solutions were de-aerated by bubbling purified hydrogen and the polarograms were recorded.

For the preparation of a working graph, different aliquots containing 40 to 205 μg of an approximately 0.05 *M* solution of A.R. cupric chloride were spotted on Whatman no. 1 filter paper impregnated with 2% KCl and subjected to the same treatment as in the case of the chromatograms.

Iron. Polarographic reduction of Fe(II) and Fe(III) ions has been investigated by several authors (*14, 22, 23*). Good polarographic curves have been obtained by complexing iron with citrate, tartrate and oxalate (*9*). In the present work, a 1:1 solution of 0.5 *M* potassium citrate and 0.5 *M* potassium chloride served as the complexing agent and the supporting electrolyte, respectively. Iron gave two waves with $E_{1/2}$ at 0.75 V and 1.03 V vs S.C.E. As the pH of the solution influences the half wave potential, all precautions were observed to keep the acidity almost constant in all determinations. The first wave being steeper than the second was selected for calculations. For the preparation of the working graph, aliquots containing 30 to 150 μg were used and the heights of the first wave ($E_{1/2} = 0.75$ V) were plotted against the respective concentrations.

Ferro and ferricyanides were not sprayed on chromatograms used in the polarographic estimations, since these developing agents would interfere with the estimations. The regions containing Fe(II) and Fe(III) were established from pilot chromatograms, marked, cut out, and subjected to the same treatment as the standards.

DISCUSSION

Copper. Copper is spotted on the paper in the form of its anionic complexes, $K_2(CuCl_3)$ and $K_2(CuCl_4)$. Cu(I) in $K_2(CuCl_3)$ is assumed to have a trigonal planar structure and the Cu(II) in $K_2(CuCl_4)$ a square coplanar structure. The solvent ligands link at the additional free coordinating sites, involving a change in structure for the complex. In the presence of large excess of solvent, the existing ligands may also be replaced by the solvent ligands to produce cationic solvo complexes, though this is only a remote possibility.

Addition of *one* solvent molecule to the trigonal coplanar Cu(I) complex causes a change to a tetrahedral configuration. The Cu(II) square coplanar structure has two free coordination sites which can accommodate two solvent ligands to give an octahedral configuration. The entry of *two* solvent molecules into Cu(II) should cause greater solubilization for the Cu(II) and consequently greater migration than Cu(I). This fact has been proved experimentally with methanol as solvent, see item 1, Table 2, where Cu(II) has an R_f value of 0.9 and Cu(I) an R_f value of 0.7.

TABLE 1

POLAROGRAPHIC DATA FOR WORKING GRAPH PREPARATION [a]

	Vol. of soln. spotted (ml)	Quantity in the spot (μg)		Height of wave (cm)	
		Cu	Fe	Cu	Fe
1.	.01	29.4	38.32	.7	.3
2.	.02	—	76.64	—	1.3
3.	.03	88.4	114.96	1.8	2.3
4.	.04	—	153.28	—	3.4
5.	.05	147.0	—	2.8	—
6.	.07	205.8	—	3.7	—
7.	.09	264.6	—	4.9	—

[a] Molarity of spotted solution: Cu, 0.0463; Fe, 0.0686.

TABLE 1

RESOLUTION OF COPPER AND IRON VALENCIES

Paper: Whatman no. 1 (Impregnated with 2% KCl for Cu).

Spotted: Cu(I) and (II), 127.14 μg each (20 μl of 0.1 M w.r.t. each); Fe(II)and Fe(III), 111.7 μg each (20 μl of 0.1 M w.r.t. each).

Redox ratio [d] (before chromatogram) $= \dfrac{M^{(x+1)+}}{[M^{(x)+} + M^{(x+1)+}]} = 50\%$.

Item no.	Solvents [a]	R_f [b]				Resolution factor [c] (ratio of R_f)		Estimated wt. (polarography)				Error on total metal (%)		Redox ratio after chromatogram	
		Cu(I)	Cu(II)	Fe(II)	Fe(III)	Cu	Fe	Cu(I)	Cu(II)	Fe(II)	Fe(III)	Cu	Fe	Cu	Fe
1.	MeOH:conc.HCl (100:1.0)	0.73	0.91	0.95	0.95	1.25	1.0	60	195	—	—	0.28	—	76	—
2.	EtOH:conc.HCl (100:1.0)	0.90	0.68	0.45	0.75	1.32	1.66	57	192	112	116	–2.08	2.06	77	50
3.	PrOH:conc.HCl (100:1.0)	0.84	0.40	0.35	0.75	2.10	2.14	48	213	108	118	2.64	1.16	81	52
4.	BuOH:conc.HCl (100:1.0)	0.69	0.29	0	0.73	2.38	0.73/0	39	219	98	118	1.46	–3.31	84	54
5.	MeOH:Ketone [e]:conc.HCl (50:50:1.0)	0.91	0.82	0.70	0.95	1.11	1.36	96	150	114	116	–3.26	2.95	60	50
6.	EtOH:Ketone:conc.HCl (50:50:1.0)	0.91	0.65	0.29	0.91	1.40	3.14	90	162	108	124	0.90	3.85	64	53
7.	PrOH:Ket:conc.HCl (50:50:1.0)	0.91	0.54	0	1.0	1.69	1.0/0	81	177	104	126	1.46	2.95	68	54
8.	BuOH:Ket:conc.HCl (50:50:1.0)	0.74	0.40	0	1.0	1.85	1.0/0	42	222	94	140	3.82	4.74	84	59
9.	MeOH:Etacet:conc.HCl (50:50:1.0)	0.93	0.75	0.50	0.90	1.25	1.8	87	156	102	120	–4.44	–0.63	64	54
10.	EtOH:Etacet:conc.HCl (50:50:1.0)	0.90	0.58	0.29	1.0	1.55	3.45	93	168	104	116	2.64	–1.50	64	52
11.	PrOH:Etacet:conc.HCl (50:50:1.0)	0.72	0.42	0.27	1.0	1.71	3.70	75	172	106	130	–2.86	2.95	69	56
12.	BuOH:Etacet:conc.HCl (50:50:1.0)	0.70	0.28	0.24	0.91	2.50	3.79	54	192	90	138	–3.26	2.06	78	60
13.	MeOH:Ket:Etacet:conc.HCl (50:50:50:1.5)	1.0	0.55	0.52	0.94	1.81	1.81	90	168	112	114	1.46	1.16	65	50
14.	EtOH:Ket:Etacet:conc.HCl (50:50:50:1.5)	0.9	0.57	0.34	0.98	1.58	2.88	87	159	106	112	–3.26	–2.42	64	51
15.	PrOH:Ket:Etacet:conc.HCl (50:50:50:1.5)	0.76	0.38	0.36	1.0	2.0	2.78	69	189	96	120	1.46	–3.31	73	55
16.	BuOH:Ket:Etacet:conc.HCl (50:50:50:1.5)	0.54	0.26	0.25	0.70	2.08	2.80	39	222	80	140	2.64	–1.52	85	63

[a] Solvent ratio is by volume.

[b] Values in the Table are averages of at least 3 chromatograms.

[c] Resolution factor is the ratio between the R_f of the faster moving species to the R_f of the slower species. A resolution factor of 1 would therefore mean that there is no separation, and values higher than 1 would indicate resolution.

[d] Redox ratio percentage is a quantitative index of separation of the two ions. The number 50 indicates 100% separation without oxidation or reduction. A number higher or lower than 50 indicates that oxidation or reduction, respectively, took place during chromatography.

[e] Ketone is to be understood as acetone in the case of Cu and MeEt Ketone in the case of Fe.

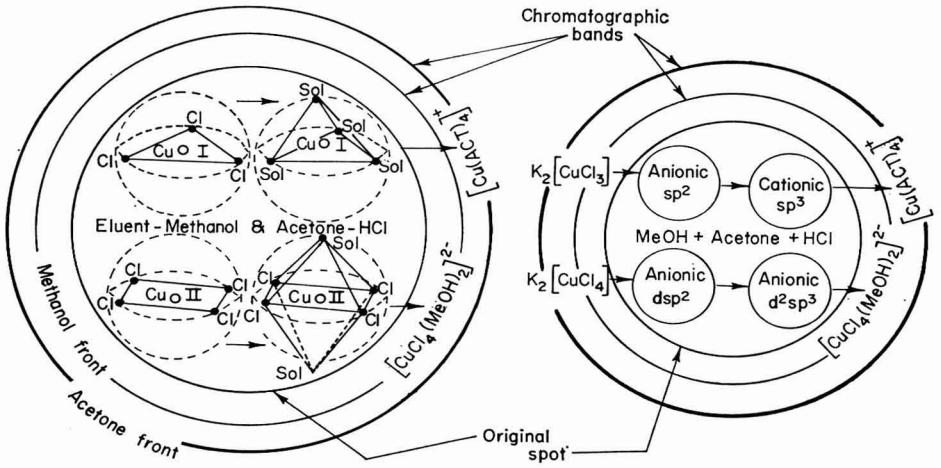

FIG. 2. Scheme of resolution of Cu(II) from Cu(I) using eluent, MeOH:Acetone:HCl.

As the size of the solvent ligand increases, steric hindrance comes into play, and the displacement of the existing ligands becomes increasingly difficult. This is all the more likely in the case of Cu(II) and the uptake of higher alcohols of the series will decrease in Cu(II). However this does not arise in the case of Cu(I) where there is no steric hindrance to going from coordination number 3 to 4. Hence Cu(I) becomes more solubilized and migrates faster in less polar solvents. As the solvent is changed from MeOH to EtOH, there is a reversal in the speed of migration of Cu(I) and Cu(II) as can be witnessed from Table 2 items 1 and 2. This idea has been diagrammatically illustrated in Figure 2. In a binary solvent Cu(I) prefers the less polar of the two components, and Cu(II) conversely the more polar. When a mixture of MeOH and acetone was used for elution, Cu(I) moved with acetone with an R_f of 0.9, whereas Cu(II) moved with MeOH with an R_f of 0.8, see item 5, Table 2.

The reluctance of the Cu(II) ion to take up the less polar solvents was demonstrated by using a series of solvents in which acetone was a common factor and the polarity was varied by the variation of alcohols, see items 6–9 in Table 2. Table 2 indicates how the gulf between the R_f values of Cu(I) and Cu(II) widens due to decreasing solvation of Cu(II) as the polarity of the solvent mixture decreases.

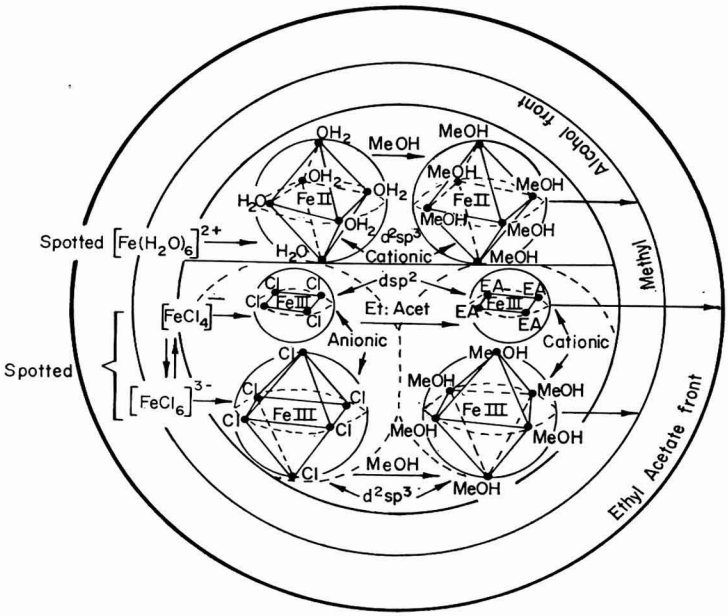

FIG. 3. Scheme of resolution of Fe(III) from Fe(II) using MeOH and Et.Acetate.

