ANALYTICAL CHEMISTRY

JANUARY 1948

The actual lot analysis is on every label

Cupric Sulfate, CP Baker's Analyzed

LARGE CRYSTAL

1lb (453.6g) $CuSO_4.5H_2O$ M. W. 249.71

Analysis of Lot No. 21047

Insoluble Matter	0.003 %
Cl	0.0005%
Subst. not ppt. by H_2S	0.03 %
Ammon. Hydrox. ppt. (Fe,etc.)	0.006 %
Ammon. Sulfide Metals other than Iron (as Ni)	0.004 %

Meets A.C.S. Standard

Made in U. S. A. J. T. Baker Chemical Co., Phillipsburg, New Jersey

"Baker's Analyzed"

THE HIGHEST TYPE OF

Chemical Reagents

When a chemist picks up a bottle of Baker's Analyzed Reagents he may well say:

"This lot analysis tells me the exact degree of purity achieved. I have a precise basis to check my own calculations and findings quickly. I can work with speed, accuracy and safety—and more economically."

That's why surveys indicate that more chemists specify Baker's Analyzed Reagents – *with the actual lot analysis* on the label – than any other brand.

Nearly a half century of chemical skill and precision lies behind Baker's Analyzed C. P. Chemicals. When you order reagent chemicals send your order to your favorite laboratory supply house and be sure to specify Baker's Analyzed by name.

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ANALYTICAL CHEMISTRY

Volume 20, Number 1

Issued January 20, 1948

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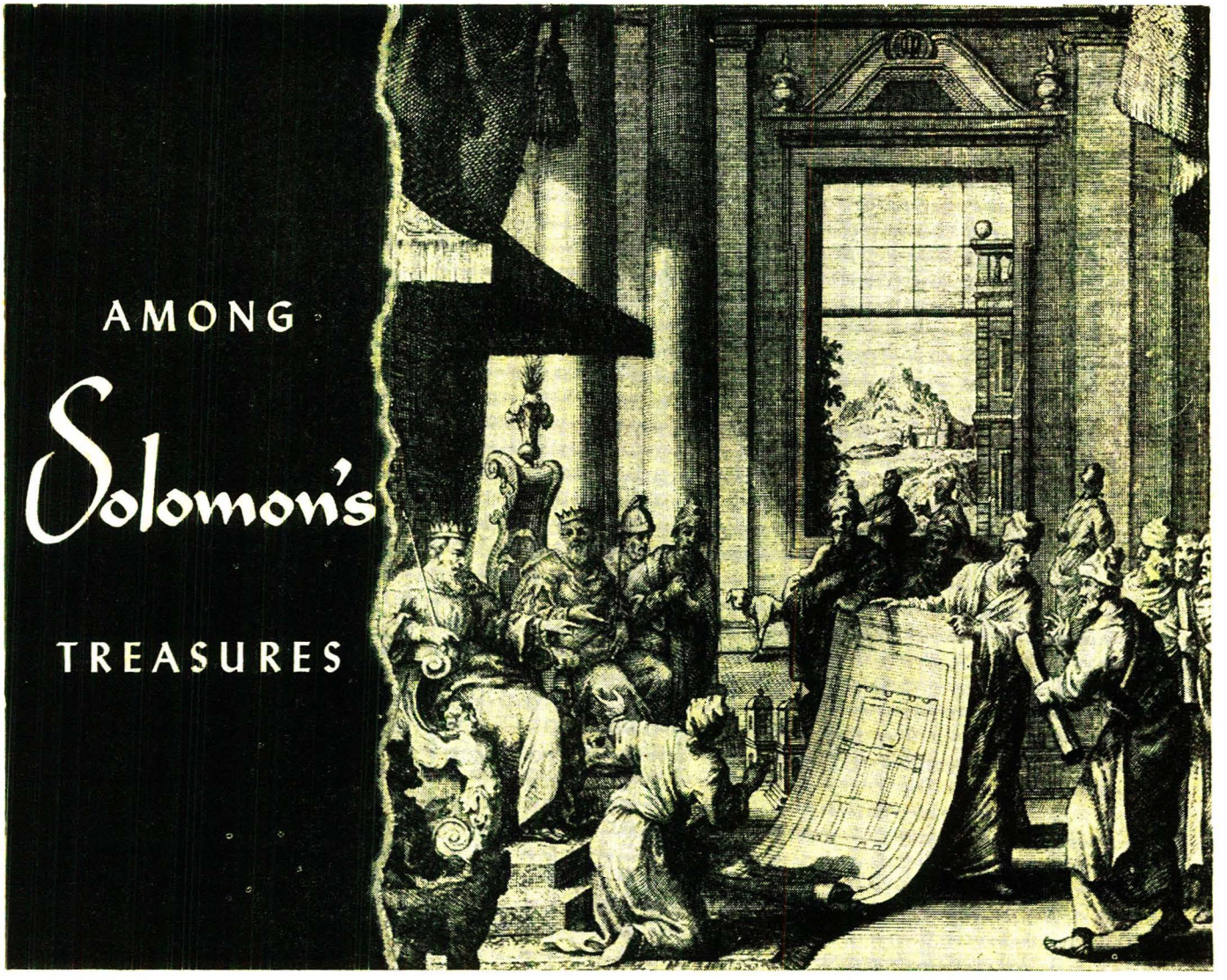

Lead, the low-melting-point metal of many uses, is mentioned in the Old Testament as being found among Solomon's treasures. In ancient times, the Romans mined lead when they controlled Wales. Today, the British Isles still produce a large percentage of the world's supply of this essential element.

Lead compounds, in addition to their applications in the laboratory, are vital to the manufacture of pigments, alloys, protective coatings, storage batteries, insecticides, and numerous other important industries that make possible our way of life.

The wide variety of analytical and industrial uses of lead compounds demands chemicals of maximum purity and dependability. Merck & Co., Inc., for almost four generations, has set the pace in the production of fine laboratory chemicals. The Merck label, which conforms to A.C.S. methods of stating maximum impurities, always has been the chemist's assurance of uniformly high quality.

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LEAD CARBONATE REAGENT
LEAD CHLORIDE REAGENT
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LEAD NITRATE REAGENT
LEAD OXIDE REAGENT
LEAD SUBACETATE REAGENT

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Los Angeles, Calif. • *In Canada:* MERCK & CO., Ltd. Montreal • Toronto • Valleyfield

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ANALYTICAL CHEMISTRY

Volume 20, Number 2

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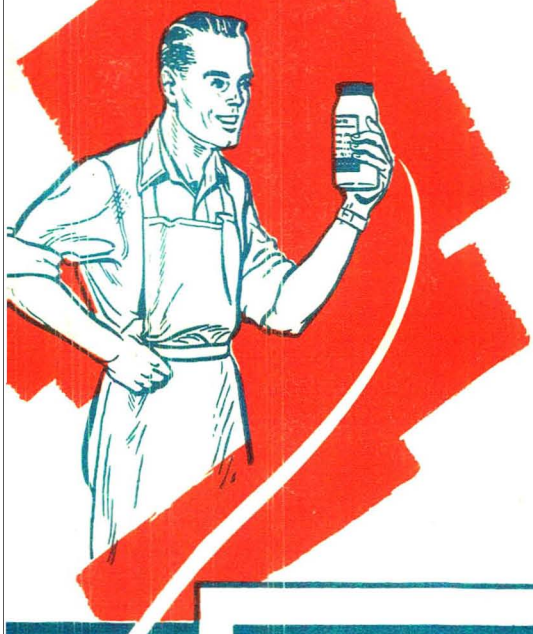
Safety with Accuracy



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And, when you can get the best at no extra cost, why not insist upon Baker's Analyzed?

Survey after survey indicates that chemists buy more Baker's Analyzed C. P. Chemicals than any other brand. So, specify Baker's Analyzed by name.

Sod. Hydroxide, C.P. Baker's Analyzed Pellets

1lb (453.6g) NaOH M.W. 40.005

Analysis of Lot No. 101347

Assay	97.9 %	Cl ..	0.001 %
PO_4	0.0003%	N ..	0.0004%
SO_4	0.003 %	Fe ..	0.0002%
Sodium Carbonate (Na_2CO_3) .	1.3 %		
H. Metals (as Ag)	0.000 %		
SiO_2 & NH_4OH ppt.	0.005 %		
K	0.00 %		

Meets A. C. S. Standard

POISON

ANTIDOTE--*External*--Flood with water, then wash with vinegar. *Internal*--Give vinegar, or juice of lemon, grapefruit, or orange, copiously. Follow with olive oil. *Eyes*--Wash out with 5 per cent boric acid solution *Call physician.*

Made in U. S. A. J. T. Baker Chemical Co., Phillipsburg, New Jersey

"Baker's Analyzed"

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Chemical Reagents

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ANALYZED REAGENTS Baker's FINE CHEMICALS

ANALYTICAL CHEMISTRY

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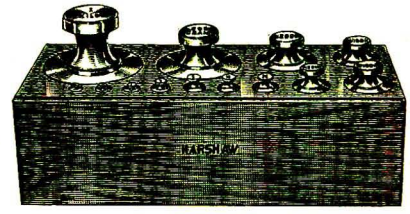
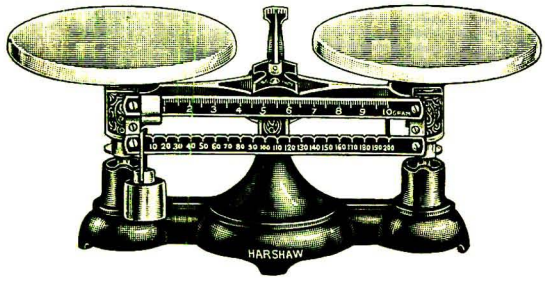
HARSHAW HARVARD TRIP SCALES

H-2550 Balance—Harvard Trip, double beam, round opal glass plates. Capacity, 2 kilos; sensitivity, 1/10 gram. Weighs to 210 grams without use of loose weights. Agate bearings are mounted in self-aligning blocks which insure continuous contact to the knife edges, thus assuring greater sensitivity and longer wear. All working parts cadmium plated and all bearings are protected by dust-proof shields. Beam, poise, dial and pointer are nickel silver. Upper beam graduated 10 grams by 1/10-gram divisions; lower beam graduated 200 grams by 10-gram divisions; plates are 6″ diameter.

EACH . $19.20 EACH (Lots of 3) . $17.28



H-3420



H-2550

H-3420 Balance Weights—Metric, brass. These weights are for use with Harvard Trip, Torsion and similar balances where extreme accuracy is not essential. Each set is contained in open hardwood box and supplied without fractions.

Sets from 1 gram to. . . .	50 gr.	100 gr.	200 gr.
Per set	$1.80	$2.40	$3.60
Per set (6 sets)	1.62	2.16	3.24

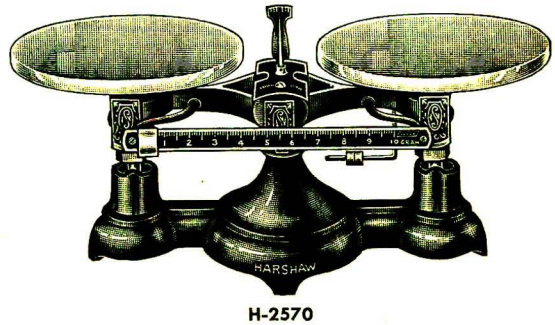
Sets from 1 gram to. . . .	500 gr.	1000 gr.
Per set	$4.45	$7.70
Per set (6 sets)	4.00	6.92

H-3440

H-3440 Balance Weights — Single metric, brass. These weights are the same as used in set No. H-3420.

Grams	1	2	5	10	20	50
Each	.10	.10	.12	.14	.24	.36
Each (Lots of 12)	.09	.09	.11	.12	.21	.32

Grams	100	200	500	1000	2000
Each	.48	.90	1.50	3.00	5.40
Each (Lots of 12)	.43	.81	1.35	2.70	4.86



H-2570

H-2570 Balance — Harvard Trip, single beam. Round opal glass plates. Capacity, 2 kilos; sensitivity, 1/10 gram. This balance is identical with No. H-2550 except that it is equipped with a single beam graduated to 10 grams by 1/10 gram divisions.

EACH . . . . . $15.90 EACH (Lots of 3) $14.31

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ANALYTICAL CHEMISTRY

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This *actual lot analysis* on the label is an extra service rendered for speed and precision—and at no extra cost. When you order Reagent Chemicals, get this extra value by specifying Baker's Analyzed.

Bromine, CP Baker's Analyzed

1lb (453.6g) Br At. Wt. 79.916

Analysis of Lot No. 1.2747

Nonvolatile Matter	0.002 %
Chlorine (Cl)	<0.3 %
Iodine (I)	<0.05 %
Organic Bromine Comp.	Passes ACS Test
Sulfur Comp. (as S)	0.000 %

Meets A. C. S. Standard

POISON

ANTIDOTE:--*Inhalation*--Fresh air, inhalation of ammonia. If swallowed, emetic of mustard, starch, followed by magnesia, sodium bicarbonate, white of eggs, flour and water

Made in U. S. A. J. T. Baker Chemical Co., Phillipsburg, New Jersey

"Baker's Analyzed"

THE HIGHEST TYPE OF

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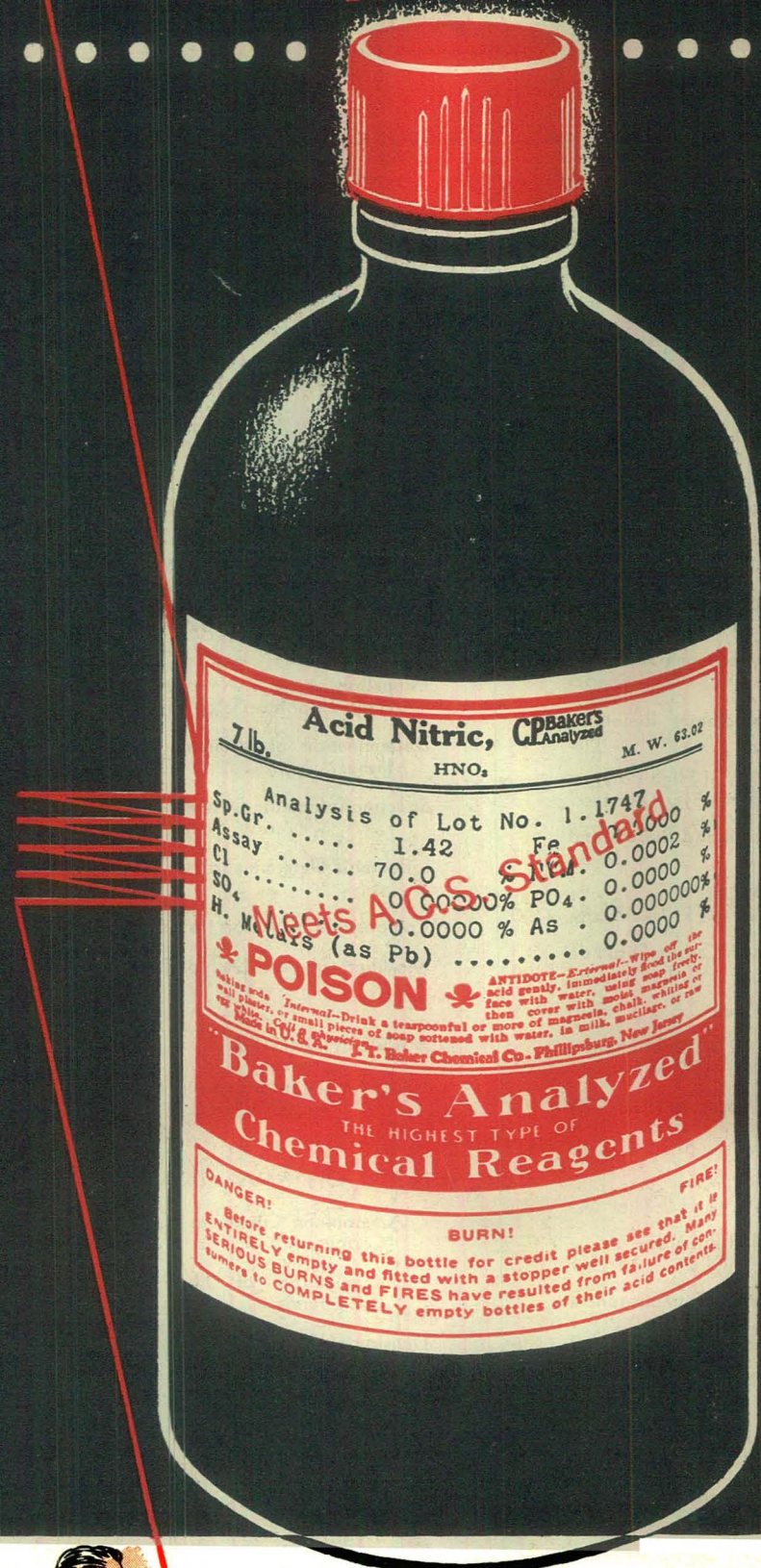
Accuracy with Safety



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just maximum limits of impurities

BUT...

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ANALYTICAL CHEMISTRY

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ANALYTICAL CHEMISTRY

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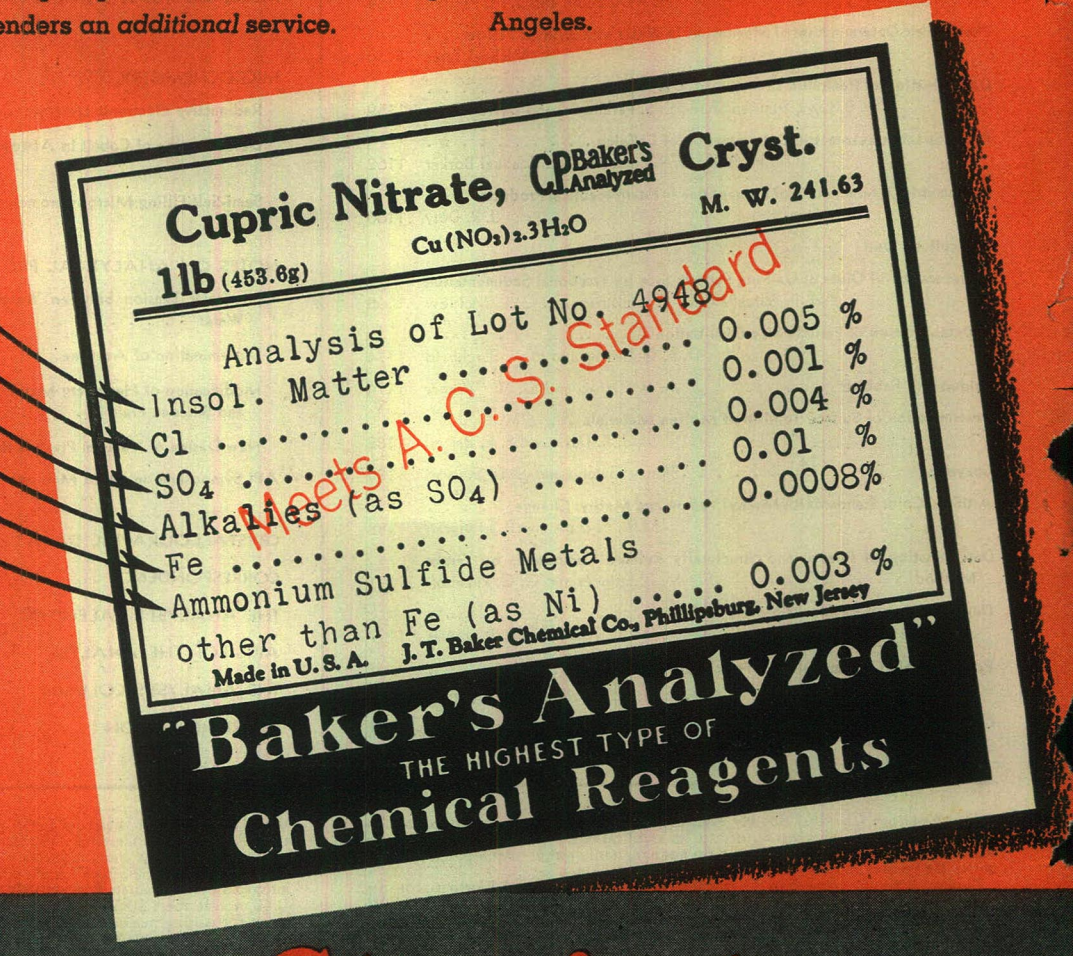

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ANALYTICAL CHEMISTRY

Walter J. Murphy, Editor

Official at Last

THE official name of this publication is now ANALYTICAL CHEMISTRY, replacing the long established and very familiar designation, the Analytical Edition of *Industrial and Engineering Chemistry*.

The change in title comes as no surprise to you, our loyal readers. We believe, however, that you will be interested to know that under the new circulation plan, whereby separate subscriptions are maintained for *Industrial and Engineering Chemistry* and ANALYTICAL CHEMISTRY, the January print order for the latter is 32,600 copies.

This is a pleasant surprise to the staff, for the survey conducted last summer to determine whether or not it was advisable to effect complete separation of the two publications, indicated a circulation of approximately 22,500 for Analytical. Even this figure was somewhat of a surprise to many but not to us, for the Opinion Research Corporation's survey, conducted as a part of the Hancock Report, indicated that 57% of the members of the AMERICAN CHEMICAL SOCIETY read or referred regularly to the Analytical Edition. This is not the whole story, of course, since approximately one quarter of the circulation of the Industrial and Analytical Editions, through 1947 sold only as a combination, is nonmember subscriptions. However, the figure of 57% was to us indicative of the large reader acceptance of the Analytical Edition and confirmed our personal impressions gathered through many contacts with the readers of the publication.

We cannot help but feel that the unexpected increase in circulation is an endorsement of the editorial policies, but let us hasten to add that it does not instill in us any feeling of smug complacency. We intend to improve and expand constantly the editorial services performed for our readers.

In line with this policy we call attention to a new department—The Analyst's Calendar on page 93, news about coming meetings of groups associated with the broad field of analytical chemistry. It is our intention to expand the news coverage of ANALYTICAL CHEMISTRY in 1948.

The Role of the Analyst

THE comments presented here bring to a close the expressions of opinion on the questions: "What is analysis?" and "What is the role of the analyst?" begun in the November issue and continued in December. The editors feel that publication of these communications has brought out many valuable suggestions which will have a very real part in improving instruction in analytical chemistry, enabling the analytical chemist to play a more effective role, and increasing appreciation of his contributions.

James J. Lingane, Harvard University

AT THE forum on the education of analytical chemists scheduled for the Chicago meeting next April it is more than likely that a good deal of discussion will revolve around "instrumental analysis." We may even hope that a more apt generic term can be invented for the physicochemical determinative techniques embraced by this pseudonym. No one will deny the increasing importance of physicochemical determinations in modern analytical practice, and the concomitant need for more systematic and more extensive education in these methods. Instructions in the established techniques, such as potentiometric and conductometric analysis, polarography, and spectrophotometry, presents no great difficulties and probably can continue to be provided within the framework of existing chemistry curricula, preferably on the graduate level.

But the science of instrumentation itself presents a larger problem. Month by month in this journal for two years our colleague Ralph Müller has been presenting convincing evidence that instrumentation in the broad sense has grown to such proportions that it merits recognition as a new branch of knowledge. Many others share the belief that haphazard instruction in this subject is no longer adequate if we wish to realize its potentialities fully. Perhaps graduate courses in

instrumentation will suffice, although there are some who believe that special curricula will be required. Müller has offered some detailed suggestions which deserve careful study [*Instruments*, **19**, 261 (1946)].

I venture the opinion that it is neither desirable nor feasible to attempt much serious instruction in "instrumental analysis" in the undergraduate course in quantitative analysis. True, one can place potentiometers, spectrophotometers, pH meters, polarographs, and the like in the laboratory and have the student "make determinations" with them, carefully selecting the "unknowns," of course, so no "difficulties" are encountered. But since the undergraduate quantitative course is peopled chiefly by sophomores and juniors, who have not begun the study of physical chemistry, and whose background in physics and mathematics is meager, the educational value of such a scheme is questionable. Too much superficial "modernization" of this kind tends to dilute the instruction in more fundamental aspects of analytical chemistry, which many will agree are still as essential to the education of an analytical chemist as they ever were.

In our justifiable enthusiasm for the truly great accomplishments of "instrumental analysis" it is easy to lose our sense of proportion and forget that the most important factor in a chemical analysis is the chemical experience of the analytical chemist rather than the final determinative techniques at his disposal.

Since first impressions are important, it will not aid in attracting students to analytical chemistry if the elementary course is presented merely as a series of unrelated determinations, with little or no discussion of theory or chemical analysis in its fuller sense. It is hard to agree with a view which is clearly expressed in the Professional Training Committee's recommended minimum standards, that an undergraduate course can be taught merely as a laboratory art. In sharp contrast to the committee's recommendations for general, organic, and physical chemistry, which in each case include 3 lecture hours per week for 30 weeks, no lecture time at all is recommended for the quantitative analysis course!

In considering ways and means for improving instruction in analytical chemistry we would do well to acquaint ourselves with the scheme employed so successfully by E. H. Swift at the California Institute of Technology, which is described in his excellent book, "A System of Chemical Analysis" (Prentice-Hall, 1939). Swift's course, which combines the traditional qualitative and quantitative courses without sacrificing the didactic virtues of either, comes closer to teaching what Lundell has so aptly termed "the chemical analysis of things as they are" than any other scheme that the writer has seen.

Clyde Williams, Battelle Memorial Institute

As late as the 17th century, among the investigators of science, it was considered unethical and very improper to substantiate theories and philosophies by experimentation and analysis. For this reason chemical research during this period lay wholly in the hands of the philosophers and was considered only from a purely speculative point of view. Sometimes, to be sure, limited experiments were performed, but any fact uncovered which conflicted with the premediated theories were discarded at once or more favorably reinterpreted. Thus, any scientific analytical chemistry was discouraged and frowned upon. However, eventually so many analytical facts were exposed that obvious interpretation could no longer be avoided, and the gap between alchemy and chemistry was bridged.

Analytical chemistry had thus become the fundamental basis of chemical progress. As time went on, to accumulate new facts, new techniques became necessary and analytical chemistry expanded into neighboring scientific fields to obtain new tools.

In 1929, a well-known chemist gave the following definition of a good analyst: "After all is said and done, a good analyst is primarily a chemist who can quantitatively manufacture pure chemicals." Analytical techniques have come a long way since 1929. The above definition would be true only to a very limited extent today. Instrumental or physicochemical methods of analysis employing the spectrograph, spectrophotometer, flame photometer, polarograph, electrotitrators, etc., are playing major roles in the modern analytical laboratory. Every analytical group must include personnel, or have available those who can intelligently use, design, repair, and on occasion build such instruments; and of course, coordination of these techniques must be carefully and objectively balanced by the individual analyst for the efficient function of the entire group.

However, perhaps by inertia, a little bit of the 17th century mode of thinking creeps into some of our present-day scientific conjectures. Thus, the analytical chemist may, at times, be very unpopular because of his (or her) unsportsmanlike slaughter with proved analytical data of a proposed scientific theory. Of course, for the same reason, confirming data should render him popular. However, it is often the theorist in this case who becomes famous all by himself.

Present-day scientific and industrial research revolves about the analytical accumulation of facts, and, although rarely admitted, the analytical division of a successful scientific research organization is the hub of the wheel, and progress will be made only through the smooth functioning of this member.

W. T. Nichols, Westvaco Chlorine Products Corporation

The chemical industry has undergone enormous growth and change in the past three decades. So has analytical chemistry. While it might not be proper to say that the growth and change of the industry are due to the transformation of the science, certainly one would not be possible without the other.

Even up to comparatively recent times, industrial analytical laboratories were staffed principally with analytical chemists who were usually very familiar with most of the theory and techniques available for use. Today, the workers in an industrial analytical laboratory are, for the most part, technicians. These workers are supervised by and use methods, techniques, and apparatus developed by the real analytical chemists, who are of high professional caliber. This evolutionary process parallels the similar effect which may be noted in the mechanical industries, where engineers have devised automatic or semiautomatic machines that need to be tended only by workers of limited skill.

This transformation, which has been gradual, has produced the need for analytical chemists with training far more elaborate than is usually available in our universities. Time was, when those specializing in chemistry could be taught the analytical courses during undergraduate years. The usual curriculum contained qualitative and quantitative analysis courses, with perhaps the opportunity to take some advanced work. Some of the better curricula offered gas and fuel analysis, chemical microscopy, etc. Chemical analysis at that time comprised principally numerous, tedious, involved, and time-consuming manipulations.

The application of new scientific theory to chemical analysis

by first-line research men has resulted in the development of methods, techniques, and apparatus which, more often than not, permit reproducible results to be obtained rapidly and cheaply. This sort of activity is now really the principal occupation of the true analytical chemist. Actual routine determinations are usually done by trained technicians who need to possess only what might be termed manipulative skill and the ability to follow instructions rigidly and to do over and over again, with minimum deviation, operations prescribed by the analytical chemist. Results obtained by technicians are interpreted by analytical chemists possessing specialized training.

The widespread use of the newer techniques places a premium upon intensive, specialized training in up-to-date analytical science. Extensive knowledge of chemistry, physics, and mathematics is a vital requirement if the full benefit of modern techniques is to be made available to industrial operations. The development of instruments and techniques dependent upon radiation and electrical phenomena and their adaptation to industrial analysis, for instance, call for a deep understanding of physics not ordinarily possessed by the usual undergraduate. The availability of rapid, cheap methods has brought about much more extensive control analysis, and the interpretation of results so as to secure full knowledge of their meaning calls for unusual mathematical ability, particularly with respect to methods of statistical analysis.

It is important to differentiate between the true analytical chemist and the analytical technician. The latter may be recruited from among high school graduates. They are by no means unimportant employees. Good routine analytical practice depends upon a supply of competent technicians, but they need competence only within a limited area. An active analytical chemist, on the other hand, to deserve the name, operates at a high professional level and must possess and use intensive and extensive knowledge of his field.

Although the large and complex structure of industry requires a very large number of analytical workers, relatively few of them are analytical chemists. Relatively few are needed to do the basic work required. Even so, the supply of real analytical chemists is much too small. The curriculum for training a modern analytical chemist is available at only a very few of the American universities. Industry has had to depend upon on-the-job, postgraduate training to meet its needs.

By no means all of industry takes advantage of up-to-date analytical practice. This is due in part, no doubt, to the pitifully small supply of thoroughly trained analytical chemists. Perhaps it is due in some measure to lack of understanding of the function and ability of the analytical specialist.

It is to be hoped that more adequately trained analytical chemists will be made available to industry in the future. If the challenge is to be met, existing curricula are likely to undergo substantial change.

J. K. Roberts, Standard Oil Company (Indiana)

A PROPER definition of analysis as used in industry must recognize the dual function which it fulfills. On the one hand, we have research analysis which yields information with respect to new developments. In this function analysis may be considered as the eyes through which research sees its way forward. The starting materials used and the products formed in research experiments must be analyzed before the results of research on either new processes or new products can be evaluated. In turn, the direction of the successive experiments will be governed by this evaluation. There have been many classic examples, of which the plutonium project was one, wherein the entire success of the processing step was based on the work of the analytical chemist. This type of analysis is used in solving the problems of sales and manufacturing technical service as well as those of research.

In its second function in industry, analysis may be considered as the guardian of manufacturing operations. Through the routine testing of incoming raw materials, the laboratory serves as a screen to protect the processes and equipment from receiving goods of inferior quality. The control testing of partially or completely finished products serves to guide the manufacturing operations and maintain the quality of the finished products.

The research type of analysis requires the most exacting skill and technical knowledge on the part of the analyst. His results must be of the highest quality, in order that research developments may be properly evaluated. Furthermore, correct analysis of new and unexpected by-products requires a broad background in the chemistry of the process materials as well as in the analysis thereof. In research analysis, no cost of technical labor or equipment can be considered too high if the potentialities of the process under development are great enough. On the other hand, the cost of control analysis bears a direct relation to total manufacturing cost. Workers in this field should have an appreciation of the broad aspects of the control problem, the significance of the values they report, and the uses made of such data by others. They should also be constantly alert to the possibilities of short-cut methods, automatic control, etc.

Although we should recognize the dual function of analysis, it would be unrealistic to attempt a sharp demarcation in all cases. In the average industrial laboratory, it is customary for the same analyst, using the same equipment, to carry out work on both types of analysis. This naturally increases the complexity of the role he must play in the industrial laboratory.

The primary requisite for an analyst is that he have the ability to carry out analyses and be technically expert in so doing. He may not actually carry out all of the manipulation, but where nontechnical assistants work under his supervision he must thoroughly master all phases of their work. He must be able to recognize and evaluate unusual phenomena observed during the course of the analysis, and he must continually be aware of features of individual analytical methods which will limit or favor their use in particular cases. Thus a broad knowledge of the many analytical methods and techniques now available for use must be at his finger tips. In addition, where no suitable method is at hand, he should be capable of developing one. He must be familiar with chemical literature and the techniques of literature searching. With this knowledge he should be able to lay out the necessary experiments in analytical research directed toward developing the new method. He should particularly understand the significance of accuracy and precision. A complementary requirement, often woefully lacking in the undergraduate applicant today, is the ability to report the results of his own work in concise and readable form. An additional asset is the ability to train and supervise nontechnical people. The opportunities for the chemist with such talents are often not recognized outside the industrial laboratory.

The conventional training in general chemistry and in qualitative and quantitative inorganic analysis does not fit a man to fill the role outlined above. Actually, in the industrial laboratory of today, conventional qualitative inorganic analysis has very little place; it should be given corresponding treatment in the curricula of our colleges. On the other hand, increased emphasis should be placed on organic analysis and particularly on the general methods of approach as used in such industries as food, drugs, and petroleum. The analytical,

chemist should receive a general review of the newer physical methods of analysis, either by a survey course or by a seminar and laboratory course that will give him at least some experience in the techniques of these methods, including spectroscopy and polarography. He should also be introduced to the electrical and electronic measuring devices now commonly used in such conventional laboratory operations as titration. The development of new analytical methods or the comparison of existing ones requires the application of statistics to the analysis of experimental data and to sampling problems. Finally, the analyst must have had enough chemical physics to understand the structure of matter and the nature of the particles or radiations which may form the basis of his newer analytical tools.

This level of training adds up to more specialized education than will fit into the normal four-year college curriculum. It leads to the conclusion that, if analytical chemistry is to come into its own as a new career, the analyst will require postgraduate work either in the academic world or in industry.

W. C. McCrone, Armour Research Foundation

The questions (1) What is analysis? and (2) What is the role of the analyst? presented in the November issue of the Analytical Edition have been under discussion at the Armour Research Foundation for some time in an attempt to name more precisely one of the important groups in the organization. This group has been in operation for more than two years, during which time the divergence between its function and its traditional name has become increasingly acute.

Analysis has become so diversified and analysts include so many types of chemists and physicists that neither can be defined in simple terms. The analytical chemist has been replaced not by a man having a different training but by a group of specialists in the determination of physical properties. In general, these specialists resent being referred to as analysts. They are instead physicists or chemists trained in the study of electron microscopy, tracer techniques, infrared spectrophotometry, emission spectroscopy, x-ray diffraction, mass spectrometry, chemical microscopy, polarography, etc. A group of people qualified in each of these phases of analytical work make up the modern analytical laboratory. The most appropriate name for such a group has not yet been found; the Armour Research Foundation has used Analytical Section, some groups prefer Chemical Physics, other possibilities are Instrumental Analysis, Analytical Physics, and Physical Analysis. It is desirable to have a new name, more dignified than "analytical" alone, yet it is essential to retain the word, or at least the connotation, "analytical." Instrumental Analysis Laboratory is perhaps the best compromise.

It is essential to emphasize that the best "analyst" in such a group may not know what happens when sodium bicarbonate and hydrochloric acid are mixed. He must know everything possible about the scope, limitations, and techniques with his instrument and he must be able to judge whether he can solve a given analytical problem on the basis of the above factors. He can usually handle problems altogether impossible for the classical chemical analyst. The emission spectroscopist, for example, can analyze quantitatively for a few parts per million of most of the cations; tracer techniques often give accurate results in terms of parts per billion; infrared will give analytical results on mixtures of geometrical or substitution isomers indistinguishable by chemical means; x-ray diffraction differentiates between polymorphic forms or hydrates of the same compounds and, in addition, gives a measure of the particle size of each crystalline phase. These are but a few examples which could be mentioned.

Probably the greatest single advantage of instrumental methods is due to the fact that no isolation of the components in the literal sense is required. Many chemical methods are based on the complete separation of a component by precipitation and determination of the weight by an analytical balance (gravimetric analysis). Although volumetric methods often allow determination of the amount of certain components by titration without actual isolation, the instrumental methods almost without exception boast this very great advantage. They are possible through the development of instruments capable of measuring accurately other physical properties besides mass. These properties include discharge potential (polarograph), infrared absorption spectra, crystal lattice dimensions (x-ray or electron diffraction), magnetic susceptibility, mass-charge relation (mass spectrometer), dipole moment, refractive index, optical rotation, etc.

The development of instrumental methods has made it possible to consider analytical problems that would not have been attempted by classical chemical methods. These are analyses of complex polycomponent mixtures such as lubricating oil or even the lighter fractions in petroleum. Even here light absorption methods give the composition of a hypothetical average molecule by showing the relative amounts of the different chemical bonds present. The problem, however, is to separate such a mixture into individual compounds. This is now possible through the use of recently developed fractionation methods and especially by cross-fractionation procedures. These methods include distillation in all of its forms (vacuum, azeotropic, and molecular), solvent extraction, fractional crystallization, ion exchange, chromatography or adsorption methods, thermal diffusion, gaseous diffusion, etc., as well as a few less general methods such as the mass spectrometer, dialysis, and flotation. The basis for separation by each of the above methods is different; boiling point for one, molecular type for another, density or molecular weight for a third, etc.

Cross-fractionation makes use of two or more of the above procedures. It is, for example, much easier to separate pure compounds from a close boiling fraction of different compound types than from a broad boiling range fraction. A very effective combination is distillation and silica gel absorption, techniques applied with great success by Rossini and his coworkers at the Bureau of Standards. Distillation gives a mixture of a few molecules of each of a number of compound types boiling over the same temperature range; these are then separated by silica gel adsorption roughly in order of their dipole moments. A third potentially extremely useful technique to supplement the above is thermal diffusion which separates compounds very efficiently, probably on the basis of relative densities. Eventually these procedures, by closer and closer cross-fractionation, give fractions which cannot be changed by further fractionation; these are then the pure compounds which can then be characterized by infrared, etc., for rapid detection in subsequent work.

It is apparent, finally, that all analytical methods consist of two stages: "isolation" and "weighing." "Isolation" may be carried out literally or figuratively; the latter, for example, includes isolation of spectral emission lines or infrared absorption bands of the compound or element. "Weighing" may also include measurement of physical properties other than mass—e.g., intensity of x-ray diffraction spectra or magnitude of diffusion current with amperometric analyses on the polarograph.

It seems that in the same way that we speak of an average molecule in a lubricating oil which is a composite of all the types of molecules present, we must also speak of an average analyst who is a composite of all the men necessary in a modern analytical laboratory.

Mass Spectrometer Analysis of Some Liquid Hydrocarbon Mixtures

R. A. BROWN, R. C. TAYLOR, F. W. MELPOLDER, AND W. S. YOUNG
The Atlantic Refining Company, Philadelphia, Pa.

Applications of the mass spectrometer method of analysis to normally liquid hydrocarbons in the C_5 to C_8 range are discussed. Accuracies attainable for individual compounds in a number of different synthetic mixtures are given and typical analyses of several hydrocarbon fractions in gasoline boiling range are shown. It is concluded that a substantial number of the paraffins, cycloparaffins, and aromatics occurring in this region may be individually determined. Olefins and cyclo-olefins, on the other hand, may be determined individually only to a limited extent at present.

APPLICATIONS of the mass spectrometer to the analysis of normally gaseous hydrocarbon mixtures, such as those occurring in petroleum refinery practice, have been covered thoroughly in previous publications (*3, 6, 7*) where accuracies attainable with various mixtures have been experimentally established. Similar data for normally liquid mixtures in the C_5 through C_8 range, however, have been insufficient to permit an accurate evaluation of the method when applied to mixtures of practical interest. This paper presents mass spectrometer analyses of a number of known synthetic mixtures in this range for the purpose of establishing accuracy, and to illustrate by several examples the application of the method to actual mixtures.

Broadly, the hydrocarbon types which must be considered because of their known presence in petroleum products include the paraffins, cycloparaffins, mono- and diolefins, and aromatics. It would be possible, however, at present to determine only a limited number of the many compounds which might conceivably be present, even in the relatively narrow C_5 through C_8 range, for a twofold reason: The lack of pure calibrating materials of the olefinic type constitutes a serious handicap to the extension of direct mass spectrometer analysis to these compounds, and it is questionable, in view of the present status of instrumental stability and the probable similarity of olefinic mass spectra, whether many of the isomers could be identified as individuals.

In spite of the somewhat unfavorable outlook for direct olefin analysis, however, the most promising approach to the general problem at present seems to lie in the separation of olefinic compounds from a complex mixture of hydrocarbons by some means such as silica gel percolation or solvent extraction. This may be followed by hydrogenation of the olefinic portion, fractional distillation, and then identification of the resulting paraffins and cycloparaffins by the mass spectrometer method, whereupon certain branched structures may be assigned to a substantial portion of the parent olefins. This method has the obvious limitations that nothing can be learned concerning the positions of the double bonds, and that it must be presumed no rearrangement or polymerization occurs during the manipulations. By utilizing other techniques, however, such as deuteration or ozonolysis, in conjunction with the mass spectrometer method, it may be possible to assign positions to double bonds in a number of cases, thus complementing the structure data.

It is evident from the foregoing that the successful spectrometer analyses of olefins in the C_6 through C_8 range at present awaits considerable experimental investigation.

What can be done, however, with the remaining hydrocarbons may be summarized as follows:

Paraffin isomers can be identified individually except for a few pairs, which, because of pattern similarity, must be grouped occasionally in complex mixtures.

Cycloparaffin isomers can for the most part be identified individually through C_7 range, but generally must be grouped in the C_8 range.

Olefin isomers can be determined at present only in the C_5 and lower range.

Cyclo-olefins can be determined in only a few instances, primarily because suitable calibrating compounds are not available.

Aromatics such as benzene, toluene, and ethylbenzene are readily determined individually, but xylenes must be grouped, as a rule.

APPARATUS AND PROCEDURE

Mass Spectrometer Analysis. The mass spectrometer used was manufactured by the Consolidated Engineering Corp., Pasadena, Calif. The general techniques of operation and computation have been adequately described (*6, 7*).

Liquid samples and liquid calibrating compounds were introduced into the instrument by using the sintered-glass valve system previously described (*5*). The known gaseous mixtures of the C_5 hydrocarbons were prepared using manometric pressure measurements in the conventional manner, while liquid mixtures of C_6, C_7, and C_8 hydrocarbons were prepared by a semimicro-weighing method (*5*).

In addition to the synthetic mixtures, a series of alkylate fractions was analyzed (Table X), whose composition had previously been determined from fractional distillation and physical property measurements (*1*).

Generally, calibrations and mixtures were run on the same day in order to minimize errors due to pattern fluctuations. An outline of the computational method used accompanies each table.

Distillation and Preparation of Naphtha Samples. The hydrocodimer analyzed (Table II) was first separated into ten fractions on a Podbielniak high-temperature column having 60 theoretical plates operated at a reflux ratio of 100 to 1.

The three cracked naphtha samples (Tables XII and XIII) were first treated with nitrogen peroxide according to the nitrosation procedure of Bond (*2*) for the removal of olefinic materials. Aromatics and traces of nitrosates were then removed from the treated naphtha by silica gel percolation (*4*) and the resulting paraffin-cycloparaffin mixtures were distilled into 2% fractions on 100-plate columns operating at 100 to 1 reflux ratio.

Blends were then made of these near-boiling fractions, wherever possible to reduce the number of samples requiring analysis.

DISCUSSION AND RESULTS

Analysis of C_5 Hydrocarbons. Presented in Table I are three analyses of a nine-component C_5 mixture containing known amounts of iso- and *n*-pentane, cyclopentane, five pentenes, and isoprene (2-methyl-1,3-butadiene). These data show that the concentration of isoprene, 2-methyl-2-butene, and the pentanes are within a mean of 0.1 to 0.6 mole % of the known composition.

Table I. Analysis of a Synthetic C_5 Mixture

Component	Known Composition	Determined Composition 1	2	3	Mean Difference
		Mole per cent			
Isoprene	4.3	4.2	4.3	4.1	0.1
3-Methyl-1-butene	10.5	10.4	8.5	11.8	1.1
1-Pentene	10.2	11.9	9.9	7.5	1.6
2-Methyl-1-butene	9.7	6.7	10.5	9.9	1.3
trans-2-Pentene	9.6	13.3	13.4	10.0	2.6
2-Methyl-2-butene	11.1	10.9	10.2	10.5	0.6
Cyclopentane	10.3	8.2	9.4	12.7	1.8
Isopentane	19.0	19.1	18.3	18.3	0.5
n-Pentane	15.3	15.3	15.5	15.2	0.1
Total mole %	100.0	100.0	100.0	100.0	

Method of computation. Iso- and *n*-pentane were first resolved by solving two simultaneous equations based on masses 57 and 72. Isoprene, cyclopentane, and the pentenes were then resolved from seven simultaneous equations based on residual peaks at masses 39, 41, 42, 55, 68, 69, and 70.

Table II. Analysis of a Synthetic C_6 Mixture

Component	Known Composition	Determined Composition	Difference
	Mole per cent		
Cyclopentane	4.2	5.1	0.9
2,2-Dimethylbutane	4.4	4.2	−0.2
2,3-Dimethylbutane	13.3	12.3	−1.0
2-Methylpentane	28.8	29.9	1.1
3-Methylpentane	40.2	40.2	0.0
n-Hexane	9.1	8.3	−0.8
Total mole %	100.0	100.0	

Method of Computation. Six simultaneous equations based on masses 42, 43, 57, 70, 71, and 86.

Cyclopentane and the remaining pentenes agree within 1.1 to 2.6 mole %.

Although this mixture contained no cyclopentene, 1,3-pentadiene, or 1,4-pentadiene, it is believed that the presence of such compounds would introduce no additional error in the pentane, cyclopentane, or pentene analysis. It would necessitate, however, the grouping of pentadienes, cyclopentene, and other compounds of molecular weight 68.

The method of computation involves the solution of one set of two simultaneous equations and one of seven equations.

Analysis of C_6 Hydrocarbons. Table II compares the mass spectrometer analysis of a six-component C_6 hydrocarbon synthetic with its known composition, the maximum difference found being 1.1 mole %. This analysis differs from a similar mixture previously reported (*6*) by including both 2,3-dimethylbutane and cyclopentane. Although no methylcyclopentane or cyclohexane was included, a consideration of the known mass spectra of these compounds indicates that their presence would probably not affect the accuracy of the analysis for the other components. Methylcyclopentane and cyclohexane binary mixtures may be analyzed with an accuracy of approximately 0.5 mole %.

Analysis of C_7 Hydrocarbons. A ten-component mixture comprising the nine heptane isomers plus 2,2,4-trimethylpentane was analyzed by means of the mass spectrometer. The proportions of the major components in the mixture correspond approximately to those found in C_8 alkylate. The results obtained for two different analyses of this mixture are compared in Table III with the known composition. It may be seen that the mass spectrometer analysis agrees to within a mean deviation of 0.1 to 1.6 mole %. Because of the similarity in the mass spectra of 2,2-dimethylpentane and 2,2,3-trimethylbutane, however, it was found necessary to group these two compounds. Experience has shown that the average cracking pattern of these two hydrocarbons may be used for any relative concentration of the two hydrocarbons without introducing a significant error.

Based also on past experience with similar mixtures and the known behavior of the pure compounds, it is felt that the presence of cycloparaffins boiling in the C_7 paraffin range would have no adverse effect on the accuracy of the paraffin analysis. The determination of the cycloparaffins, however, would probably be limited to cyclohexane and 1,1-dimethylcyclopentane, with *trans*-1,2- and 1,3-dimethylcyclopentanes grouped in certain cases.

Analysis of C_8 Hydrocarbons. Octanes in the 99.2° to 115.6° C. Range. Tables IV and V show the results obtained on three different blends of nine octane isomers. Nine simultaneous equations were solved based on mass spectra data at masses 42, 43, 56, 57, 70, 71, 85, 99, and 114. Pattern coefficients are so similar for many of these isomers that stability of the mass spectrometer appears to be an important factor in obtaining reproducibility. In several cases it was necessary to group two isomers in order to stay within a reasonable limit of error. Thus in Table IV,

Table III. Analysis of a Synthetic C_7 Mixture

Component	Known Composition	Determined Composition 1	2	Mean Difference
		Mole per cent		
2,2-Dimethylpentane	3.5 } 5.7	5.8	5.7	0.1
2,2,3-Trimethylbutane	2.2			
2,4-Dimethylpentane	50.7	48.9	49.3	1.6
3,3-Dimethylpentane	1.9	1.9	1.8	0.1
2,3-Dimethylpentane	31.7	33.0	33.6	1.6
2-Methylhexane	1.7	1.3	0.9	0.6
3-Methylhexane	3.8	4.6	3.0	0.8
3-Ethylpentane	1.8	1.6	2.5	0.5
n-Heptane	1.3	1.6	2.0	0.5
2,2,4-Trimethylpentane	1.4	1.3	1.2	0.2
Total mole %	100.0	100.0	100.0	

Method of Computation. Nine simultaneous equations based on masses 42, 43, 55, 57, 70, 71, 85, 99, and 100. 2,2-Dimethylpentane and 2,2,3-trimethylbutane were grouped by using an average cracking pattern.

Table IV. Analysis of a Synthetic C_8 Mixture Boiling between 99.2° and 115.6° C.

Component	Known Composition	Determined Composition 1	2	3	4	5	Mean Difference
		Mixture 1A, Mole Per Cent					
2,2,4-Trimethylpentane	7.7 } 26.6	4.2			10.5	15.0	
2,2-Dimethylhexane	18.9	23.2	24.0	25.7	18.0	13.4	1.6
2,2,3-Trimethylpentane	9.9	9.8	10.8	10.0	10.5	9.6	0.4
2,5-Dimethylhexane	17.1	15.7	19.8	16.2	16.1	16.1	1.4
2,4-Dimethylhexane	8.0	7.9	11.3	14.9	7.0	4.8	2.9
3,3-Dimethylhexane	15.7 } 22.3	17.5	2.5	1.7	15.9	22.2	
2,3,3-Trimethylpentane	6.6	4.3	20.6	17.9	6.0	0.9	1.1
2,3,4-Trimethylpentane	7.0	7.1	10.4	13.5	6.2	4.2	2.7 } 2.2
2,3-Dimethylhexane	9.1	10.3	0.6	0.1	9.8	13.8	4.8
Total mole %	100.0	100.0	100.0	100.0	100.0	100.0	
		Mixture 1B, Mole Per Cent					
2,2,4-Trimethylpentane	12.3 } 20.4	12.4		5.6		7.0	
2,2-Dimethylhexane	8.1	7.6	18.7	12.7	25.1	13.2	1.9
2,2,3-Trimethylpentane	12.5	13.6	13.6	14.2	13.1	13.2	1.0
2,5-Dimethylhexane	8.6	9.1	10.5	8.4	9.1	7.9	0.8
2,4-Dimethylhexane	7.2	5.6	6.7	9.1	12.8	9.3	2.3
3,3-Dimethylhexane	17.5 } 29.3	16.0	13.0 } 31.6	10.8 } 30.1	23.5 } 28.1	11.1	
2,3,3-Trimethylpentane	11.8	16.4	18.6	19.3		17.0	2.7
2,3,4-Trimethylpentane	9.1	8.6	8.9	10.9	14.7	12.4	2.3 } 2.8
2,3-Dimethylhexane	12.9	10.7	10.0	9.0	1.7	8.9	4.8
Total mole %	100.0	100.0	100.0	100.0	100.0	100.0	

Method of Computation. Nine simultaneous equations based on masses 42, 43, 56, 57, 70, 71, 85, 99, and 114.

Table V. Analysis of a Synthetic C_8 Mixture (1C) Boiling between 99.2° and 115.6° C.

Component	Known Composition	Determined Composition 1	Determined Composition 2	Mean Difference
		Mole Per Cent		
2,2,4-Trimethylpentane	38.0	41.0	39.7	2.4
2,2-Dimethylhexane	0.7	0.8	1.3	0.4
2,2,3-Trimethylpentane	4.0	0.2	0.3	3.8
2,5-Dimethylhexane	12.8	12.7	13.9	0.6
2,4-Dimethylhexane	14.2	14.3	17.1	1.5
3,3-Dimethylhexane	2.1	1.1	0.0	1.6
2,3,3-Trimethylpentane	8.1	8.7	9.0	0.8
2,3,4-Trimethylpentane	16.4	17.4	18.7	1.7
2,3-Dimethylhexane	3.7	3.8	0.0	1.9
Total mole %	100.0	100.0	100.0	

Method of Computation. Nine simultaneous equations based on masses 42, 43, 56, 57, 70, 71, 85, 99, and 114.

runs 2, 3, 4, and 5 were reported with 2,2,4-trimethylpentane and 2,2-dimethylhexane, and 3,3-dimethylhexane and 2,3,3-trimethylpentane grouped. It also was found desirable to group 2,3,4-trimethylpentane and 2,3-dimethylhexane in order to improve accuracy in most cases. Table V indicates, however, that no such grouping was necessary in a mixture of somewhat different relative concentrations. It is felt that this difference in composition had little effect on the accuracy, the improvement being due to more stable operation of the mass spectrometer during these runs. The errors reported in Table IV are attributed to a 1% fluctuation in the octane patterns. On this basis a satisfactory analysis of all the isomers requires that fluctuations in the mass spectrometer cracking patterns shall not exceed 1%.

OCTANES IN THE 106.8° TO 113.5° C. RANGE. Table VI shows the analysis of four mixtures containing six components. Here an appreciable increase in over-all accuracy was obtained as a result of reducing the number of components from nine to six. In two cases out of six, however, it was necessary to group 2,2-dimethylhexane and 2,2,3-trimethylpentane, presumably because of unstable mass spectrometer operation.

OCTANES IN THE 111.9° TO 115.6° C. RANGE. Four different synthetic mixtures of four components each were analyzed. The data in Table VII show that it was unnecessary to group any isomers and also that the average difference between the known and mass spectrometer analysis was approximately 1 mole %.

OCTANES IN THE 113.5° TO 125.7° C. RANGE. The data in Table VIII show the analysis of three different blends containing eight components. These data indicate that it was necessary to group 2,3,4-trimethylpentane, 2,3-dimethylhexane, and 4-methylheptane. The resulting agreement between known and mass spectrometer compositions is from 0.5 to 1.6 mole %. Reference to Table VIII also shows that the ratio of 2,3,4-trimethylpentane to 2,3-dimethylhexane can be approximated by assuming that 4-methylheptane is absent from a given mixture. This was permissible, since most samples of interest were found to contain little or no 4-methylheptane.

ANALYSIS OF HYDROCARBONS OCCURRING IN ALKYLATE. The alkylate analyzed was one submitted to this laboratory by the A.P.I. Project 6 for cooperative spectrographic analysis. The distillation analysis of the alkylate was determined by A.P.I. Project 6 from distillation and physical property measurements (*1*). Table IX shows the mass spectrometer analysis of 39 cuts

Table VI. Analysis of Four Synthetic C_8 Mixtures Boiling between 106.8° and 113.5° C.

	Sample 3A, Mole Per Cent				Sample 3B, Mole Per Cent			Sample 4A, Mole Per Cent				Sample 4B, Mole Per Cent		
Component	Known composition	Determined composition 1	Determined composition 2	Mean difference	Known composition	Determined composition	Difference	Known composition	Determined composition 1	Determined composition 2	Mean difference	Known composition	Determined composition	Difference
2,2-Dimethylhexane	1.3	0.9	1.4	0.3	1.7	11.9	0.3	6.5	5.8	8.2	1.2	7.4	24.0	1.1
2,2,3-Trimethylpentane	12.0	13.2	11.7	0.8	9.9			31.5	33.6	30.8	1.4	15.5		
2,5-Dimethylhexane	34.6	33.5	34.9	0.7	31.9	30.7	1.2	14.1	14.1	13.2	0.5	22.1	20.4	1.7
2,4-Dimethylhexane	30.1	29.1	30.3	0.6	35.3	37.3	2.0	31.1	29.7	30.1	1.2	29.7	31.8	2.1
3,3-Dimethylhexane	3.6	4.4	2.8	0.8	3.0	1.1	1.9	5.0	5.3	5.6	0.5	6.4	4.0	2.4
2,3,4-Trimethylpentane	18.4	18.9	18.9	0.5	18.2	19.0	0.8	11.8	11.5	12.1	0.3	18.9	19.8	0.9
Total mole %	100.0	100.0	100.0		100.0	100.0		100.0	100.0	100.0		100.0	100.0	

Method of Computation. Six simultaneous equations based on masses 56, 57, 70, 85, 99, and 114.

Table VII. Analysis of Four Synthetic C_8 Mixtures Boiling between 111.9° and 115.6° C.

	Sample 2A, Mole Per Cent			Sample 2B, Mole Per Cent				Sample 5A, Mole Per Cent				Sample 5B, Mole Per Cent		
Component	Known composition	Determined composition	Difference	Known composition	Determined Composition 1	Determined Composition 2	Mean difference	Known composition	Determined Composition 1	Determined Composition 2	Mean difference	Known composition	Determined composition	Difference
3,3-Dimethylhexane	2.9	3.1	0.2	13.4	12.9	12.8	0.6	3.0	4.4	3.5	1.0	5.5	6.2	0.7
2,3,4-Trimethylpentane	50.5	49.6	0.9	37.0	36.4	38.0	0.8	42.5	43.5	42.7	0.6	57.2	55.7	1.5
2,3,3-Trimethylpentane	35.3	36.4	1.1	32.2	33.5	32.4	0.8	37.5	35.5	36.1	1.7	25.1	26.1	1.0
2,3-Dimethylhexane	11.3	10.9	0.4	17.4	17.2	16.8	0.4	17.0	16.6	17.7	0.6	12.2	12.0	0.2
Total mole %	100.0	100.0		100.0	100.0	100.0		100.0	100.0	100.0		100.0	100.0	

Method of Computation. Four simultaneous equations based on masses 70, 71, 85, and 114.

Table VIII. Analysis of Three Synthetic C_8 Mixtures Boiling between 113.5° and 125° C.

	Sample 6A, Mole Per Cent				Sample 6B, Mole Per Cent				Sample 6C, Mole Per Cent			
Component	Known composition	Determined composition 1	Determined composition 2	Mean difference	Known composition	Determined composition 1	Determined composition 2	Mean difference	Known composition	Determined composition 1	Determined composition 2	Mean difference
2,2,3-Trimethylpentane	12.1	14.9	11.5	1.7	6.9	6.7	4.0	1.5	2.4	1.9	2.2	0.4
2,3,4-Trimethylpentane	25.2		29.6		8.0		14.6		0.0		0.3	
2,3-Dimethylhexane	35.6	65.6	35.0	1.6	10.9	28.0	13.4	1.6	9.3	6.9	7.9	1.9
4-Methylheptane	5.9		[a]		10.7		[a]		0.0		[a]	
3,4-Dimethylhexane	10.5	11.9	9.6	1.2	8.9	8.4	7.1	1.2	19.5	19.0	18.7	0.7
2-Methylheptane	5.3	5.2	7.0	0.9	10.8	12.8	13.5	2.4	5.0	7.2	7.1	2.2
3-Methylheptane	5.4	2.4	4.9	1.8	6.6	7.0	8.2	1.0	27.1	27.0	26.8	0.2
n-Octane	0.0	0.0	2.4	1.2	37.2	37.1	39.2	1.1	36.7	38.0	37.0	0.8
Total mole %	100.0	100.0	100.0		100.0	100.0	100.0		100.0	100.0	100.0	

Method of Computation. Six simultaneous equations based on masses 56, 71, 84, 85, 99, and 114 with 2,3,4-trimethylpentane, 2,3-dimethylhexane, and 4-methylheptane grouped by using an average pattern.

[a] The approximate ratio of 2,3,4-trimethylpentane to 2,3-dimethylhexane was determined by assuming the absence of 4-methylheptane. Method of computation involved seven simultaneous equations based on mass 70 in addition to those listed above.

Table IX. Analysis of an Alkylate from 39 Distillate Fractions

Component	Distillation Analysis (*I*)	Mass Spectrometer Analysis	Difference
	Volume Per Cent		
Isobutane	8.3 ± 0.5	....	0.1
n-Butane		0.34	
Neopentane		0.06	
Isopentane		8.02	
Pentenes	.....	0.09	..
n-Pentane	0.6 ± 0.3	0.35	0.2
2,2-Dimethylbutane	0.0	0.0	0.0
2,3-Dimethylbutane	4.7 ± 0.8	4.84	0.1
2-Methylpentane	1.1 ± 0.5	1.02	0.1
3-Methylpentane	0.4 ± 0.2	0.42	0.0
2,2-Dimethylpentane	0.2 ± 0.2	....	..
2,4-Dimethylpentane	3.4 ± 0.9	3.55	0.2
2,2,3-Trimethylbutane	0.2 ± 0.2	0.25	0.1
2,3-Dimethylpentane	2.3 ± 0.6	2.32	0.0
2-Methylhexane	0.3 ± 0.2	0.18	0.0
3-Methylhexane		0.15	
2,2,4-Trimethylpentane	24.3 ± 0.9	24.4	0.1
2,2-Dimethylhexane	0.2 ± 0.2		
2,5-Dimethylhexane	6.6 ± 1.4	4.48	0.6
2,4-Dimethylhexane		2.70	
2,2,3-Trimethylpentane	1.2 ± 0.6	1.21	0.0
2,3,4-Trimethylpentane	13.0 ± 1.8	12.4	0.6
2,3,3-Trimethylpentane	12.3 ± 1.8	12.3	0.0
2,3-Dimethylhexane	3.0 ± 1.4	3.0	0.0
3,4-Dimethylhexane	0.4 ± 0.3		
4-Methylheptane		17.92	0.0
2-Methylheptane	17.5		
3-Methylheptane and higher			
Total vol. %	100.0	100.0	

after the individual results had been combined into an over-all analysis. The agreement between the "known" composition and mass spectrometer analysis in general is within a few tenths of 1 mole %.

ANALYSIS OF HYDROCARBONS AND CRACKED NAPHTHAS. To illustrate the applicability of the mass spectrometer method to actual unknown mixtures, the data in Table X show an analysis of a typical hydrocodimer in the boiling range of 79° to 119° C. Here the analysis of ten cuts is shown combined on the basis of the total sample. It can be seen that the only compounds present in measurable amounts are the paraffin hydrocarbons.

As pointed out previously, the cracked naphthas were treated prior to distillation to remove olefinic and aromatic material, in order to eliminate interference caused by the presence of a large number of olefins for which pure calibrating compounds are not available. The aromatic fraction in the boiling range covered would not interfere with the analysis and was only removed incidentally.

Table XI shows the analyses of the paraffin-cycloparaffin portions of two cracked naphthas. The analysis of each of the six individual cuts into which the treated naphthas were split by distillation are included to show the overlap in concentrations of individual compounds between successive cuts. In all cases it was necessary to group the di- and trimethylcyclopentanes.

Table XII gives the same data for a third cracked naphtha over a somewhat wider temperature range. Here, too, most of the cycloparaffins had to be grouped, although some of the dimethylcyclopentanes could be individually determined.

CONCLUSIONS

A review of the application of mass spectrometer analysis to the determination of C_5, C_6, C_7, and C_8 hydrocarbons in known and unknown mixtures is given. From the accumulated data it was concluded:

All the C_6 and C_7 paraffin and cycloparaffin hydrocarbons for which pure standards are available may be individually determined, with the exception of 2,2-dimethylpentane and 2,2,3-trimethylbutane which normally must be grouped and the 1,2- and 1,3-dimethylcyclopentanes which frequently must be grouped.

Various octanes must be grouped according to the complexity of the sample and/or stability in operation of the mass spectrometer. Under ideal conditions for narrow boiling fractions, however, it is necessary to group only 4-methylheptane with 2,3,4-trimethylpentane and 2,3-dimethylhexane. In the absence of 4-methylheptane each of the remaining isomers can be resolved individually.

In general, most of the C_8 cycloparaffin isomers must be grouped, because of similarity in their cracking patterns.

Benzene, toluene, ethylbenzene, and grouped xylenes may be resolved from most hydrocarbon mixtures.

Table X. Analysis of a Hydrocodimer from Eight Distillate Fractions Boiling between 79° and 119° C.

Component	Volume Per Cent
2,2-Dimethylpentane	0.39
2,4-Dimethylpentane	0.15
2,2,3-Trimethylbutane	0.0
2,3-Dimethylpentane	1.07
2-Methylhexane	0.04
3-Methylhexane	0.22
2,2,4-Trimethylpentane	30.6
2,2-Dimethylhexane	
2,5-Dimethylhexane	1.28
2,4-Dimethylhexane	2.28
2,2,3-Trimethylpentane	15.1
3,3-Dimethylhexane	....
2,3,4-Trimethylpentane	32.9
2,3,3-Trimethylpentane	5.52
2,3-Dimethylhexane	4.68
4-Methylheptane	0.15
3,4-Dimethylhexane	1.51
2-Methylheptane	0.13
3-Methylheptane	0.22
Higher boiling compounds	3.76
Total volume %	100.00

Table XI. Paraffin and Cycloparaffin Hydrocarbon Content of Thermal Cracked Naphtha Boiling between 80° and 105° C.

	Fraction No.							Fraction No.						
	1	2	3	4	5	6	Total	1	2	3	4	5	6	Total
	Boiling Point, ° C.							Boiling Point, ° C.						
	80–90	90–92	92–96	96–99	99–101	101–105	80–105	80–90	90–92	92–96	96–99	99–101	101–105	80–105
Hydrocarbon	Naphtha A, Volume Per Cent							Naphtha B, Volume Per Cent						
2,4-Dimethylpentane	0.37	...	..	...	...	..	0.37	0.08	..	..	...	...	..	0.08
3,3-Dimethylpentane	0.53	...	..	...	...	..	0.53	0.21	..	..	...	...	..	0.21
2,3-Dimethylpentane	0.89	2.47	0.24	...	...	..	3.60	1.82	0.28	0.73	...	...	..	2.83
2-Methylhexane	1.64	6.26	0.50	...	...	..	8.40	2.24	2.78	1.25	...	...	..	6.27
3-Methylhexane	..	6.06	1.76	2.94	...	..	10.76	..	4.74	3.57	1.10	...	..	9.41
3-Ethylpentane	..	...	0.26	...	...	..	0.26	..	..	0.82	...	...	..	0.82
n-Heptane	..	...	1.62	11.92	3.56	0.48	17.58	..	..	2.26	12.78	15.31	0.15	30.50
2,5-Dimethylhexane	..	...	..	...	...	0.23	0.23	..	..	..	...	...	0.25	0.25
2,4-Dimethylhexane	..	...	..	...	...	0.22	0.22	..	..	..	...	...	0.23	0.23
Cyclohexane	7.25	0.08	..	...	...	..	7.33	5.25	0.14	..	...	...	..	5.39
Dimethylcyclopentanes	1.91	10.60	2.85	2.39	...	..	17.75	1.73	5.22	6.24	0.69	...	..	13.88
Methylcyclohexane	..	...	..	2.22	17.55	7.76	27.53	..	..	..	0.45	12.08	10.98	23.51
Ethylcyclopentane	..	...	..	...	1.40	2.69	4.09	..	..	..	...	1.41	4.15	5.56
Trimethylcyclopentanes	..	...	..	...	0.39	0.96	1.35	..	..	..	0.03	...	1.03	1.06
							100.00							100.00

Table XII. Paraffin and Cycloparaffin Hydrocarbon Content of Catalytic Cracked Naphtha Boiling between 28° and 120° C.

Hydrocarbon	Fraction No. 1	2	3	4	5	6	7	8	9	Total
	Boiling Point, ° C. 28–49	49–67	67–82	82–93	93–99	99–103	103–111	111–118	118–120	28–120
	Volume Per Cent									
2,2-Dimethylbutane	0.14	0.14	..	..	..	..	..	...	..	0.28
2,3-Dimethylbutane	0.72	4.57	..	..	..	..	..	...	..	5.29
2-Methylpentane	..	18.07	0.11	..	..	..	..	...	..	18.18
3-Methylpentane	..	10.52	..	..	..	..	..	...	..	10.52
n-Hexane	..	1.13	3.81	..	..	..	..	...	..	4.94
2,2,3-Trimethylbutane	..	...	1.03	0.11	..	..	..	...	..	1.14
2-Methylhexane	..	..	..	5.81	..	..	..	...	..	5.81
3-Methylhexane	..	..	..	6.02	1.53	..	..	...	..	7.55
n-Heptane	..	...	..	..	1.43	0.94	..	...	..	2.37
2,2,4-Trimethylpentane	..	...	..	..	0.20	0.05	..	...	..	0.25
2,5-Dimethylhexane	..	...	..	..	..	..	0.47	...	..	0.47
2,4-Dimethylhexane	..	...	..	..	..	..	0.58	..	..	0.58
2,2,3-Trimethylpentane	..	...	..	..	..	..	0.26	...	..	0.26
2,3,4-Trimethylpentane / 2,3-Dimethylhexane / 4-Methylheptane	..	...	..	..	..	..	0.10	0.81	..	0.91
3,4-Dimethylhexane	..	...	..	..	..	..	..	1.06	..	1.06
2-Methylheptane	..	...	..	..	..	..	..	1.58		
3-Methyl-3-ethylpentane / 3-Ethylhexane	..	...	..	..	..	..	..	0.04	3.06	4.68
Cyclopentane	0.47	0.52	..	..	..	..	..	...	..	0.99
Methylcyclopentane	0.18	0.80	9.48	1.24	..	..	..	...	..	11.70
Cyclohexane	..	...	0.67	0.06	..	..	..	...	..	0.73
1,1-Dimethylcyclopentane	..	...	0.56	0.71	..	..	..	...	..	1.27
1,3-Dimethylcyclopentane / 1,2-Dimethylcyclopentane	..	...	..	6.75	1.90	0.79	0.36	...	..	9.80
Methylcyclohexane	..	...	..	..	0.28	2.58	1.47	...	..	4.33
Ethylcyclopentane	..	...	..	..	0.10	0.80	1.04	0.38	..	2.32
Trimethylcyclopentane	..	...	..	..	..	0.06	1.31	...	..	1.37
Other C_8 cycloparaffins	..	...	..	..	..	..	1.37	1.83	..	3.20
										100.00

C_6 and heavier olefins and cyclo-olefins cannot be analyzed directly on the mass spectrometer because of pattern similarity and unavailability of pure calibrating samples.

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Infrared Analytical Techniques for Analyzing C_5 Mixtures

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Mixtures of C_5 hydrocarbons have been analyzed in the vapor phase rapidly and with sufficient precision for plant control. The analytical techniques were greatly simplified by the discovery that the extinction per unit pressure was constant over a pressure range of 50 to 650 mm. of mercury at 30° C. for the two C_5 paraffins and six C_5 olefins under consideration. Small variations in the concentrations of components appearing in fractionation products were quickly measured by infrared method, even though the components possessed relatively weak absorption bands, by comparing with a reference mixture.

WHEN the problem of applying infrared analytical techniques to the control of plant operations was considered for streams containing mixtures of C_5 hydrocarbons, the literature had little to offer in the way of developed methods.

The difficulty of confining these volatile samples in conventional liquid absorption cells is evident and most laboratories have spent considerable effort toward building a satisfactory liquid cell capable of containing samples under small pressures (*1–4*).

In view of this difficulty it was decided to investigate the possibility of analyzing C_5 mixtures in the vapor phase. To this end highly purified samples of each hydrocarbon listed in Table I were scanned at desirable pressures in conventional type gas absorption cells of suitable length over the rock salt range of a Perkin-Elmer Model 12-A spectrometer equipped with photoamplifier and Brown recorder.

From these scannings, extinctions were measured at twenty-three wave lengths, corresponding to key absorption bands of the components. These extinctions, measured at several pressures, were plotted against uncorrected pressures and in every case a straight line through the origin resulted. Figure 1 shows this linear relationship for a paraffin, *n*-pentane at the 8.7-micron band, for an olefin, 2-methyl-1-butene at the 8.2-micron band, and for a mixture of 2-methyl-1-butene(52%) and 1-pentene (48%), at the 8.2-micron band. This linear relationship was the most that could be hoped for and somewhat surprising to one who had analyzed C_4 mixtures by a similar procedure.

Key wave lengths were chosen from the spectrograph records and pressure-extinction curves plotted for each of the eight materials shown in Table I at each of the key wave-length positions.

From the slope of these curves the extinction coefficients needed to set up the usual set of simultaneous equations were obtained. Table II shows one such set of extinction coefficients. The underscored extinction coefficients are those of the principal absorber at the spectral positions indicated. The most unfavorable

Table I. Components of a C_5 Cut

Compound	Boiling Point, ° C.	Compound	Boiling Point, ° C.
3-Methyl-1-butene	18.8	*trans*-2-Pentene	35.85
Isopentane	27.89	*n*-Pentane	36.0
1-Pentene	30.1	*cis*-2-Pentene	37.0
2-Methyl-1-butene	31.05	2-Methyl-2-butene	38.49

Table II. Extinction Coefficients of C_5 Hydrocarbons

Components	Wave Length, Microns	3-Methyl-1-butene ($E/PL\times10^{-6}$)	Iso-C_5	1-Pentene	2-Methyl-1-butene	n-C_5	*trans*-2-Pentene	*cis*-2-Pentene	2-Methyl-2-butene
3-Methyl-1-butene	14.6	33.8	0.36	3.51	1.04	0.92	0.72	101.0	13.7
Isopentane	8.5	8.87	10.5	4.90	2.02	3.41	4.47	1.82	3.12
1-Pentene	5.45	16.8	1.72	18.0	1.57	1.33	3.35	2.86	6.37
2-Methyl-1-butene	11.4	5.27	0.82	36.6	135.0	5.24	7.96	4.52	5.00
n-Pentane	13.75	1.39	0.32	4.16	1.32	16.3	1.66	41.32	1.70
trans-2-Pentene	10.36	20.0	7.14	22.5	5.67	1.07	161.0	9.64	18.0
cis-2-Pentene	14.4	43.0	0.44	3.32	1.12	1.15	0.55	112.0	1.38
2-Methyl-2-butene	12.5	8.12	3.58	2.43	6.76	1.11	5.89	10.93	81.0

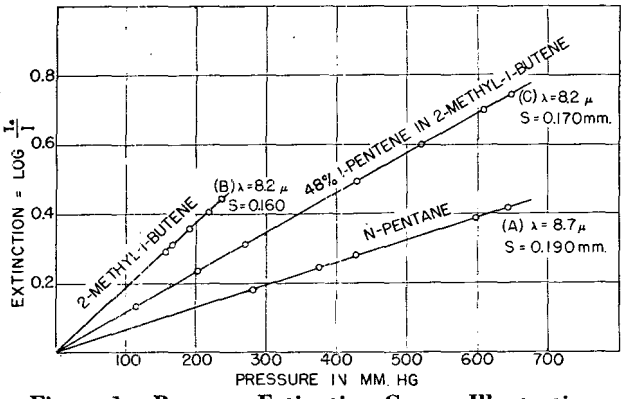

Figure 1. Pressure-Extinction Curves Illustrating Linearity

A. C_5 paraffin. *B.* C_5 Olefin. *C.* C_5 mixture

case is that of 3-methyl-1-butene in the presence of *cis*-2-pentene. Fortunately, these two compounds differ in boiling point by 18° C. and there is little chance of finding both in the same fraction.

Comparison of results obtained on different days with different operators on the same sample are shown in Table III.

The time involved in sampling, instrument running, and computing was about 1.5 hours.

Sample pressures and path lengths were chosen to give extinction measurements between 0.250 and 0.650 whenever possible. The range of workable pressures was 50 to 650 mm. of mercury at 30° to 33° C. Cell lengths used were 5 and 10 cm.

To control fractionating columns adequately, the concentration of a key component at some point in the tower may be desired at frequent intervals and in such cases the above method of analysis becomes too slow. To this requirement may be added the request for a more accurate determination of a component appearing in amounts of less than 1%. Obviously, special methods must be employed to handle such requirements.

As one example, in a fractionator producing a mixture with the composition shown in Table IV, it was necessary to control the tower so that the n-pentane component remained less than 0.5%.

A conventional five-component infrared analysis was neither fast enough nor accurate enough to be used for this control. The tower was controlled, however, by comparing a sample drawn off the liquid phase of an intermediate tray every 2 hours with a sample having the approximate composition of the tray but with zero n-pentane content.

By measuring the extinction at the spectral position of the key absorption band of n-pentane first for the standard sample and then for the tray sample without altering the instrument settings, small changes in the n-pentane content of the tray sample could be detected. Since it had previously been determined that the n-pentane content of the sample should be kept between 0 and 2%, the column engineers could keep the column operating properly from results handed them 20 minutes after sampling. The results of 7 days' successful operation are shown in Figure 2, in which the per cent of n-pentane determined at approximately 2-hour intervals, as described above, is plotted against time.

Table III. Comparison of Infrared Analyses

(Lab. A.W.R. No. 55)

Components	Analyzed 8-29-46	Analyzed 8-30-46
Isopentane	17.4	18.4
1-Pentene	54.2	53.7
2-Methyl-1-butene	1.4	1.6
n-Pentane	11.6	12.4
trans-2-Pentene	3.7	3.7
cis-2-Pentene	2.6	2.6
2-Methyl-2-butene	5.5	5.7

Table IV. Composition of Product from Fractionator

	%
n-Pentane	<0.5
2-Methyl-1-butene	7.5
1-Pentene	18.0
3-Methyl-1-butene	8.8
Isopentane	65.5

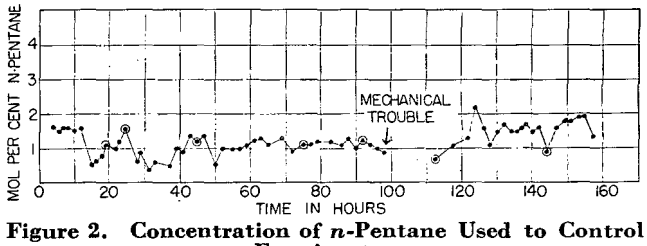

Figure 2. Concentration of n-Pentane Used to Control Fractionator

If the composition of the tray sample changed, each component which changed would contribute to the measured change at the key band of n-pentane. To check any such change and make proper corrections, a complete infrared analysis was run at fairly regular intervals. Samples so analyzed have been designated in Figure 2 by encircled points. The curve may be roughly divided into three equal parts. During the first 50 hours, the concentration of n-pentane oscillated about the 1% line as the manual controls were operated to keep the product within the desired limits. The second 50 hours show ideal operation, during which no control was necessary. At the point indicated by "mechanical trouble" the column was allowed to operate under total reflux conditions until repairs permitted normal operation. During the remaining time shown on the curve the first leveling out process was repeated approximately.