The slight fall in the R_f of Cu(I) in the butanol may be attributed to the viscosity of the solvent.

Satisfactory resolutions of Cu(I) and Cu(II) have been achieved making use of solvent mixtures, butanol:acetone, butanol:ethyl acetate, and propanol:ethyl acetate:acetone. However in certain cases a slight oxidation of Cu(I) is observed; and this is minimum in the case of lower members of the alcoholic series, probably because of the shorter time for chromatographic migration. A mixture of MeOH:acetone: ethyl acetate (item 13, Table 2) gave a good resolution with minimum oxidation.

Iron. The same principles considered under copper also hold good in the separation of Fe(II) from Fe(III). In a highly polar solvent such as acidified methanol, both Fe(II) and Fe(III) assume octahedral configuration undergoing almost equal solubilization and showing similar migration (item 1, Table 2). When the size of the ligand increases (the functional group remaining the same) the polarizability of the ligand becomes greater. This, added to the triple charge on the Fe(III)

ion, brings about strong polarization leading to covalent bonds. Fe(II) with a lesser charge on itself polarizes less, and its tendency to change over to covalent configuration is correspondingly less than in Fe(III). Fe(II) is therefore, less solvated by less polar solvents. Table 2 shows how the R_f values of Fe(II) rapidly fall from 0.95 to zero, when the solvents are varied from methanol to butanol.

In summary it may be mentioned that in order to bring about an effective resolution of Fe(II) and Fe(III), it is necessary to keep Fe(II) in ionic state and Fe(III) in covalent state, and use an eluent mixture of not too high a polarity. Propanol and butanol are found to be suitable for the purpose. Addition of solvents of lower dielectric constants such as ketones and ethyl acetate reduces the polarity of the eluent and results in better resolution.

SUMMARY

Separation of copper and iron valencies has been achieved using paper chromatographic technique. An explanation for the differential chromatographic behavior of the oxidation states has been given based on the differences in the ionic nature of their coordination complexes. The resolved ions are estimated polarographically.

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Methods for the Isolation and Characterization of Constituents of Natural Products

VIII. Gas-Liquid Chromatographic Resolution of Alcohol Ester, Amide, and Thioester Derivatives of Pyruvic Acid 2,6-Dinitrophenylhydrazone

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Parts I through VI of this series described the preparation, class separation, and thin-layer liquid-liquid partition chromatography of alcohol ester, amide, and thioester derivatives of pyruvic acid, 2,6-dinitrophenylhydrazone (*9–13, 15*). Part VII was devoted to purification of the derivatives with an anion exchange resin as a prerequisite to gas-chromatographic resolution of the derivatives (*14*). The present report is concerned with the resolution of homologous series of the derivatives by gas-liquid chromatography using both a flame ionization detector and an electron capture detector. The extreme sensitivity of the latter for polynitro compounds facilitates the detection of the derivatives in the millimicrogram range.

Resolution of phenylhydrazones (*3*), 2,4-dinitrophenylhydrazones of carbonyl compounds (*4, 16*), 3,5-dinitrobenzoates (*4*) and of *N*-2,4-dinitrophenyl amino acid esters (*1, 2, 5, 6, 8*) by gas chromatography using flame ionization detectors has already been described. Landowne and Lipsky (*7*) reported high orders of sensitivity for gas-liquid chromatography (GLC) of separated *N*-2,4-dinitrophenyl amino acid esters monitored by electron affinity spectrometry.

EXPERIMENTAL METHOD

Samples and solvent. Homologous series of esters of primary, secondary, and tertiary alcohols, amides of primary amines, and thio-

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ester derivatives of pyruvic acid 2,6-dinitrophenylhydrazone were obtained from stocks prepared and described by Schwartz and Brewington (*9, 11, 13*). Except for the tertiary alcohols, stock solutions of the first 10 members of each class were prepared in carbon disulfide and ethyl acetate to contain approximately 0.55 μM/ml of each member of each class. The following tertiary alcohol derivatives were made up also in concentrations of approximately 0.55 μM/ml each; *t*-butyl; *t*-amyl; 2-methyl-2-pentyl; 3-methyl-3-pentyl; 3-methyl-3-hexyl; 3-methyl-3-heptyl; 3-methyl-3-octyl.

In addition, a series of $C_{1\text{-}15}$ primary alcohol derivatives was prepared to contain approximately 0.55 μM/ml of each. Dilutions of the hydrazone solutions were made to establish lower limits of detection.

Solvents. Spectral grade of carbon disulfide was employed as the solvent for all of the hydrazone derivatives analyzed with the hydrogen flame detector and ACS grade ethyl acetate was used with the electron capture detector.

Apparatus.[2] An Aerograph model 204 gas chromatograph (Varian-Aerograph Co., Walnut Creek, Calif.) with a hydrogen flame ionization detector and an electron capture detector and a 0–1 mv Brown-Honeywell recorder were employed.

Samples were injected with a No. 701 Hamilton 10-μl syringe. Syringes with teflon bushings were found to be unsatisfactory. The derivatives appeared to adhere to some extent to the teflon.

Chromatographic conditions and procedure. The GLC columns and instrument operating conditions for all analyses are presented in Table 1. From 1–3 μl of the CS_2 solutions were injected into the column when the hydrogen flame detector was employed and 1–5 μl of ethyl acetate solutions with the electron capture detector.

Mixtures of each homologous series of the derivatives previously described, "Samples and Solvents," were separated by at least one of the sets of conditions described in Table 1.

To illustrate separation of higher molecular weight derivatives, a series of the pyruvic acid ester derivatives of the first 15 members of the primary alcohols was made up to contain approximately 0.55 μM/ml each. This series was separated by conditions described in Table 1, Analysis 6.

[2] Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

TABLE 1

CHROMATOGRAPHIC CONDITIONS FOR SEPARATION OF ESTERS, THIOESTERS, AND AMIDE DERIVATIVES OF PYRUVIC ACID, 2,6–DINITROPHENYLHYDRAZONE

Analysis no.	Type of detector	Column[a] length (feet)	Mode of operation	Column packing	Temperature °C			Gas flow rate (ml/min)		
					Inj.	Det.	Column	N_2	H_2	O_2
1	Flame	6.5	Isothermal	3% J × R on 100/120 mesh gas chrom Q	250	235	230	84	45	84
2a		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	225	225	175–240 at 4°C/min	180	70	138
b		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	225	225	195–240 at 4°C/min	180	70	138
3	Electron capture	4	Isothermal	3% J × R on 100/120 mesh gas chrom Q	235	210	230	168	—	—
4a		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	220	210	200–235 at 2°C/min	168	—	—
b		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	220	210	180–235 at 4°C/min	168	—	—

Table 1 *(Continued)*

Analysis no.	Type of detector	Column [a] length (feet)	Mode of operation	Column packing	Temperature °C			Gas flow rate (ml/min)		
					Inj.	Det.	Column	N_2	H_2	O_2
5a	Flame	1	Temperature program	5% SE 30 on 80/100 mesh AW–DMCS chrom W	220	240	150–220 at 4°C/min	50	40	80
b		1	Temperature program	5% SE 30 on 80/100 mesh AW–DMCS chrom W	220	240	135–200 at 4°C/min	50	40	80
6		2	Temperature program	5% SE 30 on 80/100 mesh AW–DMCS chrom W	215	240	150–235 at 4°C/min	80	52	84

[a] All columns were ⅛ inch o.d. stainless steel.

Response of the flame and EC detectors was compared by analyzing a series of secondary alcohol derivatives with each detector (see Table 1, Analyses 2b and 4b).

RESULTS AND DISCUSSION

Isothermal. Table 2 shows the relative retention times of the first 10 members of primary and secondary alcohol and thiol derivatives separated isothermally by conditions described in Table 1 (Analyses 1 and 3).

TABLE 2

RELATIVE RETENTION TIMES OF ESTER AND THIOESTER DERIVATIVES OF PYRUVIC ACID 2,6–DINITROPHENYLHYDRAZONE BY ISOTHERMAL (230°C) GLC ANALYSIS [a] ON 6.5 OR 4-FOOT J × R COLUMN

Carbon	Primary alcohols		Secondary alcohols		Mercaptan	
atoms	F	EC	F	EC	F	EC
1	0.04	0.05			0.09	0.10
2	0.06	0.06			0.11	0.11
3	0.07	0.08	0.05	0.06	0.14	0.14
4	0.09	0.10	0.07	0.07	0.18	0.19
5	0.12	0.13	0.09	0.09	0.24	0.24
6	0.16	0.17	0.12	0.11	0.32	0.32
7	0.20	0.21	0.16	0.15	0.42	0.44
8	0.27	0.28	0.20	0.20	0.56	0.58
9	0.35	0.36	0.27	0.26	0.78	0.75
10	0.47	0.47	0.34	0.33	1.00 [b]	1.00 [c]
11			0.47	0.44		
12			0.60	0.58		

[a] Conditions for analysis with the flame detector (F) are in Table 1, Analysis 1, and with the electron capture (EC) Analysis 3.

[b] Based on a retention time of 21.2 minutes.

[c] Based on a retention time of 15.6 minutes.

Although the actual retention time of the C_{10} mercaptan derivative is longer by the conditions described in Analysis 1 than by Analysis 3 (21.2 vs 15.6 minutes), the relative retention times based upon the C_{10} mercaptan derivative being 1.00 are almost identical.

It was apparent from the poor resolution of the first five members of each of the homologous series analyzed isothermally that temperature programming would be helpful in resolving these compounds.

TABLE 3

RELATIVE RETENTION TIMES OF ESTER AND THIOESTER DERIVATIVES OF PYRUVIC ACID 2,6–DINITROPHENYLHYDRAZONE BY TEMPERATURE PROGRAMMING (175–240°C AT 4°C/MIN) GLC ANALYSIS [a] ON 4–FOOT J × R COLUMN

Carbon atoms	Primary alcohols	Secondary alcohols	Mercaptans
1	0.14		0.26
2	0.17		0.30
3	0.22	0.17	0.37
4	0.28	0.23	0.45
5	0.35	0.29	0.53
6	0.42	0.36	0.63
7	0.50	0.42	0.72
8	0.58	0.50	0.81
9	0.67	0.57	0.91
10	0.75	0.66	1.00 [b]
11		0.75	
12		0.83	

[a] For complete operating conditions see Table 1, Analysis 2a.
[b] Based on a retention time of 13.1 minutes.

Temperature programming. Table 3 presents relative retention times of derivatives separated by the conditions described in Table 1, Analysis 2a. Temperature programming from 175–240°C on the 4-foot J × R column gave better resolution of the early peaks and sharpened the later peaks. There was evidence on the inside of the flame detector cylinder of a considerable amount of column bleed at high temperature. Despite this, response was good and a suitable baseline could be maintained even without dual differential flame and columns. Later work showed, however, that column retention times changed with progressive column bleed.

Temperature programming the same J × R column but with an electron capture detector gave results similar to that obtained with the flame detector. These results (from conditions described in Table 1, Analysis 4a) are shown in Table 4. The differences in relative retention

times, Tables 3 and 4 (Analyses 2a and 4a) can be understood if one considers the different program temperature ranges and rates.

TABLE 4

RELATIVE RETENTION TIMES OF ESTER AND THIOESTER DERIVATIVES OF PYRUVIC ACID 2,6–DINITROPHENYLHYDRAZONE BY TEMPERATURE PROGRAMMING (200–235°C AT 2°C/MINUTE) GLC ANALYSIS[a] ON 4–FOOT J × R COLUMN

Carbon atoms	Primary alcohols	Secondary alcohols	Mercaptans
1	0.13		0.22
2	0.14		0.24
3	0.18	0.15	0.30
4	0.22	0.19	0.37
5	0.28	0.22	0.43
6	0.33	0.27	0.53
7	0.41	0.33	0.62
8	0.48	0.39	0.71
9	0.57	0.47	0.83
10	0.66	055	1.00[b]
11		0.64	
12		0.73	

[a] For complete operating conditions see Table 1, Analysis 4a.
[b] Based on a retention time of 16.1 minutes.