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Analysis of Natural and Synthetic Rubber by Infrared Spectroscopy

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This report describes an investigation of the applicability of infrared spectroscopic methods for quantitative determination of the elastomer composition of industrial rubber products. Generally satisfactory techniques for obtaining a valid spectrum of the elastomer content of any rubber specimen have been developed and evaluated, have been applied in developing detailed analytical procedures for identification and qualitative analysis of elastomer mixtures, quantitative analysis of natural-GR-S blends, and determination of the nitrile content of Buna-N copolymers. An accuracy corresponding to ±1% average error has been obtained for these determinations. The methods can be extended to analogous problems with a minimum of alteration, and the same order of accuracy can be expected in general. A discussion of the correlations between spectral absorption and molecular structure is also included.

INFRARED spectroscopy is finding increasing application as a means for identification of organic materials and analysis of complex mixtures (*2, 8, 13, 16*). Not only are spectroscopic methods rapid and generally conservative in sample requirements, but the results are often more specific than those obtained by chemical analysis, distillation, etc., and in many cases furnish detailed structural information of importance to research and development. The advantages of infrared analysis lie in the facts that (1) the spectrum of a molecule is a unique physical property which is not altered by any changes in which molecular identity is maintained, and that (2) the individual features of the spectrum relate in a definite manner to the chemical bonds comprising the molecule and can thus be interpreted regardless of the total configuration.

Procedures for accurate determination of the identities and proportions of the elastomers present in a finished product are needed throughout the rubber industry for control and specification. With the ever-growing number and variety of rubber substitutes and special-purpose synthetics incorporated in manufactured goods either singly or as blends, the problem facing the rubber analyst has become increasingly complex and well beyond the scope of conventional chemical methods. In addition, analytical methods allowing study of polymer structure should prove significant in the development of new polymers and in the evaluation of polymerization techniques.

Several investigators have recognized the advantages of employing a physical means of investigation by which the various types of chemically inert molecular structures in polymers may be studied without changing their identities or proportions. Infrared methods have been significant, for example, in exploring the mechanism of polymer formation (*15*) and in studying vulcanization (*12*) and oxidation (*5*). Considerably less has been done to develop infrared methods into routine procedures for specification of rubber products. This is undoubtedly due, in a large part, to the experimental difficulties encountered in spectroscopic examination of vulcanized and reinforced rubbers. An attempt to overcome these difficulties was made by Barnes and co-workers (*4*) who developed a procedure for the analysis of natural–Buna-S blends in tread, carcass, and tube stocks. While their results were satisfactory for the purposes of the study, the accuracy did not appear as good as might be expected and the data presented did not permit conclusive evaluation of the procedure, particularly in regard to extension of the method to other elastomers.

This paper describes the results of an extensive study of the applicability and limitations of infrared methods for the identification and quantitative analysis of the elastomer content in commercial articles of natural rubber and some of the common synthetics. The first phase of the work was concerned with determining valid absorption spectra of the various pure elastomers. These reference spectra were required to evaluate the spectral basis for identification of elastomers and for later use in identifying any spectral effects not directly related to elastomer composition. A second phase was concerned both with the development of a satisfactory technique for spectral examination of a compounded and cured rubber and with the determination of the extent to which the spectral absorption so obtained is related to elastomer composition, as distinguished from other spectral effects of sample history or state, etc. The necessity of removing fillers which render the sample too opaque for quantitative spectral study and the additional advantages of eliminating all effects of nonrubber components from the spectral results dictated the use of procedures involving isolation of the elastomer content as a preliminary step. In this connection, solvents for cured elastomers and methods for removal of colloidal carbon from rubber solutions have received special attention. A third phase was concerned with the development and evaluation of detailed procedures for certain analyses which are of practical importance and which illustrate the general feasibility of quantitative applications.

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GUM ELASTOMERS

The problem of determining reference spectra—i.e., the correct spectrum of the pure rubber hydrocarbon—is best approached by the study of gum rubbers wherein the elastomer chains are already large compared with the polymerizing units (or monomers) but where modifying effects such as may accompany compounding, curing, aging, etc., have not yet been introduced. Thirteen gum samples comprising seven different classes of elastomers were used for this purpose. The samples were 97% pure elastomer, the remainder being chiefly antioxidant, and are listed in the tabulated summary of studies on gum elastomers in Table I.

Sample Preparation. The gum samples were prepared for spectroscopic examination by (1) solvent extraction of antioxidant and all soluble impurities, (2) solution of the elastomer in a suitable solvent, (3) removal of excess solvent by evaporation, and (4) preparation of films on rock salt or potassium bromide plates for spectroscopic study. Commercial grade ethyl alcohol (95%) was used for extraction in the standard A.S.T.M. rubber extraction assembly (*1*) consisting of 400-ml. flask, siphon cup, and water-cooled tin-coil condenser. Solubility and ensuing gelation prohibited the use of other solvents such as benzene, chloroform, and acetone. A 2-gram sample was cut into thin strips and placed in parallel rows on a filter paper, so as not to be in contact when the paper was rolled. Two or three 80-ml. portions of solvent were used and the progress of extraction was followed by noting the coloration. On the basis of subsequent spectra, continuous extraction for 24 hours was shown to be sufficient.

Solutions of the various elastomers were made at room temperature in order to minimize possible decomposition or alteration of the elastomer. Solvents used were standard technical grade

Table I. Summary of Investigations of Gum Rubber

Sample			Preparation				Spectral Study		
Elastomer class	Representative type	Sample No.	Extraction[a]	Solvent	Solution method[b]	Solution[c] characteristics	Film No.[d]	Wave length range, microns	Spectral characteristics[e]
Natural rubber	Natural smoked sheet	1	L	Xylene + nitroethane (8:3)	S	Gray, W	20	2–14	E
							22	2–14	E
							70	14–23	*f*
							149	2–14	E
	Lee pure gum cement	1	..		..	Colorless	432	2–14	E
							434	14–23	*f*
	Tube reclaim[g]	1	M	*p*-Cymene + xylene (4:1)	R	Yellow, W+	66	2–14	E
							67	2–14	E
							71	2–14	E
		2	M	*p*-Cymene + xylene	R	Yellow, W+	71	2–14	A
							82	2–14	A
		3	M	*p*-Cymene + xylene	R	Yellow, W+	120	2–14	A
							121	7–14	A
Isobutylene-isoprene copolymer	GR-I	1	L	Benzene	S	Tan, W	50	2–14	E
Chloroprene polymers	Neoprene-GN	1	L	Xylene + nitroethane	S	Brown, G	D	...	..
		2	..	Ethylene dichloride	S	Brown, W	47	2–13	E
							157	14–23	*f*
		3	L	Ethylene dichloride	B	Brown, W	59	2–14	E
	Neoprene-ILS	1	L	Xylene + nitroethane	S	Brown, G	D	...	..
		2	L	Ethylene dichloride	B	Brown, P	52	2–14	E
		3	L	Ethylene dichloride	B	Brown, P	61	2–14	E
							157	14–23	*f*
		4	..	Ethylene dichloride	B	Brown, P	61	2–14	E
Butadiene-styrene copolymer	GR-S	1	L	Xylene + nitroethane	S	Tan, W	29	2–14	E
Butadiene-acrylonitrile copolymers	Chemigum-N-1	1	L	Xylene + nitroethane	S	Brown, W	30	2–14	E
	Hycar-OR-15	1	L	Xylene + nitroethane	H	Yellow, P	32	2–14	A
		2	L	Methyl ethyl ketone	B	Yellow, W	94	2–14	E
		3	..	Methyl ethyl ketone	B	Yellow, W	94	2–14	E
	Hycar-OR-25	1	L	Xylene + nitroethane	H	Yellow, P	41	2–14	E
		2	L	Methyl ethyl ketone	B	Yellow, W	56	2–14	E
		3	..	Methyl ethyl ketone	B	Yellow, W	97	2–14	E
	Perbunan-35	1	L	Nitroethane + xylene	S	Yellow, P	D	...	..
		2	L	Methyl ethyl ketone	B	Yellow, W	53	2–14	E
		3	L	Methyl ethyl ketone	B	Yellow, W	114	6–14	E
							119	2–14	E
	Butaprene-NF	1	L	Xylene + nitroethane	H	Yellow, P	33	2–14	E
Ethylene polysulfide polymer	Thiokol-Fa	1	L	Xylene + nitroethane	S	Brown, W	24	2–14	E
							26	2–14	E
							27	2–14	E
							28	6–14	E
							97	6–12	E
Copolymer of butadiene, acrylonitrile, and unsaturated ketone	Thiokol-RD	1	L	Xylene + nitroethane	S	Brown, N	D	...	..
		2	L	Ethylene dichloride	B	Brown, P	62	2–14	E

[a] L. Extraction by 95% ethyl alcohol.
M. Extraction by 68–32 chloroform-acetone.
[b] S. Mechanical shaking at room temperature.
H. Same as S, followed by heating, below 90° C.
R. Refluxing, above 100° C.
B. Blenderization, after preliminary swelling at room temperature.
[c] W. Complete solution; no gel.
P. Over 50% solution; remainder gel.
G. Nearly all gel; very little solution.
N. Solvent ineffective; very little gel or solution.
+. Removal of filler by centrifugation.
[d] D. Sample discarded; no spectrum obtained.
[e] E. Entire spectrum satisfactory and considered valid.
A. Spectrum satisfactory except for additional band obviously attributable to impurities.
f No appreciable absorption in this region.
[g] Contained red iron oxide filler.

and contained no nonvolatile matter. Of the solvents investigated only ethylene dichloride satisfactorily dissolved all elastomers studied, including neoprenes. Methyl ethyl ketone was very effective for Buna-N copolymers, but not for the others. Natural rubber, Thiokol-FA, GR-I (Butyl), and GR-S (Buna-S) dissolved readily in several solvents (see Table I). The average requirement was 40 to 60 ml. of solvent per gram of rubber. The solutions obtained were transparent but exhibited light color, which probably resulted from oxidation. Solution of the more soluble elastomers was accomplished merely by 6 to 8 hours' mechanical shaking of the rubber with solvent. This simple technique did not prove satisfactory for neoprene and Buna-N types because of the persistence of gelatinous structure despite use of excessive quantities of solvent. Solution was accomplished far more rapidly and effectively by mixing rubber and solvent in a Waring Blendor. [The Waring Blendor, Cenco No. 17,233 with No. 17,244 (small-size) jar was used. All rubber gaskets were replaced by gaskets of felt between outer ones of lead.] This technique readily reduced gels to thin homogeneous mixtures and permitted satisfactory preparation of all samples in a minimum quantity of solvent. Generally, the rubber was allowed to soften and swell in the solvent for 2 hours, and then blenderized for 10 to 20 minutes. Tube reclaim was treated as an exception to the gum samples, as it contained a mineral filler and was considerably less soluble. It was extracted by a 32–68 blend of acetone-chloroform, and was dissolved by refluxing with a 20–80 blend of xylene–*p*-cymene. The filler was removed by centrifugation.

The solution of polymer was concentrated to a viscous mucilage suitable for film spreading by evaporation on an A.S.T.M. gum bath at 80° C. Stirring (by hand) adequately eliminated gel formation due to localized surface evaporation. While vacuum distillation was also employed successfully, the former method was preferred as it permitted simultaneous evaporation of several solutions. Films of uniform thickness were prepared on sodium chloride or potassium bromide plates by spreading the concentrated mucilage between parallel spacers, as shown in Figure 1. Thirty minutes' standing was allowed for final solvent evaporation. The appropriate film thickness for useful spectroscopic measurements was determined by trial preparation of films of several thicknesses and subsequent spectral measurement at the common 6.9 mu carbon-hydrogen band where the absorption should be between 80 and 90%. Three sets of spacers—0.05, 0.10, and 0.20 mm.—enabled suitable films to be obtained in all cases. In the case of Buna-N samples, solvent removal was generally stopped prematurely in order to avoid serious gelation, and films of suitable thickness were obtained by spreading successive films on one another.

All spectroscopic measurements were made using a large recording prism spectrometer of high resolving power (*10*). Spectra as recorded, consisted of a pair of traces measuring (as percentage full-scale) the energy transmitted by the sample and by a blank plate as a function of wave length (on a nonlinear scale). A percentage transmittance spectrum of the sample was obtained by measuring ratios of corresponding ordinates, and the spectrum was transcribed (using calibration data) to linear wave-length charts. Under normal operating conditions wave-length measurements were considered accurate to ±0.01 mu from 2 to 15 mu, and per cent transmittance measurements were reproducible to about ±1.0%.

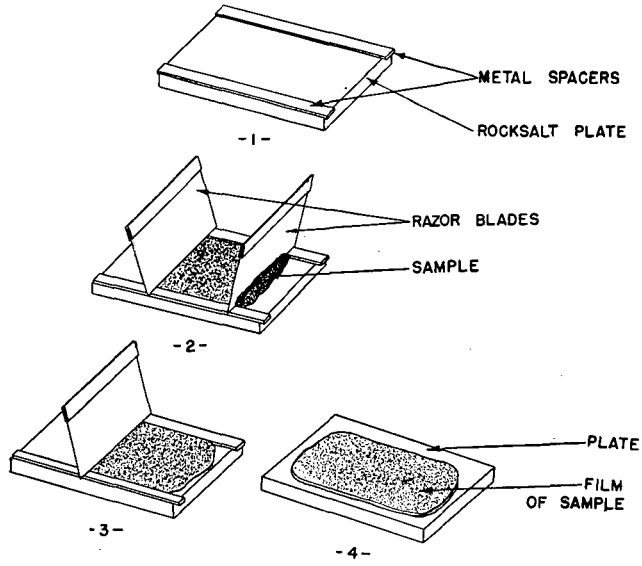


Figure 1. Method of Film Spreading

Discussion of Results. Several satisfactory spectra were obtained over the 2- to 14-micron range for each elastomer studied. Beyond 14 mu the absorption was too weak for analytical purposes and the results have not been included. A set of these reference spectra shown in Figure 2 establishes the fact that the seven different classes of rubber examined possess characteristically different spectra. The qualitative spectral differences correspond to basic differences in polymer structures and furnish an adequate basis for spectroscopic identification of each class. On the other hand, close similarity exists among the spectra of elastomers which are members of the same basic class of polymer or copolymer, as for the two neoprene polymers or the five Buna-N copolymers (not all shown). The differences here are quantitative and, though less obvious, are significant if the proportions of the various structures comprising the elastomer are of interest.

In addition to the fact that the use of pure samples assured good reference spectra, the validity of the spectra obtained was established by their close reproducibility during extensive duplication of sample preparation and spectral measurement. For nearly all preparations duplicate films were studied in order to determine the reproducibility in film preparation and spectroscopic measurement. In several cases the entire preparation was carried out in duplicate in order that the reproducibility of the over-all method of study might be established. Finally, a number of samples were prepared by alternative procedures, using different solvents and solution methods, in order that the influence, if any, of particular preparation features on the final spectrum might be shown. In all cases (in which the method of preparation was adequate) virtually perfect reproducibility of spectra was obtained, showing definitely the reliability of the over-all method of study and the sole dependence of the spectrum on the elastomer itself. The spectra obtained, therefore, are considered accurate reference spectra of the pure rubber hydrocarbons.

COMPOUNDED AND CURED ELASTOMERS

The problem of developing suitable procedures for preparing a commercial rubber product for spectral examination and of determining to what extent it is possible to obtain accurate, reproducible spectral data for cured elastomers was solved by extensive experimental work involving both considerable duplication and numerous variations in procedure. In investigating this twofold problem eighteen compounded specimens were studied. These specimens, listed in Table II, were compounded from the gum elastomers previously studied and included a variety of mixes and types of cure, given in Table III, which were considered representative of industrial products in variety and character. The method used for preparation and study of these specimens comprised: (1) preliminary milling of the sample, (2) extraction of organic additives, (3) solution of the elastomer, (4) removal of the filler, and (5) evaporation of the solvent to obtain a transparent film of pure polymer for spectroscopic examination.

Experimental. Extraction and solution of the sample were facilitated by preliminary milling. This comprised several passes through a standard roll mill at room temperature, after which the shredded rubber was chopped in a Wiley laboratory mill to pass through a No. 20 screen. A 32–68 blend of acetone-chloroform was used with the standard A.S.T.M. extraction assembly (*1*) for extraction of organic additives and impurities. Continuous extraction for 24 hours with two or three 80-ml. portions of solvent was satisfactory.

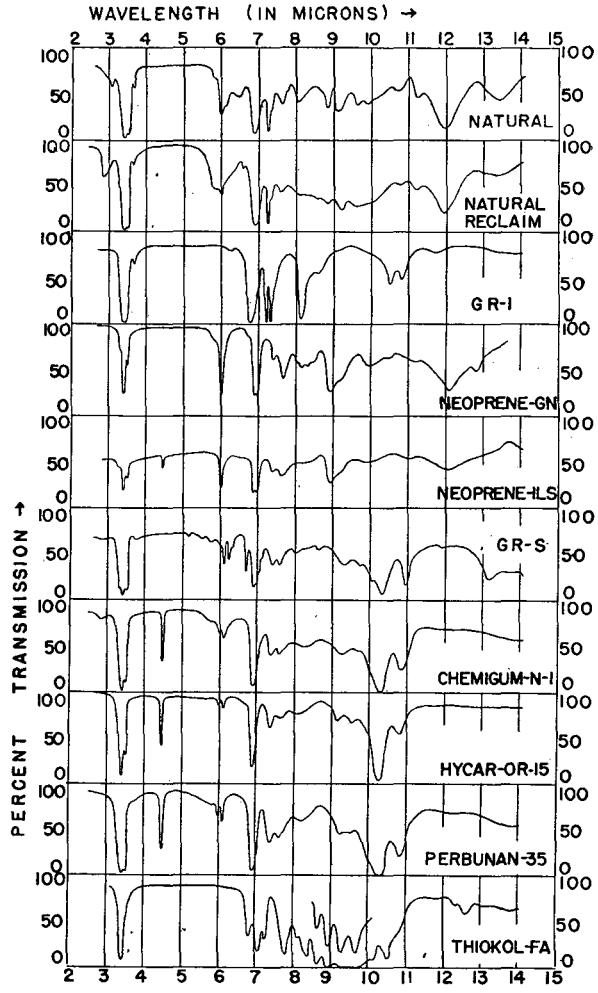


Figure 2. Spectra of Pure Gum Elastomers

Reflux temperatures above 100° C. appeared necessary to dissolve all cured rubbers, especially the neoprene and Buna-N types. Solvents boiling above 190° C., however, were not considered because of the difficulty of subsequent solvent removal and the importance of minimizing decomposition of rubber or solvent which might accompany severe heating. Technical grade solvents were distilled before use to eliminate higher-boiling components and gum-forming impurities. Of the solvents investigated, only *o*-dichlorobenzene successfully dissolved all samples, including Neoprene-ILS and the Buna-N copolymers, and was eminently satisfactory with respect to purity and inertness on prolonged heating. Tetrachloroethane was used extensively for all cured elastomers except Neoprene-ILS and Buna-N types, and, despite traces of free hydrochloric acid which persisted on

Table II. Summary of Investigations of Compounded and Cured Rubber

Sample				Preparation[a]			Spectral Study		
Elastomer class	Representative type	Sample No.	Composition[b]	Solvent[c]	Filler separation[d]	Solution characteristics[e]	Film No.[f]	Wavelength range, microns	Spectral characteristics[g]
Natural rubber	Natural rubber	1	F-2-1	Tetrachloroethane	..	Yellow, W	172	2–14	E
		2	F-2-1	*p*-Cymene	..	Yellow, W	267	2–14	E
		3	F-4	*o*-Dichlorobenzene	F	Yellow, W	386	2–14	E
							434	14–22	[h]
	Tube reclaim	1	(None)	Tetrachloroethane	C	Amber, W	164	2–14	E
		2	(None)	Tetrachloroethane	C	Amber, W	276	2–14	E
Isobutylene-isoprene copolymer	GR-I	1	F-1002	Tetrachloroethane	F	Amber, W	211	2–14	E
		2	F-1002	*p*-Cymene	F	Amber, W	268	2–14	E
		3	F-1010	*o*-Dichlorobenzene	C	Amber, W	388	2–14	E
							437	14–22	[h]
Chloroprene polymers	Neoprene-E	1	F-206	Tetrachloroethane	F	Amber, W	182	2–14	E
		2	F-206	*p*-Cymene	F	Amber, W	285	2–14	A
		3	F-206	*o*-Dichlorobenzene	F	Amber, W	424	2–14	E
	Neoprene-GN	1	F-206	*o*-Dichlorobenzene	F	Amber, W	405	2–14	E
		2	F-210-2	*o*-Dichlorobenzene	F	Amber, W	416	2–14	E
							435	14–22	[h]
	Neoprene-ILS	1	F-207	Tetrachloroethane	F	N	D	...	..
		2	F-207	Methyl isobutyl ketone	F	Dark, U	240	2–14	X
		3	F-207	*p*-Cymene	F	Brown, P	281	2–14	E
		4	F-207	Pyridine	F	Brown, P	355	2–14	E
		5	F-207	*o*-Dichlorobenzene	F	Brown, P	355	2–14	E
		6	F-207	*o*-Dichlorobenzene	F	Brown, P	396	2–14	E
		7	F-207	*o*-Dichlorobenzene	F	Brown, P	425	2–14	E
							435	14–22	[h]
Butadiene-styrene copolymer	GR-S	1	F-602	Tetrachloroethane	..	Brown, W	180	2–14	E
							438	14–22	[h]
		2	F-606	Tetrachloroethane	F	Brown, W	2–16	2–14	E
		3	F-606	*p*-Cymene	F	Brown, W	288	2–14	A
		4	F-606	*o*-Dichlorobenzene	F	Brown, W	391	2–14	E
Butadiene-acrylonitrile copolymers	Chemigum-N-1	1	F-1003	Tetrachloroethane	F	Amber, P	173	2–14	E
		2	F-1003	*p*-Cymene	F	Amber, P	287	2–14	X
		3	F-1003	Pyridine	F	Amber, P	...	4– 5	A
		4	F-1003	*o*-Dichlorobenzene	F	Amber, P	368	2–14	E
		5	F-1003	*o*-Dichlorobenzene	F	Amber, P	426	2–14	E

[a] All samples milled and extracted with 68–32 chloroform-acetone.
[b] See Table III for composition and cure of vulcanizates.
[c] Solution by refluxing.
[d] F. Filtration with Celite; C = centrifugation.
[e] W. Complete solution of specimen.
P. Partial solution (over 40%).
N. No appreciable solution.
U. Extent of solution uncertain.
[f] D. Sample discarded; no spectrum obtained.
[g] E. Entire spectrum satisfactory.
A. Spectrum satisfactory except for additional bands obviously attributable to impurities.
X. Unsatisfactory spectrum, due mainly to impurities.
[h] No appreciable absorption in this region.

Table III. Composition and Cure

Specimen Designation	Gum Elastomer	Specific Gravity	*Parts by weight*	Sulfur	Zinc Oxide	Stearic Acid	Accelerators	Antioxidants	Plasticizers, Softeners, Tackifiers
F-2-1	Natural smoked sheet	0.92	100	3.0	5.0	1.0	Methyl zimate, 0.1 Rotox, 1.0	Age Rite Hipar, 1.0	Reogen, 2.0
F-4	Natural smoked sheet	0.92	100	3.0	5.0	0.5	Captax, 0.5	Neozone D, 1.0	.....
F-206	Neoprene-E	..	100	1.0	10.0	0.5	.....	Neozone D, 2.0	Circo X Oil, 10.0 F. F. wood rosin, 5.0
F-207	Neoprene-ILS	..	100	..	10.0	0.5	.....	Neozone D, 2.0 Permalux, 1.0	Dibutyl phthalate, 20
F-210-2	Neoprene-GN	1.25	100	..	10.0	0.5	No. 552, 0.2	Neozone A, 2.0 Parazone, 1.0 Permalux, 2.0	Circo X Oil, 10.0
F-1002	Butyl (GR-I)	..	100	1.5	5.0	3.0	Monex, 1.0 Captax, 0.5	.....	Dibutyl sebacate, 10.0
F-1010-1	Butyl (GR-I)	..	100	1.5	5.0	3.0	Captax, 1.5 Tuex, 1.5	.....	Circo X oil, 5.0
F-606	Buna-S (GR-S)	0.94	100	..	5.0	0.5	Selenac, 2.0	.....	.....
F-602	Buna-S (GR-S)	0.94	100	3.0	5.0	1.0	Methyl zimate, 0.1 Rotox, 1.0	Age Rite Hipar, 1.0	Reogen, 2.0
F-804	Thiokol-FA[a]	1.34	100	..	10.0	0.5	D.P.G., 0.1 Altax, 0.3	.....	.....
F-801	Thiokol-ST	..	80	..	0.5	0.5	G.M.F., 1.5	.....	L.P. No. 2, 20.0
F-803	Thiokol-RD	1.03	100	1.5	5.0	1.0	Altax, 1.0	Neozone D, 2.0	Dibutyl sebacate, 20.0
F-1003	Chemigum-N-1	1.03	100	2.0	5.0	1.0	Tuads, 0.5	.....	Circo X Oil, 10.0
F-1302	Perbunan-35	..	100	2.0	5.0	0.5	Altax, 1.0	.....	Dibutyl sebacate, 17.5
F-407	Hycar-OR-15	1.00	100	1.25	5.0	0.5	Altax, 1.5	.....	Tributoxyethylphosphate, 17.5 S.C., 15.0 tributoxyethylphosphate, 15.0
F-402	Hycar-OR-25	0.98	100	1.25	5.0	0.5	Altax, 1.5	.....	S.C., 15.0
F-416	Hycar-OR-25	..	100	2.0	5.0	0.5	Altax, 1.5 Tetrone A, 0.75	.....	Tributoxyethylphosphate, 15.0 .....
F-205	Butaprene-NF	..	100	2.5	3.0	..	Altax, 1.0	.....	Tributoxyethyl phosphate, 25
F-10	Natural smoked sheet	..	100	2.0	5.0	1.0	Bardol, 5.0 Thionex, 0.5	Age Rite resin, 1.0	.....
F-10-1	Tube reclaim	..	100	2.0	5.0	1.0	Altax, 1.0 Thionex, 0.5	Age Rite resin D, 1.0	.....
F-620	GR-S	..	100	2.0	5.0	1.0	Bardol, 5.0 Thionex, 0.5	Age Rite resin D, 1.0	.....
F-421	Buna-N	..	100	1.5	5.0	1.0	Ethyl zimate, 0.1 Altax, 1.5	.....	Triethyl phosphate, 15.0

[a] Basic oxides in this column are curing agents.

Table II. (Contd.)

Table II. Summary of Investigations of Compounded and Cured Rubber

Sample				Preparation[a]			Spectral Study		
Elastomer class	Representative type	Sample No.	Composition[b]	Solvent[c]	Filler separation[d]	Solution characteristics[e]	Film No.[f]	Wavelength range, microns	Spectral characteristics[g]
Butadiene-acrylonitrile copolymer (*contd.*)	Hycar-OR-15	1	F-407	Tetrachloroethane	F	N	D	...	..
		2	F-407	Cyclohexane	F	Dark, U	194	2-14	X
		3	F-407	Diisobutyl ketone	F	Dark, U	212	2-14	X
		4	F-407	Pyridine	F	Dark, P	339	2-14	E
		5	F-407	*o*-Dichlorobenzene	F	Amber, P	339	2-14	E
		6	F-407	*o*-Dichlorobenzene	F	Amber, P	404	2-14	E
	Hycar-OR-25	1	F-402	Tetrachloroethane	F	N	D	...	..
		2	F-402	Diisobutyl ketone	F	Dark, U	197	2-14	X
		3	F-402	*o*-Dichlorobenzene	F	Amber, P	428	2-14	E
		4	F-416	*o*-Dichlorobenzene	F	Amber, P	457	2-14	E
	Perbunan-35	1	F-1302	Tetrachloroethane	F	N	D	...	..
		2	F-1302	*p*-Cymene	F	Dark, U	282	2-14	X
		3	F-1302	*o*-Dichlorobenzene	F	Amber, P	375	2-14	E
							437	14-22	h
		4	F-1302	Pyridine	F	Dark P	...	4- 5	A
		5	F-1302	*o*-Dichlorobenzene	F	Amber, P	427	2-14	E
	Butaprene-NF	1	F-205	Tetrachloroethane	F	N	D	...	..
		2	F-205	*o*-Dichlorobenzene	F	Amber, P	394	2-14	E
	Ethylene polysulfide polymers	1	F-804	Tetrachloroethane	F	Red, W	186	2-14	E
		2	F-804	*p*-Cymene	F	Red, W	291	2-14	A
		3	F-804	*o*-Dichlorobenzene	F	Red, W	403	2-14	E
	Thiokol-ST	1	F-801	*o*-Dichlorobenzene	F	Red, W	458	2-14	E
							438	14-22	h
Copolymer of butadiene, acrylonitrile and unsaturated ketone	Thiokol-RD	1	F-803	Tetrachloroethane	F	N	D	...	..
		2	F-803	Diisobutyl ketone	F	Dark, U	231	2-14	X
		3	F-803	*p*-Cymene	F	Dark, P	269	2-14	A
		4	F-802	Pyridine	F	Dark, P	...	4- 5	A
		5	F-803	*o*-Dichlorobenzene	F	Amber, P	369	2-14	E

refluxing, was satisfactorily inert toward the rubber hydrocarbon. *p*-Cymene dissolved all samples except Buna-N types, but its use at prolonged refluxing led to oxidation and serious gum formation which contaminated the sample. Pyridine and nitropropane were ineffective for Buna-N, and several high-boiling ketones precipitated gums in the sample during refluxing.

A 4-gram sample and 250 ml. of solvent were refluxed for 24 to 36 hours in an A.S.T.M. condenser and flask assembly or in a similar all-glass assembly, using a water-cooled condenser (Figure 3) designed for this purpose. The glass condenser can be used interchangeably with the A.S.T.M. tin-coil condenser and is preferred for chemical inertness and ease of cleaning. Complete solution of some of the less soluble elastomers was best accomplished by successive treatment with two or three 100-ml. portions of solvent. Blenderization decreased the time required for solution, but was not essential since serious gelling never occurred. Absence of appreciable residue after an hour of settling indicated complete solution of the elastomer.

Comparatively coarse fillers such as clays, iron oxide, etc., were separated by simple centrifugation. This method was ineffective for colloidal carbon blacks. The procedure used by Barnes (*4*), employing dilution with *n*-hexane, was tested on a series of natural

of Compounded Specimens

Inhibitors, Waxes Extenders	Carbon Blacks	Basic Oxides[a], Clays	Total Parts by Weight	Cure	Physical Measurements			
					Sp. Gr.	Dur.	Tensile	Elongation
Parts by weight				*Min.*			*Lb.*	%
.....	.....	.....	113.1	15, 300° F.	..	30–40	3200	725
Heliozone, 2.0	P-33, 0.5	.....	112.5	30, 300° F.	0.97	35–40	2125	775
.....	Thermax, 100	L.C. magnesium oxide, 10.0	238.5	40, 300° F.	..	65	1800	300
.....	Thermax, 100	L.C. magnesium oxide, 10.0	243.5	40, 300° F.	..	70	1875	300
Acrawax, 2.0	P-33, 91	L.C. magnesium oxide, 4.0	222.7	30, 300° F.	1.39	60–65	1225	650
.....	P-33, 30	.....	151.0	45, 300° F.	..	45	1800	825
Paraffin, 3.0	.....	Superex clay	220.5	45, 310° F.	1.34	45–50	1550	785
Heliozone, 1.0	Furnex, 16.0	.....	150.0	07, 375° F.	..	45	1500	750
Mineral rubber, 7.5	Micronex, 18.0							
.....	.....	.....	113.1	20, 300° F.	..	...	..	..
.....	Furnex, 40	.....	150.9	40, 300° F.	1.52	60–65	665	400
.....	Furnex, 60	.....	162.5	30, 290° F.	..	65	1300	325
.....	Furnex, 40	.....	170.5	15, 300° F.	..	55	1375	300
.....	Furnex, 50	.....	168.5	30, 300° F.	..	65	1600	260
.....	Thermax, 130	.....	273.5	15, 300° F.	..	60	1675	610
.....	Thermax, 70	.....	208.25	30, 300° F.	1.22	45–50	1700	700
.....	Furnex, 70	.....	208.25	30, 300° F.	1.20	45–50	2000	550
Selastic SR-66, 100	Wyex, 35 Furnex, 35	.....	279.75	30, 300° F.	1.43	75–80	950	125
.....	P-33, 50	.....	181.5	50, 300° F.	..	48	600	300
.....	Statex B, 50	.....	164.5	15, 300° F.	1.13	60	3200	500
.....	.....	.....	110.5	15, 300° F.	..	...	..	..
.....	Statex B, 50	.....	164.5	15, 300° F.	..	...	..	..
.....	Furnex, 50	.....	174.1	30, 300° F.	..	...	..	.

and Buna-N samples and found unreliable because of selective precipitation of the elastomer. Filtration with adsorbents such as bentonite, fuller's earth, alumina, and silica gel was also unsatisfactory. Celite analytical filter aid (Johns-Manville Co.), however, permitted complete removal of colloidal carbon in every case investigated. After the polymer solution had been diluted to 300 ml. with benzene, about 50 to 100 cc. of Celite were added and the slurry was filtered through a semifine paper. Filtration proceeds most rapidly if sufficient Celite is added so that free carbon does not clog the paper. Trial filtrations with small portions of the slurry readily allow a proper Celite content to be found. Quantitative work with solutions of mixed elastomers conclusively showed no selectivity toward the elastomers and the solution holdup in the filter aid can be nearly completely recovered by adding hot benzene and refiltering.

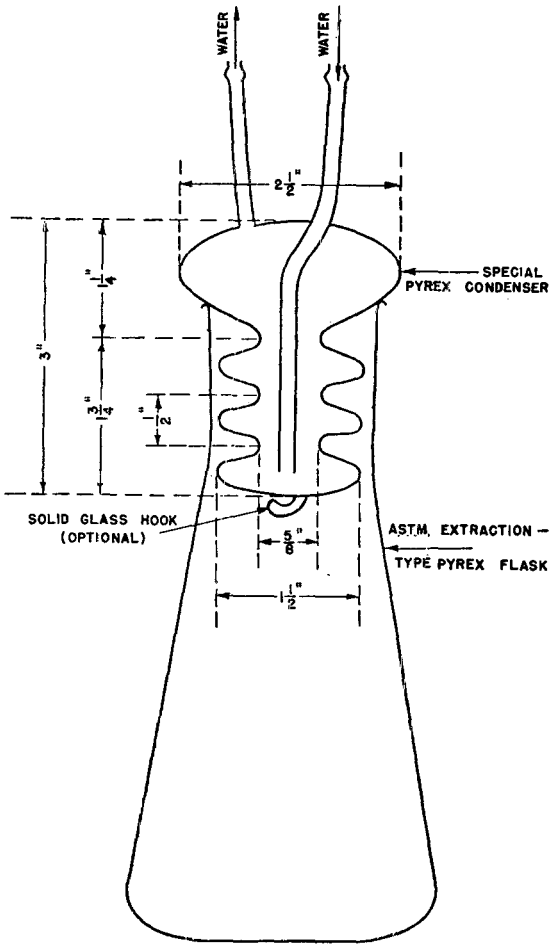


Figure 3. All-Glass Reflux Assembly

The elastomer solutions were concentrated by vacuum distillation at 100° to 110° C., using a water aspirator and conventional Claisen-type flask immersed in an oil bath. The viscous concentrate was diluted with a few milliliters of benzene and transferred to a small container for storage; the benzene was removed later by evaporation at room temperature or in a vacuum oven at 60° C. Films for spectral examination were spread as before and 40 minutes in a vacuum oven at 80° C. were allowed for final solvent evaporation. Films of natural and Thiokol rubber were sticky but satisfactorily rigid and those of other elastomers were solid and nearly free of tackiness.

Discussion. The work on cured specimens is summarized in Table II, and representative spectra of the cured elastomers are shown in Figure 4. Comparison with Figure 2 shows the spectra of a pure gum elastomer and its vulcanizate to be nearly identical, except for minor differences which are discussed below. This significant result was obtained consistently throughout more than fifty spectra of separate sample preparations and alone is adequate evidence of the validity of all spectra obtained! In addition, almost perfect spectral reproducibility was found for the spectra of each elastomer obtained from duplicate preparations in *o*-dichlorobenzene and in most cases from additional preparations in *p*-cymene and in tetrachloroethane. The overall method was thus shown to be reliable and the spectral results obtained were shown to be nearly independent of the sample history—i.e., of compounding and curing effects and of the method of preparation for study.

The complete spectral identity of cured and uncured rubbers is not inconsistent with current concepts of the vulcanization process, which is considered to introduce comparatively few cross linkages in building the three-dimensional vulcanizate. Since the infrared spectrum of a polymer is determined almost entirely by the small recurring structural units and not by the gross size or configuration, the net result of vulcanization, consisting mainly of changes which involve relatively few of the recurring polymer units, does not alter the rubber spectrum to any appreciable extent. Only two minor differences between the spectra of gums and their vulcanizates (Figures 2 and 4) were noticed. In general, oxidation during vulcanization produces hydroxyl absorption near 3.0 mu and carbonyl absorption between 5.6 and 6.0 mu. These extra bands do not interfere, however, with any bands useful for analysis. A more consistent difference is the poorer definition of bands themselves and the increased background absorption beyond 8 mu in the spectra of most cured rubber. This effect has been observed in homologous series of simple hydrocarbons and is consistent with the increased molecular weight of the vulcanizate. This absorption was not reproducible for different sample preparations and, though serious only in natural vulcanizates, it cannot be disregarded in quantitative work.

The method of study leaves little to be desired from the spectroscopist's viewpoint, since isolation of the rubber hydrocarbon assures valid results. The method applies to all elastomers investigated and to blends of these elastomers, and a single procedure remarkably free of exacting techniques may be used. Routine application requires about 6 man-hours per sample.

IDENTIFICATION AND QUALITATIVE ANALYSIS

The fact that the spectra of the various elastomers, when obtained by the procedures described, are unique and experimentally reproducible regardless of the past history of the sample constitutes a sound basis for characterization of the elastomer content of commercial rubber products. The individual elastomers are identified by the following absorptions:

Natural and neoprene are distinguished from all other classes by a broad band at 12 mu, and are differentiated from one another by the exact position of this band, by the 7.25 mu band in natural, by the strength and position of the 6.0 mu band, and by the appearance of the 9 mu region. Butyl (GR-I) is distinguished by a strong unique band at 8.13 mu and by a strong doublet at 7.20, 7.30 mu. Buna-N (Chemigum, Hycar, Perbunan) and Buna-S (GR-S) are characterized by strong bands at 10.3 and 10.9 mu (common to butadiene polymers), and Buna-S is readily identified by unique bands at 6.23, 6.70, and 14.3 mu, while Buna-N is identified by a band at 4.47 mu. Thiokol-FA and -ST possess identical spectra, characterized by a unique envelope of strong bands at 8.36, 8.64, 8.98, 9.32, and 9.68 mu. Thiokol-RD possesses typical Buna-N bands at 4.47, 10.25, and 10.86 mu, but is readily set apart by a strong band at 5.86 mu and by a doublet at 7.25, 7.38 mu.

Thus, identification of a rubber specimen containing a single elastomer is always possible simply by empirical comparison of the spectrum with reference spectra, such as those of Figures 2 and 4.

For mixtures of elastomers the ease of qualitative analysis depends upon the degree of differences existing in the spectra of the components. For example, less than 1% of a nitrile (Buna-N) copolymer may be identified in any mixture by absorption at

4.47 mu, since all other elastomers are highly transparent in this region. Less than 5% of Thiokol-FA may be detected in any mixture by its characteristic bands in the region of 7.5 to 10.5 mu, since these bands, though not isolated, are much stronger than those of other elastomers in this region. Since blends of more than two components are seldom encountered in commercial products, it is generally possible to identify any component which exists in 5%, or greater, concentration. The limit of detection of neoprene in natural, however, is probably nearer 10%, since great spectral similarity exists for these elastomers. The major component(s) is best identified by examining a film which transmits about 10 to 20% at 6.8 mu. Thicker films may then be examined over selected wave-length regions to establish the presence or absence of minor components.

Exact spectral correspondence may not always be found for some elastomers because of differences in component proportions and modes of addition in copolymers or oxidation of the sample. These differences do not hinder the analysis and, in addition, furnish valuable information if interpreted in terms of molecular structure. Thus, the relative intensity of the 4.47 mu nitrile absorption distinguishes between high- and low-nitrile Buna-N; and emulsion polymers may be differentiated from sodium polymers since the former are predominantly 1,4-addition, with absorption at 10.35 mu, while the latter are predominantly 1,2-addition, with absorption at 10.95 mu.

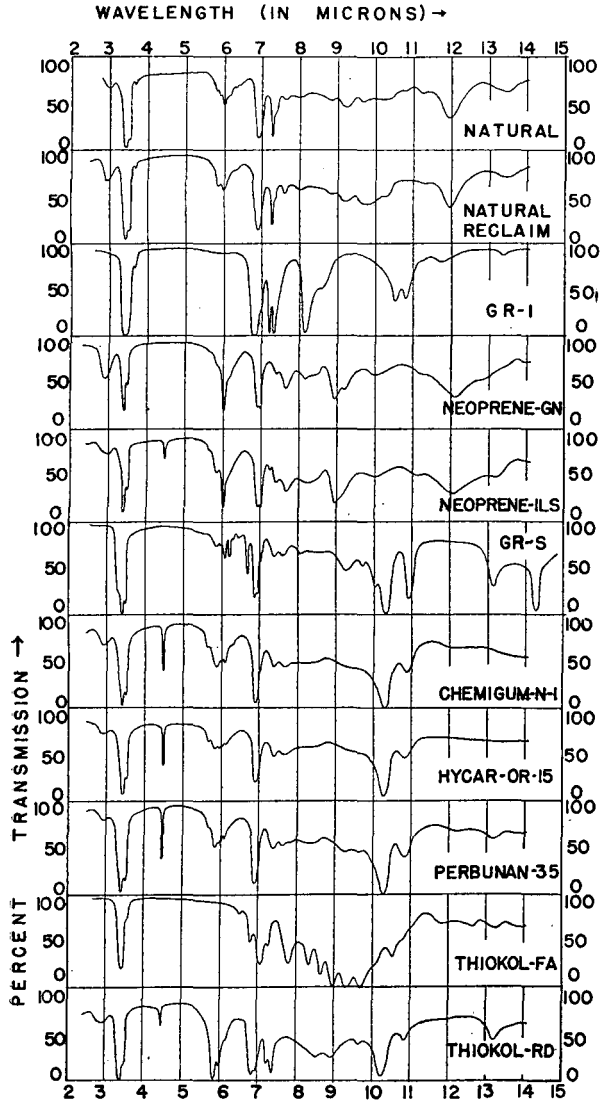


Figure 4. Spectra of Cured Elastomers

QUANTITATIVE ANALYSIS OF NATURAL-GR-S MIXTURES

Basis of Method. Quantitative spectral analysis of a binary mixture generally involves (1) selection of a spectroscopic measurement or function which is sensitive to the composition, (2) construction of a working curve expressing the relationship between the spectroscopic function and sample composition for a set of accurately known mixtures, and (3) subsequent analysis of samples by applying analogous measurements to this working curve. The working curve method requires the utmost in experimental reproducibility of the over-all procedure, and the results obtained with uncured and cured elastomers show the methods presented to be fully adequate in this respect. The analytical problem relates, therefore, largely to determining the most satisfactory spectroscopic function for the particular analysis. The case of natural–GR-S mixtures is considered here, but since similar spectral differences exist for other binary mixtures analogous procedures and similar results may be obtained in other cases.

Comparison of the spectra of natural and GR-S indicates that the former can be detected in the mixture by methyl absorption at 7.25 mu or by 11.95 mu absorption due to the isoprene structure, $-(CH_3)C{=}CH-$, and that the latter can be detected by the 6.70 mu phenyl absorption or by butadiene absorptions at 10.35 and 10.95 mu due to 1,4- and 1,2-addition, respectively. The 7.25 and 6.70 mu bands appear most suitable for natural and GR-S determination, since both are independent of olefinic bonds which might be expected to change under certain conditions—for example, with vulcanization, molecular weight, and mode of addition. Since the styrene content of GR-S is maintained as nearly constant as possible by all manufacturers, the number of phenyl and methyl groups present, and hence the intensities of their absorptions, are proportional to the GR-S and natural content, respectively, of the mixture. The use of the shorter wave-length bands avoids any effects due to variable background absorption at longer wave lengths.

The spectroscopic function to be employed generally includes the transmittance, T, of the sample as the experimentally measured quantity. This is usually expressed in terms of extinction, E, by the equations:

$$E \equiv \log_{10}(1/T) = \log_{10}(I_0/I) = KCt$$

where I_0 and I represent the energy transmitted by the blank and sample, respectively, K is a proportionality constant, C is the concentration, and t is the sample thickness.

Since it is impractical, if not impossible, to measure or control the thickness of rubber films with sufficient accuracy, it is convenient to eliminate t from the spectroscopic function. This can be done by employing the ratio of extinctions determined at different wave lengths, since only t is independent of wave length. It would be convenient, then, to employ as the spectroscopic function the ratio $E^{7.25}/E^{6.70}$ where the superscripts refer to wave length. In practice, however, this ratio can be determined accurately only for mixtures which contain appreciable concentrations of both components, for only under these conditions is it possible to spread a film of given thickness which yields transmittance values in the accurately measurable range of 20 to 65% at each wave length. This difficulty may be resolved by selecting, as an internal standard, a third band common to both components and of such intensity that the transmittance, both at the internal standard wave length and at the analytical wave length of the minor component, will always fall within the accurately measurable range. For this purpose the common band of medium strength at 7.60 mu is especially well suited, since it occurs in both natural and GR-S at so nearly the same intensity as to be almost

independent of the composition of the blend. The extinction ratios $E^{6.70}/E^{7.60}$ and $E^{7.25}/E^{7.60}$ thus may be used as functions only of composition to determine GR-S and natural, respectively, and together enable analysis of all mixtures from 0 to 100%. Both ratios are linear with composition over the useful range (see Figure 5).

Analytical Procedure. The following method is presented for routine analysis of finished rubber articles composed of natural rubber and GR-S. The procedures are in no way exacting, and the time requirement is about 6 man-hours per sample.

SAMPLE PREPARATION. A 4-gram sample, which has been milled to pass a No. 20 screen, is extracted for 24 hours with two 80-ml. portions of a 32–68 blend of acetone-chloroform. The extracted sample is completely dissolved in 250 ml. of distilled *o*-dichlorobenzene by refluxing for 24 hours, and the solution is diluted to 300 ml. with benzene. Carbon blacks are removed by Celite filtration, and mineral fillers, if present, by centrifugation. A thick mucilage for film spreading is obtained by vacuum distillation of the solvent, and films are spread on sodium chloride or potassium bromide plates. If the sample is fairly viscous, the films can be checked for thickness immediately (since *o*-dichlorobenzene is transparent at this wave length) by measurement at 7.60 mu where the transmittance should be between 40 and 60%. After adjustment to an appropriate thickness the film is dried 40 minutes in a vacuum oven at 80° C., or for 4 hours at room temperature before analytical measurements are made. Films of decided irregularity must not be used.

MEASUREMENTS AND CALCULATIONS. Percentage transmittance of each film is measured at 7.60, 7.25, and 6.70 mu. Care must be taken to measure at the center of the bands. Duplicate films and measurements are recommended for accuracy, and transmittance values outside the range 20 to 65% are not used. The extinction ratio $E^{7.25}/E^{7.60}$ or $E^{6.70}/E^{7.60}$ (or both) is obtained, depending upon the suitability of the measurements, and plotted against composition for a series of standard mixtures. Subsequent samples are analyzed by the converse procedure of applying the measured ratios to the appropriate working curve.

Experimental Results.

WORK WITH STANDARD BLENDS. The analytical method was tested by applying it to eleven standard samples of accurately known composition. These were made by curing appropriate mixtures of two master batches, natural (F-10) and GR-S (F-620) (see Table III), compounded with 30% carbon black as filler. Samples were prepared in triplicate, and several films of each preparation were studied. The analytical results, presented in Table IV, were obtained by plotting the measured extinction ratios against the known composition of each sample, and applying the corresponding values to the working curve (Figure 5) drawn through the average value of each group of points. For each blend the several analyses differ from the average only rarely by more than 2% and the average deviation for the 58 results is 1%. The reproducibility of the analytical values for the three separate preparations of each sample is equal to that obtained for duplicate films of any one preparation. This proves conclusively the adequacy of the preparation method on the one hand, and shows the importance of the quality of the films on the other.

While some films were perfectly smooth, others were somewhat uneven, and this probably accounts for what little variation was obtained. For evaluation of the effect of film thickness, a number of films of greatly different thickness were purposely studied, although fairly close control of thickness was easily possible.

Table IV. Analysis of Standard Natural–GR-S Blends

Sample[a]		Measurements[b]			Natural Analysis[c] 7.25		GR-S Analysis[d] 6.70	
Blend	No.	7.60 mu	7.25 mu	6.70 mu	7.60	%	7.60	%
F-620 (100% GR-S)	1	36.0/0.445	52.0/0.285	18.0/0.745	0.64	00.0	1.68	[e]
	2	52.0/0.283	66.5/0.178	32.0/0.498	0.63	00.0	1.76	[e]
1 (90% GR-S) (10% natural)	1	35.0/0.456	32.5/0.488	20.0/0.700	1.07	10.5	1.54	[e]
	2	22.5/0.647	21.5/0.667	12.3/0.910	1.03	9.5	1.41	[e]
	3a	39.5/0.404	37.0/0.432	29.0/0.542	1.07	10.5	1.34	[e]
	3b	49.5/0.306	47.0/0.328	38.0/0.417	1.07	10.5	1.36	[e]
	3c	45.0/0.349	42.0/0.377	33.5/0.474	1.08	10.7	1.36	[e]
	3d	32.0/0.492	29.0/0.535	21.0/0.672	1.08	10.7	1.37	[e]
2 (80% GR-S) (20% natural)	1	30.0/0.523	17.5/0.755	20.0/0.700	1.44	20.0	1.34	[e]
	2	34.0/0.470	20.0/0.700	21.5/0.667	1.49	21.5	1.42	[e]
	3a	28.0/0.556	17.0/0.774	18.0/0.742	1.39	19.0	1.33	[e]
	3b	38.0/0.424	25.0/0.603	27.0/0.573	1.41	19.5	1.35	[e]
	3c	49.5/0.305	37.5/0.428	39.0/0.409	1.40	19.0	1.35	[e]
	3d	62.5/0.206	50.0/0.301	52.0/0.284	1.46	20.5	1.38	[e]
3 (70% GR-S) (30% natural)	1	38.0/0.420	16.3/0.788	29.0/0.538	1.87	31.0	1.28	72.0
	2	22.5/0.647	–	15.7/0.804	–	–	1.24	69.0
	3a	40.5/0.394	20.0/0.701	31.5/0.500	1.78	29.0	1.27	71.5
	3b	50.0/0.301	27.0/0.567	41.0/0.388	1.88	31.5	1.29	73.0
	3c	35.0/0.458	14.5/0.842	–	1.84	30.5	–	–
	3d	46.0/0.339	23.0/0.642	37.0/0.428	1.89	31.7	1.24	69.0
4 (60% GR-S) (40% natural)	1	29.5/0.530	–	25.0/0.603	–	–	1.13	60.0
	2	38.5/0.415	–	34.0/0.470	–	–	1.13	60.0
	3a	48.0/0.320	20.0/0.703	–	2.20	40.0	–	–
	3b	36.5/0.438	–	31.0/0.512	–	–	1.17	63.0
	3c	70.5/0.152	46.0/0.339	67.0/0.174	2.23	40.5	1.14	61.0
	3d	39 0/0.408	–	35.0/0.456	–	–	1.12	59.0
5 (50% GR-S) (50% natural)	1	32.0/0.495	–	33.0/0.482	–	–	0.975	47.5
	2	25.5/0.595	–	26.0/0.587	–	–	0.990	48.5
	3a	31.5/0.514	–	31.0/0.506	–	–	0.980	48.0
	3b	27.5/0.558	–	26.5/0.576	–	–	1.03	52.0
	3c	57.5/0.242	26.0/0.584	57.0/0.244	2.40	45.0	1.01	50.0
	3d	59.5/0.224	27.0/0.566	58.5/0.232	2.53	48.0	1.03	52.0
	3e	41.0/0.384	–	41.5/0.382	–	–	1.00	50.5
6 (40% GR-S) (60% natural)	1	20.5/0.690	–	24.5/0.610	–	–	0.885	40.0
	2	41.5/0.380	–	45.5/0.340	–	–	0.885	40.0
	3a	31.0/0.506	–	35.0/0.456	–	–	0.900	41.0
	3b	38.5/0.413	–	43.0/0.368	–	–	0.890	40.5
	3c	42.0/0.376	–	46.0/0.335	–	–	0.890	40.5
	3d	32.5/0.490	–	37.0/0.428	–	–	0.875	39.5
7[f] (30% GR-S) (70% natural)	3a	36.0/0.444	–	45.5/0.342	–	–	0.770	31.0
	3b	32.0/0.494	–	40.5/0.393	–	–	0.795	32.5
	3c	23.0/0.642	–	32.0/0.492	–	–	0.765	30.0
	3d	29.5/0.532	–	39.0/0.410	–	–	0.770	31.0
	4a	33.5/0.472	–	42.0/0.378	–	–	0.800	33.0
	4b	59.0/0.232	–	65.5/0.184	–	–	0.790	32.0
8 (20% GR-S) (80% natural)	1	25.5/0.595	–	43.0/0.365	–	–	0.610	18.0
	2	27.0/0.570	–	44.0/0.355	–	–	0.620	18.5
	3a	27.5/0.558	–	43.5/0.361	–	–	0.650	21.0
	3b	32.0/0.494	–	47.5/0.324	–	–	0.655	21.5
	3c	34.0/0.465	–	51.5/0.286	–	–	0.615	18.2
	3d	30.0/0.524	–	47.0/0.330	–	–	0.630	19.5
9 (10% GR-S) (90% natural)	1	25.5/0.595	–	50.5/0.297	–	–	0.500	09.5
	2	38.0/0.420	–	60.5/0.218	–	–	0.520	10.5
	3a	41.0/0.387	–	64.5/0.191	–	–	0.495	08.5
	3b	37.5/0.428	–	60.0/0.220	–	–	0.515	10.0
	3c	45.0/0.346	–	67.5/0.171	–	–	0.495	08.5
	3d	28.0/0.549	–	53.5/0.273	–	–	0.500	09.5
	3e	24.0/0.620	–	49.5/0.305	–	–	0.490	08.0
F-10 (100% natural)	1	22.5/0.647	–	55.0/0.260	–	–	0.400	00.5
	2	36.0/0.445	–	65.5/0.185	–	–	0.415	02.0

[a] Numbers refer to sample preparation; letters indicate several films measured.
[b] Percentage transmittance, T, and extinction, E, at each wave length given as T/E.
[c] Using indicated ratio of extinction values and curve 2 of Figure 4.
[d] Using indicated ratio of extinction values and curve 1 of Figure 4.
[e] Extinction ratios (though measurable) beyond linear range of working curve.
– Absorption too great for accurate measurement.
[f] Preparations 1 and 2 of blend 7 ruined prior to film preparation.

Despite threefold differences in the 7.60 mu extinction, the analytical results proved without exception to be independent of this factor. Finally, the reproducibility is the same for all concentrations on the linear portion of the working curve. In view of the facts that these results include all data obtained in development of the method, and that no exceptional degree of regulation was used in any steps of the analysis, it is considered that a quantitative accuracy expressed by an average deviation of 1% is generally obtainable in application of the method to mixtures of high-quality natural–GR-S stocks.

ANALYSIS OF RECLAIM-CONTAINING MIXTURES. Analysis of the standard samples described above necessarily constituted favorable conditions for application of the method, in order that limitations pertaining solely to the experimental procedure could be established. The general usefulness of the method, however, depends upon the consistency of the analytical results when stocks of different origin are encountered. Although different stocks will exhibit the same general spectral features, slight quantitative differences in polymer structure may exist and produce corresponding differences in band intensities which will affect the analytical results.

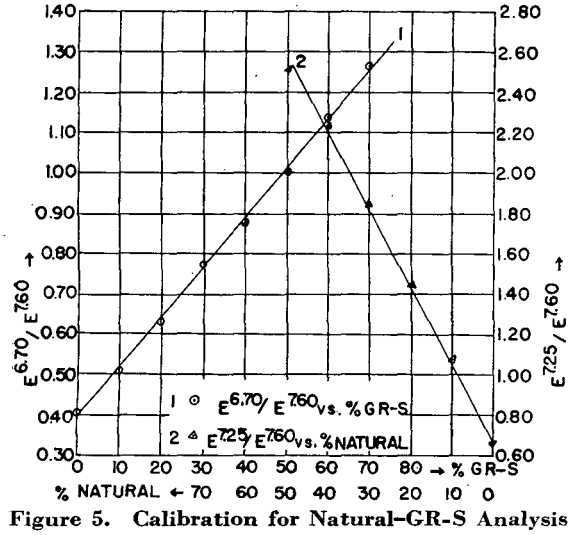


Figure 5. Calibration for Natural–GR-S Analysis

To determine the maximum effect which would be encountered, attention was directed toward natural–GR-S blends containing a natural reclaim stock. A different GR-S stock was not studied, as little variation of GR-S would be expected. Six reclaim-containing blends, made by curing appropriate mixtures of natural (F-10), GR-S (F-620), and a tube reclaim (F-10-1), were examined. Several preparations and analyses of each of these samples were made using the working curves previously determined (Table V). Although no apparent difficulties were encountered in sample preparation, both the reproducibility and the correctness of the results are much inferior to the accuracy previously reported. The errors are roughly proportional to the reclaim content, and far greater interference is found in the natural (plus reclaim) determination than in the GR-S determination.

For GR-S concentrations of 10 to 70% an accuracy of ±5% or better can be obtained by using the GR-S working curve and determining natural by difference. For other compositions as much as 10% error may be obtained. However, if it is assumed that reclaim is always used with at least equal quantities of natural rubber, the over-all accuracy of the method may be put at ±5% for reclaim-containing mixtures. The seriousness of interference by reclaim is minimized somewhat by the fact that the presence of reclaim can be recognized in important cases. In the absence of reclaim both natural and GR-S can be measured directly only for concentration near 50%, and in these cases the concentrations total 100% within the limits of error. However, when reclaim is present in sufficient quantity to cause serious interference it becomes possible to determine both components over a much wider range, and in every case the interference is indicated by a corresponding deviation of total concentration from 100%.

Although the results obtained with reclaim-containing blends were not unreasonably erroneous considering the severity of the test, an attempt to obtain more satisfactory results was made by employing a different choice of analytical wave lengths. Results based upon measurements at the 11.95 mu absorption of natural were found unreliable and other procedures employing the functions $E^{10.35}/E^{10.70}$, $E^{10.35}/E^{11.40}$, and $E^{10.95}/E^{11.40}$ yielded satisfactory results for high-quality stocks but no improvement in results for reclaim-containing mixtures. The original method therefore appears to yield the best accuracy for infrared analysis of natural–GR-S mixtures.

Table V. Analysis of Reclaim-Containing Mixtures

Sample[a] Blend	No.	Measurements[b] 7.60 mu	7.25 mu	6.70 mu	Natural Analysis[c] 7.25/7.60	%	GR-S Analysis[d] 6.70/7.60	%
F-10 (100% natural)	1a	36.0/0.442	–[e]	66.3/0.179	–	–	0.405	01.0
	1b	57.5/0.241	13%	80%	–	–	–	–
	2a	39.5/0.404	–	68.0/0.168	–	–	0.417	02.0
	2b	57.5/0.241	13%	80%	–	–	–	–
F-10-4 (50% natural) (50% reclaim)	1a	32.2/0.492	–	62.0/0.209	–	–	0.425	02.5
	1b	55.3/0.258	18.2/0.739	77.4/0.112	2.86	*f*	0.435	03.5
	2a	37.8/0.422	–	64.3/0.192	–	–	0.455	05.0
	2b	51.7/0.286	13%	74.0/0.132	–	–	0.460	05.5
F-10-1 (100% reclaim)	1a	40.0/0.398	–	67.0/0.175	–	–	0.440	04.0
	1b	58.2/0.232	24.3/0.614	78.0/0.108	2.64	54.0	0.465	06.0
	2a	33.9/0.470	–	53.3/0.273	–	–	0.580	15.0
	2b	48.8/0.311	22.2/0.653	64.5/0.191	2.10	36.0	0.615	18.0
	3	45.5/0.342	14.0/0.844	65.0/0.187	2.46	47.0	0.550	13.0
	4	48.8/0.311	15.0/0.824	66.5/0.177	2.64	54.0	0.570	14.5
F-10-2 (33.3% natural) (33.3% reclaim) (33.3% GR-S)	1a	31.5/0.499	–	41.5/0.383	–	–	0.770	30.5
	1b	54.0/0.266	25.5/0.594	64.5/0.197	2.23	40.0	0.740	28.5
	2a	34.0/0.470	–	41.8/0.379	–	–	0.805	33.5
	2b	51.0/0.292	18.3/0.737	58.5/0.234	2.52	49.0	0.800	33.0
	3	48.8/0.311	15.5/0.808	55.5/0.256	2.60	52.0	0.825	35.5
F-10-5 (25% natural) (25% reclaim) (50% GR-S)	1a	63.0/0.200	37.2/0.430	64.5/0.190	2.15	38.5	0.950	45.5
	1b	44.2/0.354	17.8/0.750	45.5/0.342	2.12	38.0	0.965	46.5
F-10-3 (50% reclaim) (50% GR-S)	1a	34.0/0.470	14.0/0.860	37.0/0.432	1.83	30.0	0.920	43.0
	1b	53.0/0.276	29.8/0.525	55.8/0.253	1.90	32.0	0.920	43.0
	2	49.4/0.306	26.2/0.582	50.5/0.296	1.90	32.0	0.970	47.0
	3	47.8/0.321	22.2/0.653	46.7/0.331	2.03	35.0	1.03	50.5
F-10-6 (15% natural) (15% reclaim) (70% GR-S)	1a	59.2/0.228	41.0/0.386	52.2/0.282	1.69	26.5	1.24	69.0
	1b	45.5/0.343	26.5/0.575	37.2/0.430	1.68	26.5	1.25	70.0
F-620 (100% GR-S)	1a	37.2/0.430	49.2/0.308	23.2/0.634	0.715	01.0	1.47	*f*
	1b	50.5/0.296	60.5/0.219	37.0/0.431	0.740	01.5	1.45	*f*
	2	50.0/0.301	57.8/0.238	34.0/0.470	0.790	03.0	1.56	*f*

[a] Numbers refer to sample preparations; letters indicate several films measured.
[b] Percentage transmittance, T, and extinction, E, at each wave length given as T/E.
[c] Using indicated ratio of extinction values and curve 2 of Figure 4.
[d] Using indicated ratio of extinction values and curve 1 of Figure 4.
[e] Absorption too great for accurate measurement.
f Extinction ratios (though measurable) beyond linear range of working curve.

DETERMINATION OF ACRYLONITRILE IN BUNA-N COPOLYMERS

Basis of Method. The general procedure described for the spectral analysis of elastomer mixtures may also be used to

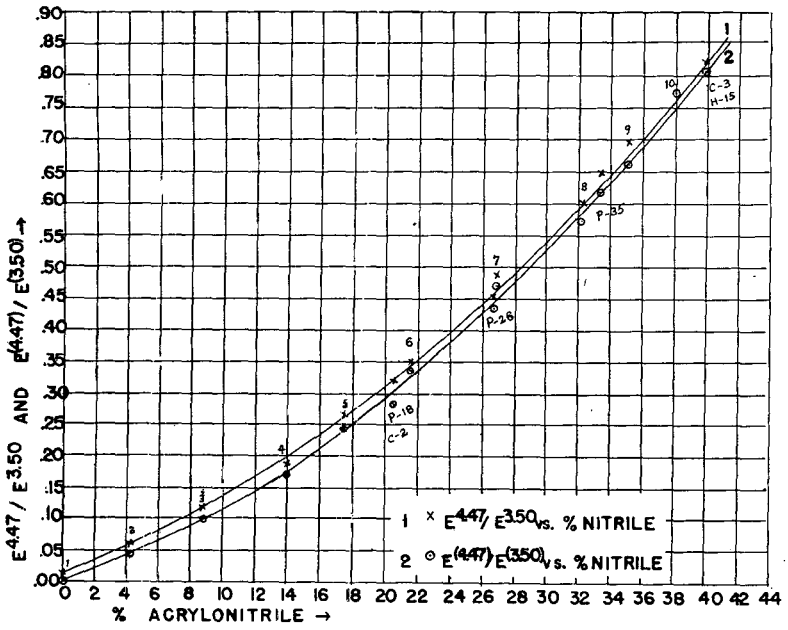


Figure 6. Calibration for Nitrile Determination

determine the structural units comprising a given elastomer. Determination of the nitrile (nitrile and acrylonitrile are used interchangeably throughout the discussion) content of Buna-N copolymers is considered in detail, but similar methods may readily be developed for analogous problems, such as determination of the styrene content of Buna-S or the ratio of 1,2- to 1,4-addition structures in polybutadienes.

Satisfactory procedures for sample preparation of cured and uncured Buna-N copolymers have been presented, so that only the spectroscopic function need be considered. The ratio $E^{4.47}/E^{3.50}$ obtained by measuring the transmittance at the 4.47 mu nitrile band (due to the C≡N valence vibration) and at the 3.50 mu C—H absorption, is a satisfactory function and can be determined accurately for samples containing 0 to 50% nitrile, which range includes all Buna-N rubbers of industrial importance. A working curve is constructed by plotting the spectroscopic function versus composition for a series of standard samples and subsequent analyses are made by applying analogous measurements to the curve.

If the transmittance is measured relative to a blank plate at the analytical wave lengths, an error due to reflection and scattering losses in the sample film will result and the calibration curve will not read zero at 0% nitrile (pure polybutadiene). Although this effect is scarcely noticeable for nitrile concentrations above 15%, it gives appreciable positive error at low nitrile concentrations. This error may be eliminated entirely by choosing as "100% transmittance" the transmittance of the sample film itself at a near-by wave length of maximum transmittance—i.e., at 4.60 mu—rather than the transmittance of a blank plate at the analytical wave length. With this modification polybutadiene will show 0% absorption at 4.47 mu, and valid results will be obtained for low nitrile samples, irrespective of wide variations in general transparency of films.

Analytical Procedure. Sample Preparation. For gum rubber, a 2-gram sample is cut into thin strips and extracted for 24 hours with two 80-ml. portions of 95% ethanol. The extracted sample is allowed to soften and swell in 100 ml. of ethylene dichloride for 2 hours and then thoroughly mixed in a Waring Blendor. Excess solvent is removed by evaporation on an A.S.T.M. gum bath at 70° C., but evaporation must be stopped before gelling occurs. For cured rubber, a 4-gram sample, which has been milled to pass a No. 20 screen, is extracted for 24 hours with two 80-ml. portions of a 32–68 blend of acetone-chloroform. The extracted sample is dissolved in 300 ml. of distilled *o*-dichlorobenzene under reflux, the solvent being used successively in two portions, and blenderization being employed if necessary. Carbon blacks are removed by Celite filtration, and mineral fillers by centrifugation. Vacuum distillation is employed for solvent removal. Films are spread on sodium chloride or potassium bromide plates and residual solvent is removed by evaporation at room temperature or more rapidly in a vacuum oven at 80° C.

Spectroscopic Measurements. Percentage transmittance of each film relative to a blank plate is measured at 4.60, 4.47, and 3.50 mu, care being taken to locate the maxima of the bands (except at 4.60 mu). Films of satisfactory thickness give about 20 to 25% transmittance at 3.50 mu. The percentage transmittances at 3.50 and 4.47 mu are corrected for film scattering by dividing by the decimal transmittance at 4.60 mu and the extinction ratio $E(4.47)/E(3.50)$ is calculated, parentheses being used to denote the use of corrected transmittance values. This ratio is plotted versus composition for several known samples and subsequent samples are analyzed by the converse procedure of applying the measured ratios to this curve. Films and measurements are made in duplicate as a check on accuracy.

Experimental Results. The analytical method was tested by applying it to over fifty samples prepared from a variety of Buna-N copolymers. Preliminary measurements were made on gum samples of Perbunan-18, -26, and -35, Chemigum-N-1, -2, and -3, and Hycar-OR-15 and -25. A working curve was constructed using the average values of the uncorrected analytical function and nitrile values were determined by Kjeldahl analysis (total nitrogen assumed to be nitrile nitrogen). All values were then applied to the working curve and the results are presented as set 1 in Table VI. Despite neglect of the scattering correction and the use of films of widely different quality and thickness, the analytical results show a mean deviation of ±1% and a maximum error only rarely twice this value. The error is in no sense consistent with film thickness, and can be attributed entirely to differences in film quality. The over-all method was then critically tested by using the same working curve to analyze a set of the same Buna-N stocks which had been compounded with carbon black and cured. The agreements of the results (set 4, Table VI) with those for gum samples are fully as close as the reproducibility obtained in either set for duplicate films of the same preparation. This severe test of the over-all method shows its complete reliability on the one hand and the importance of obtaining high-quality films on the other.

For extension of the calibration below 20% nitrile a specially prepared set of ten Buna-N gum elastomers (supplied by the Firestone Rubber Company) covering the range 0 to 40% nitrile were prepared in duplicate and several films of each preparation were measured at 3.50, 4.47, and 4.60 mu. Extinction ratios were calculated both with and without correction for scattering and the average values for each sample were plotted versus nitrile values based upon the manufacturer's Kjeldahl analyses to obtain the working curves shown in Figure 6. Curve 1 (uncorrected ratios) was identical with that previously obtained for the commercial elastomers and established the equivalence both of the spectral results and of the chemical analyses for both series of samples. The analytical results obtained by applying the ratios for each film to the proper curve of Figure 6 are presented in Table VII. For each curve an average deviation of ±1% was obtained for samples containing 15% or more nitrile; for samples of less than 15% nitrile no decrease in accuracy resulted when the corrected ratio was used (curve 2), whereas the uncorrected ratio gave considerable error. The errors were independent of film thickness and were attributed entirely to differences in film quality. Some films obtained from gelati-

nous preparations were of such low transparency that scattering losses contributed half of the measured "absorption." Even for such inferior films use of the scattering correction gave results within about 2% of the correct value.

In view of the superior reliability of the corrected function the analysis of both the cured and uncured samples of the eight commercial Buna-N rubbers was repeated, using both corrected and uncorrected measurements. The accuracy of the results (set 3, Table VI) corresponded to ±1% average error for either function except for one sample, Perbunan-35, where gelation caused larger errors in the results obtained with the uncorrected function. An additional preparation of the gum samples was made but without the ethanol extraction. The results (set 2, Table VI) exhibited excellent reproducibility but deviated from the previous results in some cases. Extraction appears to be necessary for highest accuracy, although this is surprising in view of the fact that the method is based upon the specific nitrile absorption. However, with adequate sample preparation and avoidance of gelation, an average deviation of ±1% in results is readily obtainable in the application of the method either to gum or to cured Buna-N copolymers.

SPECTRAL-STRUCTURAL RELATIONSHIPS

It remains impossible to assign many of the bands in the spectra of polyatomic molecules to exact molecular vibrations. However, certain functional groups in the molecule frequently control certain vibrations and thus it is possible in many cases to correlate spectral absorption with molecular structure. In the case of rubber spectra, these relationships are incomplete and are mostly empirical, but because of their importance in connection with analytical work,

Table VI. Acrylonitrile Analysis of Commercial Buna-N Copolymers

Sample		Measurements[a]					Nitrile Determination[b]			
							4.47		(4.47)	
Copolymer[c]	Set[d] and run	4.60 mu	4.47 mu	(4.47)	3.50 mu	(3.50)	3.50	%	(3.50)	%
Perbunan-10	1 a	98.0%	83.5/0.78	85.0/0.070	16.7/0.777	17.0/0.770	0.100	07.2	0.091	08.2
	b	96.0%	86.7/0.062	90.0/0.046	34.0/0.468	35.2/0.453	0.132	09.4	0.101	08.8
	2 a	99.0%	85.5/0.068	86.5/0.063	26.7/0.574	27.0/0.568	0.108	08.0	0.101	08.8
	b	100.0%	84.5/0.072	84.5/0.072	20.0/0.699	20.0/0.699	0.103	07.4	0.103	09.0
Perbunan-16	1 a	98.5%	72.8/0.138	74.0/0.130	20.6/0.686	20.9/0.680	0.201	14.0	0.191	14.8
	b	99.5%	86.7/0.072	87.2/0.060	47.8/0.320	48.0/0.318	0.194	13.6	0.189	14.6
	2 a	97.0%	70.0/0.155	72.5/0.140	20.0/0.699	20.7/0.684	0.222	15.2	0.205	15.6
Perbunan-18 (20.5%)	1 a	—	59.2/0.227	—	17.3/0.761	—	0.298	19.6	—	—
	b	—	68.0/0.167	—	28.8/0.541	—	0.309	20.0	—	—
	c	—	63.8/0.196	—	22.0/0.658	—	0.298	19.6	—	—
	2 a	99.0%	77.8/0.108	78.5/0.106	36.0/0.444	36.5/0.438	0.244	16.6	0.242	17.6
	b	97.5%	66.7/0.176	69.0/0.160	21.1/0.675	21.8/0.662	0.261	17.4	0.244	17.6
	3 a	97.5%	61.0/0.214	63.0/0.200	17.8/0.750	18.3/0.738	0.285	18.6	0.271	19.0
	b	95.5%	60.5/0.218	63.3/0.198	18.9/0.724	19.8/0.704	0.301	19 5	0.282	19.5
	4 a	—	62.0/0.208	—	22.8/0.642	—	0.324	20.6	—	—
	b	—	63.8/0.195	—	24.7/0.607	—	0.321	20.4	—	—
	c	87.5%	58.3/0.234	67.0/0.174	18.3/0.738	21.0/0.678	0.317	20.2	0.257	18.4
Perbunan-26 (26.7%)	1 a	—	54.7/0.262	—	25.5/0.593	—	0.442	26.2	—	—
	b	—	45.8/0.339	—	17.2/0.764	—	0.444	26.4	—	—
	c	—	62.7/0.203	—	33.0/0.483	—	0.420	25.2	—	—
	d	—	61.0/0.214	—	33.0/0.485	—	0.442	26.2	—	—
	2 a	97.5%	63.5/0.197	65.0/0.187	32.8/0.484	33.7/0.472	0.407	24.6	0.396	24.8
	b	95.5%	45.5/0.342	47.5/0.323	15.5/0.810	16.3/0.788	0.422	25.4	0.410	25.4
	3 a	98.0%	51.7/0.286	52.8/0.78	22.8/0.642	23.2/0.632	0.445	26.4	0.440	26.6
	b	98.0%	52.8/0.277	54.0/0.268	26.0/0.583	26.7/0.574	0.475	27.8	0.467	27.7
	4 a	—	47.8/0.320	—	17.6/0.755	—	0.425	25.4	—	—
	b	—	51.7/0.287	—	22.7/0.644	—	0.447	26.4	—	—
	c	88.0%	47.8/0.320	54.5/0.264	17.8/0.750	20.2/0.696	0.427	25.6	0.378	—
Perbunan-35 (33.5%)	1 a	—	37.5/0.426	—	21.6/0.664	—	0.643	34.6	—	—
	b	—	50.8/0.294	—	35.4/0.452	—	0.651	34.8	—	—
	c	—	40.1/0.397	—	23.8/0.624	—	0.638	34.4	—	—
	2 a	80.5%	39.0/0.408	48.3/0.316	24.4/0.612	30.3/0.518	0.667	35.4	0.610	33.2
	b	84.5%	54.0/0.267	64.0/0.194	39.5/0.403	46.6/0.330	0.662	35.2	0.590	32.4
	3[e] a	68.5%	31.6/0.500	46.3/0.334	20.6/0.686	30.0/0.523	0.730	37.6	0.640	34.2
	b	73.4%	35.0/0:456	47.7/0.322	23.3/0.633	31.8/0.498	0.720	37.4	0.647	34.5
	4 a	—	38.3/0.416	—	22.8/0.642	—	0.650	34.8	—	—
	b	—	51.1/0.292	—	35.0/0.456	—	0.640	34.4	—	—
	c	81.0%	44.5/0.352	54.8/0.260	27.8/0.566	34.2/0.466	0.633	34.2	0.557	31.2
Chemigum-N-1 (30.8%)	1 a	—	46.7/0.328	—	24.5/0.608	—	0.540	30.6	—	—
	b	—	51.0/0.292	—	28.4/0.544	—	0.537	30.4	—	—
	c	—	36.2/0.440	—	15.7/0.804	—	0.548	30.8	—	—
	2 a	97.0%	44.0/0.356	45.5/0.342	16.7/0.777	17.3/0.762	0.458	27.0	0.448	27.0
	b	98.5%	56.7/0.264	57.6/0.240	27.8/0.556	28.2/0.550	0.442	26.2	0.436	26.4
	3 a	95.0%	43.3/0.364	45.5/0.342	20.0/0.700	21.0/0.678	0.520	29.6	0.505	29.2
	b	96.5%	51.1/0.292	53.0/0.276	26.6/0.575	27.6/0.560	0.508	29.2	0.493	28.8
	4 a	—	47.2/0.326	—	25.8/0.588	—	0.55	31.0	—	—
	b	—	52.7/0.287	—	31.6/0.515	—	0.558	31.2	—	—
	c	93.5%	51.0/0.292	54.8/0.260	29.0/0.538	31.0/0.510	0.542	30.6	0.510	29.4
Chemigum-N-2 (20.7%)	1 a	—	65.6/0.181	—	24.8/0.604	—	0.300	19.2	—	—
	b	—	52.7/0.276	—	14.6/0.834	—	0.332	21.0	—	—
	c	—	56.8/0.244	—	16.2/0.788	—	0.310	19.8	—	—
	2 a	98.5%	74.5/0.128	75.7/0.120	30.0/0.523	30.5/0.516	0.245	16.2	0.232	17.0
	b	97.5%	67.8/0.168	69.7/0.156	21.6/0.665	22.3/0.652	0.253	16.6	0.240	17.4
	3 a	98.0%	63.3/0.198	64.8/0.188	20.0/0.700	20.4/0.690	0.283	18.2	0.272	19.0
	b	93.5%	60.0/0.222	64.3/0.192	19.4/0.712	20.8/0.682	0.312	20.1	0.282	19.5
	4 a	—	54.0/0.268	—	15.0/0.820	—	0.327	20.6	—	—
	b	—	64.5/0.191	—	26.4/0.578	—	0.330	20.8	—	—
	c	90.0%	64.0/0.194	71.0/0.150	24.0/0.620	26.5/0.576	0.313	20.0	0.260	18.4
Chemigum-N-3 (40.0%)	1 a	—	53.5/0.270	—	44.0/0.354	—	0.764	39.0	—	—
	b	—	34.4/0.462	—	25.8/0.586	—	0.790	39.8	—	—
	2 a	90.0%	21.6/0.655	24.0/0.620	15.0/0.824	16.7/0.777	0.806	40.2	0.798	39.8
	b	94.5%	39.5/0.403	41.8/0.380	31.7/0.500	33.5/0.475	0.806	40.2	0.800	40.0
	3 a	94.0%	32.8/0.484	35.0/0.456	26.1/0.584	27.8/0.556	0.830	41.0	0.820	40.6
	b	90.0%	27.8/0.556	30.8/0.511	22.2/0.654	24.7/0.608	0.850	41.7	0.840	41.2
	4 a	—	42.2/0.375	—	33.7/0.472	—	0.795	39.8	—	—
	b	—	51.2/0.291	—	44.0/0.356	—	0.820	40.8	—	—
	c	94.5%	34.5/0.462	36.5/0.438	27.8/0.556	29.5/0.530	0.830	41.0	0.828	41.0
Hycar OR-15 (41.8%)	1 a	—	26.5/0.574	—	18.0/0.740	—	0.776	39.2	—	—
	b	—	32.8/0.484	—	24.5/0.611	—	0.793	39.8	—	—
	c	—	44.8/0.348	—	38:0/0.420	—	0.830	41.0	—	—
	2 a	96.0%	31.0/0.508	32.4/0.490	21.6/0.665	22.6/0.646	0.780	39.4	0.758	38.6
	b	98.0%	54.5/0.264	55.5/0.256	46.7/0.330	47.7/0.322	0.800	40.0	0.795	39.8
	3 a	97.0%	32.2/0.492	33.4/0.476	25.6/0.592	26.4/0.578	0.830	41.0	0.825	40.8
	b	95.5%	32.8/0.484	34.3/0.464	27.2/0.566	28.5/0.545	0.870	42.3	0.853	41.7
	4 a	—	35.4/0.451	—	27.2/0.566	—	0.798	40.0	—	—
	b	—	54.5/0.264	—	47.8/0.321	—	0.822	40.8	—	—
	c	87.0%	39.0/0.410	44.8/0.348	30.5/0.516	35.2/0.454	0.795	39.8	0.785	39.4
Hycar OR-25 (33.0%)	1 a	—	42.2/0.373	—	20.2/0.692	—	0.540	30.4	—	—
	b	—	38.8/0.411	—	18.0/0.745	—	0.552	31.0	—	—
	c	—	49.7/0.303	—	28.2/0.549	—	0.553	31.0	—	—
	2 a	95.5%	59.0/0.230	61.8/0.210	37.2/0.430	39.0/0.408	0.535	30.4	0.515	29.6
	b	96.0%	39.0/0.408	40.5/0.392	17.8/0.750	18.5/0.732	0.544	30.6	0.535	30.4
	3 a	95.5%	42.2/0.375	44.2/0.355	22.2/0.654	23.2/0.634	0.575	32.0	0.560	31.2
	b	92.5%	40.5/0.392	44.0/0.356	21.7/0.664	23.5/0.629	0.590	32.6	0.566	31.5
	4 a	—	42.0/0.377	—	20.3/0.692	—	0.545	30.6	—	—
	b	—	45.3/0.344	—	24.2/0.616	—	0.559	31.4	—	—
	c	87.5%	46.7/0.331	53.8/0.269	25.6/0.592	29.4/0.532	0.560	31.2	0.506	29.2

[a] Percentage transmittance and extinction at each analytical wave length given as T/E. Parentheses at head of column indicate values corrected to 100% transmittance at 4.60 mu.
[b] Using indicated extinction ratio and appropriate working curve of Figure 5.
[c] Nitrile content as determined by Kjeldahl analysis given in parentheses.
[d] Numbers refer to sample preparations; letters designate several films studied. Preparations 1,2,3 are of gum samples; preparation 2 is without extraction; preparation 4 is of cured and compounded samples.
[e] Gelatinous sample and correspondingly unsatisfactory films.