Table 5 shows the relative retention times of derivatives separated on a 1-foot SE 30 column temperature programmed from 150–220°C at 4°C per minute or 135–200°C (for tertiary alcohol derivatives). Complete operating conditions are described in Table 1, Analyses 5a and b. This short column could be operated at a lower temperature than the J × R column and thus primary alcohol derivatives with 15 carbons in the parent alcohol could be analyzed with good resolution before column bleed became a factor (see Fig. 1).

For some reason that has not been established the EC detector did not respond with the short SE 30 column.

Lower limits of detection are illustrated by Figs. 2 and 3. Although no attempts were made to relate peak size with concentration, it was obvious that one might quantitate the method. As can be seen from Fig. 2 there is no problem in analyzing solutions containing 0.055 μM/ml of the secondary alcohol derivatives with the hydrogen flame de-

TABLE 5

RELATIVE RETENTION TIMES OF PRIMARY, SECONDARY AND TERTIARY ALCOHOL ESTER, AMIDE AND THIOESTER DERIVATIVES OF PYRUVIC ACID 2,6–DINITROPHENYLHYDRAZONE BY TEMPERATURE PROGRAMMING (150–220°C AT 4°C/min) ON 1–FOOT SE 30 COLUMN [a]

Carbon atoms	Primary alcohols	Secondary alcohols	Mercaptans	Amines
1	0.19		0.31	0.26
2	0.22		0.34	0.30
3	0.29	0.25	0.42	0.37
4	0.35	0.30	0.50	0.44
5	0.43	0.36	0.58	0.52
6	0.50	0.43	0.66	0.60
7	0.57	0.50	0.75	0.67
8	0.65	0.57	0.83	0.76
9	0.73	0.66	0.92	0.84
10	0.81	0.73	1.00 [b]	0.92
11		0.81		
12		0.90		
		Tertiary alcohols		
t-Butyl		0.45 [c]		
t–Amyl		0.55		
2–Methyl–2–pentyl		0.62		
3–Methyl–3–pentyl		0.62		
3–Methyl–3–hexyl		0.73		
3–Methyl–3–heptyl		0.80		
3–Methyl–3–octyl		1.00 [d]		

[a] For complete operating conditions see Table 1, Analyses 5a and 5b (for tertiary alcohols only).

[b] Based on retention time of 15.7 minutes.

[c] Temperature programming started at 135°C instead of 150°C.

[d] Based on a retention time of 12.8 minutes.

tector. Operating conditions for this analysis are shown in Table 1, Analysis 2b.

Even less material could be distinguished with the electron capture detector. Figure 3 shows the response from 5 μl of a solution containing 0.0055 μM/ml of each of the secondary alcohol derivatives. This represents only 10–15 mμg of the derivative and 1–5 mμg of the parent

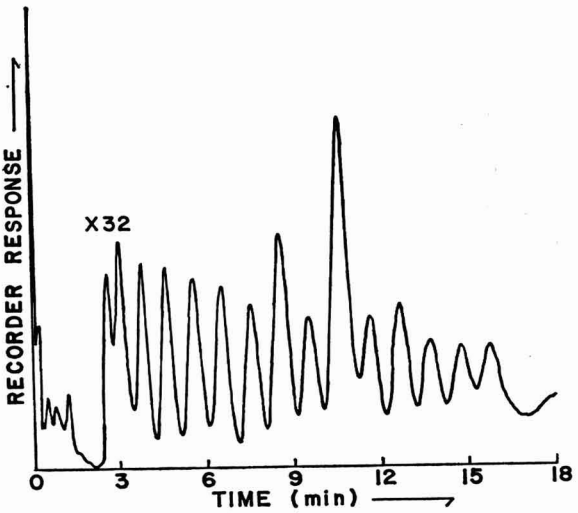

FIG. 1. The GLC pattern from 1.1 $\times$ 10^{-9} moles of each of $C_{1\text{-}15}$ primary alcohol derivatives separated by temperature programming 150–240°C on a 2–foot SE 30 column and using a flame ionization detector.

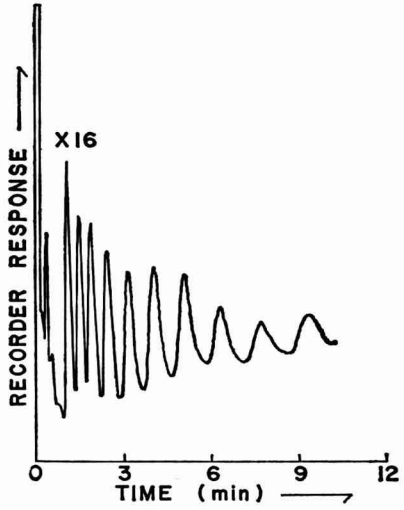

FIG. 2. The GLC pattern from 1.1 $\times$ 10^{-10} moles of each of $C_{3\text{-}12}$ secondary alcohol derivatives separated by temperature programming 195–225°C on a 4–foot J $\times$ R column and using a flame ionization detector.

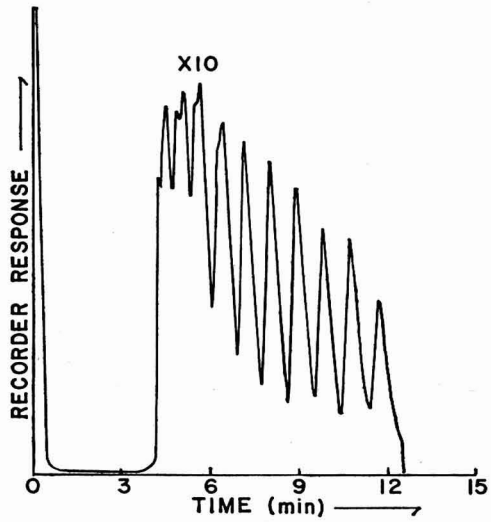

FIG. 3. The GLC pattern from 2.5×10^{-11} moles of each of $C_{3\text{-}12}$ secondary alcohol derivatives separated by temperature programming 180–245°C on a 4–foot J × R column and using an electron capture detector.

alcohol. Operating conditions to obtain the chromatogram in Fig. 3 are presented in Table 1, Analysis 4b.

Initially, glass liners were employed in the injection block but it was found that when the injector port was kept clean, glass liners were not essential. Care was taken also that the flame tips in particular and the detector in general were kept clean.

Changes in retention times were found to occur with extensive use of these columns. Although the retention times did change, conditions could be altered to maintain the good resolution obtained with the new column. It is very possible that silicone oil columns less inclined to bleed may eliminate or reduce this problem.

It would be necessary, of course, for identification purposes, to run standard solutions during the same sequence that unknowns are being analyzed. Since these derivatives are stable and easily maintained, this presents no problem.

Double peaks. When conditions were employed to increase separation of the first three members of each homologous series, each was

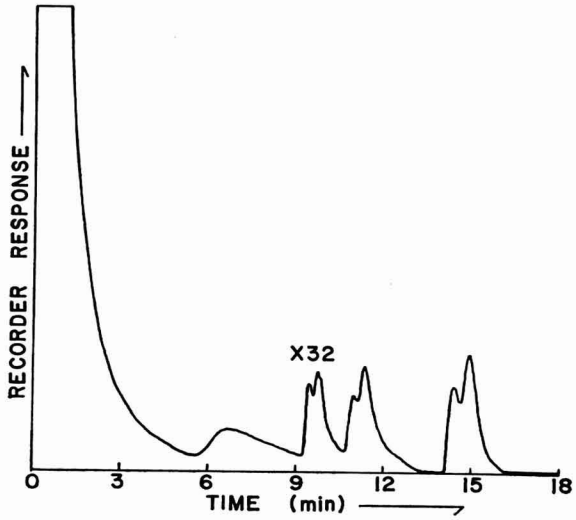

FIG. 4. The GLC pattern showing separation of each of the first three primary alcohol derivatives (C_{1-3}) each into two peaks isothermally at 200°C on a 6.5–foot J × R column and using a hydrogen flame detector.

found to separate into two peaks. Figure 4 illustrates this condition. With the 6.5-foot J × R column described in Table 1, Analysis 1 operated at a column temperature of 200°C, the first three members of primary alcohol derivatives shown in Fig. 4 separate clearly into two peaks. These doublet peaks were merged into single peaks by increasing the column temperature to 230°C.

With the small sample employed to determine lower limits of detection with the electron capture detector, the doublet peaks also were apparent. This can be seen in the chromatogram (Fig. 3) of the homologous series of millimicrogram levels of secondary alcohol derivatives monitored with the EC detector.

The authors feel that each of the single peaks represented in the figures could be resolved into two peaks on the J × R column with an appropriate column temperature. To simplify interpretation of the results, conditions were employed to yield one peak from each member of each homologous series.

It is possible that the doublet peaks represent isomeric forms of the derivatives present in the original crystals. However, we have been unable to obtain any evidence for isomers by thin-layer or

column adsorption chromatography on silica gel or on magnesium oxide, or by thin-layer and column liquid-liquid partition chromatograms. The appearance of doublet peaks has not been apparent by gas-liquid chromatography using 6 foot × 0.25-inch J × R columns in some other instruments (Research Specialties and Barber-Coleman) under conditions which produce the double peaks on the Aerograph 204.

The question of whether the crystalline derivatives used in this study contain 2 isomers or whether the isomers are produced during the gas chromatographic operation has not been resolved as yet. Additional experiments are underway to settle this question. Either way, the problem is not serious from an analytical standpoint since conditions can be chosen during gas chromatography to eliminate the appearance of the double peaks, and no evidence for double spots or bands has been encountered during thin-layer or column chromatography.

SUMMARY

Gas-liquid chromatographic techniques have been employed for the resolution of homologous series of primary, secondary, and tertiary alcohol ester– primary amine amide– and thioester derivatives of pyruvic acid 2,6–dinitrophenylhydrazone. The derivatives can be separated isothermally or by temperature programming on 6.5 or 4–foot J × R or on a 1–foot SE 30 column and can be detected by flame or electron capture detectors. The latter permits the detection of from 10–15 mμg of a derivative which is equivalent to about 1–5 mμg of the parent compound.

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MICROCHEMICAL JOURNAL 13, 310–315 (1968)

Methods for the Isolation and Characterization of Constituents of Natural Products

IX. Separation of Alcohol, Primary Amine and Thiol Derivatives of Pyruvic Acid 2,6-Dinitrophenylhydrazone into Classes

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Methods for the preparation of alcohol (*5*), amine (*7*), and thiol (*9*) derivatives of pyruvic acid 2,6-dinitrophenylhydrazone [1] were described in previous reports from this Laboratory. We have also presented methods for the separation of the alcohol derivatives into tertiary, secondary, and primary classes (*10*), and for the separation of primary from secondary amine derivatives (*8*). The analysis of the individual members of the separated classes by liquid-liquid thin-layer (*6,8,9*), and by gas-liquid chromatography of these, and also of a series of thiol derivatives (*1*) has been described.

The present report is concerned with the separation of alcohols (as a class), primary amine, and thiol derivatives of pyruvic acid 2,6-dinitrophenylhydrazone from each other. Both quantitative column adsorption and qualitative thin-layer adsorption systems are described.

APPARATUS AND MATERIALS [2]

Magnesium oxide (cat. no. 2477) suitable for chromatographic use was obtained from the J. T. Baker Company, Phillipsburg, N. J. The powder had an adsorption index (Food and Drug Yellow No. 4)

[1] Pyruvic acid chloride 2,6-dinitrophenylhydrazone, the reagent employed for preparation of these derivatives, is now available from the J. T. Baker Co., Phillipsburg, N. J.