Table VII. Acrylonitrile Analysis of Special Buna-N Copolymers

Sample		Measurements[a]					Nitrile Determination[b]			
							4.47		(4.47)	
Copolymer[c]	Set[d] and run	4.60 mu	4.47 mu	(4.47)	3.50 mu	(3.50)	3.50	%	(3.50)	%
1 (0.9%)	1 a	90.0%	89.0/0.050	99.0/0.00	19.0/0.720	21.0/0.678	0.070	04.8	0.00	0.0
	b	93.5%	92.5/0.034	99.5/0.00	17.2/0.764	18.5/0.732	0.040	02.4	0.00	0.0
	2 a	96.5%	96.0/0.020	99.5/0.00	09.0/01.05	09.1/01.04	0.020	00.0	0.00	0.0
	b	98.0%	97.5/0.010	99.5/0.00	19.0/0.720	19.3/0.715	0.010	00.0	0.00	0.0
2 (4.2%)	1 a	98.5%	91.0/0.040	92.7/0.032	20.5/0.688	21.0/0.678	0.058	04.0	0.047	4.4
	1 b	99.0%	93.3/0.030	94.5/0.024	29.4/0.532	29.8/0.526	0.056	03.6	0.046	4.2
	2 a	99.0%	92.2/0.035	93.2/0.030	19.0/0.270	19.2/0.717	0.049	03.2	0.042	4.0
	2 b	99.0%	92.3/0.035	93.3/0.030	20.0/0.699	20.2/0.695	0.050	03.2	0.043	4.0
3 (8.7%)	1 a	97.5%	79.5/0.100	81.7/0.087	14.5/0.838	14.8/0.828	0.119	08.8	0.105	9.2
	1 b	97.5%	85.5/0.068	88.0/0.055	25.0/0.602	25.7/0.590	0.113	08.2	0.093	8.6
	2 a	99.0%	84.0/0.075	85.0/0.070	20.0/0.699	20.2/0.695	0.107	07.8	0.101	8.8
4 (14.0%)	1 a	98.0%	76.7/0.115	78.5/0.105	23.3/0.632	23.8/0.622	0.182	12.8	0.169	13.6
	1 b	98.5%	71.0/0.148	72.5/0.140	15.5/0.810	15.8/0.800	0.183	12.8	0.175	13.8
	2 a	98.5%	74.0/0.130	75.2/0.124	19.0/0.720	19.2/0.716	0.180	12.6	0.173	13.8
	2 b	99.0%	75.5/0.122	76.5/0.116	20.6/0.686	20.8/0.682	0.178	12.6	0.170	13.6
5 (17.0%)	1 a	95.5%	74.5/0.128	78.0/0.108	34.0/0.470	35.5/0.450	0.272	18.0	0.240	17.4
	2 a	97.5%	60.5/0.218	62.2/0.206	14.5/0.840	14.8/0.830	0.260	17.6	0.248	17.8
	2 b	98.0%	66.7/0.176	68.0/0.168	21.1/0.676	21.6/0.666	0.260	17.6	0.252	18.0
6 (21.7%)	1 a	97.5%	51.5/0.288	53.0/0.276	16.7/0.777	17.0/0.768	0.370	23.0	0.360	23.2
	1 b	97.5%	61.7/0.210	63.5/0.198	24.4/0.612	25.2/0.598	0.343	21.6	0.332	22.0
	2 a	96.0%	62.8/0.202	64.2/0.192	26.0/0.584	26.7/0.574	0.346	21.8	0.335	22.0
	2 b	92.5%	57.2/0.242	58.5/0.232	20.6/0.686	21.0/0.678	0.352	22.2	0.342	22.4
7 (27.0%)	1 a	96.5%	58.3/0.234	60.5/0.218	34.0/0.468	35.0/0.456	0.500	28.8	0.478	28.2
	2 a	96.0%	41.7/0.286	53.8/0.270	25.0/0.602	26.0/0.584	0.475	27.8	0.463	27.6
	2 b	92.5%	43.3/0.363	47.0/0.328	19.0/0.722	20.4/0.690	0.503	29.0	0.475	28.0
8 (32.3%)	1 a	90.0%	30.0/0.523	33.5/0.474	15.5/0.810	17.3/0.762	0.645	34.6	0.622	33.6
	2 a	93.5%	37.8/0.422	40.5/0.392	19.5/0.710	20.8/0.682	0.595	32.8	0.575	31.8
	2 b	91.0%	39.0/0.410	42.7/0.370	20.0/0.699	22.0/0.658	0.585	32.4	0.563	31.4
9[e] (35.2%)	1 a	68.0%	35.5/0.450	51.5/0.288	29.0/0.538	42.0/0.378	0.836	41.2	0.762	38.6
	1 b	54.5%	35.5/0.450	65.3/0.184	31.0/0.508	57.0/0.244	0.885	42.8	0.755	38.4
	2 a	67.0%	29.0/0.538	43.3/0.363	19.5/0.710	28.2/0.550	0.758	38.6	0.660	35.0
10 (38.2%)	1 a	85.5%	24.0/0.620	28.0/0.554	16.7/0.778	19.5/0.710	0.797	40.0	0.780	39.2
	1 b	98.0%	35.0/0.456	39.5/0.404	26.7/0.574	30.0/0.523	0.795	40.0	0.775	39.0
	1 c	88.0%	26.0/0.585	29.8/0.526	19.0/0.722	21.5/0.668	0.810	40.4	0.788	38.6
	1 d	83.0%	27.2/0.566	33.0/0.482	20.6/0.686	24.8/0.606	0.825	40.8	0.795	38.8

[a] Percentage transmittance and extinction at each analytical wave length given as T/E. Parentheses at head of column indicate values corrected to 100% transmittance at 4.60 mu.
[b] Using indicated extinction ratio and appropriate working curve of Figure 5.
[c] Nitrile content as determined by Kjeldahl analysis given in parentheses.
[d] Numbers refer to sample preparations; letters designate several films studied. Both preparations are of gum samples.
[e] Gelatinous sample and correspondingly unsatisfactory films.

as well as in the general elucidation of molecular structure, some of the correlations which have been found useful in this investigation are summarized here.

Bands due to C—H vibrations are found in the spectra of all hydrocarbon-type elastomers and these may be considered collectively before passing on to those which characterize the various elastomers. The bands in the neighborhood of 3.4 mu are due chiefly to C—H valency (stretch) vibrations, while those at 7 mu are due to C—H deformation (bending) vibrations. The study of a large number of hydrocarbons has shown that $-CH_3$, methyl, groups absorb at 3.37 mu and at 7.25 mu while $>CH_2$, methylene, groups absorb at 3.42 mu, and that both methyl and methylene groups absorb at 3.5 mu and in the 6.81 to 6.98 mu region. Greater spectral resolution has shown that the absorption in the 3.4 mu region is complex (*7*) and the presence of additional unresolved bands determines the exact contour of the bands obtained here and confuses the assignment. However, the correlations obtained under moderate resolution agree with the frequencies assigned to the stronger C—H bands by Fox and Martin (*7*) and are useful in identifying the various types of C—H bands when they are present in significant proportions and in not too great variety. These bands in Figures 2 and 4 are too strong to show the resolvable structure, but results obtained with thinner films were consistent with the expected absorptions for all of the elastomers, except for a minor difference in the case of Butyl where a band at 3.50 mu was expected but not found. Two additional bands at 3.67 and 2.32 mu are found in the spectra of most elastomers, as well as in the spectra of most hydrocarbons, and are due to combinations or harmonics of other C—H frequencies.

Natural (Hevea) rubber consists of long chainlike molecules composed of the recurring isoprene unit, I, and all the strong

$$-CH_2-\underset{\displaystyle CH_3}{\overset{|}{C}}=CH-CH_2- \qquad (I)$$

bands in the spectrum are consistent with this structure.

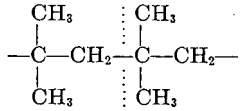
Wave Length, Mu	Absorbing Structure
3.37	$-CH_3$
3.42	$>CH_2$
3.50	$-CH_3$, $>CH_2$
6.00	C=C (valency vibration)
6.90	$-CH_3$, $>CH_2$
7.25	$-CH_3$
11.95	$\overset{CH_3}{\overset{|}{-C}}=CH-$

Sheppard and Sutherland (*12*) suggest that weak absorption around 3 mu is composed of a band at 3.1 which is to be distinguished from hydroxyl (oxidation) absorption at 3.0 mu. Increased resolution obtained here has located this band at 3.05 mu, while the oxidation impurity absorption (which always appears stronger in vulcanized and reclaimed samples) appears to be nearer 2.9 mu. The 3.05 mu band is characteristic of the rubber polymer although it is frequently masked by the hydroxyl impurity absorption. It has previously been assigned (*12*) either to a combination frequency or to the fundamental of the hydrogen vibration relative to a tertiary carbon. The same authors also report a new band at 4.98 mu which has not been confirmed here. This band was found for only one sample and apparently originates in an impurity. However, most hydrocarbons absorb weakly in this region and under proper conditions—i.e., using a thick film—a band at 4.93 mu would be expected in the spectrum of natural. All other bands reported by Sheppard and Sutherland were also found in this investigation.

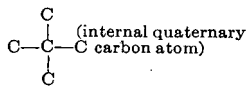
Weak bands in the region of 5.8 mu are due to C=O, carbonyl, oxygen resulting from oxidation, and, as for the 2.9 mu hydroxyl absorption, these impurity bands are stronger in vulcanizates than in gums. The 6 mu olefinic absorption varies with structure, terminal linkages (vinyl groups) absorbing at 6.10 mμ, internal double bonds absorbing at slightly shorter wave lengths and in general at much less intensity, and double bonds at branching centers usually absorbing at slightly different wave lengths than the corresponding unbranched structures. The olefinic absorption for the normal rubber skeleton absorbs at exactly 6.00 mu, and the weaker absorption at approximately 6.1 mu probably results from olefinic groups modified by removal from methyl branching centers or by proximity to the end of the polymer chain. Absorption around 6.5 mu found by Barnes (*3*) but not by Sheppard and Sutherland (*12*) was not consistent in the various samples and must be due to an impurity. Since stearates (*12*) and many soaps of other fatty acids absorb strongly at 6.5 mu, the absorption of natural gum rubber in this region probably results from the presence of unextracted vegetable soaps or esters. It is noted that this absorption becomes weaker after vulcanization, which would tend to decompose these impurity compounds.

For natural rubber, as for the other elastomers, the bands beyond 7.5 mu are generally characteristic of larger molecular groupings, so that correlation of absorption and structure is more difficult. In addition, correlations which have been found for liquid hydrocarbons are not necessarily applicable to rubber spectra, since considerable variation in position and intensity of absorption occurs with change of state. However, the strong band at 11.95 mu has been assigned by Thompson and Torkington (*15* to type $R_1R_2C{=}CHR_3$ structures and this corresponds to structure I for natural rubber, provided that R_1 or R_2 is methyl. Since the bands which characterize other olefinic structures such as those in IV and V (see below) are absent, all double bonds in natural rubber apparently occur at branching positions. Absorption at 11.25 mu may be due to type $R{-}(CH_3)C{=}CH_2$ structure at the ends of the elastomer chains. 2-Methyl-1-heptene absorbs strongly at this wave length and the terminal structure would also account for the weak olefinic absorption around 6.10 mu. The intensity of the 11.25 mu absorption varies for different samples and may be dependent upon molecular weight, although this has not been investigated. The question of *cis-trans* isomerism about the double bond (*9*) is being considered but must be deferred for the present.

Butyl rubber, or GR-I, designates a class of elastomers prepared by low-temperature copolymerization of isobutylene with a very small percentage of a diolefin—e.g., isoprene. The elastomer chains undoubtedly consist chiefly of head-to-tail polyisobutylene units, II, separated occasionally by a 1,4-addition isoprene unit, I. The small olefin content (about 0.6 mole %) contributes the necessary property of vulcanizability, but does not contribute significantly to the absorption, so that the spectrum of GR-I corresponds to that of pure head-to-tail polyisobutylene (*15*), and all the strong bands correlate entirely with structure II.

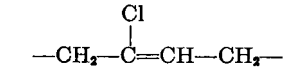


(II)

Wave Length, Mu	Absorbing Structure
ca. 3.40	$-CH_3$, $>CH_2$
6.80	$-CH_3$, $>CH_2$
7.20, 7.30	$-CH_3$ (deformation vibration; two methyls on same carbon atom)
8.13	 (internal quaternary carbon atom)
8.56	
10.52	
10.82	

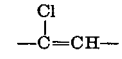
In some cases the 3.4 mu absorption was resolved into methyl and methylene bands at 3.38 and 3.44 mu, but the bands are diffuse and difficult to resolve. A band ordinarily found at 3.50 mu is missing, which indicates that the absorption in this region probably consists of several unresolved bands. Interpretation of the bands at 8.56, 10.52, and 10.82 is uncertain, but their origin certainly lies in distortion vibrations of the carbon skeleton (*15*). A quaternary carbon atom does not seem fundamentally related to these bands, however; 1,1,6-triisobutylhexane, 1,1,2-triisobutylethane, 1,1-diisobutyltridecane, and isobutylbenzene all absorb at 8.55, 10.55, and 10.85 mu (but not at 8.0 to 8.3 mu) while isopropylbenzene absorbs at 10.85 mu only.

Neoprene is a trade-name which designates a variety of elastomers formed by polymerization of chloroprene and composed of the recurring structure III, which differs from I only in that the methyl has been replaced by chlorine.



III

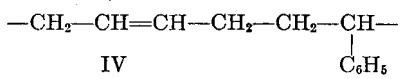
Accordingly, the spectrum of neoprene agrees with that of natural if allowance is made for the expected effects of chlorine substitution.

Wave Length, Mu	Absorbing Structure
3.42	$>CH_2$
3.50	$>CH_2$
6.02	$C{=}C$
6.90, 6.98	$>CH_2$
8.92	
12.10	

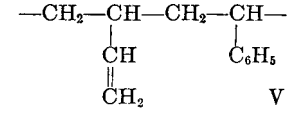
An unresolved band at 3.30 mu is probably due to a fundamental vibration of hydrogen relative to a tertiary carbon (*7*), and the 3.38 mu methyl band is absent. Oxidation impurity bands at 2.90 mu and from 5.5 to 6.0 mu are generally present, especially in cured samples. The 6.02 mu band with a shoulder at 6.12 mu is analogous to a similar band in the spectrum of natural and is correlated with the olefinic structure, although Sears (*11*) suggests that a harmonic of the 12.10 mu band may contribute here too. The 6.90 mu band is the usual C—H deformation and the 6.98 mu band has a similar origin, the shift resulting from chlorine substitution (*15*) on adjacent carbon atoms. Other reproducible bands are found at 7.42, 7.66, 8.16, 8.94, 9.20, and 10.00 mu, that at 8.94 being one of the most distinctive features of the neoprene spectrum (for analytical purposes). Sears (*11*) suggests that the weak absorption at 12.85 mu may be due to C—Cl absorption. There seem to be no strong bands between 13 and 20 mu, where C—Cl vibrations might be expected.

Neoprene-ILS is a polychloroprene which contains a small amount of acrylonitrile. Except for the nitrile absorption at 4.47 mu, the spectrum of Neopren-ILS is virtually identical with that of Neoprene-GN and Neoprene-E.

Buna-S is a generic designation for the copolymers of butadiene with styrene, of which GR-S is the particular product from emulsion copolymerization of 3 parts of butadiene with 1 part of styrene. Condensation can occur by either 1,4- or 1,2-addition, giving, respectively, the structural units IV and V, both of which are shown by the infrared spectrum.



IV

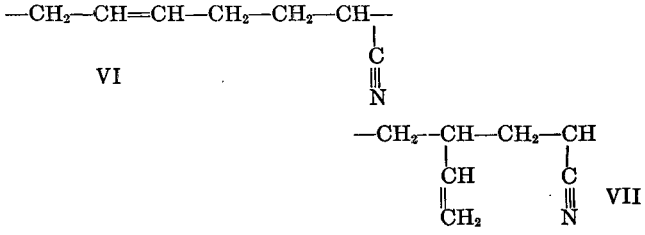


V

Wave Length, Mu	Absorbing Structure
3.42	>CH_2
3.50	>CH_2
6.10	>C=CH_2
6.23	Phenyl, C_6H_5—
6.70	Phenyl, C_6H_5—
6.88 | 6.96	>CH_2
10.35	—CH=CH—(1,4- addition)
10.97	—CH=CH_2 (1,2- addition) (vinyl group)
13.16	Phenyl, C_6H_5—
14.28	Phenyl, C_6H_5—

Aromatic C—H absorption, which usually appears as a band or doublet at 3.26 and 3.30 mu, is indicated only by a shoulder in the GR-S spectra and probably is unresolved owing to interference from an additional tertiary C—H band at 3.35 mu. The 6.10 mu band is due to terminal olefinic (vinyl) groups and a weaker band at 6.00 mu is characteristic of internal olefinic linkages. The bands from 7 to 9 mu are definitely reproducible for GR-S, but they have not been interpreted. Thompson and Torkington (*15*) have shown the strong 10.35 mu band to be associated with the structure $R_1CH{=}CHR_2$, and the 10.97 (and also the weaker band at 10.05) to be associated with the structure $RCH{=}CH_2$ of a vinyl group, all these bands arising from CH deformation vibrations. The relative intensities of these bands have been used at this laboratory and elsewhere (*6*) to derive information concerning the relative amounts of 1,4- and 1,2- addition in butadiene polymers and it is noted that 1,4- addition (structure IV) predominates in GR-S as in other emulsion polymers. The 14.3 mu band is characteristic of all monoalkyl benzenes and a band around 13.2 mu seems to appear whenever the alkyl group differs from the methylene structure at the attachment carbon.

Buna-N is a generic designation for a variety of copolymers—Hycar OR, Perbunan, Chemigum, Butaprene—formed by emulsion copolymerization of butadiene with 15 to 40% of acrylonitrile. Condensation can proceed by either 1,4- or 1,2- addition, giving, respectively, structural unit VI or VII. The spectrum is similar to that of the structurally analogous Buna-S,



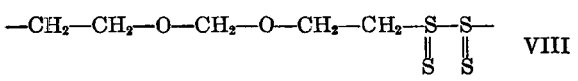
VI

VII

except that the phenyl bands are all absent and a new nitrile band due to the C≡N valency vibration (*14*) appears at 4.47 mu.

Wave Length, Mu	Absorbing Structure
3.42	>CH_2
3.50	>CH_2
4.47	—C≡N
6.10	>C=CH_2
6.90	>CH_2
10.32	—CH=CH— (1,4- addition)
10.86	—CH=CH_2 (1,2- addition) (vinyl group)

Thiokol is commonly used to designate the sulfur-containing elastic resins formed by condensation of sodium polysulfide with ethylene dichloride or its derivatives. Thiokol-FA is one variety made by reaction of sodium polysulfide with dichloroethylformal to give the recurring structure VIII.



VIII

As expected, the infrared spectrum bears no great resemblance to the spectra of the common hydrocarbon-type elastomers.

Wave Length, Mu	Absorbing Structure
3.42	>CH_2 (valency)
6.82	>CH_2 (deformation)
7.08	
7.26	
7.82	
8.36	
8.64	
8.98	
9.32	
9.68	
10.53	

Except for the S—H, mercaptan, frequency at 3.9 mu, which is not present here, very little is known about the characteristic absorption frequencies of sulfur compounds (*12*), especially these concerned in structure VIII. The aliphatic ether oxygen groups probably account for the absorption envelope in the 8.5 to 10.5 mu region, but the origins of the discrete maxima, however, are not known.

Thiokol-RD, contrary to the usual terminology, is a non-sulfur material formed by interpolymerization of butadiene, acrylonitrile, and an unsaturated ketone. The molecular units of the interpolymer can hardly be predicted with completeness, but the expected Buna-N structures VI and VII as well as additional ketonic structures are evident from the spectrum.

Wave Length, Mu	Absorbing Structure
3.42	>CH_2 (aliphatic)
3.50	>CH_2, —CH_3
4.47	—C≡N (nitrile)
5.86	C=O (carbonyl)
6.00	>C=C< (aliphatic)
6.84 to 6.94	>CH_2 and —CH_3 (deformation)
7.25	—CH_3 (deformation)
7.38	
10.25	—CH=CH—(1,4- addition)
10.86	—CH=CH_2 (1,2- addition) (vinyl group)

The band at 5.86 mu is too strong to be due to oxidation impurities and must be ascribed to the presence of carbonyl oxygen structures incorporated into the elastomer during polymerization.

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Determination of Glycerol in Fermentation Residues

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Glycerol can be satisfactorily determined by measurement of the formaldehyde formed on periodic acid oxidation of a glycerol-containing solution. Substances present in fermentation residues which produce formaldehyde on oxidation with periodic acid are removed by treatment in ethanol solution with carefully controlled quantities of lime; defecation with lead and silver salts is unnecessary. Previously suggested methods for determining of glycerol were experimentally examined for applicability in analyzing fermentation residues for glycerol.

AT THE present time no entirely satisfactory analytical method is described in the literature which is specifically adapted for the determination of glycerol in fermentation residues, such as the stillage or slop left after the alcohol has been removed by distillation from the solution resulting from an alcoholic fermentation. Similarly, there is no suitable method for the determination of glycerol in residues obtained by concentration of the slop by evaporation, or in material derived from these residues. Fermentation slop usually contains 2 to 5% or more of material other than water and may result from the fermentation of blackstrap or invert molasses, corn or other grain, or mixtures of such materials to produce as primary products ethanol, whisky, or rum. In normal alcoholic fermentation, three to seven parts of glycerol may be produced for every hundred parts of ethanol; the solid or nonvolatile material—i.e., the nonaqueous material—contains 4 to 8% of glycerol.

A method for determining the glycerol content of such residues is desirable when utilizing the slop or the solids obtained on filtration and drying of the slop for feeds, feed supplements, core binders, and other products; the glycerol content determines to a considerable extent the hygroscopic nature of the material. In processing the slop to recover specific components or to convert the material to other forms, a knowledge of the glycerol content is usually necessary. In experiments on the recycling of slop in fermentations, such as "back-slopping," the accumulation of glycerol must be known.

The methods for the determination of glycerol in soap lyes or in mixtures of other related organic compounds cannot be directly applied to the type of material derived from a fermentation because of the complicated and—to a considerable extent—unknown nature of the fermentation residue solids. Table I gives the approximate composition of the nonaqueous material in a slop derived from the ethanolic fermentation of blackstrap molasses. The problem of determining glycerol in such a mixture is largely a matter of separating or isolating the glycerol in a state where accompanying material would not interfere with the measurement of the glycerol present by chemical or physical means. The method described consists of the removal of interfering substances by treatment with controlled quantities of lime in alcoholic solution, oxidation of the final glycerol fraction with periodic acid, and isolation and measurement of the formaldehyde formed from the glycerol.

A number of methods for the determination of glycerol in fermentation samples are described by Lawrie (*7*), the best of which are discussed below.

The only material even approximately analogous to fermentation residues in nature, for which methods for the determination of glycerol have been standardized, is wine. Official A.O.A.C. method (*3*) for the determination of glycerol in wines, which is similar to the method for glycerol in spices and other commodities, is a long and tedious one, involving numerous evaporations, precipitations of interfering material with lime, silver carbonate, and basic lead acetate, extraction with anhydrous ethanol and ether, and final oxidation with dichromate solution; the assumption is made that glycerol will be the only substance remaining which will reduce dichromate. This procedure is based on one developed by Neubauer and Borgmann (*9*) in 1878.

Table I. Approximate Composition of Nonwater Material of Slop or Residue from Ethanolic Fermentation of Blackstrap Molasses

		%
A.	Nonvolatile solids on drying at 105° C.	93
	Ash after heating to 500° C. in air	23
	Ash, insoluble in water	8
B.	Total invert sugar	19
	Proteins and amino acids	6
	Yeast and other water-insoluble organic matter	20
	Glycerol	6
	Organic acids Sugars other than invert sugar Caramel substances—e.g., complex sugar anhydrides Fats Miscellaneous condensation products such as melanoidins	16
	Ammonium compounds as $(NH_4)_3PO_4$	4
	Potassium chloride	15
	Calcium phosphate	2
	Calcium sulfate	6
	Silica and iron salts	1
	Water	5

After the present authors had begun work on a method for the determination of glycerol, the work of Amerine and Dietrich (*1*) on a simpler procedure than that of the A.O.A.C. for determining glycerol in wine came to their attention. Amerine and Dietrich clarify the sample of wine by treatment with lead acetate and barium hydroxide, and oxidize the final glycerol solution with periodic acid, determining the amount of the periodic acid which is consumed; this procedure is based on the work of Fleury and Fatome (*4*, *5*) on the same determination. Both the A.O.A.C. and Amerine-Dietrich methods failed to yield satisfactory results when applied by the authors to fermentation residues and derived materials; in addition, the procedures were too tedious and subject to manipulative error to be applied to the analysis of any considerable number of samples.

The losses in glycerol which occur on the evaporation of aqueous and alcoholic glycerol solutions were studied by Raveux (*11*), who confirmed the fact that the addition of sodium hydroxide is preferable to lime as a means of decreasing the glycerol loss. Raveux also reviewed the methods which have been proposed for the isolation of a pure glycerol solution in connection with its determination in fermentation products and residues. The proposed methods usually involve one or more of three principal approaches: (1) defecation in aqueous or alcoholic solution of pro-

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teins, sugars, and other nonglycerol substances by the addition of lead acetate, lime, or barium hydroxide; (2) extraction with organic solvents; and (3) steam distillation at reduced pressure. None of the proposed methods is entirely satisfactory when applied to fermentation residues; the only satisfactory one, described by Lawrie (*7*), involves vacuum steam distillation of sufficient sample to give 4 to 6 grams of glycerol. This method was tried in the authors' laboratory and was found satisfactory, but the need of using a huge sample and the time required for each analysis made the use of the method inapplicable to the number and size of samples to be expected in consistent research or control work.

Raveux's procedure (*11*) for determining glycerol in complex mixtures such as fermentation residues was based on the separation of the glycerol from the other constituents by entrainment in the vapors of superheated ethanol passed over the dried sample which contained a small amount of alkali suspended on asbestos. The ethanol is removed by evaporation and the glycerol is measured by oxidation by dichromate in nitric acid solution. Free mineral acids and large amounts of alkali retard distillation of the glycerol, while the organic acids found in fermentation residues distill over to a greater or lesser extent. Appreciable amounts of salts also decrease the glycerol recovery. In the presence of sizable quantities of sugars a preliminary defecation with lime in alcohol solution is necessary.

Raveux (*10*) has reviewed the literature on the determination of pure aqueous solutions of glycerol, covering various physical methods and a variety of chemical methods including acetylation, reaction with hydriodic acid, conversion into acrolein, formation of a complex with copper, and oxidation by different reagents such as bromine, ceric sulfate, iodic acid, periodic acid, and dichromates in sulfuric or nitric acid solution. The oxidation procedures yield either carbon dioxide or other organic compounds; in the former case the excess of oxidizing agent is determined and in the latter, either the excess of oxidizing agent or the products formed from the glycerol are determined.

Whyte (*14*) has indicated the inherent errors of the official international acetin and dichromate methods for glycerol.

REAGENTS AND APPARATUS

The following reagents are necessary for the preliminary clarification procedure: C.P. grade lime, 95% ethanol, 95% ethanol saturated with lime, 30% sodium hydroxide solution, approximately 2 *N* sulfuric acid solution, and an indicator solution of methyl red.

The approximately 0.5 *N* periodic acid solution required in the measurement of the glycerol by oxidation to formaldehyde is prepared by dissolving 11 grams of periodic acid (G. Frederick Smith Chemical Co.) in distilled water and diluting to 100 ml. The solution is stored in a dark, glass-stoppered bottle.

The simple distilling apparatus used for isolating the formaldehyde consists of a semimicro 100-ml. Kjeldahl flask with a 25-cm. neck, a spray trap, and a vertical condenser about 35 cm. long, 20 cm. of which are water-cooled. The authors used apparatus with standard-taper glass joints and a distilling rack which held six flasks.

For the polarographic determination of the formaldehyde, any polarograph will suffice; the authors used the Fisher Elecdropode and the Leeds & Northrup Electro-Chemograph with a dropping mercury cathode and a quiet pool of mercury as the anode. The temperature of the polarographic cell should be controlled to ±0.2° C. The solutions necessary are the stock base solution of 1 *N* lithium hydroxide in 0.1 *N* lithium chloride and a stock standard solution of formaldehyde (approximately 0.8 mg. per ml.) which is prepared from commercial formalin and standardized by the hydrogen peroxide method.

The electrometric titration of the formaldehyde requires a fairly good means of measuring the pH of the titrated solution; the authors used a Beckman Model G pH meter with a glass electrode. The solutions required are standard 0.1 *N* hydrochloric acid solution, approximately 0.1 *N* sodium hydroxide solution, approximately 0.1 *N* sodium thiosulfate solution, 12% sodium sulfite solution, and thymol blue indicator solution.

The glycerol used as standard was found to be 95.5% pure with water as the only apparent impurity based on refractive index and specific gravity measurements, and the determination of water by the Karl Fischer method and of glycerol by various methods.

DEVELOPMENT OF PROCEDURE

In the systematic studies which led to the development of the procedure reported in this paper, four solutions were used as standard known samples to obtain information on the recovery of glycerol and on possible interference. These solutions were a 5% solution of glycerol in water, a solution containing 5% glycerol and 5% glucose, a sample of concentrated slop of about 50% solids content containing 3% glycerol, and a sample of processed concentrated slop of about 50% solids content containing 1.5% glycerol.

The approach to the problem of obtaining a solution in which the glycerol could be measured was determined in large part by the decision to use as the final measuring procedure, other things being equal, the measurement of the formaldehyde formed on periodic acid oxidation of the glycerol-containing solution. This procedure was decided upon, inasmuch as it would permit any organic matter which did not form formaldehyde on periodic acid treatment to be present in the final glycerol solution. Thus, while 1,2-dihydroxy compounds such as sugars would have to be absent, most organic acids and other compounds could be present. Most of the other procedures, physical or chemical, for the measurement of the glycerol content of a solution, require a practically pure aqueous solution—e.g., the usual oxidimetric procedures depend upon a measurement of the total oxidizable material present, while the acetin and similar esterification procedures measure the total reactive hydroxyl content.

The general method used in studying the separation of the glycerol was to set up systems for treating the slop so as to obtain a satisfactory glycerol fraction; the criteria for the latter were the production on periodate oxidation of the correct amount of formaldehyde for the glycerol content of the original sample and the nonproduction of glycerol for glycerol-free samples of typical compounds of the types expected to be present. The purification procedures used were the most promising ones found in the literature—e.g., those described by the A.O.A.C. (*2*), Amerine and Dietrich (*1*), and Raveux (*11*); variations of these procedures based on omission of one or more steps as well as rearrangement of steps; procedures based on the results obtained with the two previously listed types of procedures; and suitable variations of the last-mentioned type of procedures. By this more or less logical method of attack, it was possible to evaluate the efficacy of the individual steps in the procedures and to develop a simpler procedure for the type of material in which the authors were interested—i.e., fermentation residues.

A large number of procedures was examined and an enormous number of numerical results was obtained. The detailed description and discussion of these procedures and data are being omitted, since the amount of space required would probably be in excess of the value to be derived from the material; instead, the most directly pertinent results are briefly summarized.

The official A.O.A.C. method (*2*), when applied to fermentation residues, was found not only tedious but subject to manipulative errors. It was difficult to obtain a final glycerol solution free of other interfering organic material; in addition, there was a variable loss of glycerol during the process. Consequently, this procedure could not be directly applied to the problem under investigation.

Based on the work reported by Amerine and Dietrich (*1*) in which wines containing glycerol were subjected to a two-stage clarification process, using neutral lead acetate and alcoholic barium hydroxide, followed by a periodic acid oxidation, a procedure was developed in which the resulting formaldehyde was detected and determined polarographically. While consistent duplicable results could be obtained by one analyst using this procedure when a set of determinations was conducted simultaneously, the results were found to differ by as much as 100% rela-

tive, either when duplicates of the same sample were analyzed at a different time by the same analyst or when a number of analysts performed the determinations, independently, at the same time. An extensive study was made of the various steps involved in this procedure to ascertain the degree to which the results are affected by unavoidable manipulative modifications entailed in adapting the method to routine analysis. Extremely careful control and standardization of all obvious physical factors and technique gave results that showed a precision with a maximum deviation of approximately 12%, relative, from the average when the procedure was followed by different analysts or by the same analyst at different times. Modification of the various steps did not materially change these results. For example, it was found that up to a sixfold increase in the sample size did not affect the results appreciably, nor did the use of a larger volume of the lead acetate solution have a significant effect on the values found. The length of time for which the precipitate was allowed to remain in contact with the lead acetate–treated solution was varied from 5 minutes to overnight (16 hours); this factor had little effect on the precision of the determination.

Since the variables of sample size and lead acetate contact time do not appreciably affect the precision of the determination, an attempt was made to eliminate the lead acetate clarification step in order to reduce the number of possible errors in the procedure. In addition, talc, which was used by Amerine and Dietrich as a filter aid, was also omitted. From results obtained, it appears that when lead acetate is omitted from the procedure, higher values are obtained, indicating that this reagent either removes interfering substances or actually removes some of the glycerol itself by adsorption on the resulting precipitate. At any rate, this modification did not improve the precision of the determination.

Increasing the temperature of the barium hydroxide treatment from 50° to 100° C. and omitting the lead acetate clarification gave a lower average for the glycerol content of the sample and reduced the precision of the determination.

Reversing the order of the barium hydroxide and lead acetate clarification procedures resulted in the same values which were obtained when the lead acetate treatment was omitted; however, the precision was improved.