[2] Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

of 12–13 and was used without further treatment. The original container was subdivided into 6 or 7 lots and these were stored in tightly sealed containers and stored in the absence of atmospheric moisture after it was noted that MgO lost some of its activity as samples were withdrawn from the original container over a period of time.

Celite 545 was obtained from the Johns-Manville Company, Baltimore, Md. Chloroform, methanol, and benzene were ACS grade. Nitromethane was the Fisher Certified grade. Hexane was the high purity grade produced by the Phillips Petroleum Company, Bartlesville, Okla. Diethylamine (Baker) was redistilled. Silica Gel G was obtained from the Brinkmann Instrument Company, Westbury, N. Y.

The thin-layer equipment was the same as previously described (*6*). A borosilicate glass column, 3.7 cm i.d. × 28 cm was employed for column chromatography.

EXPERIMENTAL METHOD

Separation of alcohol, primary amine and thiol derivatives into classes by column adsorption chromatography. The MgO (7.5 g) and Celite 545 (30 g) are slurried in about 150 ml of 0.5% methanol in $CHCl_3$ in a 500-ml Erlenmeyer flask. The flask is stoppered tightly and shaken at high speed on a mechanical shaker (Eberbach Corp., Ann Arbor, Michigan) for 15 minutes. The slurry is poured quickly through a long-stemmed funnel into the chromatography tube and packed immediately under moderate gas pressure until a few ml of solvent remain above the bed. The sides of the tube are washed down with a few ml of solvent and the sample quantitatively transferred to the column. The classes are separated using 200 ml of 0.5% methanol in $CHCl_3$ to remove the alcohols, 500 ml of 1% methanol in $CHCl_3$ to remove the primary amines, and finally 200 ml of 25% nitromethane in $CHCl_3$ to elute the thiol derivatives.

Separation of alcohol, primary amine, and thiol derivatives into classes by thin-layer adsorption chromatography. Plates of Silica Gel G, 250 μ thick are prepared in the usual manner and activated for 1–2 hours at 100°C before use. The derivatives are spotted from benzene solution and the plate is developed in an equilibrated tank using hexane:benzene:diethylamine (3:2:5). Satisfactory separation

of the classes is effected in about 20 minutes. The solvent should be discarded each day and replaced with fresh solvent when needed. The tank should then be equilibrated 1–2 hours before insertion of a plate.

RESULTS AND DISCUSSION

Figure 1 is a chromatogram showing separation of the alcohol, primary amine, and thiol derivatives into classes by the column procedure. The following compounds were selected for the attempted separation. Tertiary alcohols: 2-methyl 2-nonanol, *tert*-amyl alcohol, and *tert*-butyl alcohol; secondary alcohols: 2-nonadecanol, 2-dodecanol, 2-heptanol, 2-butanol and 2-propanol; primary alcohols: *n*-octadecanol, *n*-dodecanol, *n*-heptanol, *n*-propanol, ethanol, and methanol; primary amines: *n*-octadecylamine, *n*-dodecylamine, *n*-octylamine, ethylamine, and methylamine; thiols: *n*-octadecylmercaptan, *n*-dodecylmercaptan, *n*-heptylmercaptan, ethylmercaptan, and methylmercaptan. Approximately 0.5μmole of each compound was included in the mixture.

Recovery studies were conducted on 0.5 μmole of the extremes in each class on individual columns. Quantitative recovery was obtained in each case. It was noted, however, that old stock solutions of the derivatives which had been exposed to light at room temperature for several months gave an unadsorbed (yellow) band on the MgO and/or a strongly adsorbed violet band which could not be eluted from the column. In both instances these bands represented only a small percentage of the total color put on the column. Nevertheless, they represent some breakdown product of the derivatives either due to light, heat, or a combination of these. Benzene solutions of the derivatives stored at —18°C for over a year do not show these artifacts, nor do freshly prepared solutions.

Separation of the classes as described takes about 4 hours. The separated classes were checked for the individual members using thin-layer partition chromatography (*6,8,9*). All of the alcohols were eluted together as shown in Fig. 1 except for the methanol derivative which was found in the primary amine fraction. All of the primary amines were eluted together except methylamine which was found in the thiol class. The anomalous behavior of these two lower members is also manifested in the thin-layer class separation (see below).

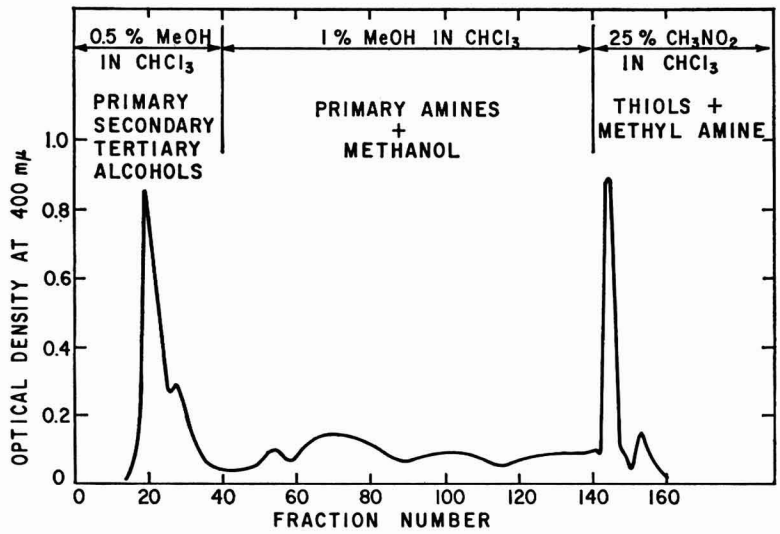

FIG. 1. Separation of alcohol, primary amine, and thiol derivatives of pyruvic acid 2,6-dinitrophenylhydrazone into classes of column chromatography.

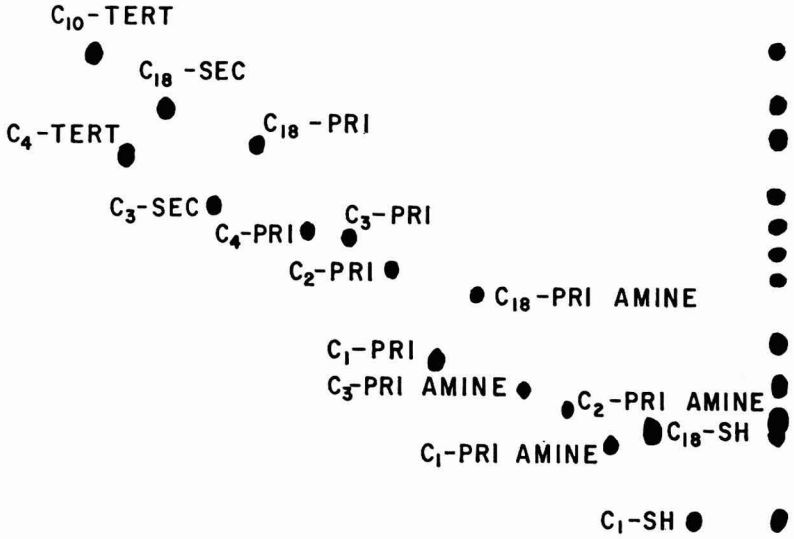

FIG. 2. Separation of alcohol, primary amine, and thiol derivatives of pyruvic acid 2,6-dinitrophenylhydrazone into classes by thin-layer chromatography on Silica Gel G. Solvent: hexane:benzene:diethylamine (3:2.5).

A difference in color exists for the three classes which aids in their classification. The alcohols are yellowish-violet, the amines are violet and the thiols are rust-red. The thiols are easily distinguishable from the amines.

Separation of the classes by thin-layer adsorption chromatography on Silica Gel G in the alkaline solvent system is shown in Fig. 2. The classes separate nicely, but the methanol derivative moves into the primary amine class and methylamine into the thiol class as with the column separation. The colors displayed on the column are also evident on the plate. Very small amounts (about 5×10^{-4} μmole) of a derivative can be seen on the plate.

The complexity of natural products, especially food products, is manifested by the large number of peaks that can be obtained by capillary gas chromatography (e.g., *2,3,4,12*). Although sophisticated instrumental techniques such as capillary gas chromatography coupled with mass spectrometry afford a great deal of information, it is doubtful whether this technique, as presently used, is capable of separating and characterizing all of the volatile constituents in a food product, for example. Evidence gathered in this Laboratory indicates that a fairly large number of constituents which we can identify in the volatiles by techniques which we have described in this series and in another report regarding the identification of carbonyl compounds (*11*) go undetected or unidentified by capillary gas chromatography–mass spectrometry. In view of this, it would seem that instrumental techniques, in their present state, could beneficially be supplemented by methods such as are described here and in our previous publications, in order to gain a more complete picture of the spectra of compounds present in natural products.

SUMMARY

Quantitative column and qualitative thin-layer chromatographic procedures are described for separating a mixture of alcohol, primary amine, and thiol derivatives of pyruvic acid 2,6-dinitrophenylhydrazone into classes. Magnesium oxide is used as the adsorbent in the column procedure and Silica Gel G in the thin-layer technique. The alcohols, amines, and thiol derivatives which are eluted in that order, show differences in color during chromatography which aid in their proper classification.

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Titrimetric Microdetermination of Alanine

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INTRODUCTION

The literature concerning the determination of alanine seems to be abundant. Workers have determined it by oxidative decomposition (*24, 26, 14*), with potassium permanganate (*20, 23*), with periodate (*1, 16*), with hypochlorite (*2, 3, 4*), by microanalysis (*5*), with chromic acid (*7*), chromatographically (*8, 12, 13*), with ninhydrin (*18, 22, 25*), conductometrically (*17*), with nitrous (*21*) acid, by glutamic pyruvictransaminase (*19*), cerimetrically (*15*), by perchloric acid (*10*), by H_2O_2 (*11*), with *p*-hydroxy biphenyl (*9*), and spectrophotometrically (*6*).

The present work deals with the determination of alanine in micro amounts with gold chloride in alkaline medium. The reaction mixture consists in adding alanine to a known excess of gold chloride and sodium hydroxide. Remaining excess of gold is titrated (27) by acidifying and adding an excess of potassium ferrocyanide when the remaining excess of potassium ferrocyanide solution is titrated against a standard solution of ceric sulfate using *N*-phenyl anthranilic acid as indicator. Probably the following reaction takes place:

$$6CH_3 \cdot CH(NH_2)\,COOH + 26HAuCl_4 + 104NaOH \rightarrow 12CO_2 + 3N_2 + 6HCOOH + 26Au + 80H_2O + 104NaCl.$$

From the above equation it appears that the equivalence is 13, i.e., 6.5 atoms of oxygen are required where formic acid is formed under these conditions. It has been observed that excess of sodium hydroxide inhibits the reaction.

EXPERIMENTAL METHOD

Chemicals employed

Alanine (E. Merck grade); gold chloride (Palmston's grade); potassium ferrocyanide (ANALAR B.D.H. grade); ferrous ammonium

sulfate (ANALAR B.D.H. grade); sodium hydroxide (E. Merck grade); sodium carbonate (ANALAR B.D.H. grade); sulfuric acid (ANALAR B.D.H. grade); ceric sulfate (Technical B.D.H. grade); and *N*-phenyl anthranilic acid (B.D.H. grade).

The 0.0041 *M* ceric sulfate (in 8 *N* H_2SO_4) is standardized by titrating against a standard solution of ferrous ammonium sulfate (in 1*N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

The 0.0214 *M* potassium ferrocyanide is standardized by titrating against a standard solution of ceric sulfate (in 8*N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

The 0.0224 *M* gold chloride solution is standardized by adding a known excess of a standard solution of potassium ferrocyanide and then titrating the remaining potassium ferrocyanide solution against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

Procedure

The reaction mixture, comprising a known solution of alanine, a known excess of a standard solution of gold chloride, sodium hydroxide and distilled water, is put on a hot plate at full heat (keeping in mind that the reaction mixture may not evaporate) for about 180 minutes. In case the volume of the reaction mixture is reduced to about 5 or 8 ml (much before 180 minutes) then again 20 ml of distilled water is

TABLE 1

MICRODETERMINATION OF ALANINE

Alanine 0.005 *M* (ml)	NaOH 0.1 *M* (ml)	H_2O (ml)	$HAuCl_4$ 0.0223 *M* (ml)	$K_4Fe(CN)_6$ 0.0214 *M* (ml)	$Ce(SO_4)_2$ 0.0041 *M* (ml)	$Ce(SO_4)_2$ 0.0041 *M* used to oxidize alanine (ml)	Amount of alanine (g/liter)
—	—	—	—	2	10.46	—	—
—	—	—	1	2	5.00	5.46	—
0.04	10	20	1	2	5.68	0.68	0.0190
0.06	10	20	1	2	6.00	1.00	0.0277
0.08	10	20	1	2	6.38	1.38	0.0387
0.10	10	20	1	2	6.70	1.70	0.0499

added. Separate beakers containing the reaction mixture must be covered with beaker covers. After cooling at room temperature, the metallic gold precipitate, corresponding to the alanine oxidized, is filtered off and thoroughly washed with distilled water. Remaining gold chloride (unused) solution in the filtrate is acidified and titrated back by adding a known excess of a standard solution of potassium ferrocyanide. Then remaining excess of potassium ferrocyanide solution is titrated against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator. At the sharp end point a red brown color appears.

RESULTS AND DISCUSSION

Results are shown in Table 1. The range in which alanine has been estimated varies from 0.0190 to 0.0499 g/liter.

The reaction between alanine and gold chloride in alkaline medium results in giving the products as HCOOH, CO_2, N_2 at equivalence 13. Under these conditions it has been observed that excess of hydroxyl ion concentration inhibits the reaction. It has also been observed that acetone, oxalic acid, malic acid, antipyrine, glycine, leucine, and DL-valine interfere.

SUMMARY

Titrimetric microdetermination of alanine with gold chloride in alkaline medium is described. Remaining gold chloride solution is titrated back with potassium ferrocyanide. Remaining potassium ferrocyanide is titrated against a standard solution of ceric sulphate using N-phenyl anthranilic acid as indicator. Excess alkali inhibits the reaction. Interference by certain organic compounds has also been observed.

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Microdetermination of L-Leucine and DL-Valine

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INTRODUCTION

Literature concerning the determination of L-leucine and DL-valine is abundant. L-leucine and DL-valine have been determined by oxidation with (*15*), Formageot and Heitz methods (*2, 16*) ninhydrin method (*12, 21, 22, 24*), microbiological methods (*1, 9, 11, 17, 18*), ion-exchange column (*19*), chromatographically (*3, 6, 8, 10, 13, 14, 25*), spectrophotometrically (*4, 5, 7*), with chloramine T and *p*-dimethylaminobenzaldehyde (*20*), and by paper electrophoresis (*23*).

Present work deals with the determination of L-leucine and DL-valine separately in micro quantities with gold chloride in alkaline medium. The reaction mixture consists in adding either L-leucine or DL-valine to a known excess of gold chloride and sodium hydroxide. Remaining excess of gold chloride is titrated (*26*) by acidifying and adding an excess of potassium ferrocyanide solution when the remaining excess of ferrocyanide is titrated against a standard solution of ceric sulfate using *N*-phenyl anthranilic acid as indicator. Probably the following reactions take place:

$$2(CH_3)_2CH \cdot CH_2 \cdot CH(NH_2)COOH + 22HAuCl_4 + 88NaOH \rightarrow 12CO_2 + 22Au + 88NaCl + 68H_2O + N_2$$

$$2(CH_3)_2CH \cdot CH(NH_2)COOH + 18\ HAuCl_4 + 72NaOH \rightarrow 10CO_2 + N_2 + 18Au + 56H_2O + 72NaCl$$

From the above equations it appears that both L-leucine and DL-valine break their components at 33 and 27 equivalence, respectively. Thus it becomes apparent that for L-leucine and DL-valine 16.5 and 13.5 atoms of oxygen are required, respectively, for complete decomposition. It has been observed that excess of sodium hydroxide inhibits the reaction.

EXPERIMENTAL METHOD

Reagents used. The reagents were L-leucine, DL-valine, and sodium hydroxide (E. Merck grade); potassium ferrocyanide, ferrous ammonium sulfate, sodium carbonate, and sulfuric acid (ANALAR B.D.H. grade); ceric sulfate (Technical B.D.H. grade) and *N*-phenyl anthranilic acid (B.D.H.); gold chloride (Palmston's grade).

The 0.0041 *M* ceric sulfate (in 8 *N* H_2SO_4) is standardized by titrating against a standard solution of ferrous ammonium sulfate (in 1 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

The 0.0382 *M* potassium ferrocyanide solution is standardized by titrating against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

The 0.0294 *M* gold chloride solution is standardized by adding a known excess of standard solution of potassium ferrocyanide solution against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator.

PROCEDURE

Since the procedure for both L-leucine and DL-valine is the same, the words "amino acid" has been used in the procedure. The reaction mixture, comprised of a known solution of amino acid, a known excess of gold chloride, sodium hydroxide and distilled water, in different beakers covered with beaker covers, is put on a hot plate at full heat (the reaction mixture should not evaporate completely) for about 150 minutes. In case the volume of the reaction mixture is reduced to about 5 or 8 ml (much before 150 minutes), 20 ml of distilled water is added. After cooling at room temperature the metallic gold precipitated, corresponding to the amino acid oxidized, is filtered off and thoroughly washed with distilled water. Remaining gold chloride (unused) solution in the filtrate is acidified with dilute sulfuric acid and titrated back by adding a known excess of a standard solution of potassium ferrocyanide. Then remaining excess of potassium ferrocyanide solution is titrated against a standard solution of ceric sulfate (in 8 *N* H_2SO_4) using *N*-phenyl anthranilic acid as indicator. At the end point a brown red color appears sharply.

TABLE 1

MICRODETERMINATION OF L-LEUCINE

L-leucine 0.001 *M* (ml)	NaOH 0.1 *M* (ml)	H_2O (ml)	$HAuCl_4$ 0.0294 *M* (ml)	$K_4Fe(CN)_6$ 0.0382 *M* (ml)	$Ce(SO_4)_2$ 0.0042 *M* (ml)	$Ce(SO_4)_2$ 0.0042 *M* used to oxidize L-leucine (ml)	Amount of L-leucine (mg/liter)
—	—	—	—	1	9.10	—	—
—	—	—	1	1	2.10	7.00	—
0.08	11	20	1	1	2.80	0.70	1.1075
0.10	11	20	1	1	2.96	0.86	1.4424
0.16	11	20	1	1	3.48	1.38	2.3145
0.20	11	20	1	1	3.80	1.70	2.8515

TABLE 2

MICRODETERMINATION OF DL-VALINE

DL-valine 0.0012 *M* (ml)	NaOH 0.1 *M* (ml)	H_2O (ml)	$HAuCl_4$ 0.0294 *M* (ml)	$K_4Fe(CN)_6$ 0.0382 *M* (ml)	$Ce(SO_4)_2$ 0.0042 *M* (ml)	$Ce(SO_4)_2$ 0.0042 *M* used to oxidize DL-valine (ml)	Amount of DL-valine (mg/liter)
—	—	—	—	1	9.10	—	—
—	—	—	1	1	2.10	7.00	—
0.08	11	20	1	1	2.76	0.66	1.2025
0.10	11	20	1	1	2.94	0.84	1.5307
0.16	11	20	1	1	3.40	1.30	2.3665
0.20	11	20	1	1	3.74	1.64	2.9864

RESULTS AND DISCUSSION

Results are shown in Tables 1 and 2. Range in which L-leucine and DL-valine have been estimated vary from 1.1075 to 2.8515 mg/liter and from 1.2025 to 2.3864 mg/liter, respectively. The reaction between L-leucine, DL-valine, and gold chloride in alkaline medium takes place in the ratio of 1:11 and 1:9, respectively. Complete decomposition of both takes place finally giving N_2,CO_2, and water. This method is useful in estimating L-leucine and DL-valine separately but not in mixtures nor containing acetone, alcohols, glycols, sugars, and hydroxy acids.

SUMMARY

L-leucine and DL-valine have been determined in micro quantities by oxidizing with gold chloride in alkaline medium. Sixteen and one-half and 13.5 atoms of oxygen are required for complete decomposition of L-leucine and DL-valine, respectively. Excess of alkali inhibits the reaction. Interference by organic compounds is observed.

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Improved Instrumental Carbon, Hydrogen, and Nitrogen Analysis with Electronic Integration

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INTRODUCTION

Classical methods of carbon, hydrogen, and nitrogen microanalysis have basically remained unchanged since their introduction during the early part of this century. These methods tend to be lengthy with two analyses being required for the determination of carbon, hydrogen, and nitrogen in organic compounds (7). During recent years instrument manufacturers have introduced carbon and hydrogen analyzers, and carbon, hydrogen, and nitrogen analyzers. The principles of combustion are the same as those outlined for the Pregl carbon and hydrogen analysis and the Dumas nitrogen analysis. The advantage of the instrumental method is a more rapid analysis because of faster gas flow rates, automation of manipulations, and an electronic measurement of combustion products. Normally the signal is fed to a strip chart recorder where peaks are drawn for the three combustion products (CO_2, H_2O, N_2). Actual measurements have been made by measuring the peak heights with a millimeter scale or by determining recorder chart divisions (*1–6*). The author's experience has been primarily with the F & M Model 180 and Model 185 Carbon, Hydrogen, and Nitrogen Analyzers. Peak height measurements using a millimeter rule have been shown to be tedious and subjective. One to 2 hours are required to measure and calculate 6 hours of analyses.

The use of electronic integration of the signal from the thermal conductivity detector of the F & M Model 185 C, H, and N Analyzer was given serious consideration in the past; however, no work was performed because of poor resolution of the component peaks and because of an unsteady baseline. Research (at F & M) with new column packings has yielded a new gas chromatographic column that gives excellent resolution and a steady baseline making electronic integration feasible.

The object then in the use of an integrator is to: (i) eliminate a tedious operation; (ii) eliminate the subjectivity from the very critical peak size measurement; (iii) reduce time required for measurement and calculation; (iv) increase precision (repeatability and reproducibility); and (v) obtain more accurate results. The results obtained during this study, as discussed later, show that these objectives have been met.

MATERIALS

The F & M Model 185 Carbon, Hydrogen, and Nitrogen Analyzer was used for this work. This Instrument was obtained from the F & M Scientific Division of Hewlett-Packard and was the standard instrument with exception of the new Polypak gas chromatographic column. The integrator used was the Infotronics Model CRS-11HSB/42 obtained from the Infotronics Corporation. Pure standards were obtained from either the National Bureau of Standards or the British Drug House. All expendable supplies normally used with the C, H, and N Analyzer were obtained from F & M.

METHOD

The carbon and hydrogen fractions of organic compounds are converted to carbon dioxide and water, in the presence of an oxidant, in a combustion tube packed with copper oxide at 1050°C. Combustion takes place in a static system for exactly 20 seconds. After the 20 second combustion cycle has been completed, the oxidation products are carried along in a stream of helium to a second combustion tube packed with reduced copper turnings and maintained at a temperature of 400°C. Here, oxides of nitrogen are converted into free nitrogen gas. The helium flow then carries the three products through the chromatographic column where the components are separated and then to the thermal conductivity detector. A peak appears on a strip chart recorder for each of the three products—carbon dioxide, water, and nitrogen. Calculations are made by comparing sample peak areas to standard peak areas.