In view of the fact that standardization of technique and the modifications described did not give reproducible results, it was concluded that certain unknown factors inherent in the method were responsible for the erratic results. This belief was strengthened by the work of Raveux (*10*, *11*) who points out that, under properly selected conditions, small quantities of glycerol can be quantitatively extracted from solutions of complex mixtures by distillation in alcohol vapors. Inasmuch as one of the steps in the original procedure of Amerine and Dietrich involves the distillation of alcohol from a solution containing glycerol, and since no precaution was exercised in preventing the loss of glycerol by entrainment in ethanol vapors, it appeared essential that this factor be considered in the scheme of analysis. One of the most effective means of reducing the loss of glycerol during the process of removing the alcohol is by the addition of a small amount of sodium or barium hydroxide to the solution prior to the distillation of the alcohol. Another source of error in the procedure originally investigated, which was discussed by Raveux, is the choice of alcoholic barium hydroxide as a clarifying agent. Because of the retention of glycerol by barium hydroxide, Raveux recommends that lime be used instead. Therefore the procedure was altered by replacing the barium hydroxide with lime and distilling the alcohol from a Kjeldahl flask containing a small amount of sodium hydroxide; the precision was greatly improved, giving a maximum deviation from the average of approximately 3%. Using this revised procedure, a study was then made of the effect of the reagents employed and the steps involved in order to ascertain the accuracy of the glycerol determination. In this regard, the effect of the lead acetate clarification, the lime clarification, and the alcohol distillation was investigated using a sample of pure glycerol in water, a synthetic mixture of glycerol and glucose in water, and a sample of processed slop.

When the solution of glycerol in water, which was found to contain 5.75% glycerol by direct periodic acid oxidation and by refractive index measurement, was subjected to the revised procedure, replicate values of 5.36, 5.31, and 5.27% glycerol were found for an average recovery of 92%. The synthetic mixture, containing 4.86% glycerol and 5% glucose in water, gave 4.56, 4.58, 4.66, and 4.54% glycerol for an average recovery of 94%. A loss of approximately 7% glycerol was therefore noted in both series of determinations. In order to determine where this loss occurs, each step in the procedure was tested.

When the lead acetate clarification was omitted, an average of 5.56% glycerol was found for the solution of glycerol in water, which is a recovery of 97%; omitting the lime clarification but using the preliminary lead clarification gave a 100% recovery of the glycerol present; the lead clarification step alone did not affect the recovery of the theoretical amount of glycerol.

The results of these experiments indicate that there are two factors responsible for the loss of glycerol: (1) the lime clarification and (2) the combination of lime and lead which probably removes some of the glycerol as a complex. Thus, the lime itself caused a loss of approximately 3% of the glycerol, and the combination of lime and lead caused a loss of approximately 7% of the glycerol.

Since the lead acetate alone does not affect the accuracy of the determination, but the combination of lead acetate and lime does reduce the recovery considerably, an attempt was made to remove the excess lead after the preliminary clarification before the addition of lime. When sodium sulfate and hydrogen sulfide were used to precipitate the lead prior to the lime clarification, there was still a loss of glycerol amounting to approximately 7%. Apparently this latter experiment contradicts the one in which it was found that by omitting the lead a quantitative recovery of glycerol can be obtained, since all the lead was first removed before the lime was added. There is a probable explanation for this unexpected result in the possible removal of a significant amount of glycerol from the solution by adsorption on the precipitated lead.

The loss of glycerol due to the lime was eliminated by reducing the quantity of lime from 2 grams to 1 and omitting the lead acetate clarification. In this way a recovery of 100% was obtained with the aqueous glycerol solution and the mixture of glycerol and glucose. The sample of processed slop, which gave a value of 1.48% by the revised procedure based on Raveux's experiments, was found to contain 1.83% glycerol when the lead was omitted and the lime was decreased to 1 gram. This latter value for the glycerol in the slop sample was increased to a higher extent than was anticipated. Therefore, it was desired to determine whether the lead acetate removed any substances from the slop which yield formaldehyde when treated with periodic acid. If no interfering substances are removed by the lead, then the preliminary clarification is unnecessary. The need for lead was tested by performing the lead clarification, collecting and washing the resulting precipitate, and submitting it to a periodic acid oxidation. The results obtained with the same sample used in the previous experiments showed that negligible quantities of formaldehyde were produced; this indicated that the processed slop does not contain substances that interfere with the periodic acid oxidation determination of glycerol. A similar test was made on the precipitate resulting from the lime clarification. In this case it was found that the precipitate from the sample of glycerol in water yielded practically no formaldehyde; the precipitate from the synthetic mixture of glycerol and glucose gave slightly higher than the theoretical amount of formaldehyde based on the amount of glucose present; and the processed slop gave an amount of formaldehyde corresponding to 1.6% glucose. The

Table II. Determination of Glycerol in Synthetic Mixtures and Concentrated Slop

	Nature of Sample			
	Glycerol, 5.14%	Glycerol, 4.94% Glucose, 4.7%	Glycerol, 5.54%	Concentrated slop
	Glycerol Found			
	%	%	%	%
Run 1	4.87	5.03	..	3.39
	4.79	5.09	..	3.52
Run 2	5.14	5.00	..	3.51
	..	5.08	..	3.28
Run 3	..	4.91	5.49	3.43
	..	..	5.46	3.36
Average, $\bar{x}$	4.93	5.02	5.48	3.42
Average deviation	0.10	0.05	0.02	0.07
Standard deviation, σ	0.18	0.09	0.04	0.11
Standard error, σ_m	0.10	0.04	0.03	0.04
Confidence range, $\bar{x} \pm 2\sigma_m$	4.93 ± 0.20	5.02 ± 0.08	5.48 ± 0.06	3.42 ± 0.08
Accuracy, $\bar{x}$ − true value	0.21	0.08	0.06	
Confidence range in terms of % recovery	96 ± 4	102 ± 2	99 ± 1	100 ± 3
Accuracy in terms of % recovery	4	2	1	..

Table III. Recovery of Glycerol Added to Concentrated Slop

Glycerol (3.31%) in Slop	Glycerol (95.5%) Added	Total Glycerol Present	Glycerol Found	Glycerol Recovered
Mg.	*Mg.*	*Mg.*	*Mg.*	%
113.6	248.7	362.3	319.0	88
116.4	169.9	286.3	294.8	103
110.9	136.4	247.3	240.0	97
47.0	93.8	140.8	150.3	107
55.0	92.3	147.3	145.2	99
49.2	137.6	186.8	194.5	104
Average, $\bar{x}$				102
Average deviation				3
Standard deviation, σ				5
Standard error, σ_m				2
Confidence range, $\bar{x} \pm 2\sigma_m$				102 ± 4
Accuracy, $\bar{x}$ − true value				2

Table IV. Determination of Glycerol in Samples of Treated Slop

Sample No.	2840	2842	2844	2937	2939	2948	3590	2591	2593	2596	3596	3597	3598	3600
Glycerol found, %	3.23	1.19	1.96	1.49	1.24	3.58	1.21	1.53	0.87	0.84	1.34	1.49	1.45	1.69
	3.31	1.15	1.93	1.37	1.26	3.68	1.29	1.45	0.88	0.91	1.42	1.54	1.41	1.71
	3.38					3.61								

and clarification process was determined by the glycol procedure described by Warshowsky and Elving (*12*), consisting of periodate oxidation, aldehyde separation by distillation, and polarographic measurement of the formaldehyde formed; for routine work, the formaldehyde can be advantageously determined titrimetrically instead of polarographically.

Although the polarographic procedure for the measurement of formaldehyde is probably the most specific under the circumstances, it requires very careful control of the conditions recommended to obtain accurate results. Accordingly, the cyanide and sulfite methods for the determination of formaldehyde as described by the A.O.A.C. (*2*) were also investigated. The precision of both these methods was good, although the value obtained by the cyanide method on aqueous glycerol solutions was usually slightly lower than the theoretical value. In addition, interference due to the liberation of iodine was encountered when the cyanide procedure was used to estimate the formaldehyde obtained by the oxidation of glycerol in slop samples. Consequently, the sulfite method, using the Beckman Model G pH meter for determining the end point, was adopted for routine work, since it proved satisfactory from the viewpoint of accuracy, reproducibility, and facility of operation. The results given in Tables II, III, and IV were obtained by titrimetric and polarographic methods.

PROCEDURE

The clarification, oxidation, and recovery steps of the procedure are the same for either of the two procedures for measuring the formaldehyde produced on oxidation of the glycerol; either measuring procedure may be used. If desired, the distillate obtained from the aldehyde recovery procedure can be divided and the formaldehyde measured by both of the procedures described.

Clarification. Weigh a sample containing 150 mg. of glycerol into a 50-ml. volumetric flask and dilute to volume with distilled water. Add a 10-ml. aliquot to 1 gram of lime in a 125-ml. Erlenmeyer flask, and heat at 50° C. on a water bath for 30 minutes. Add 50 ml. of 95% ethanol, stir, and filter by decantation through a Büchner funnel, using gentle suction and a fine filter paper. Wash the residue five times with 5-ml. portions of 95% ethanol saturated with lime. Combine the washings and filtrate, and quantitatively transfer the solution to a 250-ml. beaker with about 20 ml. of distilled water (3 × 7 ml.). Add approximately 1 ml. of a 30% sodium hydroxide solution to the mixture and evaporate on a water bath until approximately 20 ml. of the solution are left. Neutralize the solution to methyl red with 2 *N* sulfuric acid solution, transfer it to a 100-ml. Kjeldahl flask, and dilute to approximately 50 ml. with water. Only 1 or 2 drops of methyl red solution should be used; excess methyl red results in the formation of iodine during the oxidation step.

Oxidation and Formaldehyde Recovery. Add 5 ml. of the periodic acid solution to the solution in the Kjeldahl flask and a few beads to prevent bumping. Place the Kjeldahl flask in an upright position and connect it to the distilling unit. Collect the distillate in a 250-ml. beaker containing 50 ml. of distilled water, which is sufficient to cover the end of the condenser to a depth of 2 cm. Heat the contents of the Kjeldahl flask gently at first, and distill the solution over at a rate of 3 to 4 ml. per minute until about 5 ml. remain in the Kjeldahl flask. Near the end of the distillation, lower the receiver so that the end of the condenser no longer extends below the surface of the liquid in the receiver. After distillation is complete, rinse the end of the condenser with distilled water.

Titrimetric Determination of Formaldehyde. If the color of iodine is visible in the distillate, discharge the color by the cautious addition of 0.1 *N* thiosulfate solution. Carefully neutralize the distillate containing the formaldehyde and formic acid pro-

latter experiments clearly demonstrate that the lime does remove the substances which interfere with the determination.

Another experiment, performed to illustrate that the use of lead is not necessary, consisted of treating a sample of slop with the lime in the usual manner and then treating the resulting filtrate with lead acetate. It was noted that practically no precipitate was obtained, merely a slight cloudiness. This indicates that the lime removes from the slop all substances which are precipitated by neutral lead acetate, and consequently the preliminary lead clarification is not necessary for samples such as slop.

The procedure in which the lead clarification was omitted and the quantity of lime reduced to 1 gram was applied directly to samples of ethyl molasses slop. The slop itself, which contained approximately 53% by weight of solids, was found to contain 2.94 and 2.89% glycerol. When samples of this slop were diluted with water so that the percentage of glycerol was theoretically reduced to 1.54 and 0.37%, respectively, the corresponding average experimental values found were 1.59 and 0.37%.

The application of the Malaprade periodate oxidation reaction (*8*) to glycols has been reviewed by Raveux (*10*) and by Warshowsky and Elving (*12*), who covered the determination of glycols via the measurement of the aldehydes produced (*6*, *12*). Whitnack and Moshier (*13*) found that formaldehyde, in the presence of other aldehydes, is most satisfactorily determined polarographically in a base solution which is 0.1 *N* in lithium hydroxide and 0.01 *N* in lithium chloride; the removal of dissolved oxygen is unnecessary.

The glycerol in the final solution obtained in the purification

duced by the periodic acid oxidation of the glycerol with 0.1 *N* sodium hydroxide solution to a pH of 9.3 as determined electrometrically, using the Beckman pH meter and a glass electrode as the indicating electrode. Add approximately 25 ml. of a 12% sodium sulfite solution to the mixture and titrate the liberated alkali potentiometrically with standard 0.1 *N* hydrochloric acid solution to the initial pH of 9.3. An internal indicator such as thymol blue may be added in order to aid in detecting the approach of the end point. Titrate a blank, using distilled water instead of the distillate of the sample solution, and subtract the volume of standard acid used from the amount required in the titration of the sample.

$$\% \text{ glycerol} = \frac{\text{ml. of acid consumed} \times \text{normality of acid} \times 4.605}{\text{grams of sample in aliquot taken}}$$

Polarographic Determination of Formaldehyde. Quantitatively transfer the distillate to a 250-ml. volumetric flask, taking care not to exceed a total volume of 225 ml. The formaldehyde solution can be kept in this state until ready for the polarographic measurement. Immediately prior to the latter, add 25 ml. of the 1 *N* lithium hydroxide in 0.1 *N* lithium chloride solution to the flask and dilute to volume; this results in a volume which is 0.1 *N* in lithium hydroxide and 0.01 *N* in lithium chloride.

Rinse the polarographic cell and electrodes several times with the solution to be analyzed. Place a sample of the solution in the cell and permit it to come to constant temperature. Record the polarogram, using a dropping mercury cathode and a quiet mercury pool anode; alternatively, read the galvanometer deflections at applied voltages of −1.40 and −1.63 volts (dropping mercury electrode versus mercury pool electrode). The height, or diffusion current, of the formaldehyde wave is the difference between these two points. Compare this wave height to that obtained from a 250-ml. solution prepared from the stock standard formaldehyde solution. The concentration of formaldehyde in the latter solution should be approximately the same as that in the sample solution polarographed.

$$\% \text{ glycerol} = \frac{H_f \times V_s \times C_s \times 153.4}{H_s \times S}$$

where H_f = formaldehyde wave height from periodic acid oxidation of glycerol in the sample; H_s = formaldehyde wave height of standard used; V_s = volume of stock standard solution used; C_s = mg. of formaldehyde per ml. of stock standard solution; and S = weight of sample in mg. present in the aliquot.

DATA

The procedure described was applied to the determination of glycerol in synthetic mixtures of glycerol-water and of glycerol-glucose-water, and in concentrated slop of about 50% solids content from a molasses fermentation. The results obtained on these samples at different times by one analyst are shown in Table II. The indexes of precision used are the following:

$$\text{Standard deviation, } \sigma, = \frac{1}{C_2}\sqrt{\frac{\Sigma d i^2}{n-1}}$$

$$\text{Standard error, } \sigma_m, = \frac{\sigma}{\sqrt{n}}$$

$$\text{Confidence range} = \bar{x} \pm 2\,\sigma_m$$

where $\bar{x}$ is the arithmetrical average or mean, n is the number of items or results, d_i is the deviation of an individual result from the mean, and C_2 is a correction factor for the small number of items used and is taken as equal to $(n - 0.8)/n$.

The accuracy is the difference between the mean value and the true value. The value found for the glycerol content of the concentrated slop was in accord with that based on the weighed average of results obtained by a variety of methods including large-scale vacuum steam distillation as recommended by Lawrie (7). The aqueous glycerol sample (5.54% glycerol) and the concentrated slop were analyzed by a second analyst, who obtained average values of 5.48 and 3.31% glycerol, respectively, with a precision similar to that shown in Table II. Subsequent analysis of the four samples by other analysts indicated similar precision and accuracy.

A sample of the residue obtained after solvent removal from the fermentation of grain to produce ethanol was analyzed to obtain an idea of the precision obtainable; the results for the percentage of glycerol in the raw slop were 0.37 and 0.36. Samples of two wines, muscatel and sherry, were analyzed by the procedure described and the following results were obtained: muscatel, 0.75, 0.71, 0.71, and 0.79%; sherry, 0.89 and 0.86%.

To study the reliability of the method further, a series of recovery experiments was performed in which the glycerol content was determined for samples of concentrated slop to which varying amounts of glycerol were added. The results of this study, shown in Table III, were satisfactory except in one case; the fact that in this case the largest absolute amount of glycerol was present is believed to be coincidental, since similar amounts of glycerol have been successfully determined. The results obtained, omitting the result mentioned, indicate that the method probably has no systematic error and has a precision shown by an average deviation calculated from five values of ±3 parts per hundred.

As a further test of the precision of the method when applied in routine analysis, a series of samples obtained from processing concentrated slop in various ways by chemical and physical treatment was analyzed by several analysts under routine conditions over a period of several days. These results, given in Table IV, show a mean average deviation of 2 parts per hundred; the greatest average deviation of a set of results was 5 parts per hundred.

SUMMARY

Glycerol can be satisfactorily determined in simple and complex solutions, such as fermentation residues, by measurement of the formaldehyde formed on periodic acid oxidation of a glycerol solution. Substances in fermentation residues which interfere in the estimation of the glycerol by themselves producing formaldehyde on periodate oxidation are removed effectively by clarification with lime in ethanol solution. The amount of lime used must be carefully controlled for correct results. A preliminary defecation with lead or silver salts is unnecessary.

The formaldehyde can be determined by any good method; the polarographic method, which is most specific, and the sulfite method with electrometric titration, which is best for routine work, are described.

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Chemical Determination of Tryptophan

Study of Color-Forming Reactions of Tryptophan, p-Dimethylaminobenzaldehyde, and Sodium Nitrite in Sulfuric Acid Solution

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The paper describes the optimum conditions for carrying out the color-forming reaction between tryptophan, *p*-dimethylaminobenzaldehyde, and sodium nitrite in both 13.2 *N* and 19 *N* sulfuric acid, and gives several procedures for the quantitative determination of tryptophan.

PROCEDURES based on the colors formed by oxidation of the condensation products of tryptophan with various aldehydes have long been used for the qualitative and quantitative determination of tryptophan. These methods have included the use of glyoxalic acid (*1, 8*), formaldehyde (*16, 21*), benzaldehyde (*4, 5, 13, 14*), vanillin (*10, 13, 14*), salicylaldehyde (*5, 13, 14, 21*), and *p*-dimethylaminobenzaldehyde (*7, 15*).

The early history of the development of methods for the quantitative estimation of tryptophan in proteins is described by Mitchell and Hamilton (*12*). Among the many procedures proposed, the *p*-dimethylaminobenzaldehyde colorimetric method is still used. Rohde (*15*) in 1905 first observed that tryptophan gave a blue color with *p*-dimethylaminobenzaldehyde and in 1913 Herzfeld (*7*) first used this reaction for the quantitative estimation of tryptophan in proteins. Further discussion of the determination of tryptophan in proteins by this and other methods is deferred to a later paper of this series.

In procedures (*3, 9, 11, 18, 19*) recently described for the determination of tryptophan in proteins, strong hydrochloric acid was used as solvent for *p*-dimethylaminobenzaldehyde and also for the test solutions. Because of corrosive and irritating fumes, the use of hydrochloric acid is objectionable. Therefore, experiments were designed to substitute sulfuric acid for hydrochloric acid as did Tillmans and Alt (*20*) who, however, used formaldehyde. *p*-Dimethylaminobenzaldehyde was considered preferable to formaldehyde because it is easily purified and is stable in the solid state. Early in this study it became apparent that a critical investigation of the reactions involved was needed.

The original objective of this work was to determine optimum conditions for obtaining maximum color when the condensation product of tryptophan and *p*-dimethylaminobenzaldehyde, in sulfuric acid solution, was oxidized with sodium nitrite. It was then planned to adapt these conditions to the determination of tryptophan, starting with intact proteins as has been done by others. It was found that maximum color was attained in 12 to 13.2 *N* acid (throughout this paper the term "acid" refers to sulfuric acid; reference to other acids is appropriately indicated). This concentration was not strong enough to determine tryptophan starting with intact proteins, but 19 *N* acid was found suitable for this purpose. This paper, therefore, describes the optimum conditions for carrying out the color-forming reaction between tryptophan, *p*-dimethylaminobenzaldehyde, and sodium nitrite in both 13.2 *N* and 19 *N* acid. Several procedures for the quantitative determination of tryptophan, based on these studies, are also described.

The reactions were studied in two steps. The combination of tryptophan and *p*-dimethylaminobenzaldehyde to form a colorless condensation product was called reaction I, and development of the blue color by oxidation of this compound with sodium nitrite was termed reaction II.

CHOICE OF WAVE LENGTH

Wave lengths from 590 to 600 mμ gave minimum transmittancy with the tryptophan–*p*-dimethylaminobenzaldehyde color de-

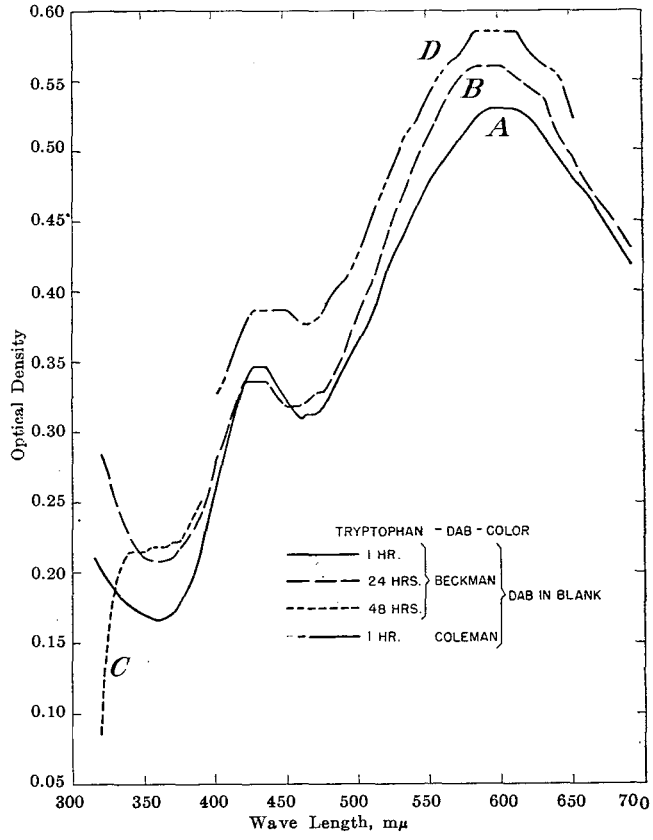

Figure 1. Absorption Curves of Tryptophan–*p*-Dimethylaminobenzaldehyde Color Obtained from Free Tryptophan

A, B, C. **Color developed by Procedure B, using 100 micrograms of tryptophan per test. Reaction I proceeded 1, 24, and 48 hours, respectively, before color developed. Transmittances determined with Beckman quartz spectrophotometer, using 10-mm. cuvettes**

D. **Color developed by Procedure B using 70 micrograms of tryptophan per test. Reaction I proceeded 1 hour before color developed. Transmittancies determined with Coleman Model 11 spectrophotometer**

Blank solutions contained 30 mg. of *p*-dimethylaminobenzaldehyde per test, 0.1 ml. of 0.04% sodium nitrite added

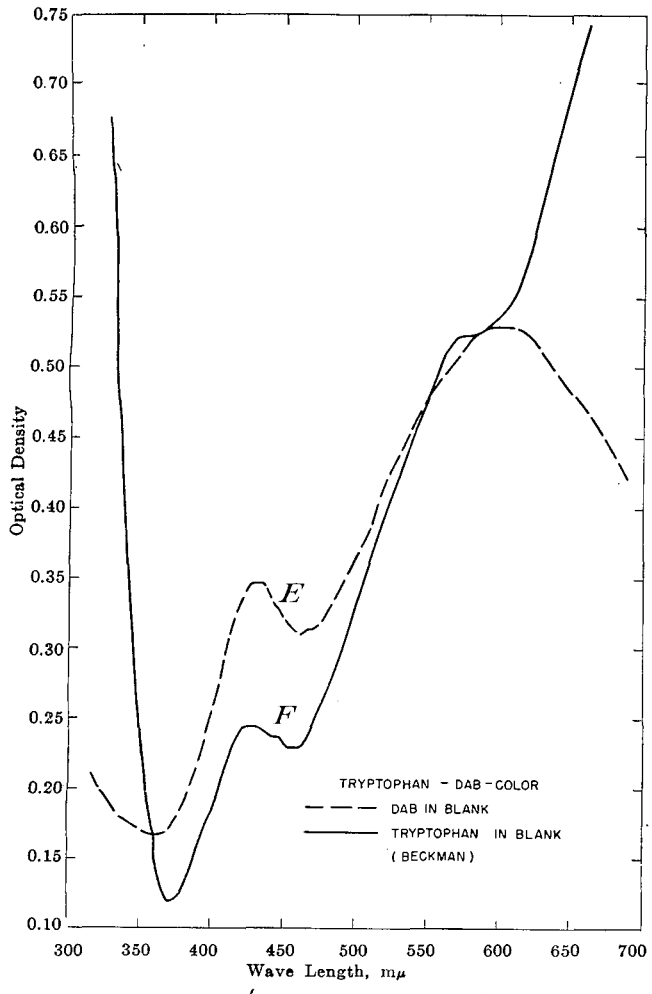

Figure 2. Absorption of Tryptophan–*p*-Dimethylaminobenzaldehyde Color from Free Tryptophan

Transmittancies determined with Beckman spectrophotometer, using 10-mm. cuvettes

veloped under optimum conditions in 13.2 *N* acid according to Procedure A. (Procedures A to H are described below.)

Wave-length–density curves of the *p*-dimethylaminobenzaldehyde–tryptophan color developed under optimum conditions in 19 *N* acid are shown in Figures 1 and 2.

Curves A and D were obtained with the Beckman and Coleman spectrophotometers, respectively. The region of maximum density ranged from 580 to 610 mμ with the Coleman and from 590 to 600 mμ with the Beckman instrument. In general, the shapes of the two curves correspond as well as would be expected, considering the differences in band widths.

Curves A, B, and C, obtained with the Beckman spectrophotometer, compare the wave-length–density relationships of the color developed after the tryptophan–*p*-dimethylaminobenzaldehyde complex had stood at 25° C. in the dark for 1, 24, and 48 hours, respectively, before addition of sodium nitrite. Curve C is shown from 320 to 380 mμ only, because over the rest of the spectrum it practically coincided with curve B. The shape of these curves corresponded closely from 380 to 690 mμ. Maximum density for the color developed after reaction I had proceeded for 1 hour was 590 to 610 mμ and 580 to 600 mμ for the color when developed after reaction I had gone for 24 to 48 hours. The unexplained departure in the shape of curve C from that of curves A and B in the spectral region from 320 to 380 mμ is of interest, but it has no bearing on the analytical method.

The effect of the composition of the blank solution on the wave-length–density curves is shown in Figure 2.

Curves E and F were obtained on the same colored solution with the Beckman spectrophotometer. Curve E was read against a blank solution containing 30 mg. of *p*-dimethylaminobenzaldehyde, and curve F was obtained with a blank solution differing only in that it contained 100 micrograms of tryptophan and no *p*-dimethylaminobenzaldehyde. Points on the curve read first were checked after the second curve was obtained to show that no change occurred during the time required for the readings.

Table I. Effect of Acid Concentration on Rate of Reaction I and on Amount of Color Formed at 25° C.

Normality of Acid	Time in Minutes									
	0	1	5	15	30	60	120	240	420	1440
	% Maximum Color Formed									
10.1	...	...	..	..	38	58	80	89	92	83
12	4.1	6.1	21	48	69	86	96	100	100	100
13.2	3.1	10	35	63	83	92	96	100	100	100
14.1	3.1	12	42	75	89	96	96	96	...	96
15.1	4.1	21	56	82	89	92	92	96	...	92
16.2	3.1	33	67	82	89	89	89	89	...	89
17.2	3.1	42	75	82	86	86	86	86	...	86
19.1	...	....	..	..	82	82	82	82	...	82

The composition of the blank solution exerted a profound effect on the density values at wave lengths ranging from 320 to 500 mμ and from 610 to 700 mμ. However, the two curves coincided at the critical wave length of 590 mμ. The coincidence of the two curves at this point is important because the tryptophan solutions to be analyzed sometimes contain some foreign color which can be eliminated by adding the test solution to the blank. This condition is usually encountered in analyzing proteins. It is concluded that a wave length of 580 to 600 mμ may be used for measurement of the tryptophan–*p*-dimethylaminobenzaldehyde color developed after reaction I had proceeded 1, 24, or 48 hours in 19 *N* acid with the blank solution containing either *p*-dimethylaminobenzaldehyde or tryptophan.

EFFECT OF SULFURIC ACID CONCENTRATION ON REACTION I

The effect of the concentration of acid on the degree of color obtained with a fixed quantity of tryptophan and *p*-dimethylaminobenzaldehyde was determined in a preliminary study of the kinetics of reaction I. The kinetic study was based on the observation that reaction I does not take place if sodium nitrite, in the concentrations used, is added to the acid solution of *p*-dimethylaminobenzaldehyde just before addition of the tryptophan. If the nitrite solution is added at any interval before reaction I is completed, no further condensation will occur, but color will be formed by oxidation of that portion of tryptophan and *p*-dimethylaminobenzaldehyde already reacted.

The relative rates of reaction I and the relative degrees of maximum color obtained in various concentrations of sulfuric acid are shown in Table I. The acid concentrations used were from 10 to 19 *N* and time intervals were from 1 to 1440 minutes.

Each test contained 100 micrograms of tryptophan and 30 mg. of *p*-dimethylaminobenzaldehyde in 10 ml. of sulfuric acid. The only variable in this series of tests was the concentration of acid used. The following example shows how tests were carried out in 13.2 *N* acid. Sample: 23.8 *N* acid, 5.5 ml.; *p*-dimethylaminobenzaldehyde, 30 mg. in 1.0 ml. of 2 *N* acid; water, 2.5 ml.; tryptophan, 100 micrograms in 1.0 ml. of distilled water; sodium nitrite, 0.1 ml. of 0.06% solution in water. The acid solution, *p*-dimethylaminobenzaldehyde, and water were mixed and cooled to 25° C. In the zero time test the sodium nitrite was added to the mixture followed by the tryptophan within 10 seconds. The transmittancy of this solution was read after it had stood for 30 minutes in the dark following addition of the sodium nitrite. In other tests the tryptophan was added and the solutions were kept at 25° in artificial light for the indicated time interval. The sodium nitrite was then added and transmittancies were read after standing 30 minutes in the dark. Blank: 23.8 *N* acid, 5.5 ml.; *p*-dimethylaminobenzaldehyde, 30 mg. in 1.0 ml. of 2 *N* acid, water 3.5 ml.; sodium nitrite, 0.1 ml. of 0.06% solution. All tests were conducted similarly except that the proportions of 23.8 *N* acid and water were varied to give

the desired acid concentrations. All blank solutions had the same acid concentration as the corresponding test solutions.

For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve A, Figure 3, and then expressed in Table I as per cent of the maximum color obtained in the series of tests. The lowest per cent transmittancy obtained in this series of tests was 10%, which was considered to represent 100% of the color. As an example with 19.1 *N* acid the lowest transmittancy obtained was 15% which represented 82% of the maximum color obtained with 13.2 *N* acid.

The rate of reaction I was more rapid in higher than in lower concentrations of acid, but more intense maximum color was obtained in the lower concentrations of acid than in the higher concentrations. Thus in 16, 17, and 19 *N* acid the reaction appeared to be complete in 30 minutes (results presented below show that there is slightly further reaction after 30 minutes in 19 *N* acid), whereas in 12 and 13.2 *N* acid the reaction required 4 hours for completion. However, maximum color was obtained in 12 and 13.2 *N* acid and the relative degrees of maximum color in 14.1, 15.1, 16.2, 17.2, and 19.1 *N* acid represented 96, 96, 89, 86, and 82%, respectively. In 10 *N* acid 7 hours were required to attain maximum color which, however, was only 92% of that obtained in 12 and 13.2 *N* acid. The condensation product was stable during 24 hours at 25° C. except in 10 *N* acid.

Nineteen normal acid is near the upper limit of concentration that can be used safely; some charring occurred in a test made with 22 *N* acid. Tillmans and Alt (*20*) used 66 to 67 weight % (21.3 *N*) sulfuric acid in their method with formaldehyde.

OPTIMUM CONCENTRATION OF *p*-DIMETHYLAMINOBENZALDEHYDE

The optimum quantity of *p*-dimethylaminobenzaldehyde required for reaction I in 13.2 *N* and 19 *N* acid was determined.

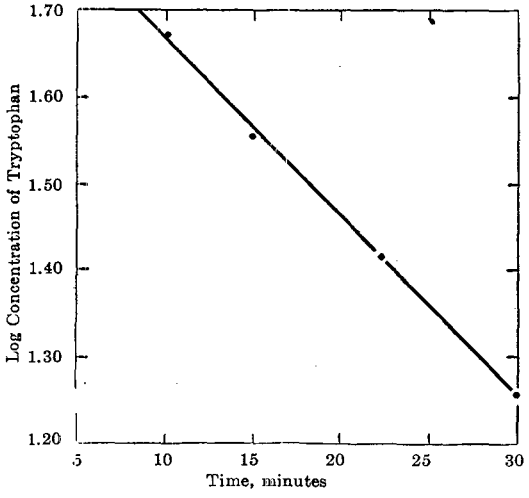

Figure 3. Pseudo First-Order Nature of Reaction I

Table II. Effect of Concentration of *p*-Dimethylaminobenzaldehyde on Amount of Color Formed in 13.2 and 19 *N* Acid

Quantity of DAB per Test, Mg.	Micrograms of Tryptophan					
	25		50		100	
	Normality		Normality		Normality	
	13.2	19.0	13.2	19.0	13.2	19.0
	% Maximum Color Formed					
1.0	32	88	32	82	36	84
2.5	56	96	56	96	61	96
5.0	80	96	78	100	82	100
10	92	100	94	100	96	100
20	100	100	100	100	100	100
30	100	100	100	100	100	100
60	92	96	92	96	96	96

Quantities ranging from 1 to 60 mg. per test were used with each of 25, 50, and 100 micrograms of tryptophan.

Tests in 13.2 *N* acid were carried out by Procedure A at room temperature. Composition of blank solutions was the same as that of test solutions, except that 1 ml. of water was substituted for the 1 ml. of tryptophan solution. Color was developed with 0.1 ml. of 0.06% sodium nitrite solution. Transmittancies were read 30 minutes after addition of the sodium nitrite. Tests in 19 *N* acid were carried out by Procedure B except that *p*-dimethylaminobenzaldehyde was added in 4 *N* acid solution in such a way that the *p*-dimethylaminobenzaldehyde solution plus the 4 *N* acid equaled 1 ml. Color was developed with 0.1 ml. of 0.06% sodium nitrite solution and transmittancies were read 40 to 60 minutes after addition of the sodium nitrite.

For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve A, Figure 3, for tests in 13.2 *N* acid and curve B, Figure 3, for tests in 19 *N* acid and then expressed in Table II as per cent of the maximum color obtained in each series of tests.

Results in Table II show that in 13.2 *N* sulfuric acid 20 and 30 mg. of *p*-dimethylaminobenzaldehyde gave maximum color with all concentrations of tryptophan, while in 19 *N* acid 10, 20, or 30 mg. per test gave maximum color. Sixty milligrams per test gave slightly less color than the maximum in both concentrations of acid. The reason for this effect is not known. Thirty milligrams per test were adopted for this work.

OPTIMUM CONCENTRATION OF SODIUM NITRITE FOR REACTION II

The kinetics and end point of reaction II were studied with both 13.2 *N* and 19 *N* acid. The data in Table III show the rate and end point of reaction II, when 100 micrograms of tryptophan and molar ratios of sodium nitrite to tryptophan ranging from 0.25 to 16.0 per test in 13.2 *N* acid were used. The rate of reaction II increased with increasing concentration of sodium nitrite. Maximum color was obtained in 60 and 180 minutes with molar ratios of sodium nitrite of 2 and 4, respectively. A curious plateau occurred when molar ratios of sodium nitrite were 8 and 16. The reaction appeared to be complete in each case in 3 minutes with color representing 86 and 79% of the maximum respectively. Then, however, a gradual increase in color occurred until after 180 minutes the color was 96 and 92% of the maximum, respectively. It is apparent that in 13.2 *N* acid the greatest sensitivity was obtained in 60 to 180 minutes when the molar ratio of sodium nitrite was 2. The color of the test solution under these conditions is blue with a violet tinge.

The data in Table IV show results of a similar kinetic study using 100 micrograms of tryptophan in 19 *N* acid. Maximum color was attained in 30 minutes with a molar ratio of sodium nitrite to tryptophan of 1.0 to 1.5. The anomalous plateau indicating apparent rapid completion of reaction II in 3 minutes when the molar ratio of sodium nitrite was 4, 8, and 16 was observed. The reaction appeared to be complete for 60 minutes but then a further intensification of color occurred between 60 and 180 minutes. On the basis of these results 0.1 ml. of 0.04% sodium nitrite reacting for 30 minutes in 19 *N* acid was adopted for tests on solutions containing only tryptophan. The pure blue color formed under these conditions is stable for at least 3 hours and shows only slight deterioration in 24 hours.

Visually, the quality of the color developed was influenced by the quantity of the oxidant used and the time of standing after addition of the oxidant. Oxidation with progressively greater quantities of sodium nitrite gave progressively deeper violet tinges to the color. The violet shade of the color tended to increase on standing, particularly in tests in which excess oxidant was present. Violet tinges were more apparent when tests were observed in ordinary artificial light than when fluorescent light was used. Violet tinges were less apparent when color was developed in 19 *N* acid than when 13.2 *N* was used. The wave length of maximum color decreased slightly when an excess of sodium nitrite was used and also after test solutions stood for 5 days.

Table III. Kinetics of Reaction II in 13.2 *N* Acid with Varying Quantities of Sodium Nitrite

Sodium Nitrite Added		Time, Minutes												
		0	1	2	3	5	7	10	15	30	60	180	1440	7200
Molar ratio[a]	%[b]	% Maximum Color Formed[c]												
0.0	...	4	3	3	3	3	3	3	4	4	...	...	5	7
0.25	0.0085	4	22	28	33	40	45	50	56	59	61	61	61	56
0.50	0.017	4	35	42	48	55	59	65	72	77	77	77	74	67
0.80	0.027	4	48	57	65	73	77	83	86	89	92	...	86	79
1.0	0.034	4	53	63	69	77	83	86	89	92	96	96	89	83
1.5	0.051	3	61	72	77	83	86	89	92	96	96	...	92	86
2.0	0.068	3	67	77	83	89	89	92	96	96	100	100	96	89
4.0	0.14	3	77	83	86	89	89	92	92	92	96	100	96	89
8.0	0.27	3	79	83	86	86	86	86	86	86	92	96	96	86
16.0	0.54	3	77	79	79	79	79	79	79	79	89	92	92	83

[a] Molar ratio: $\frac{\text{sodium nitrite}}{\text{tryptophan}}$.

[b] % concentration of sodium nitrite solution added to each test in 0.1 ml. of water solution.

[c] All tests were conducted similarly according to Procedure A at 24° to 26° C. except that transmittancies were read before and at indicated intervals after addition of sodium nitrite. For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve A, Figure 3, and then expressed as per cent of maximum color formed. The lowest % transmittancy obtained in this series of tests was 9 which represented 100% of the color. 100 γ of tryptophan used in each test.

Table IV. Kinetics of Reaction II in 19 *N* Acid with Varying Quantities of Sodium Nitrite

Sodium Nitrite Added		Time, Minutes												
		0	1	2	3	5	7	10	15	30	60	180	1440	7200
Molar ratio[a]	%[b]	% Maximum Color Formed[c]												
0.0	...	7	...	...	...	...	...	...	...	...	7	...	14	27
0.25	0.0085	7	41	53	61	68	71	75	77	79	79	...	77	77
0.50	0.017	7	68	77	82	87	90	90	93	96	96	96	93	90
1.0	0.034	7	75	84	87	90	93	96	96	100	100	100	96	90
1.5	0.051	6	84	90	93	96	96	96	100	100	96	100	96	93
2.0	0.068	5	84	90	93	93	96	96	96	96	96	100	96	90
4.0	0.14	5	87	90	93	93	93	93	93	93	93	96	96	90
8.0	0.27	5	90	93	93	93	93	93	93	93	93	96	96	90
16.0	0.54	5	90	90	90	90	90	90	90	90	90	93	93	87

[a] Molar ratio: $\frac{\text{sodium nitrite}}{\text{tryptophan}}$.