Samples are inserted into the oxidation furnace of the instrument by means of a long rod slotted at one end. The sample is weighed into a small aluminum boat, which in turn is placed into the slotted rod. Catalyst (oxidant) is added to fill the boat, and the rod is inserted about 3 inches into the instrument. Seals are screwed down and the furnace is purged for about 4 minutes. The oxidation furnace

area is sealed off by depressing a button which activates timer operated solenoid valves. The sample is then inserted into the hot furnace by pushing the rod all the way in. In exactly 20 seconds the combustion products are automatically swept from the first combustion tube through the remainder of the combustion train and the GLC section of the instrument.

The "Mode" switch on the electronic integrator is turned from its "Reset" position to its "Auto" position just after the combustion products are swept from the first combustion tube. At the completion of the GLC run, counts are totaled and the integrator "Mode" switch is placed in its "Reset" position until the next run. A "Threshold Level" of 0.05% is used on the integrator to insure that the tail on the CO_2 and the H_2O peaks are integrated. If a small noise spike causes the integrator to print out on the tail of the peak, this control will cause the integrator to continue to integrate until the baseline is reached. At this point the integrator will again print out and both areas are summed to get the total area for the peak. "Zero Printout" is an essential feature to have on the integrator so that there is adequate time between the CO_2 peak and the H_2O peak for baseline correction. A manual attenuation of 2 is used throughout the entire run. The Ratio Recording feature of the Cahn Balance is not used. All other settings on the F & M instrument are set according to the manufacturer's recommendations. The integrator is wired in series between the thermal conductivity detector and the recorder. The integrator conditions are:

Maximum range (mV)	Auto
Tracking rate up (UV/sec)	1
Tracking rate down (UV/sec)	3
Threshold level (%)	0.05
Positive printout	On
Trip level	6
Control	Direct
Slope sensitivity	1
Filter frequency	1
Mode	Reset to Auto
Recorder span (mV)	1
Attenuation (mV)	Fixed 1
Counting rate (kc)	4

Calculations using the integrator are somewhat different from those using peak height measurements. The Ratio Recording feature which is used with peak height measurements eliminates the necessity of recording or using weights in calculation because recorder response is proportional to composition and not sample size. For this reason a simple proportion is used for calculation:

$$\frac{(\text{Peak height sample})\ (\%\ \text{Element standard})}{(\text{Peak height standard})} = \%\ \text{Element sample}$$

Normally for a series of runs the ratio

$$\frac{\%\ \text{Element standard}}{\text{Peak height standard}}$$

remains constant (C) and percentage element in the sample can be determined by simply multiplying the constant (C) by the respective peak height from the sample chromatogram. A separate constant is established for each element. Peak height determinations are normally obtained by using a good quality millimeter rule.

When the integrator is used, the measurement of the peak area is completely automatic. Calculation is accomplished by using a factor for each element which is derived from one or more standard runs.

$$F = \frac{(\%\ \text{Element standard})\ (\text{Weight standard})}{(\text{Integral counts element area})}$$

Percentage composition of a sample is the determined using this factor (F).

$$\frac{(F)\ (\text{Integral counts for element in sample})}{(\text{Sample wt.})} = \%\ \text{Element in sample}$$

A separate factor is established for each element.

It is essential that the weighing pot on the Cahn Balance is not disturbed while a run is being made when the peak height method of measurement and calculation is being used. It is necessary to complete the weighing cycle of a sample after the analysis of the previous sample has been completed. Since the Ratio Recording feature of the balance is not used when peak area measurements are made, a sample can be completely weighed during the analysis of the previous

sample. The second sample cannot be introduced until the complete area of the water peak (for hydrogen) has been printed out. This is not the case with peak height determinations because the second sample can be introduced as soon as the water peak reaches its apex. No time is lost or gained at this point in the analysis because these factors offset one another.

As much as 2 hours/day in analysis time can be saved when the integrator is used. Area measurements are automatic, and calculations are simple and quick if a calculator is handy. Calculation of analyses can be made while analyses are being performed. Here, an added advantage is gained in that results are obtained soon after the sample is run so that a duplicate analysis can be made if necessary.

A time savings will also be realized by reducing the number of standards needed to calibrate the instrument; however, it is difficult to anticipate the magnitude of this savings. The number of unweighed samples can also be reduced. During this work unweighed samples were eliminated on several occasions with the results of the first samples being within the acceptable limits of $\pm$ 0.3%.

The GLC column supplied with the Model 185 Carbon, Hydrogen, and Nitrogen Analyzer gave adequate separation of the N_2, CO_2, and H_2O peaks for peak height measurements. Baseline separation was not achieved between the N_2 and CO_2 peaks (Fig. 1). This lack of baseline separation has not affected the accuracy of analysis with peak height measurements. Baseline separation is essential for accurate and precise area measurements by electronic integration. The Polypak column gives the required separation, a steady baseline, and a reduced run time of about 2–4 minutes (Fig. 2 and 3).

EXPERIMENTAL PROCEDURE

To insure that the instrument was performing satisfactorily and to evaluate the new GLC column, a series of acetanilide samples was run and calculated using peak height measurements. Deviations were silghtly higher than experienced in earlier studies (Table 1). The higher than normal deviations obtained with peak height measurements were attributed to the inability of the Polypak column to give reproducible peak heights. Area measurements were found to be very reproducible as subsequent data will show.

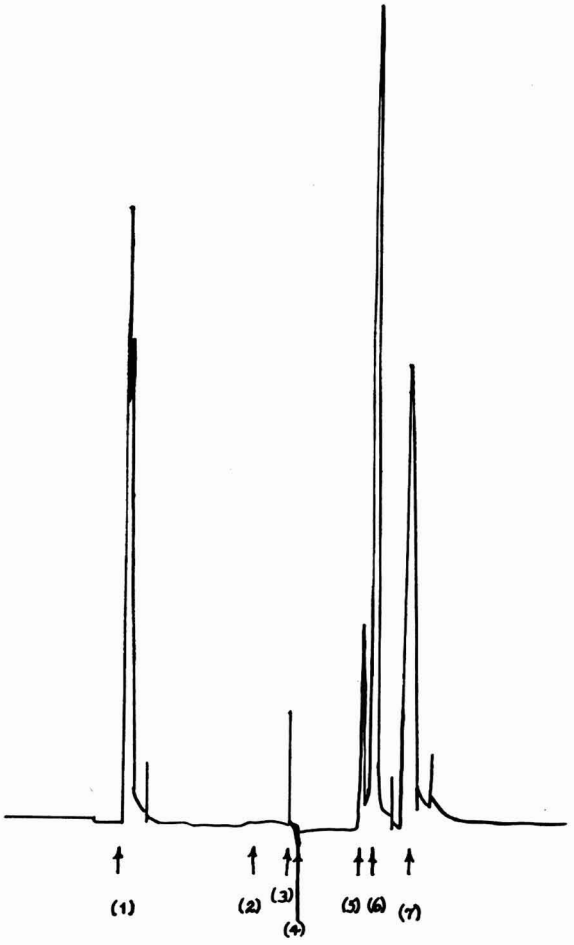

FIG. 1. Standard chromatogram. (1) Rod with sample and catalyst inserted into instrument cold zone; (2) purge peak; (3) oxidation combustion tube sealed off and sample inserted into hot oxidation combustion tube; (4) oxidation products injected into reduction combustion tube and then to GLC; (5) N_2 peak att. = 8; (6) CO_2 peak att. = 32; and (7) H_2O peak att. = 8.

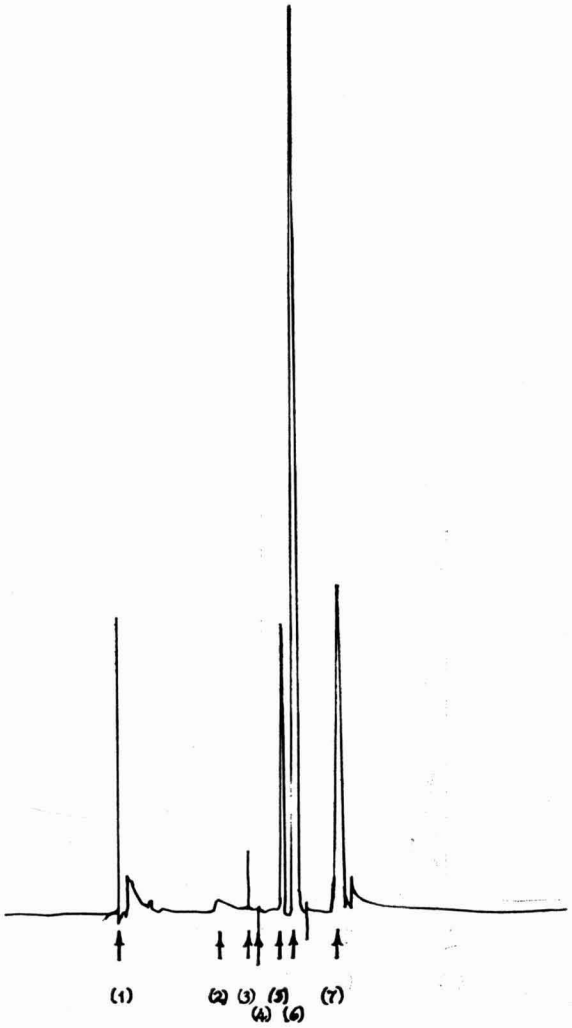

FIG. 2. Standard chromatogram using Polypak column. (1) Rod with sample and catalyst inserted into instrument cold zone; (2) purge peak; (3) Oxidation combustion tube sealed off and sample inserted into hot oxidation combustion tube; (4) Oxidation products injected into reduction combustion tube and then to GLC; (5) N_2 peak att. = 8; (6) CO_2 peak att. = 32; and (7) H_2O peak att. = 8.

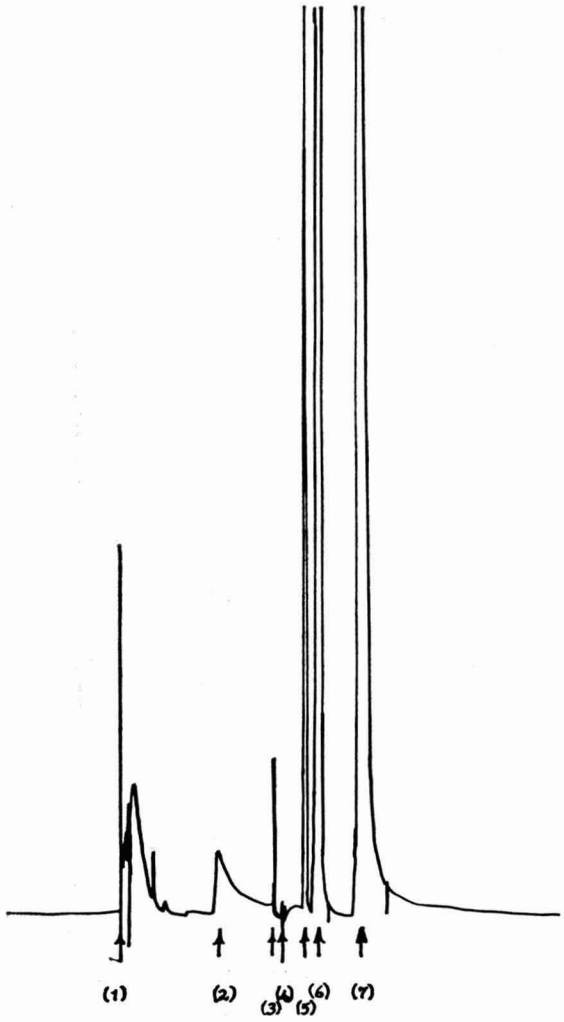

FIG. 3. Chromatogram using Polypak column and integrator. (1) Rod with sample and catalyst inserted into instrument cold zone; (2) purge peak; (3) oxidation combustion tube sealed off and sample inserted into hot oxidation combustion tube; (4) oxidation products injected into reduction combustion tube and then to GLC; (5) N_2 peak att. = 2; (6) CO_2 peak att. = 2; and (7) H_2O peak att. = 2.