[b] % concentration of sodium nitrite solution added to each test in 0.1 ml. of water solution.

[c] All tests were conducted similarly according to Procedure B at room temperature (23° to 26° C.) except that transmittancies were read before and at indicated intervals after addition of sodium nitrite. For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve B, Figure 3, and then expressed as per cent of maximum color formed. Lowest % transmittancy obtained in this series of tests was 15, which was considered to represent 100% of the color. 100 γ of tryptophan used in each test.

From results shown in Tables III and IV, it might be considered necessary to use a constant molar ratio of sodium nitrite and tryptophan. But, practically, this is not necessary, as is shown by a later experiment (Table VIII) in which standard curves were obtained with both the constant quantity of sodium nitrite determined as optimum for 100 micrograms of tryptophan and a constant molar ratio of sodium nitrite to tryptophan. Results obtained with 10 to 120 micrograms of tryptophan were nearly identical by both methods.

EFFECT OF TEMPERATURE ON REACTION I

The effect of temperature on the rate and sensitivity of reaction I was determined by a study of the kinetics of the reaction at 25°, 45°, and 85° C. with 13.2 *N* acid (Table V).

Procedure. Tests were set up according to Procedure A. The sulfuric acid, water, and *p*-dimethylaminobenzaldehyde solution were mixed and allowed to stand at the temperature of the test for about 5 minutes. The tryptophan solution was then added, and the reaction was allowed to proceed in the dark. After the desired time interval test solutions were cooled 1 minute in tap water and the sodium nitrite solution was added (0.1 ml. of 0.068%). Transmittancies were read after standing 30 minutes in the dark. The same transmittancy was obtained when the solution was kept at 45° C. for 30 minutes after addition of the sodium nitrite as when it was kept at room temperature. At 85°, however, a transmittancy of 39% was obtained when the solution was kept at 85° for 30 minutes after adding the sodium nitrite as compared to a reading of 23% when the solution was first cooled to room temperature and kept at that temperature during color development.

The solution of *p*-dimethylaminobenzaldehyde used for the 25° and 85° tests was freshly prepared, and that for the 45° tests was 2 days old.

The maximum color for each temperature was obtained at 25°, 45°, and 85° when reaction I proceeded 3.5 hours, 2 hours, and 15 minutes, respectively. The sensitivity of the reaction was greatest at 25°. If the degree of color obtained at 25° represents 100% the relative sensitivities at 25°, 45°, and 85° were 100, 85, and 61%, respectively. According to these data, the tryptophan–*p*-dimethylaminobenzaldehyde complex was stable at 25° C. for at least 24 hours and at 45° for at least 5 hours; but at 85° it was stable for only 15 minutes.

The effect of temperature on the rate and sensitivity of reaction I in 19 *N* acid was not determined, but probably both rates of condensation and destruction of tryptophan would be greater in 19 *N* than in 13.2 *N* acid at the higher temperatures. An optimum temperature of 25° C. was chosen for reaction I in both 13.2 *N* and 19 *N* acid because of the greater stability of the tryptophan–*p* - dimethylaminobenzaldehyde complex, greater sensitivity of the reaction, and greater convenience of operation at this temperature.

The importance of temperature control is apparent from data in Table V. Thus an appreciable error might be made if transmittancies obtained in tests conducted at summer temperatures (30° to 35° C.) were converted to weight of tryptophan from a standard curve prepared at winter temperatures of 20° to 25° C. However, less extreme variations in temperature would not be important if maximum accuracy is not necessary.

The color of the tests at 85° C. was reddish violet in 15 minutes, changing to pinkish violet with increased time at 85°; at 45° a violet tinge appeared in the blue color of the tests.

RATE OF REACTION I AND STABILITY OF TRYPTOPHAN–*p*-DIMETHYLAMINOBENZALDEHYDE CONDENSATION PRODUCT AT 25° C.

The rate of reaction I and the stability of the tryptophan–*p*-dimethylaminobenzaldehyde condensation product in 13.2 *N*

Table V. Effect of Temperature on Rate and Sensitivity of Reaction I in 13.2 *N* Acid

Time, Min.	Temperature		
	25° ± 0.1° C.	45° ± 0.5° C.	83°–85° C.
	% Maximum Color Formed[a]		
0	5	..	..
1	11	24	34
3	22	..	..
5	34	48	58
10	51	..	59
15	61	69	61
30	79	79	61
60	89	82	58
Hours			
2	96	85	53
3	96	85	53
3.5	100	85	..
5	100	85	..
24	100	..	..

[a] For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve A, Figure 3, and then expressed in this table as % of maximum color formed in all tests of the series. The lowest transmittancy obtained was 9 which was considered to represent 100% of the color. 100 γ of tryptophan used in each test.

Table VI. Rate of Reaction I and Stability of Tryptophane–*p*-Dimethylaminobenzaldehyde Condensation Product in 13.2 and 19 *N* Sulfuric Acid at 25° C.

Time of Reaction I, Min.	% Maximum Color Formed			
	13.2 *N*[a]	19 *N*[b]	19 *N*[c]	19 *N*[c]
0	5	5	...	...
0.5	...	49	...	...
1	11	65	...	...
2	...	79	...	...
3	22	...	...	...
5	34	87	...	...
10	50	89	...	...
15	61	89	95	96
22	72	...	...	...
30	79	93	96	96
60	89	96	98	98
Hours				
2	96	96	98	99
3	97	96	99	100
4	99	...	100	100
5	99	...	...	...
6	100	96	100	100
7	100	...	...	...
10	...	...	100	100
24	100	100	100	100
Days				
2	...	100	99	99
3	...	97	100	100
5	...	93	93	93

[a] Procedure A was used except that reaction I was allowed to proceed for indicated time interval instead of 4 hours. For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve A, Figure 3, and then expressed in column 2 as % of maximum color formed. Lowest transmittancy obtained in this series of tests was 9, which was considered to represent 100% of the color. 100γ of tryptophan used in each test.

[b] Procedure B was used except that reaction I was allowed to proceed for indicated time interval. For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve C, Figure 3, and then expressed in column 3 as % of the maximum color formed. Lowest transmittancy obtained in this series of tests was 13.0 which was considered equivalent to 100% of the color. 100γ of tryptophan used in each test.

[c] A procedure similar to E was used except that the solution contained 100γ of tryptophan per 10 ml. of test solution. 10-ml. aliquots were withdrawn at desired intervals, and color developed according to Procedure E. Results with tryptophan sample I are shown in column 4 and sample II in column 5. For comparative purposes all transmittancies were converted to micrograms of tryptophan from curve C, Figure 3, and then expressed in respective columns as % of maximum color formed. Lowest transmittancies obtained in this series of tests was 13.1, which was considered to represent 100% of color.

and 19 *N* acids at 25° C. were determined and pertinent data are shown in Table VI. The initial rate of reaction I in 19 *N* acid (column 3) was faster than that in 13.2 *N* acid (column 2). Approximately one half of the tryptophan combined in 0.5 minute in 19 *N* acid, but 10 minutes were required for an equal degree of reaction in 13.2 *N* acid. However, maximum color developed in 6 to 7 hours in 13.2 *N* acid and in between 6 to 24 hours in 19 *N* acid.

Data in columns 4 and 5 show a comparison of the two tryptophan samples I and II, respectively, used in this study. In comparing these samples 19 *N* sulfuric acid was added directly to a mixture of solid tryptophan and solid *p*-dimethylaminobenzaldehyde at 25° C. (Procedure E). It is apparent that the two samples of tryptophan possessed identical degrees of purity as revealed by this test. Reaction I was complete in 3 to 4 hours by this procedure, as compared to 6 to 24 hours when tryptophan was added in aqueous solution.

The tryptophan–*p*-dimethylaminobenzaldehyde compound was stable in 13.2 *N* acid at least 24 hours and for 2 to 3 days in 19 *N* acid at 25° C. The deterioration in color-producing capacity amounted to not over 5% in 5 days.

ORDER OF REACTION I

The structure of the condensation product or products of tryptophan and *p*-dimethylaminobenzaldehyde has been studied by Fearon (*5*) and Ghigi (*6*). No effort was made in this study to elucidate this problem further, but reaction I is probably a second- or third-order reaction. However, under the conditions of the test, reaction I probably would behave as a first-order reaction because the molar concentration of *p*-dimethylaminobenzaldehyde (30 mg. per test) was 410 times greater than that of tryptophan (100 micrograms per test). To determine how closely reaction I follows the first-order reaction equation, the log of the concentration of unreacted tryptophan was plotted against reaction time using data from Table VI, column 2. The values for the tryptophan reacted in a given time were obtained from curve A, Figure 4. These values were corrected on the basis of the observation that at zero time 5 micrograms of tryptophan appeared to have reacted. This value was obtained from the transmittancy shown when the sodium nitrite was added to the test before the addition of tryptophan, as discussed above. The amount of reacted tryptophan, therefore, was corrected in proportion to the amount of unreacted tryptophan that remained when sodium nitrite was added. Thus, at time intervals of 5, 10, 15, 22, and 30 minutes the values of tryptophan reacted were 34, 51, 62, 73, and 81 micrograms, respectively. To obtain the quantity of unreacted tryptophan, these values were subtracted from 97, 98, 98, 99, and 99 micrograms to give 63, 47, 36, 26, and 18 micrograms of unreacted tryptophan, respectively. The log of the values plotted against time from 10 to 30 minutes, as shown in Figure 3, falls on a straight line. Reaction I, therefore, may be regarded as a pseudo first-order reaction.

STABILITY OF TRYPTOPHAN IN 19 *N* ACID

The stability of free tryptophan in 19 *N* acid at 25° C. in the dark is shown by results in Table VII. On the basis of the colorimetric test, no loss of tryptophan occurred in 2 hours but a gradual loss then took place. In 48 and 120 hours the loss was

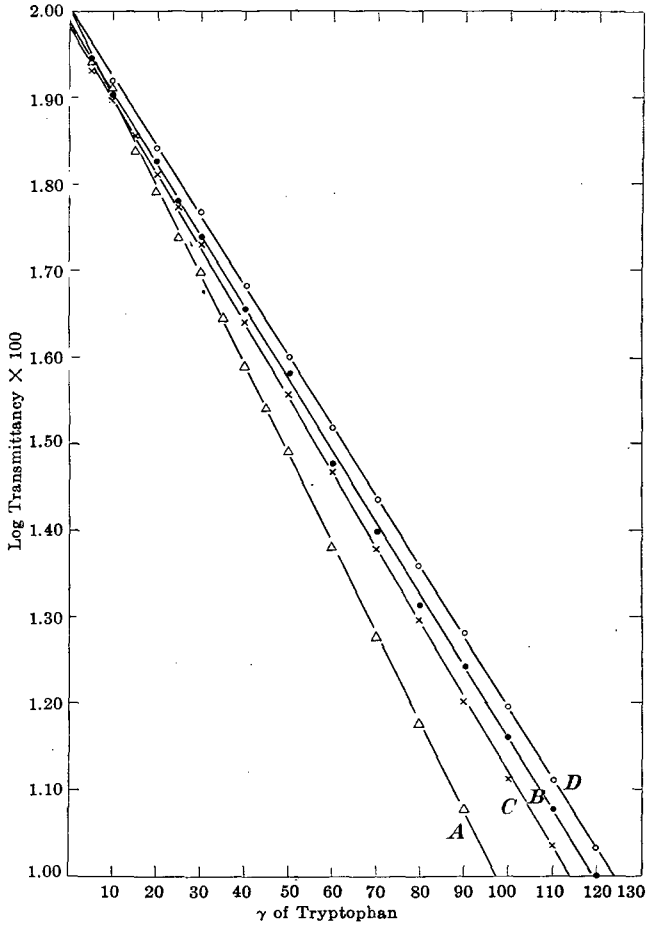

Figure 4. Standard Curves for Tryptophan

***A*. 13.2 *N* sulfuric acid, determined by Procedure A**
***B, C, D*. 19 *N* sulfuric acid, determined by Procedures B, E, and D, respectively**

Table VII. Stability of Free Tryptophan in 19 *N* Sulfuric Acid at 25° C. in the Dark[a]

Time, Hours	% Transmittancy 580 mμ	590 mμ	600 mμ	Loss of Tryptophan, %
0.1	13.7	13.1	13.2	0
0.5	13.1	13.0	13.0	0
1.0	13.2	13.1	13.1	0
2.0	13.8	13.3	13.3	0
4.0	13.9	13.9	13.9	3
6.0	14.0	13.9	13.9	3
12	14.9	14.8	14.8	6
24	14.9	14.8	14.9	6
48	15.6	15.3	15.6	8
120	16.9	16.8	16.8	12
264	18.0	18.2	18.9	16
504	22.9	23.0	23.7	28

[a] Procedure. 145.6 ml. of 19 *N* sulfuric acid added to 1.456 mg. of tryptophan. This solution was kept at 25° C. in the dark. At indicated intervals 10-ml. aliquots of solution were withdrawn and added to 30 mg. of DAB. Tryptophan determined by Procedure C. Transmittancies may be converted to weight of tryptophan from curve C, Figure 4.

8 and 12% compared with 0 and 5%, respectively, when the tryptophan was bound with *p*-dimethylaminobenzaldehyde as shown in Table VI. The loss of free tryptophan was 28% in 21 days. As shown in Table VII, the wave length of maximum color formed with *p*-dimethylaminobenzaldehyde and the aged tryptophan solutions shifted from 590–600 mμ to 580 mμ after 264 and 504 hours' standing.

These results show that, under the conditions of the analytical methods described below, practically no tryptophan is destroyed by the acid before it combines to form the relatively more stable tryptophan–*p*-dimethylaminobenzaldehyde compound.

PREPARATION OF STANDARD CURVES

Standard curves for 5 to 120 micrograms of tryptophan were determined by several representative procedures which embody optimum conditions for reactions I and II. A straight line was always obtained when the log of the per cent transmittancy was plotted against weight of tryptophan, as shown in Figure 4. The excellent conformity to Beer's law over a range of transmittancies from 10 to 88% is thus demonstrated.

Curve A, Figure 4, shows the transmittancy-concentration relationship obtained with 13.2 *N* acid by Procedure A. Although Procedure A gave maximum sensitivity, most of the subsequent tests were made in 19 *N* acid, because this concentration was regularly used in later studies on the determination of tryptophan in proteins.

Curve B, Figure 4, was obtained with 19 *N* acid by Procedure B. This procedure is very convenient because it requires only 1.5 hours.

To obtain curve C, Figure 4, reaction I was carried out in 19 *N* acid for 7 hours as described in Procedure E, in which tryptophan (sample II) and *p*-dimethylaminobenzaldehyde were added to the acid solution in the solid state. A curve practically identical to C was obtained by Procedure C, in which tryptophan was added to the test in water solution and reaction I was allowed to proceed for 24 hours. Curve C is used as a standard curve in the determination of tryptophan starting with intact proteins, as will be described in a later paper.

Curve D, Figure 4, was obtained by Procedure D. Tryptophan was added to the test in 1 *N* sodium hydroxide solution, and reaction I was carried out for 1 hour. Procedure D is similar to Procedure B except for the difference in solvent for tryptophan. It is apparent that a very slight loss of tryptophan occurs when tryptophan is added in the alkaline solution. This loss is probably caused by the presence of a trace of oxidant in the sodium hydroxide and not by the slightly greater heat produced on adding the tryptophan in alkaline rather than in water solution to the test. Procedure D is used in determining the tryptophan content of alkaline hydrolyzates of proteins, as will be described in a later paper.

The curves shown in Figure 4 are indicative of the relative sensitivity of the various procedures, because all tests were made under comparable conditions with the same cuvettes and with pure tryptophan samples.

Results shown in Figure 4 were all obtained with the constant optimum quantity of sodium nitrite (determined for 100 micrograms of tryptophan) for all concentrations of tryptophan. From results shown in Tables III and IV it might be expected that it would be necessary to use a constant ratio of sodium nitrite to tryptophan. However, as shown in Table VIII, the transmittancy values obtained, with either the constant molar ratio or constant optimum quantity of sodium nitrite, are in excellent agreement. Therefore, because of greater convenience, the constant optimum quantity of sodium nitrite, as shown in Tables III and IV, was used for all quantities of tryptophan over the range of the test.

EFFECT OF ADDED SUBSTANCES

Indole, Skatole, and Tryptamine. The effects of indole, skatole, and tryptamine on the analysis of tryptophan, are shown in Table IX. The appearance of each test, as well as the wave length of maximum color, is also shown.

Indole is without significant effect on the test, as shown by a transmittancy of 36.0% for 50 micrograms of tryptophan alone and 36.1% when the test contained 50 micrograms of indole plus 50 micrograms of tryptophan.

Skatole, 50 micrograms, gave a transmittancy equivalent of 23 micrograms of tryptophan. The tryptophan equivalence of skatole, 50 micrograms, and tryptophan, 50 micrograms, combined was 80 micrograms, an increase of 7 micrograms over the sum of the two substances when determined alone.

Tryptamine, 50 micrograms, gave a transmittancy equivalent to 68 micrograms of tryptophan. On a molar basis 50 micrograms of tryptamin are equivalent to 60.4 micrograms of tryptophan. Therefore, mole for mole tryptamine produces about 13% more color than does tryptophan. When tryptamine and tryptophan were combined, the transmittancy was 9.0%, which is equivalent to about 130 micrograms of tryptophan. But the sum of the two determined separately was 118 micrograms. So here, as with skatole, the effectiveness in color production is

Table VIII. Comparison of Transmittancies

(Constant optimum quantity of sodium nitrite and a constant optimum ratio of sodium nitrite and tryptophan)

Procedure A				Procedure B			
Tryptophan per Test, γ	Constant molar ratio, sodium nitrite to tryptophan, 2:1 — 0.1 ml./test[a], %	% T[b]	Constant quantity of sodium nitrite, 0.1 ml. of 0.068% solution % T[b]	Tryptophan per Test, γ	Constant molar ratio, sodium nitrite to tryptophan, 1.2:1 — 0.1 ml./test[a], %	% T[b]	Constant quantity of sodium nitrite, 0.1 ml. of 0.04% solution % T[b]
5	0.0034	90	85	5	0.002	89	89
10	0.0068	80	80	10	0.004	82	82
15	0.010	71	69	15	0.006	74	74
20	0.014	64	62	20	0.008	67	67
25	0.017	57	55	25	0.01	61	60
30	0.020	51	49	30	0.012	54	55
35	0.024	45	43	40	0.016	46	45
40	0.027	40	39	50	0.020	38	37
45	0.031	35	34	60	0.024	31	30
50	0.034	31	30	70	0.028	26	25
60	0.041	25	24	80	0.032	21	21
70	0.048	19	19	90	0.036	17	17
80	0.054	15	15	100	0.040	14	14
90	0.061	12	12	110	0.044	12	11+
100	0.068	9.3	9.3	120	0.048	10	10

[a] Per cent concentration of sodium nitrite added, in 0.1 ml. to each test.
[b] Transmittancy, 600 mμ.

Table IX. Effects of Indole, Skatole, and Tryptamine on Tryptophan Determination[a]

Sample		Trypto-	Wave Length of Maximum	Transmittancy		Appearance of Test Solution		
				At max.	At 600	Immediately after addition	1 hour after addition to test	30 min. after addition of sodium
Substance	Quantity, γ	phan, γ	Color[b], mμ	color, %	mμ %	to test solution[c]	solution[c]	nitrite[d]
Indole	50	0	570	93.4	93.9	Canary yellow	Intense canary yellow	Pale yellow
Indole	50	50	590–600	36.1	36.1	Canary yellow	Intense canary yellow	Dull blue green
Skatole	50	0	550	60.8	63.0	Canary yellow	Orange yellow	Pale pink grayish cast
Skatole	50	50	580	20.4	20.8	Canary yellow	Orange yellow	Blue-violet
Tryptamine[e]	50	0	590	26.0	26.1	Faint orange yellow	Fainter orange yellow	Blue with violet cast
Tryptamine[e]	50	50	580–610	9.0	9.0	Faint orange yellow	Fainter orange yellow	Deep purple blue
	50	50	590–600	36.0	36.0	Pink	Colorless	Pale blue
	100	100	590–610	14.0	14.0	Pink	*Colorless*	Deep blue

[a] Procedure B used. Blank solution contained 23.8 *N* acid, 8.0 ml.; 2 *N* acid containing 30 mg. of DAB, 1.0 ml.; water 1.0 ml.; 0.04% sodium nitrite solution 0.1 ml. Transmittancies may be converted to weight of tryptophan from curve B, Figure 4.
[b] Determined over wave-length range of 550 to 630 mμ.
[c] Ordinary artificial light.
[d] Fluorescent light.
[e] 62 γ of tryptamine hydrochloride.

Table X. Effect of Glucose and Fructose on Tryptophan Determination

Time of Reaction I, Hours	Carbohydrate, 5.0 Mg. per Test	Molar Ratio of Sodium Nitrite to Tryptophan Required for Max. Color	Blank Used	Transmittancy at Maximum Color[a], %	Tryptophan Found, γ	Tryptophan Loss, %	Appearance Blank B1	Appearance Test before color developed	Appearance Test after color developed
1	Glucose	1.2	B1[b]	14.2	101	0	Colorless	Colorless	Blue
		1.2	B2[c]	14.1	102	0	Colorless	Colorless	Blue
4	Glucose	1.2	B1	14.0	97	3	Colorless	Colorless	Blue
		1.2	B2	13.2	100	0	Colorless	Colorless	Blue
30	Glucose	1.2	B1	14.2	96	4	Colorless	Colorless	Blue
		1.2	B2	13.7	98	2	Colorless	Colorless	Blue
1	Fructose[d]	1.2	B1	15.2	97	3	Light yellow	Colorless	Blue
		1.2	B2	15.0	98	2	Colorless	Colorless	Blue
	Fructose	3.0	B1	16.0	90	10	Orange	Colorless	Slight greenish-blue
		2.0	B2	14.3	96	4	Colorless	Colorless	Slight greenish-blue
30	Fructose	>6.0	B1	24.8	69	31	Brown	Greenish-orange	Greenish-blue
		4.0	B2	16.8	88	12	Colorless	Greenish-orange	Greenish-blue

[a] Transmittancies read after reaction I had proceeded for 1 hour were converted to weight of tryptophan from curve B, Figure 4; those read after 4 and 30 hours were converted using curve C, Figure 4.
[b] B1 test read against an individual blank containing carbohydrate, tryptophan, and the same quantity of sodium nitrite used in corresponding test.
[c] B2 test read against blank containing only acid and DAB.
[d] Test with fructose set up similarly to that with glucose.

slightly enhanced when tryptamine and tryptophan are in the same test.

The wave lengths of maximum color of indole, skatole, tryptamine, and tryptophan, when determined separately, were 570, 550 (or lower), 590, and 590 to 610 mμ, respectively. When determined in the presence of equal weights of tryptophan, the wave lengths of maximum color were 590 to 600 mμ, 580 mμ, and 580 to 610 mμ for indole, skatole, and tryptamine, respectively.

Glucose and Fructose. The effect of carbohydrate on the estimation of tryptophan was determined using both glucose and fructose, which behave differently. Table X shows the recoveries of tryptophan in test solutions containing 100 micrograms of tryptophan and 5000 micrograms of glucose or fructose. The tests were carried out by Procedure E in which reaction I was allowed to proceed 1, 4, and 30 hours. Also shown in Table X is the effect of the presence of carbohydrate on the quantity of sodium nitrite required to produce maximum color.

Glucose did not produce interfering color in the test nor in the blank solutions and no additional sodium nitrite was required to develop maximum color. Using blank tests containing no glucose, 98 to 100% of the tryptophan was recovered when 1, 4, or 30 hours were allowed for reaction I. When glucose and tryptophan were in the blank, 100% recovery was attained when reaction I proceeded 1 hour, and 97 and 96% when reaction I proceeded for 4 to 30 hours, respectively.

The color of blank solutions containing fructose progressively deepened from light yellow in 1 hour to deep brown in 30 hours. However, test solutions containing fructose were colorless up to 4 hours but then became greenish-orange in 30 hours. As shown in Table X, tests containing fructose required progressively more sodium nitrite to produce maximum color with increased time of standing. Test solutions which had stood 1, 4, and 30 hours required 1.2, 3.0, and >6.0 equivalents of sodium nitrite (referred to tryptophan) when read against blank solutions containing fructose and 1.2, 2.0, and 4.0 equivalents, respectively, when read against blanks containing no fructose. The color of blank tests containing fructose was progressively lightened by increased amounts of sodium nitrite, and doubtless this reductive competition of the brown color for sodium nitrite caused the increased requirement of sodium nitrite in tests containing fructose. Recoveries of 98, 96, and 88% of the tryptophan were obtained in 1, 4, and 30 hours, respectively, when tests were read against blanks containing no fructose and recoveries of 97, 90, and 69% were likewise obtained when the blanks contained fructose.

The procedure used was similar to Procedure E. To 1.820 mg. of tryptophan, 546 mg. of *p*-dimethylaminobenzaldehyde, and 91 mg. of glucose were added 182.0 ml. of 19 *N* acid at 25° C. The reaction mixture was kept at 25° C. in the dark and at desired time intervals 10-ml. aliquots were removed for testing. Individual blanks: To 1.629 mg. of tryptophan and 81.5 mg. of glucose were added 162.9 ml. of 19 *N* acid at 25° C. This solution was set up at the same time as the test solution. At desired intervals 10-ml. aliquots were removed to be used with corresponding test solutions. Sodium nitrite of the desired concentration was added to each blank in 0.1 ml. of solution. Blank with no glucose: 19 *N* acid, 10.0 ml.; *p*-dimethylaminobenzaldehyde, 30 mg.; water 0.1 ml.

Fructose is regarded as representative of types of carbohydrate which give adventitious color under the conditions of the tryptophan test. But in the tests described in Table X the ratio of carbohydrate to tryptophan is probably higher than will ordinarily be encountered in analytical work. Even under these severe conditions 98 to 100% recoveries of tryptophan were obtained in the presence of fiftyfold quantities of carbohydrate when reaction I was allowed to proceed 1 hour and when the

quantity of sodium nitrite required to give maximum color was determined. Furthermore, the brown coloration produced by fructose would undoubtedly be much less if tryptophan was determined in 13.2 *N* acid by Procedure A. This procedure can be used, however, only if the tryptophan is not bound in protein linkages.

Tryptophan-Free Proteins. Tryptophan is usually determined in the presence of other amino acids, such as are encountered in proteins. It was of interest, therefore, to determine the degree of recovery that could be attained when tryptophan was added to gelatin plus tyrosine (added to make up for the tyrosine deficiency of gelatin) and also to a tryptophan-free allergenic protein fraction (CB-1C76) obtained from castor beans. The effect of the composition of the blank solution on the quantity of tryptophan recovered under the conditions of the test was also determined.

Tables XI and XII show the recovery of added tryptophan (50 micrograms per test) when determined in the presence of gelatin (10 mg. per test) plus tyrosine (500 micrograms per test) and also CB-1C76 (10 mg. per test). Blank solutions of various compositions were used. In Tables XI and XII tryptophan values of 48.4 and 51.3 micrograms, respectively (with blank solution B2, test T4), are regarded as the ones to be used for comparison with the other values in respective tables because they were obtained with the same blank solution which would normally be used in a tryptophan determination.

A tryptophan value of 51.2 micrograms was obtained in the test made in the presence of gelatin and tyrosine (B3, T5, Table XI) using the blank solution that would normally be used in such a determination. If this value is corrected by subtracting 1.3 micrograms (the tryptophan content of this sample of gelatin was determined as 0.013% in a later study), then the quantity of added tryptophan becomes 49.9 micrograms which deviates by +3.0% from the amount actually present.

Likewise a tryptophan value of 51.2 micrograms was obtained in the presence of CB-1C76 using the normal blank (B3, T5, Table XII). This value deviates by only −0.2% from the amount actually determined (B2, T4, Table XII).

Deviations of from −5.6 to +5.8% from the added tryptophan were obtained with blank solutions of various compositions, as shown in the tables.

EFFECT OF AGING ON SODIUM NITRITE AND *p*-DIMETHYLAMINOBENZALDEHYDE SOLUTIONS

Desired dilutions of sodium nitrite were prepared from a 1% aqueous stock solution. The effect of aging on the color-developing capacity of dilutions of the stock solution was determined. In comparative tests by Procedure A, final transmittancies of 9.2, 9.2, 9.2, and 9.5% were obtained in tests with 100 micrograms of tryptophan with an 0.068% dilution of a freshly prepared 1% solution of sodium nitrite, an 0.068% dilution of a 42-day-old 1% solution, a 42-day-old 0.068% dilution, and an 0.068% dilution of an 89-day-old 1% solution, respectively.

As a further test of the stability of dilute solutions of sodium nitrite the rate of oxidative capacity of 0.1 ml. of 0.0085% dilutions of sodium nitrite of various ages was determined. This test provides a delicate measure of oxidative capacity because the quantity of sodium nitrite used is insufficient for maximum color development (*21*). Therefore the rate of oxidation as well as the end point may be compared. Results in Table XIII show that an 0.0085% dilution of an 89-day-old 1% solution of sodium nitrite caused a slightly faster rate of oxidation and gave a transmittancy of 18% as compared to a transmittancy of 20% for an 0.0085% dilution of a freshly prepared 1% solution. A transmittancy of 18% was obtained with a freshly prepared 0.0085% dilution of a 42-day-old 1% solution of sodium nitrite as compared to 21% for a 42-day-old 0.0085% dilution of the same solution. These results are probably all within the experimental error of the method. It is apparent, therefore, that aging up to three months does not cause appreciable deterioration in color-producing capacity of sodium nitrite solutions stored in glass-stoppered Pyrex flasks exposed to indirect artificial light.

Table XI. Effect of Gelatin Plus Tyrosine and Effect of Composition of Blank Solution on Tryptophan Determination

Blank Solution[a]					Test Solution[b]				Tryptophan		
Blank No.	Tryptophan	Tyrosine	Gelatin[c]	DAB	Test No.	Tryptophan	Tyrosine	Gelatin[c]	Found	Corrected[d]	Deviation
	γ	γ	*Mg.*	*Mg.*		γ	γ	*Mg.*	γ	γ	%
B1	0	500	10	30	T1	50	500	10	51.2	49.9	+3.0
					T2	50	0	0	45.6	...	−5.6
B2	0	0	0	30	T3	50	500	10	52.7	51.3	+5.8
					T4	50	0	0	48.4	...	0.0
B3	50	500	10	0	T5	50	500	10	51.2	49.9	+3.0
					T6	50		0	47.2	...	−2.4
B4	0	500	10	0	T7	50	500	10	52.7	51.3	+5.8
					T8	50	0	0	48.4	...	0.0

[a] Composition of blank solution dissolved in 10 ml. of 19 *N* acid. To each blank was added 0.1 ml. of 0.06% sodium nitrite solution.
[b] Tests conducted by Procedure C. Tryptophan added in 0.5 ml. of 0.1 *N* sodium hydroxide solution and gelatin-tyrosine added in 0.5 ml. of same solvent. Color was developed with 0.1 ml. of 0.06% sodium nitrite solution. Transmittancies read 30 minutes after adding sodium nitrite.
[c] Ash- and water-free basis. Nitrogen content 18.52 (ash- and water-free basis).
[d] Corrected on basis of tryptophan content of 0.013% as determined by method to be described.

Table XII. Effect of a Tryptophan-Free Protein (CB-1C76)[a] from Castor Beans and Effect of Composition of Blank Solution on Tryptophan Determination

Blank No.	Blank Solution[b]			Test No.	Test Solution[c]		Tryptophan Found	Deviation
	Tryptophan	CB-1C76	DAB		Tryptophan	CB-1C76		
	γ	*Mg.*	*Mg.*		γ	*Mg.*	γ	%
B1	0	10	30	T1	50	10	51.5	+0.4
				T2	50	0	51.5	−0.2
B2	0	0	30	T3	50	10	52.5	+2.4
				T4	50	0	51.3	0.0
B3	50	10	0	T5	50	10	51.2	−0.2
				T6	50	0	49.7	−3.2
B4	0	10	0	T7	50	10	52.7	+2.8
				T8	50	0	51.6	+0.6

[a] CB-1C76 was a subfraction of castor bean allergenic polysaccharidic-protein fraction CB-1C. CB-1C76 contained 19.1% nitrogen and 0.37% carbohydrate. CB-1C is similar to CB-1A. Properties and amino acid content of CB-1A have been previously recorded (*17*).
[b] Composition of blank solution dissolved in 10 ml. of 19 *N* acid. To each blank solution was added 0.1 ml. of 0.045% sodium nitrite solution.
[c] Tests conducted by Procedure C. Tryptophan added to test in 0.5 ml. of water and CB-1C76 added in 0.5 ml. of water. Color developed with 0.1 ml. of 0.045% sodium nitrite solution.

Table XIII. Effect of Aging on Rate and Total Oxidizing Capacity of 0.0085% Solutions of Sodium Nitrite[a]

Age of 1% Stock Solution and Dilution Prepared from It		Time, Minutes									
Stock solution, days	0.0085% dilution, days	% Transmittancy (600 mμ)									
		0	1	2	3	5	7	10	15	30	60
89	0	93	58	48	41	34	29	26	22	19	18
42	42	93	62	53	47	40	35	31	27	23	21
42	0	93	60	50	43	35	30	27	23	19	18
0	0	92	61	52	46	38	33	29	25	22	20

[a] Tests conducted by Procedure A, like those described in Table III, using 100 γ of tryptophan. Transmittancies convertible to weight of tryptophan from curve A, Figure 4.

The effect of aging on solutions of *p*-dimethylaminobenzaldehyde in 2 *N* acid, stored in glass-stoppered Pyrex flasks in the dark, was also determined. Comparative tests with solutions of *p*-dimethylaminobenzaldehyde from 0 to 60 days old are shown in Table XIV. *p*-Dimethylaminobenzaldehyde solutions 1 day old gave maximum color with tryptophan, but losses of 2 to 10% were observed on 5 to 60 days' standing. Even freshly prepared solutions of *p*-dimethylaminobenzaldehyde should never be exposed, even briefly, to sunlight or bright artificial light, as will be shown in a later paper.

APPARATUS AND MATERIALS

A Coleman Universal spectrophotometer, Model 11 (wave band width 35 mμ), was used for most of this work. The wave-length scale was checked with a didymium filter. Round 19-mm. cuvettes were used. The open ends of the cuvettes were squared so that they fitted into the holder with enough tension to prevent even slight movement. The cuvettes were always placed in the holder in the same position and the same cuvette was always used for the blank and another for the test solution. Per cent transmittancy was read directly from the galvanometer scale.

A Beckman quartz spectrophotometer was used to obtain the absorption curves shown in Figures 1 and 2. Square 10-mm. cuvettes were used.

A calibrated 1-ml. hypodermic syringe was used for measuring tryptophan solutions. Tests showed this instrument to be both more accurate and more precise than glass pipets. A 0.25-ml. syringe was used for measuring sodium nitrite solutions.

Tryptophan. Two samples were used. Sample I was Eastman crystalline *l*-tryptophan, melting point 286–288° C. decomposed (a standardized Anschütz thermometer was used). Analysis: calcd. for $C_{11}H_{12}O_2N_2$, N, 13.72; C, 64.67; H, 5.93; found, N, 13.77, 13.73; C, 64.97, 64.61; H, 6.10, 6.11.

For sample II, 1 gram of Eastman crystalline *l*-tryptophan was recrystallized three times from 70% ethanol used in the proportion of 40 ml. per gram of tryptophan. Yields of 0.47, 0.23, and 0.14 gram were obtained from respective recrystallizations. The final product was ground in an agate mortar and dried in a platinum boat in a vacuum Abderhalden dryer at 110° C. for 2 hours. Analysis: found, N, 13.71, 13.76; C, 64.55, 64.65; H, 5.79, 5.86. Nitrogen was determined by the Kjeldahl micromethod. Carbon and hydrogen were determined by a micromethod. Standard solutions of tryptophan in water were freshly prepared on the same day used.

***p*-Dimethylaminobenzaldehyde.** Reagent grade *p*-dimethylaminobenzaldehyde was further purified by the procedure described by Adams and Coleman (*2*). The purified product was washed three times with water until free from chlorides and then dried to constant weight in a vacuum desiccator over calcium chloride. In a typical experiment 74.1 grams of *p*-dimethylaminobenzaldehyde yielded 43.5 grams of an almost colorless crystalline product, melting point 73–74° C. (literature value 73° C.). Analysis: calcd., N, 9.37; found, 9.46, 9.33. Solutions of *p*-dimethylaminobenzaldehyde in 2 *N* sulfuric acid were freshly prepared each day unless otherwise stated.

Sulfuric Acid. Reagent grade 36 *N* sulfuric acid was used to prepare desired dilutions, which were standardized against pure sodium carbonate with methyl red as indicator. The sulfuric acid used in this study was so free from oxidizing agents that purification was not necessary. However, it is possible that some lots of sulfuric acid might require distillation to remove traces of oxidizing agents. This point will be discussed in a subsequent paper.

Sodium Nitrite. Reagent grade sodium nitrite was used, which assayed 97% according to the manufacturer. Dilutions were usually prepared from a 1% aqueous solution on the day used, although tests showed that sodium nitrite in solution retained its full color-producing strength indefinitely.

Other Substances. Eastman reagent grade indole (nitrogen calculated, 11.97; found, 12.04, 12.07), skatole, and tryptamine hydrochloride were used. The nitrogen content of the *l*-tyrosine used was 7.64%; theoretically it is 7.74%. The glucose used was a National Bureau of Standards' standard sample. The fructose was a purified sample. The gelatin contained 18.52% nitrogen (ash- and water-free basis). The tryptophan-free protein CB-1C76 was obtained in a fractionation of an allergenic fraction CB-1C obtained from castor beans. CB-1C is similar to CB-1A, the amino acid composition of which has been previously reported (*17*). CB-1C76 contained 19.1% nitrogen and 0.49% carbohydrate (ash- and water-free basis).

General Considerations. A total volume of 10.1 ml. (including sodium nitrite solution) was used in all tests. Tests were carried out in 25- or 50-ml. Pyrex, standard-tapered, glass-stoppered Erlenmeyer flasks.

Blank solutions were always set up as nearly as possible similar in composition to the test solution. An equal volume of the same solvent used for the tryptophan in the test was added to blank solutions when pure tryptophan was analyzed. It made no difference in the value of the transmittancy observed whether or not the blank solution contained *p*-dimethylaminobenzaldehyde or sodium nitrite. An equal volume of 2 *N* acid could be substituted for the 2 *N* acid solution of *p*-dimethylaminobenzaldehyde. Likewise 0.1 ml. of water could be added to the blank instead of the 0.1 ml. of the appropriate concentration of sodium nitrite. When the solution to be analyzed contained constituents other than tryptophan, *p*-dimethylaminobenzaldehyde was omitted from the blank and a volume of 2 *N* acid solution equal to that used in the test was added to the blank. In these cases sodium nitrite was added to the blank solution.