TABLE 1

ANALYSIS OF ACETANILIDE USING PEAK HEIGHT MEASUREMENTS AND THE EXPERIMENTAL POLYPAK GLC COLUMN [a]

Sample	Carbon (%)		Hydrogen (%)		Nitrogen (%)	
	Found	Deviation	Found	Deviation	Found	Deviation
1	71.24	+0.15	6.73	+0.02	10.23	—0.13
2	70.90	—0.19	6.75	+0.04	10.50	+0.24
3	71.33	+0.24	6.82	+0.09	10.33	—0.03
4	70.76	—0.33	6.84	+0.07	10.60	+0.24
5	71.57	+0.46	6.57	—0.14	10.22	—0.14
6	70.71	—0.38	6.55	—0.16	10.30	—0.06
	Theory C 71.09				SD C ±0.35	
	H 6.71				H ±0.11	
	N 10.36				N ±0.18	

[a] Peak heights were measured with a millimeter rule; no blank corrections used.

A series of ten acetanilide standards was run using the integrator to measure peak areas; precision was excellent (Table 2). This data shows that carbon, hydrogen, and nitrogen analyses are more reproducible when an electronic integrator is used to measure peak areas than when manual peak height measurements are made.

TABLE 2

ANALYSIS OF ACETANILIDE USING ELECTRONIC INTEGRATION OF PEAK AREAS AND THE EXPERIMENTAL POLYPAK GLC COLUMN

Sample	Carbon (%)		Hydrogen (%)		Nitrogen (%)	
	Found	Deviation	Found	Deviation	Found	Deviation
1	71.16	+0.07	6.75	+0.04	10.33	—0.03
2	71.24	+0.15	6.73	+0.02	10.43	+0.07
3	70.81	—0.28	6.69	—0.02	10.29	—0.07
4	71.12	+0.02	6.75	+0.04	10.43	+0.07
5	71.24	+0.15	6.70	—0.01	10.46	+0.10
6	71.05	—0.04	6.72	+0.01	10.32	—0.04
7	71.19	+0.10	6.69	—0.02	10.40	+0.04
8	71.02	—0.07	6.67	—0.04	10.32	—0.04
9	71.12	+0.03	6.73	+0.02	10.34	—0.02
10	70.96	—0.13	6.68	—0.03	10.29	—0.07
	Theory C 71.09				SD C ±0.14	
	H 6.71				H ±0.03	
	N 10.36				N ±0.06	

Peak area measurements should be less dependent upon sample size than peak height measurements. Several acetanilide samples were analyzed that were out of the 0.6–0.8-mg sample size range recommended for the F & M C–H–N Analyzer (Table 3). Previously determined factors were used for calculation. These analyses show

TABLE 3

ANALYSIS OF ACETANILIDE USING SAMPLE WEIGHTS OUT OF THE 0.6–0.8-*mg* RANGE SUGGESTED BY F & M

Sample	Weight (mg)	Carbon (%)		Hydrogen (%)		Nitrogen (%)	
		Found	Deviation	Found	Deviation	Found	Deviation
1	0.5693	71.16	+0.07	6.71	0.00	10.35	—0.01
2	0.7194	71.17	+0.08	6.68	—0.03	10.44	+0.08
3	0.9281	71.42	+0.33	6.65	—0.06	10.40	+0.03
4	0.3270	70.87	—0.22	6.82	+0.11	10.30	—0.07
5	0.1362	72.86	+1.77	6.18	—0.55	10.27	—0.10
6	0.5568	71.32	+0.23	6.65	—0.06	10.32	—0.05
7	0.6722	70.97	—0.12	6.68	—0.03	—	—

that a wider range of weights can be used. The only significant error in this series of samples was the one very low weight, 0.1362 mg, and this may be in part because weighing errors become more of a factor as sample weights decrease. An attempt was made to reduce weighing errors by increasing sample weights. Although an

TABLE 4

ANALYSIS OF ACETANILIDE USING HIGH SAMPLE WEIGHTS

Sample	Weight (mg)	Carbon (%)		Hydrogen (%)		Nitrogen (%)	
		Found	Deviation	Found	Deviation	Found	Deviation
1	2.416	71.35	+0.24	6.69	—0.02	10.35	—0.01
2	1.994	71.05	—0.04	6.72	+0.01	10.21	—0.15
3	1.665	70.89	—0.20	6.71	0.00	10.46	+0.10
4	1.647	70.74	—0.35	6.67	—0.04	10.19	—0.17
5	1.745	71.36	+0.25	6.75	+0.04	10.61	+0.25
					SD C	±0.27	
					H	±0.03	
					N	±0.18	

TABLE 5

ANALYSIS OF VARIOUS STANDARDS USING ACETANILIDE TO CALCULATE FACTORS [a]

Sample	Theory (%)			Found (%)			Difference (%)		
	C	H	N	C	H	N	C	H	N
Acetanilide	71.09	6.71	10.36	70.77	6.70	10.31	−0.32	−0.01	−0.05
				71.14	6.67	10.34	+0.03	−0.04	−0.02
				71.21	6.77	10.35	+0.12	+0.06	−0.01
				71.08	6.77	10.49	−0.01	+0.06	+0.13
				71.17	6.63	10.37	+0.08	−0.08	+0.01
				71.00	6.75	10.33	−0.09	+0.04	−0.03
				71.20	6.69	10.35	+0.11	+0.02	−0.01
Cyclohexanone–2:4–dinitrophenylhydrazone	51.79	5.07	20.14	51.72	5.09	20.36	−0.07	+0.02	+0.22
				51.76	5.19	20.40	−0.03	+0.12	+0.26
				51.77	5.19	20.42	−0.02	+0.12	+0.28
Benzoic Acid	68.86	4.96	—	68.98	5.03	—	+0.12	+0.07	—
				68.71	4.96	—	−0.15	0.00	—
Acetanilide	71.09	6.71	10.36	71.42	6.64	10.37	+0.33	−0.07	+0.01
				71.27	6.74	10.29	+0.18	+0.03	−0.07
				71.11	6.76	10.31	+0.02	+0.05	−0.05
Benzoic Acid	68.86	4.96	—	68.77	5.02	—	−0.09	+0.06	—
				69.04	4.98	—	+0.18	+0.02	—
Cyclohexanone–2:4–dinitrophenylhydrazone	51.79	5.07	20.14	51.68	5.17	20.44	−0.11	+0.10	+0.30
S-Benzyl-thiuronium chloride	47.40	5.47	13.82	47.36	5.53	13.83	−0.04	+0.06	+0.01
				47.69	5.61	13.98	+0.29	+0.14	+0.16
Benzoic acid	68.86	4.96	—	68.74	5.02	—	+0.08	+0.07	—
Acetanilide	71.09	6.71	10.36	71.42	6.78	10.36	+0.33	+0.07	0.00
				SD C ±0.17					
				H ±0.07					
				N ±0.14					

[a] No blank corrections used.

increase in percision was not gained (Table 4), higher weights can be used when sample uniformity becomes a significant problem. The recommended working range of between 0.6- to 0.8-mg sample size remains as a good choice for integrator work.

A check on the excellent precision obtained earlier (Table 2) was made to check continued good operation of the system. Standard deviations of C $\pm$ 0.16, H $\pm$ 0.05, and N $\pm$ 0.06 were found to be nearly identical to those obtained earlier (C $\pm$ 0.14, H $\pm$ 0.03, and N $\pm$ 0.06).

All work to this point dealt with one standard, acetanilide; therefore, no blank corrections were necessary. To investigate the effect of blanks on sample runs where carbon, hydrogen, and nitrogen content differed from that of the standard, a series of different standards was run using factors calculated from several acetanilide runs. No blank corrections were made (Table 5). The ability to calculate samples without the necessity of making blank runs and using blank corrections results in a substantial time savings with only a slight decrease in accuracy. Blank corrections were subsequently eliminated throughout this investigation.

One day's evaluation was spent analyzing samples in a manner similar to a normal day's operation. Pure standards were used in place of samples so that a continuous check on accuracy could be maintained throughout the day (Table 6). No unweighed samples were run in the morning or after lunch. Unweighed samples are normally run after extended periods of downtime to "condition" the instrument. If unweighed samples can be eliminated or reduced, output can be increased. Runs 1 and 2 (Table 6) were the first two runs of the day and run 25 was the first run after lunch. The deviations of these runs indicate that it is still desirable to run two unweighed samples each morning, but it is not necessary to run any after lunch or any other idle period during the day. Calculation factors were obtained from an average of runs 3, 4, and 5. With a calculator nearby, there was sufficient time to calculate each sample as the next sample was being run.

Up to this point all data (Tables 1–6) was obtained by only one analyst. Previous work on carbon, hydrogen, and nitrogen analysis has shown that one analyst can usually reproduce his own analyses better than he can check his co-worker with peak height measure-

TABLE 6

ANALYSIS OF VARIOUS STANDARDS DURING 1 DAY'S WORK [a]

No.	Sample	Theory			Found			Difference		
		C (%)	H (%)	N (%)	C (%)	H (%)	N (%)	C (%)	H (%)	N (%)
1	Acetanilide	71.09	6.71	10.36	70.76	6.52	10.39	—0.33	—0.19	+0.03
2					70.80	6.64	10.34	—0.29	—0.07	—0.02
3					70.90	6.69	10.35	—0.19	—0.02	—0.01
4					70.94	6.71	10.36	—0.15	0.00	0.00
5					71.43	6.73	10.37	+0.32	+0.02	+0.01
6					71.61	6.73	10.42	+0.52	+0.02	+0.06
7					71.53	6.68	11.04	+0.44	—0.03	+0.58
8					70.83	6.67	10.41	—0.28	—0.04	+0.05
9					71.04	6.71	10.39	—0.05	0.00	+0.03
10					71.26	6.77	10.69	+0.15	+0.06	+0.33
11					71.02	6.68	10.40	—0.07	—0.03	+0.04
12					70.98	6.70	10.41	—0.11	—0.01	+0.05
13					70.95	6.65	10.37	—0.14	—0.06	+0.01
14					71.00	6.70	10.51	—0.09	—0.01	+0.15
15					71.01	6.66	10.44	—0.08	—0.05	+0.08
16					70.85	6.66	10.37	—0.24	—0.05	—0.01
17					71.21	6.71	10.41	+0.12	0.00	+0.05
18					71.00	6.71	10.37	—0.09	0.00	+0.01
19					71.03	6.73	10.42	—0.06	+0.02	+0.06
20					71.08	6.71	10.43	—0.01	0.00	+0.07
21					71.17	6.70	10.42	+0.08	—0.01	+0.06
22					71.09	6.76	10.44	0.00	+0.05	+0.08
23					70.97	6.75	10.40	—0.12	+0.04	+0.04
24					71.02	6.74	10.46	—0.07	+0.02	+0.10
25					71.13	6.77	10.42	+0.04	+0.06	+0.06
26					70.85	6.74	10.41	—0.24	+0.03	+0.05

TABLE 6—*(Continued)*

No.	Sample	Theory C (%)	Theory H (%)	Theory N (%)	Found C (%)	Found H (%)	Found N (%)	Difference C (%)	Difference H (%)	Difference N (%)
27					71.09	6.78	10.35	0.00	+0.07	—0.01
28					71.18	6.68	10.45	+0.09	—0.03	+0.09
29	Cyclohexanone–2:4–dinitrophenylhydrazone	51.79	5.07	20.14	52.09	5.10	20.23	+0.30	+0.03	+0.09
30					51.37	5.14	20.66	—0.42	+0.07	+0.52
31					51.63	5.23	20.44	—0.16	+0.16	+0.30
32	S-benzyl-thiuronium chloride	47.40	5.47	13.82	47.23	5.52	13.87	+0.17	+0.05	+0.05
33	Benzoic acid	68.86	4.96	—	68.63	5.05	—	—0.23	+0.09	—
34					68.76	4.94	—	—0.10	—0.02	—
35	S-benzyl-thiuronium chloride	47.40	5.47	13.82	47.51	5.60	13.99	+0.11	+0.13	+0.17
36					47.19	5.58	13.93	—0.21	+0.11	+0.11
37	Cyclohexanone–2:4–dinitrophenylhydrazone	51.79	5.07	20.14	51.79	5.28	20.72	—0.08	+0.11	+0.58
38	Benzoic acid	68.86	4.96	—	68.82	4.93	—	—0.04	—0.03	—
39	S-benzyl-thiuronium chloride	47.40	5.47	13.82	47.33	5.58	13.92	—0.07	+0.11	+0.10
40	Acetanilide	71.09	6.71	10.36	71.14	6.80	10.48	+0.05	+0.09	+0.12
41					71.21	6.80	10.45	+0.12	+0.09	+0.09
					SD C ±0.20					
					H ±0.07					
					N ±0.16					

[a] Runs 1 and 2 used to calculate factors; no unweighed samples run; no blank corrections used.

ments. Several analysts were trained in the use of the Carbon, Hydrogen, and Nitrogen Analyzer with the Infotronics Integrator so that precision could be investigated with area measurements. Each analyst ran a series of acetanilide standards to check his own reproducibility prior to analyzing a series of actual routine samples. Table 7 summarizes the standard deviations on acetanilide obtained by all analysts.

The same analysts then ran a series of eight routine samples. Since some samples were run only singly by some analysts, a cumulative standard deviation was calculated to demonstrate reproducibility among analysts. All values were not obtained under what can be considered optimum conditions so the standard deviations can be considered as a realistic average for varying conditions. No values were excluded (Table 8). Average standard deviations were then calculated for the three elements and were compared to previous results obtained by the peak height method. Three analysts were used for the previous peak height study as compared to the five analysts used during this current investigation (Table 9). This data shows that reproducibility among analysts is greatly improved when the integrator is used.

TABLE 7

STANDARD DEVIATIONS OBTAINED BY SEVERAL ANALYSTS USING AN ACETANILIDE SAMPLE [a]

Analyst	Carbon (%)	Hydrogen (%)	Nitrogen (%)
A	±0.14	±0.03	±0.06
A	±0.16	±0.05	±0.06
B	±0.19	±0.06	±0.09
B	±0.16 [b]		
C	±0.27	±0.12	±0.09
C	±0.19 [b]		
C	±0.22	±0.08	±0.04
C	±0.14 [b]		
D	±0.12	±0.08	±0.03
D	±0.27	±0.09	±0.07
D	±0.18 [b]		
E	±0.12	±0.10	±0.18

[a] Carbon ±0.19; hydrogen ±0.08; and nitrogen ±0.08 cumulative standard deviation calculated from all values; total of 80 determinations.

[b] One stray result eliminated.

TABLE 8

ANALYSIS OF VARIOUS ROUTINE SAMPLES BY DIFFERENT ANALYSTS [a]

Analyst	Carbon (%)	Hydrogen (%)	Nitrogen (%)
		Sample K-1	
A	64.53	7.77	6.28
A	64.42	7.71	6.23
A	64.31	7.62	6.26
B	64.66	7.74	6.29
B	64.48	7.66	6.26
C	64.56	7.63	6.24
D	64.64	7.69	6.23
D	65.07	7.50	6.37
D	65.06	7.78	6.30
D	64.27	7.59	6.23
D	64.68	7.71	6.26
E	64.61	7.66	6.38
SD	±0.25	±0.09	±0.05
		Sample K-2	
A	51.85	4.12	12.95
A	51.73	4.61	12.87
A	51.94	4.58	12.93
B	51.60	4.61	12.94
C	51.45	4.48	12.80
D	51.61	4.48	12.80
E	51.71	4.55	12.97
SD	±0.17	±0.17	±0.07
		Sample K-3	
A	50.57	4.62	12.91
A	50.75	4.84	13.04
A	50.35	4.86	13.04
A	50.50	4.98	13.01
B	50.33	4.69	13.03
C	50.11	4.58	12.91
D	50.40	4.77	12.96
E	50.17	4.73	13.00
SD	±0.22	±0.14	±0.05

TABLE 8—*(Continued)*

Analyst	Carbon (%)	Hydrogen (%)	Nitrogen (%)
		Sample K-4	
A	32.44	2.58	12.59
A	32.41	2.74	12.65
A	32.92	2.93	12.66
A	32.51	2.86	12.63
B	32.82	2.98	12.67
C	32.70	2.77	12.65
D	32.72	2.90	12.77
E	32.60	2.61	12.75
SD	±0.18	±0.15	±0.06
		Sample K-5	
A	58.99	6.52	34.46
A	58.76	6.43	34.64
A	58.80	6.49	34.69
B	58.76	6.42	34.45
C	58.94	6.35	34.64
D	59.32	6.46	34.77
D	59.31	6.56	34.77
D	58.99	—	34.48
E	58.92	6.51	34.83
SD	±0.21	±0.07	±0.14
		Sample K-6	
A	40.33	4.81	—
A	40.35	5.35	—
A	40.02	5.21	—
A	40.15	5.26	—
B	40.20	5.58	—
C	40.26	5.39	—
D	40.02	5.17	—
E	40.32	5.31	—
SD	±0.13	±0.25	—
		Sample 0-1	
B	60.44	4.70	7.91
C	60.14	4.69	7.93
C	60.27	—	7.85
D	60.08	4.53	7.77
D	60.37	4.38	7.85
SD	±0.15	±0.15	±0.06

TABLE 8—*(Continued)*

Analyst	Carbon (%)	Hydrogen (%)	Nitrogen (%)
		Sample 0-2	
A	48.95	4.35	6.26
B	49.08	4.28	6.38
C	48.93	4.20	6.37
SD	±0.08	±0.08	±0.07

[a] Acetanilide used to calculate factors.

TABLE 9

AVERAGE STANDARD DEVIATIONS CALCULATED FROM THE ANALYSIS OF ROUTINE SAMPLES BY VARIOUS ANALYSTS

	Peak Height	Area
Carbon	±0.34	±0.17
Hydrogen	±0.13	±0.11
Nitrogen	±0.29	±0.07

SUMMARY

The use of the new Polypak gas chromatographic column in the F & M Model 185 Carbon, Hydrogen, and Nitrogen Analyzer has given improved resolution of component peaks making electronic integration now feasible. Electronic integration using the Infotronics Integrator has improved accuracy and precision as well as reducing total analysis time.

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Book Reviews

The Formation and Properties of Precipitates. By ALAN G. WALTON. Wiley (Interscience), New York, 1967. ix + 232 pp. $11.00.

This book is an integrated study of the features of precipitation, nucleation, growth, coprecipitation, surface properties, and multicomponent systems from as quantitative a viewpoint as possible. Recent experimental and theoretical advances have enabled formulation of a quantitative approach to the mechanism of precipitate formation and development. Until the last decade or so, most information in the literature has only been of a qualitative nature.

The volume consists of six chapters. Chapters 1–5 deal with the molecular mechanism theme of precipitation. Chapter 6 is a review of the work performed in the area of precipitation by the Jugoslav school and has been contributed by Dr. Füredi from the Ruder Boškovic Institute Zagreb, Yugoslavia.

The first five chapters explore in detail the mechanism of precipitate formation and growth. They reveal that the surface, shape, composition, and structure of a precipitate are determined by the interaction of many processes, such as homogeneous and heterogeneous nucleation, crystal growth, agglomeration, adsorption of constituent or foreign ions on the surface of the precipitate, and coprecipitation. Which process will be dominant depends on the experimental conditions under which precipitation occurs. Chemical equilibria have also to be taken into account in order to predict whether a precipitate is to be formed.

The final chapter, which is mainly empirical in approach, is included for two reasons. First, it represents a method or methods for tackling complex applied problems. Second, because many of the original papers were in Croatian journals and were not readily available in translated form. This chapter examines in detail the methodology of obtaining, presenting, and evaluating experimental data. It deals with experimental methods and techniques, evaluation of equilibrium constants, the solubility curve, the precipitation boundary, graphical presentation of precipitation data, and applications.

The book is well illustrated with many excellent drawings as well as numerous fine photomicrographs. In addition to the usual author index and subject index it contains an inorganic compound index and an organic compound index. Each chapter is followed with a rather complete set of references.

Anyone interested in the mechanism of precipitation from solution, whether it be in analytical or physical chemistry, physiology, or geophysics, will find this volume a worthwhile contribution to his library.

LEO K. YANOWSKI, *Department of Chemistry, Fordham University, Bronx, New York*

The Particle Atlas. By Walter C. McCrone, Ronald G. Draftz, and John Gustav Delly. Ann Arbor Science Publishers, Ann Arbor, Michigan, 1967. xv + 406 pp. $125.00.

Nearly two decades ago, Dr. Walter C. McCrone felt that the microscope had a greater application in air pollution than was generally appreciated. This magnificent book is the result. At first glance, the task of classifying airborne particles by microscopic observations seems impossible. However, rather than millions of different kinds, only a small number of species account for approximately 90% of the particles that cause these gloomy days in our urban areas. Actually, identifying a few hundred substances under the microscope is well within reason.

The book is divided into three parts: Principles and Techniques, covers the optics of the microscope, the collection and preparation of samples, and the analytical techniques for their identification; the Atlas, consists of 170 pages of photomicrographs in full color; and Analytical Tables. At the end there is a glossary of terms and a list of abbreviations and symbols, together with 1085 references and a list of photographic equipment needed. There is also a good author and subject index.

Too much cannot be said for the photomicrographs. They are beautiful—clear and well reproduced—and the best this reviewer has ever seen. The colors are perfect (as proved by a spot check by the reviewer).

In these days of increased emphasis on environmental control, more and more is going to be required of the microscopist. This book will not only prove to be a handy tool, it will be practically indispensable. In fact, it is an absolute must.

David B. Sabine, *484 Hawthorne Avenue, Yonkers, New York 10705*

Titrations in Nonaqueous Solvents

By **Walter Huber**
Ludwigshafen a/Rhein
West Germany

This book discusses the theory, basic principles, techniques and instrumentation of the titration in nonaqueous solvents. The application of various analytical methods for different chemical groups of substances is considered in detail, enabling the reader to select the optimal method to satisfy his specific purpose. The book presents the pK values of the compounds in tabulated form, and contains a thorough description of the use of automatic titration instruments, sources of error, reagents, solvents, and indicators. The practical section of the book is devoted to an extensive compilation of instructions and working procedures for determination of specific functional groups. Special attention is given to micro and ultramicro determination and the reactions of Lewis acids with Lewis bases.

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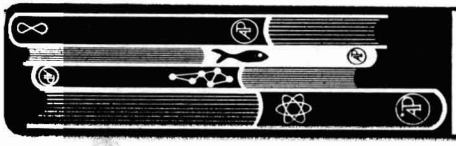

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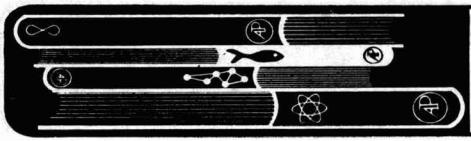

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