Table XIV. Effect of Age on Solutions of *p*-Dimethylaminobenzaldehyde in 2 *N* Acid[a]

Age of DAB Solution, Days	Transmittancy, 600 mμ, %	Tryptophan Recovered, γ	Loss, %
0	9.8	100	0
1	9.8	100	0
5	10.1	98	2
7	10.5	96	4
12	11.0	94	6
19	11.0	94	6
26	11.0	94	6
35	12.0	90	10
60	12.9	90	10

[a] Tests made by Procedure A with 30 mg. of DAB and 100 γ of tryptophan per test. Transmittancies convertible to weight of tryptophan from curve A, Figure 4.

Reaction I was carried out at 25° ± 0.1° C. in the dark in all tests except some preliminary ones which were conducted at room temperature. Reaction II was carried out at room temperature in the dark. Protection from light, especially sunlight and bright artificial light, is important for reaction I because destruction of tryptophan in acid solution is accelerated by light. Protection from artificial light during reaction II was practiced but it is not essential. Acid solutions of *p*-dimethylaminobenzaldehyde should not be exposed to light. The photochemistry of these reactions will be the subject of a later paper.

The wave length of maximum color varied slightly over the range of 590, 600, or 610 mμ; consequently, in the analytical procedures the lowest transmittancy obtained at one of these wave lengths was used. The variation in transmittancy value over this wave-length range is very slight and for filter photometers either 590 or 600 mμ would be satisfactory.

The colorimetric reactions involved in the following procedures are very sensitive and may be influenced by contaminants. Therefore, care should be taken to use purified reagents, distilled water, and carefully cleaned glassware, and even to avoid impure air. Tillmans and Alt (*20*) also recognized and emphasized

these precautions in connection with the formaldehyde-tryptophan colorimetric reactions.

RECOMMENDED PROCEDURES FOR DETERMINATION OF TRYPTOPHAN

Procedure A (5 hours, 13.2 *N* acid, tryptophan added in water solution). Seven milliliters of 18.7 *N* acid, 1.0 ml. of 2 *N* acid containing 30 mg. of *p*-dimethylaminobenzaldehyde, and enough to equal 10 ml. (including the tryptophan solution) are mixed and cooled to 25° C. The tryptophan solution is added and mixed, and the resulting solution is cooled to 25° and kept in the dark at this temperature for 4 hours. To this solution is then added 0.1 ml. of 0.068% sodium nitrite solution in water. The solution is shaken and kept at room temperature in the dark for 60 minutes; then the transmittancy is read. Transmittancy may be converted to weight of tryptophan from curve A, Figure 4.

Procedure B (1.5 hours, 19 *N* acid, tryptophan added in water solution). Eight milliliters of 23.8 *N* acid and 1.0 ml. of 2 *N* acid containing 30 mg. of *p*-dimethylaminobenzaldehyde are mixed and cooled to 25° C. To this solution is added 1.0 ml. of a water solution of the tryptophan. The solution after shaking and cooling to 25° is kept in the dark at 25° for 1 hour. To this solution is added 0.1 ml. of 0.04% sodium nitrite solution. The solution is shaken, and the color is allowed to develop for 30 minutes at room temperature in the dark. Transmittancy is then read and may be converted to weight of tryptophan from curve B, Figure 4.

Procedure C (24 to 48 hours, 19 *N* acid, tryptophan added in water solution). Procedure C is the same as Procedure B except that reaction I is allowed to proceed for 24 to 48 hours before color is developed. In the presence of substances other than tryptophan it would be necessary to determine whether or not standing caused destruction of the tryptophan–*p*-dimethylaminobenzaldehyde compound. Transmittancies are convertible to weight of tryptophan from curve C, Figure 4.

Procedure D (1.5 hours, 19 *N* acid, tryptophan added in 1 *N* sodium hydroxide solution). Procedure D is the same as Procedure B except that the tryptophan is added in 1 *N* sodium hydroxide solution. Other concentrations of alkali could be used if the standard curve was determined with the corresponding concentrations. Transmittancies may be converted to weight of tryptophan from curve D, Figure 4.

Procedure E (4 to 72 hours, 19 *N* acid, tryptophan and *p*-dimethylaminobenzaldehyde added in solid form). This procedure was used to obtain curve C. To 1.392 mg. of tryptophan (sample II) and 348 mg. of *p*-dimethylaminobenzaldehyde (30 mg. per 10 ml. of test solution) in a 125-ml. glass-stoppered Erlenmeyer flask were added 116.0 ml. of 19 *N* acid at 25° C. (solution A). To 300 mg. of *p*-dimethylaminobenzaldehyde were added 100 ml. of 19 *N* acid at 25° (solution B). Appropriate volumes of solutions A and B were mixed at once to give quantities of tryptophan ranging from 5 to 120 micrograms per 10 ml. of test solution. After mixing, the test solutions were reserved in the dark at 25° for 7 hours (4 to 72 hours would give the same results according to Table VI). To each test solution was then added 0.1 ml. of 0.04% sodium nitrite solution. The solutions were shaken and kept in the dark at room temperature for 30 minutes, then transmittancies were read. Results are plotted in curve C, Figure 4.

Procedure F (1.5 hours, 19 *N* acid, tryptophan added in water solution, *p*-dimethylaminobenzaldehyde in solid form). To 30 mg. of *p*-dimethylaminobenzaldehyde are added 9.0 ml. of 21.4 *N* acid. To this solution at 25° C. is added 1.0 ml. of tryptophan in water solution. After mixing, Procedure B is followed, using curve B, Figure 4, to convert transmittancies to weight of tryptophan. This procedure, like G and H, is useful for conducting a few tests where it is not desired to make up a solution of *p*-dimethylaminobenzaldehyde.

Procedure G (24 to 48 hours, 19 *N* acid, tryptophan added in water solution). Procedure G is the same as Procedure F except that reaction I is allowed to proceed from 24 to 48 hours. Curve C, Figure 4, is used to convert transmittancies to weight of tryptophan.

Procedure H (1.5 hours, 19 *N* acid, tryptophan added in 1.0 *N* sodium hydroxide solution). Procedure H is the same as Procedure F except that the tryptophan is added in 1 *N* sodium hydroxide solution. Curve D, Figure 4, is used to convert transmittancies to weight of tryptophan.

DISCUSSION AND SUMMARY

The color-forming reaction between tryptophan, *p*-dimethylaminobenzaldehyde, and sodium nitrite can be carried out satisfactorily in sulfuric acid solution, thus eliminating the objectionable features of using strong hydrochloric acid. Maximum color is attained in 12 to 13.2 *N* acid but the reaction is more rapid in 19 *N* acid and this latter concentration is suitable for the determination of tryptophan in proteins starting with the intact protein. Optimum conditions for conducting the reaction in both 13.2 and 19 *N* acid have been determined. In 13.2 *N* acid transmittancies ranging from 88 to 10% were obtained with 5 to 98 micrograms of tryptophan per 10 ml. of test solution and in 19 *N* acid transmittancies of 88 to 10% were obtained with 5 to 114 micrograms of tryptophan with 19-mm. cuvettes in the Coleman, Model 11, spectrophotometer. Conformity to Beer's law over the entire range of concentration was observed.

Under the conditions of the test, free tryptophan is stable in 19 *N* sulfuric acid for 2 hours. This period is long enough for completion of the reaction between tryptophan and *p*-dimethylaminobenzaldehyde to form the much more stable condensation product which suffers no loss in color-forming capacity on standing for 2 days in the dark at 25° C.

Tryptophan can be satisfactorily determined in the presence of an equal quantity of indole, a fiftyfold quantity of glucose or fructose, and a 200-fold quantity of tryptophan-free protein. Equal quantities of skatole and tryptophan gave color equivalent to 1.6 times the quantity of tryptophan present. When equal quantities of tryptamine and tryptophan were determined together, color equivalent to 2.6 times the quantity of tryptophan was obtained.

The determination of free tryptophan usually involves preliminary procedures to get the tryptophan into a solution suitable for analysis. Therefore several alternative procedures have been presented in this paper which can be adapted to a variety of needs by the analyst. Tryptophan in solid form or dissolved in water, in sodium hydroxide, or in acid solution can be determined. Procedure A, which gives maximum sensitivity, requires 5 hours. Procedure B can be completed in 1.5 hours, while Procedures C and E can be set up one day and finished the next day. Even more variations can be devised from the comprehensive data concerning the reaction presented. Thus, procedures consuming only a fraction of an hour could be devised to meet special requirements.

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Determination of Theobromine and Caffeine in Cacao Materials

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Rapid and accurate methods for the determination of theobromine and caffeine in cacao materials have been developed. The theobromine and caffeine are extracted from the plant materials by percolation with hot water in the presence of magnesium oxide, and the extract is clarified by treatment with zinc acetate–potassium ferrocyanide reagents. The alkaloids in the clarified extract are adsorbed on a column of English XL fuller's earth and the theobromine is selectively eluted with dilute sodium hydroxide. The alkaline solution is adjusted to pH 6.4 with sulfuric acid and treated with an excess of silver nitrate, and the nitric acid formed by the reaction between theobromine and silver nitrate is titrated electrometrically with sodium hydroxide. For determination of caffeine, the clarified solution following the zinc acetate–potassium ferrocyanide treatment is made alkaline with sodium phosphate and the caffeine is selectively extracted by shaking with chloroform. The chloroform is evaporated and the caffeine is measured by a Kjeldahl nitrogen determination.

NUMEROUS methods have been proposed for the estimation of theobromine and caffeine in cacao materials, but none of the available methods combines the speed and reliability that are highly desirable in analytical methods used for production control purposes. The objective of the current investigation was the development of a procedure that would afford rapid and accurate analytical data and would be adaptable to production control.

A procedure for the determination of a specific plant component such as theobromine usually involves the following four steps:

1. Preparation of the samples
2. Extraction of the compound from the plant material
3. Purification of the compound
4. Quantitative measurement of the compound

Several schemes have been presented in the literature for carrying out each of the steps outlined above for the analysis of theobromine. Wadsworth (*16*) made a careful criticism of several procedures and introduced extraction with tetrachloroethane in the presence of magnesium oxide as a distinct improvement. Criticism and comments on Wadsworth's procedure and other methods are given below.

PROCEDURE FOR THEOBROMINE DETERMINATION

Reagents and Equipment. All reagents should be of C.P. grade unless otherwise specified.

Zinc Acetate. Dissolve 219 grams of crystallized zinc acetate, $Zn(C_2H_3O_2).2H_2O$, and 30 ml. of glacial acetic acid in water and make up to 1 liter.

Potassium Ferrocyanide. Dissolve 106 grams of crystallized potassium ferrocyanide, $K_4Fe(CN)_6.3H_2O$, in water and make up to 1 liter.

Silver Nitrate, 1 *N* and 0.1 *N*. Dissolve 170 grams (1 *N*) or 17 grams (0.1 *N*) of crystalline silver nitrate in water and make up to 1 liter.

Sodium Hydroxide, 0.1 *N* and 0.025 *N*, prepared from carbonate-free sodium hydroxide. Prepare as described by the A.O.A.C. (*2*) from 50% sodium hydroxide solution after standing 10 days or longer.

Sulfuric Acid, 1 *N* and 0.1 *N*.

Potassium Acid Phthalate Buffer, 0.05 molar.

Sodium Nitrate Solution. Add about 100 grams of sodium nitrate to 100 ml. of water.

Magnesium Oxide, heavy U.S.P.

Nitrazine Indicator Paper (E. R. Squibb and Sons, New York) or Alkacid Indicator Paper (Fisher Scientific Co., Pittsburgh, Pa.).

Folded Filter Paper, 27 cm. Green's No. 488½ or equivalent.

Mixed Indicator, 0.625 gram of methyl red and 0.412 gram of methylene blue. Dissolve methyl red in 450 ml. of 95% ethanol. Filter through asbestos. Dissolve methylene blue in 50 ml. of distilled water. Combine methyl red and methylene blue solutions, make to 500 ml., and mix. pH range, 5.34 (acid) to 5.65 (alkaline). Reddish purple to green.

Fuller's Earth–Celite Absorption Mixture. Mix thoroughly equal quantities by weight of English Superfine XL fuller's earth and Celite 535 or 545. Samples of the fuller's earth were obtained from L. A. Salomon and Brothers, 216 Pearl St., New York, N. Y. The fuller's earth should be tested for its capacity to adsorb theobromine. The following test is recommended: Stir 2 grams of the fuller's earth for 10 minutes with 200 ml. of a neutral water solution containing 100 mg. of theobromine. Centrifuge and determine the theobromine in a 100-ml. aliquot of the supernatant solution by the silver nitrate titration procedure described below. The fuller's earth should absorb at least 75 mg. of the theobromine.

Extraction Tubes. Attach 100-mm. lengths of 8-mm. glass tubing to the bottom of 25 × 200 mm. test tubes.

Absorption Tubes. Attach 80-mm. lengths of 6-mm. glass tubing to the bottom of 18 × 150 mm. test tubes.

Salt Bridge for Electrometric Titration. Seal a thread of asbestos into the end of an 18 × 100 mm. test tube.

All other equipment are standard laboratory items.

Method. For the best accuracy, sample weights should be adjusted so that the materials going through the clarification and adsorption steps contain about 40 mg. of theobromine.

EXTRACTION AND CLARIFICATION. Place a weighed portion of the prepared sample (usually 2.0 to 3.0 grams) and 1 gram of heavy magnesium oxide in a 200-ml. casserole. Add 4 grams of Celite 545 and mix thoroughly with sufficient hot water (about 10 ml.) to make a smooth paste. Dilute to about 50 ml. with hot water, mix thoroughly, and transfer to a 25 × 200 mm. extraction tube containing a glass wool plug and Celite 545 filter bed (about 1 gram) placed in a 1-liter filter flask. Connect the extraction tube to a supply of boiling distilled water and attach a vacuum line to the filter flask. Regulate the vacuum on the filter flask to percolate boiling water through the bed at such a rate that 500 ml. of extract will be collected in 30 to 40 minutes. The tube should not be allowed to go dry at any time during the percolation. The water on top of the sample should not be more than 2.5 cm. (1 inch) deep in order to avoid cooling. The temperature of the sample bed should be 80° to 90° C.

Neutralize the extract to about pH 6 with 1 *N* sulfuric acid, using universal indicator paper. Transfer the extract to a 1-liter beaker and concentrate to about 150 ml. Transfer the concentrate to a 200-ml. volumetric flask, using a small amount of wash water and keeping the total volume to about 170 ml. Add 7 ml. of zinc acetate reagent and mix, then immediately add with swirling 7 ml. of potassium ferrocyanide reagent. Make to volume with water and mix thoroughly by shaking. After a minimum of 3 or a maximum of 5 minutes' standing, filter the solution through a dry paper (Green's No. 488½). Discard the first 5 to 8 ml. of the filtrate and collect the remainder. Place 150 ml. of the theobromine solution in a separatory funnel equipped with a rubber stopper to fit the top of the adsorption tube.

ADSORPTION OF THEOBROMINE BY FULLER'S EARTH. Place a plug of glass wool in the bottom of the adsorption tube and press it firmly and evenly in place by use of a glass rod. Then intro-

duce about 0.5 gram of Celite 545 to make a bed about 10 mm. thick. On top of the Celite bed, place about 6 grams of the 1 to 1 fuller's earth–Celite mixture. To obtain a compact uniform column that will not channel, tap the tube to distribute the packing, then compress the bed by drawing air through the dry fuller's earth column. This is conveniently carried out by placing the adsorption tube on a 500-ml. suction flask and drawing a vacuum on the flask. Test the column for channeling by adding water to the tube while the vacuum is released. Turn the vacuum on slowly. If no channeling is observed, immediately connect to the adsorption tube the separatory funnel holding the clarified solution containing preferably 40 mg., and not more than 60 mg., of theobromine. Draw the theobromine solution through the fuller's earth column. The filtration time for the adsorption step should be 20 to 30 minutes. As soon as the theobromine solution is drawn through, all except 4 to 5 ml. on top of the clay bed, wash the fuller's earth column with 50 ml. of water. It is desirable to keep some liquid on top of the adsorption column at all times to avoid channeling.

ELUTION OF THEOBROMINE FROM FULLER'S EARTH COLUMN. Remove the suction flask containing the alkaloid-free filtrate and wash water and replace it with a clean 250-ml. suction flask. Place 75 ml. of 0.1 *N* sodium hydroxide in the separatory funnel and elute the theobromine by connecting the funnel to the adsorption tube and drawing the alkali through the column by applying vacuum to the suction flask. The time for the elution step should be 10 to 20 minutes, making the total time for the absorption, washing, and elution about 40 to 60 minutes.

TITRATION OF THEOBROMINE. Add 2 drops of mixed indicator to facilitate the rough adjustment of the pH, then neutralize the sodium hydroxide eluate with 1 *N* sulfuric acid to a faint red end point. Transfer the neutralized sodium hydroxide eluate to a 250-ml. beaker, using water to rinse the flask. In the beaker place a glass electrode and a calomel electrode, making contact with the solution by means of a salt bridge filled with a saturated solution of sodium nitrate. Adjust the solution to pH 6.40 ± 0.05, using 0.025 *N* sodium hydroxide for the final adjustment. The total volume should be about 125 ml. Add 25 ml. of 0.1 *N* silver nitrate solution from a graduate or automatic pipet and stir thoroughly. With thorough stirring titrate to pH 6.40 ± 0.05. For best results, this titration should be made without interruptions and should not require more than 5 minutes for its completion. Record the amount of 0.025 *N* sodium hydroxide required for the titration.

BLANKS AND CONTROLS. Carry a sample of water through the zinc acetate–potassium ferrocyanide treatment, adsorption, elution with sodium hydroxide, neutralization, and titration in the presence of silver nitrate. Perform each operation in the exact manner prescribed for unknown samples. This blank titration should require not more than 0.20 ml. of 0.025 *N* sodium hydroxide.

Carry a sample containing 40 mg. of theobromine through the same procedure to establish the quantitative nature of all steps involved.

CALCULATIONS.

$$\% \text{ theobromine} = \frac{100\ (\text{vol. of NaOH for sample} - \text{vol. of NaOH for blank}) \times \text{theobromine factor}}{\text{weight of sample clarified}}$$

$$\text{Theobromine factor} = \text{normality of NaOH} \times 0.180 \times \frac{\text{total volume clarified}}{\text{aliquot analyzed}}$$

(The addition of a 3% correction as recommended in the discussion may be made by substituting 103 for 100 in the above formula.)

For 0.025 *N* sodium hydroxide and using a 150-ml. aliquot from a total volume of 200 ml. clarified, the factor is 0.006.

ANALYSIS OF PURE OR CRUDE THEOBROMINE. In the analysis of pure or crude theobromine samples the clarification and adsorption steps may be omitted.

Pulverize the pure or crude theobromine samples with a mortar and pestle and weigh accurately about 0.1 gram into a 500-ml. Erlenmeyer flask. Add about 250 ml. of water, place a small funnel in the flask, and boil for about 30 minutes. Filter while still hot through a coarse filter paper into a 400-ml. beaker. Filtration may be omitted for samples containing 90 to 100% theobromine. Wash the filter paper thoroughly with about 50 ml. of hot water. Cool the filtrate and washings to room temperature, adjust to pH 6.40, add 5 ml. of 1 *N* silver nitrate, and titrate back to pH 6.40 with 0.025 *N* sodium hydroxide. Correct for water blank, using 250 ml. of boiled water and 5 ml. of 1 *N* silver nitrate.

PROCEDURE FOR CAFFEINE DETERMINATION

Reagents. Chloroform, U.S.P. redistilled.

Sodium Phosphate, 0.5 Molar. Dissolve 190 grams of trisodium phosphate, $Na_3PO_4.12H_2O$, in 1 liter of water.

The reagents for the extraction and clarification are the same as those specified under the theobromine procedure.

The reagents for the nitrogen determination are specified by the A.O.A.C. (*2*).

Method. EXTRACTION AND CLARIFICATION. Mix 4 grams of the sample with 2 grams of heavy magnesium oxide and 8 grams of Celite 545 and extract by the same procedure used for theobromine. Neutralize the extract, concentrate to 150 ml., and clarify with zinc ferrocyanide. Add 10 ml. of 0.5 *M* sodium phosphate to 150 ml. of filtrate, make to 200 ml., and filter on fluted paper. Collect 150 ml. and transfer to a 500-ml. separatory funnel.

CHLOROFORM SEPARATION. Shake the clarified water extract five successive times for 1 minute each with 30-ml. portions of chloroform. After each shaking, draw off the chloroform solution into a 250-ml. separatory funnel. After the chloroform extraction is completed, add 5 ml. of 1 *N* sulfuric acid to the chloroform solution in the 250-ml. separatory funnel. Mix thoroughly, let stand about 10 minutes, and draw off the chloroform solution through a cotton plug in the stem of the separatory funnel into a 650-ml. Kjeldahl flask. Wash the acid with one 30-ml. portion of chloroform and add the washings to the Kjeldahl flask. Recover the major portion of the chloroform by distillation. Do not allow the distillation to proceed too rapidly or go to dryness, but stop when the Kjeldahl flask contains 10 to 15 ml. of solution.

NITROGEN DETERMINATION. Determine the total nitrogen in the chloroform extract by the Kjeldahl-Gunning method (*2*). Run a nitrogen blank on the same reagents used for the determination, starting with 180 ml. of chloroform.

CALCULATIONS.

$$\% \text{ caffeine} = \frac{100\ (\text{vol. of NaOH for blank} - \text{vol. of NaOH for sample}) \times N \text{ of NaOH} \times 0.0485}{\text{weight of sample analyzed}}$$

DISCUSSION OF METHODS AND EXPERIMENTAL RESULTS

The theobromine method described herein involves several new and original features not found in other theobromine methods. The most significant departures from existing methods are: use of the percolation extraction technique, purification of the extracted theobromine by adsorption and elution from fuller's earth, modification of the volumetric silver nitrate method to include an electrometric titration procedure for measuring the purified theobromine, and use of an aqueous solution buffered at a high pH for the selective liquid-liquid separation of caffeine from theobromine.

Preparation of Samples. The recommendations of Wadsworth (*15*, *16*) on the preparation of samples of cacao materials for analysis have been found satisfactory. In the laboratory, samples can be ground readily in a Wiley mill or any comparable type of grinding equipment. Samples of whole beans, nibs, or cacaos which contain more than 15% fat should be extracted with a fat solvent, such as hexane or pentane, to facilitate the extraction of the theobromine and the subsequent clarification and absorption steps. No theobromine could be detected in either hexane or pentane extracts of cacao nibs.

Extraction of Theobromine. The large number of different extraction techniques reported in the literature (*5*, *10*, *11*, *12*, *15*) indicates the unsatisfactory nature of extraction procedures previously used and the extreme difficulty of this step. Some early authors contended that an acid treatment was necessary to

liberate the theobromine from its natural complex, usually considered at that time to be a glucoside compound. The Kunze (*10*) method using 5% sulfuric acid was tried, but considerable difficulty in filtration was encountered. A more drastic acid treatment, involving the use of warm concentrated sulfuric acid, was proposed by Cortes (*4*) in a method for estimating caffeine in coffee. In applying this technique to cacao materials it was found that the theobromine is not readily extracted from the acid digest and is apparently held in the carbonized residue by adsorption.

Other authors including Dekker (*5*), Wadsworth (*15*), and Moir and Hinks (*12*) believed that theobromine occurs in combination with tannin materials and added magnesium oxide to the sample to facilitate the extraction and the decomposition of the natural theobromine complex. Other alkalies would serve the same purpose, but theobromine is decomposed at a high pH, particularly in hot solutions. The low pH (about 10) of a magnesia extract is much safer than the high pH (about 12) of an extract made with calcium hydroxide. No loss of pure theobromine was detected in a saturated solution of magnesium oxide held at 100° C. for 1 hour. A saturated lime solution containing 40 mg. of theobromine per 100 ml. showed a decomposition rate of 2.6% per hour at 90° C. This decomposition continued for at least 14 hours when the rate study was discontinued. A loss of 10% of the theobromine was found in 0.1 *N* sodium or barium hydroxide solutions held for 2 hours at 100° C., but no loss was observed at room temperature. On the basis of extensive experience in the extraction of theobromine from cacao materials, a high quality magnesium oxide (U.S.P. or C.P. grade) is recommended for use in the analytical extraction procedures.

The batch extraction steps in the Dekker method using water and in the Moir and Hinks method using 80% ethanol do not give complete extraction of the theobromine unless the number of extraction steps or the amount of solvent is increased. The Wadsworth extraction procedure provides quantitative extraction only if the conditions are very carefully controlled. The degree of extraction is dependent upon the effectiveness of the magnesium oxide treatment and upon the moisture distribution in the sample during the solvent extraction. Both factors require exacting attention on the part of the analyst in order to provide the optimum conditions for extraction.

Experimental evidence indicates that cacao solids will act as an adsorbent for theobromine. A similar observation has been made by Watson, Sheth, and Sudborough (*17*) on the adsorption of caffeine by tea waste. When extracted cacao solids containing 0.004% theobromine were held in contact with a saturated lime solution containing 0.412% theobromine, the theobromine content of the cacao solids increased to 0.23% and decreased in the solution to 0.316%. This observation regarding adsorption is highly significant in all quantitative extraction procedures for the alkaloids theobromine and caffeine. The theobromine dissolved in a solution which is in contact with a sample of cacao solids will be in equilibrium with the theobromine adsorbed by the solids. This means that in order to have quantitative extraction of the theobromine from a sample of cacao solids, the solvent in contact with the sample at the end of the extraction should contain essentially no theobromine.

The continuous hot water percolation procedure finally selected takes advantage of several factors known to be important in the extraction of theobromine.

The intimate mixing of the sample with magnesium oxide forms the soluble magnesium salt of theobromine, acts as an aid in liberating the alkaloids from the natural plant compounds, and prevents the extraction of some of the interfering substances.

The procedure provides a method for continuously bringing alkaloid-free solvent into contact with the sample, thereby maintaining a high extraction rate.

It provides the optimum conditions for desorbing theobromine which may be adsorbed by the sample.

It provides a simple manipulative technique suitable for routine analysis.

The theobromine is usually completely extracted in the first 250 ml. of extract, but in order to provide a reasonable safety factor for all types of samples it is recommended that the percolation be continued until 500 ml. of filtrate are collected.

The filtrate is neutralized to about pH 6 before concentration, to avoid any possible loss of theobromine. No loss of theobromine was found in concentrating 500 ml. of a pure theobromine solution to 100 ml. This concentration step is desirable, since the time required for the concentration, about 40 minutes, is less than the additional time which otherwise would be required to clarify and run the fuller's earth adsorption step on the larger volume of extract. An excess of acid should be avoided because it will slow down filtration through the fuller's earth column. This condition is apparently caused by physical or chemical changes in the fuller's earth, which cause it to swell and pack tightly in the column.

Purification of Alkaloids. The use of zinc acetate and potassium ferrocyanide as clarifying reagents for the aqueous solutions of the alkaloids, as in the Moir-Hinks method, proved effective as the first step in the purification scheme. However, certain precautions must be observed to avoid a significant loss of theobromine. The time interval between adding the reagents should be a minute or less and the time between adding the reagents and filtering should be held to 3 to 5 minutes. The filter paper used for filtering the zinc ferrocyanide should permit fairly rapid filtration, so that 150 ml. are collected within 10 to 15 minutes. These precautions are necessary because a small amount of theobromine is apparently precipitated by, or adsorbed on, the clarifying reagents. This loss of theobromine is greater in pure solution than in cacao extracts. This effect would be expected if the theobromine is lost by adsorption. The nontheobromine substances in the extract might be expected to reduce the adsorptive activity of the reagent for theobromine. The loss of theobromine will depend upon the concentration of theobromine in the solution at the time of clarification, and for this reason the volume of solution and the amount of theobromine should be held fairly constant (40 mg. in 150 to 170 ml.).

Thorough agitation during the addition of the reagents is recommended to minimize the precipitation, occlusion, or adsorption of the theobromine. Agitation also gives a fine flocculent precipitant which is effective in removing the substances that interfere in the subsequent steps for theobromine and in the liquid-liquid separation of caffeine. Without the clarification step, the extracts will not filter readily during the adsorption and elution steps. The clarification is also necessary to reduce emulsions during the separation of the caffeine by chloroform.

The quantitative recovery of caffeine from aqueous solutions by extraction with chloroform is relatively easy, but the recovery of theobromine in this manner is a slow and tedious process. For complete recovery of the theobromine from the concentrated solution used in the Moir-Hinks method, a series of 15 shakings with 30 ml. each of chloroform was found necessary. The Wadsworth method relies upon the selectivity of the tetrachloroethane extraction to obtain a pure alkaloid extract. The theobromine obtained in this method is claimed by Wadsworth to have a purity of 98% theobromine. In the analysis of English expeller cakes, the authors never have been able to approach the purity claimed by Wadsworth for the theobromine precipitated by ether. The theobromine obtained at the same stage in current work varied from 80 to 90% as measured by both Kjeldahl nitrogen determination and silver nitrate titration. His method of purification applied to the crude theobromine obtained in the analysis of English expeller cakes (dissolving the theobromine in the residue with hot water, drying, weighing, and determining the ash in the soluble residue) gave a purified theobromine residue weight which was close to the theobromine found by silver nitrate titration of the solvent residue. However, this purification procedure, which is considered necessary for accurate results by the Wadsworth method, requires considerable manipulative time.

Table I. Adsorption and Elution of Theobromine and Caffeine from Fuller's Earth Column[a]

Theobromine Added	Caffeine Added	Theobromine in 0.1 *N* NaOH Elution			Total Theobromine Recovered
		First 25 ml.	Second 25 ml.	Third 25 ml.	
Mg.	*Mg.*	*Mg.*	*Mg.*	*Mg.*	%
40.8	0.0	38.8	1.2	0.5	99.3
40.8	0.0	33.2	6.8	0.5	99.3
0.0	40.3	0.0	0.0	0.0	0.0
0.0	40.3	0.0	0.0	0.0	0.0
30.6	10.1	27.9	2.1	0.0	98.0
30.6	10.1	27.9	2.1	0.0	98.0
20.4	20.2	18.9	0.9	0.0	97.2
20.4	20.2	16.6	3.3	0.9	102.0
10.2	30.2	9.5	0.5	0.0	98.0
10.2	30.2	9.5	0.5	0.0	98.0

[a] Analyses of all samples made by Kjeldahl nitrogen determination.

Kay and Haywood (*8*) also found that the purity of the theobromine extracted by the Wadsworth method was only 80%, based on the nitrogen content measured by the Kjeldahl method. They proposed a modification of the Wadsworth method in an effort to avoid the purity determination on the extracted theobromine. By using their modification, a purity of 98% is reported for the extracted theobromine. Even with the elimination of the purity test, as proposed by Kay and Haywood, the modified Wadsworth method will still require considerably more manipulative time than the new method presented in this paper.

In order to provide a rapid analytical procedure for theobromine analysis, it was necessary to find some other means of obtaining a purified solution of the theobromine. Previous work in this laboratory had demonstrated that theobromine and caffeine are adsorbed by various types of fuller's earth and the theobromine can be effectively recovered by desorbing with sodium hydroxide solutions. These observations suggested the use of fuller's earth as a means of purifying theobromine solutions for analysis. The familiar chromatographic adsorption technique was found to be adaptable for the present purpose. The solution of theobromine is filtered through a column of fuller's earth and Celite, the Celite being added to obtain favorable percolation rates while the fuller's earth adsorbs the theobromine. The solution remaining on the fuller's earth and some of the impurities are removed by washing the column with water. The theobromine is recovered by elution with 0.1 *N* sodium hydroxide solution. The theobromine is then free from interfering impurities and can be measured accurately in a short time.

The design and size of the adsorption column were determined primarily by the amount of fuller's earth required for quantitative adsorption of the theobromine plus a reasonable safety factor. It was also necessary to have a column that would permit rapid filtration during both the adsorption and elution steps. Extensive testing indicated that an 18 × 150 mm. column holding 6 grams of a 1 to 1 fuller's earth–Celite mixture would permit quantitative adsorption and elution of theobromine in the range of 20 to 60 mg. with a good filtration rate. Samples containing 150 mg. have been carried through the adsorption and elution steps with good accuracy. The adsorption and elution steps were found to be quantitative for theobromine in extracts of cacao solids as well as pure solutions (see Tables I, II, and V). It was necessary to wash the column after adsorption to remove the original extract remaining with the fuller's earth. It was found that 50 ml. of water would effectively wash the fuller's earth column without a detectable loss of the adsorbed theobromine.

Small losses of theobromine have been observed when solutions of this alkalinity stood for 1 day or longer at room temperature, but no loss of theobromine could be detected in 0.1 *N* solutions of sodium hydroxide standing 2 hours at room temperature. It is recommended that the elution solution be neutralized within the first hour to avoid decomposition of theobromine.

Measurement of Theobromine. Four different methods for measuring the purified theobromine were used extensively in laboratory experiments.

The silicotungstic acid method of Griebel (*7*) was modified and improved in this laboratory for the determination of caffeine and showed promise as a rapid and time-saving procedure for theobromine. Richardson and Campbell (*14*) have proposed a gravimetric method based on the precipitate formed with phosphotungstic acid. However, these methods were found to have limitations which made their use undesirable. The conditions which affect the turbidity, such as time, temperature, acidity, and salt concentration, require very exact control. These methods will not distinguish between caffeine and theobromine. Silicotungstic and phosphotungstic acids are useful for rapid qualitative or semiquantitative estimations of the alkaloids in relatively pure solutions, but further work is needed to make them accurate quantitative methods.

Good precision and accuracy in the measurement of pure alkaloids were obtained with the iodometric method of Emery and Spencer (*6*), by careful control of the conditions affecting the precipitation of the periodide. This method requires time and cooling for complete precipitation of the periodide, while the silver salt forms rapidly at room temperature. The iodine precipitate must be removed before titrating the excess iodine, which means an extra filtration or centrifugation step not required in the silver nitrate method.

Moir and Hinks have indicated some of the precautions necessary to obtain accurate results by the Kjeldahl procedure for total nitrogen. Theoretical nitrogen values were obtained for pure theobromine when sucrose and selenium were added and the digestion was continued for 3 hours after clearing. This long digestion period increases the time and equipment required for completing large numbers of analyses. The total nitrogen determination does not provide as much specificity as the silver precipitation procedure. The theobromine as measured by the silver nitrate titration accounted for 93.5% of the total nitrogen in the sodium hydroxide eluate obtained in the analysis of a sample of English expeller cake.

The silver nitrate method of Boie (*3*) for measuring theobromine is based on the reaction:

$$C_7H_8N_4O_2 + AgNO_3 = AgC_7H_7N_4O_2 + HNO_3$$

The nitric acid formed by the reaction is titrated with standard sodium hydroxide solution. In the tentative A.O.A.C. method for theobromine in drugs (*2*), a sample containing about 0.2 gram of theobromine is dissolved in warm water. Acid is added and the solution is heated to boiling. Phenol red indicator is added and the solution made slightly alkaline. An excess of silver nitrate is then added and the solution titrated immediately to the end point with 0.1 *N* sodium hydroxide. The visual end point used in this procedure limits the method to solutions that are essentially colorless. By introducing an electrometric titration the procedure can be used in colored solutions with even greater precision than is possible with the colorimetric end point. With this modification the silver nitrate titration became a convenient and rapid procedure capable of good precision and accuracy.

The usual combination of glass electrode and calomel electrode placed directly in the titration solution cannot be used very long in the presence of silver nitrate. The reaction between silver nitrate and the potassium chloride at the liquid junction of the calomel electrode interferes with the measurement of the potential. In order to eliminate this difficulty a salt bridge was inserted between the calomel electrode and the titration solution. A saturated solution of sodium nitrate possesses the required properties of being a highly dissociated salt with a neutral pH and not reactive with potassium chloride or silver nitrate. The level of the solution in the salt bridge should be above the point to which the bridge is immersed in the titration solution.

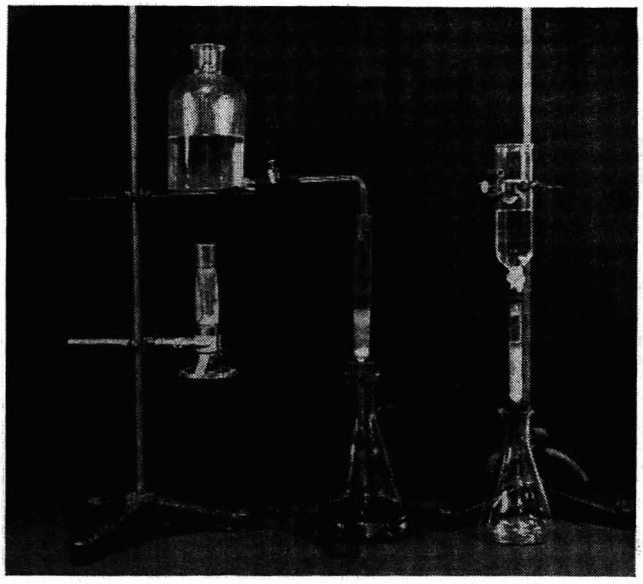

Figure 1. Theobromine Extraction and Absorption Apparatus

The amount of silver nitrate added must be controlled, since any large change in concentration of the excess silver nitrate present during the titration will affect the solubility of the silver theobromine precipitate through mass action effect of the silver ion. The amount of silver nitrate specified in the method is calculated to give a concentration of excess silver nitrate comparable to the concentration found in the A.O.A.C. procedure (*2*). This concentration of about 0.015 *N* silver nitrate during the titration has been found satisfactory.

The titration end point was determined empirically by using pure theobromine and noting the rate of pH change under the conditions of the titration. The maximum slope of the titration curve was observed at a pH of 6.4, as shown in Figure 2. This pH should also provide the highest specificity for the silver precipitation, since several of the silver salts which might interfere have a relatively high solubility in this pH range, but would be precipitated along with the theobromine if the pH were raised. The silver theobromine salt formed under the conditions of the analysis contained 99.3% of the amount of silver required by the chemical formula. Chlorides and sulfates do not interfere with the reaction unless they are present to such an extent that the concentration of excess silver nitrate is altered. If a large amount of chloride is present, the silver nitrate should be increased by an amount equivalent to the chloride present, so that the final titration is made with essentially the same excess of silver nitrate in all samples. Carbonates will interfere with the reaction by forming silver carbonate, the liberated acid resulting in an increase in the apparent theobromine titration. For this reason, the amount of carbonates should be kept as low as possible. Since solid sodium hydroxide usually contains some carbonate, the dilute solutions of sodium hydroxide used in the procedure should be prepared from a 50% solution which has stood for 10 days or longer. Most of the carbonate is precipitated from a sodium hydroxide solution prepared in this manner. The blank titration, which should be run daily, takes care of traces of carbonate and other interfering substances present in the reagents and materials used in the analysis.

Extraction and Purification of Caffeine. The initial steps in the theobromine procedure, including the preparation of sample, the extraction, and the zinc ferrocyanide clarification, have been used successfully in a method for measuring caffeine in cacao materials.

Separation of Theobromine and Caffeine. Experimental results indicated that a fuller's earth column can be used for the separation of theobromine and caffeine.

In the examples shown in Table II, varying proportions of the two alkaloids were adsorbed by percolating 150 ml. of the alkaloid solutions through the columns. After washing with 50 ml. of water, the theobromine was eluted with 75 ml. of 0.1 *N* sodium hydroxide solution and measured by the silver nitrate titration as prescribed in the theobromine method. The caffeine was then eluted by percolating through the column two successive 100-ml. portions of a solution containing by volume 40% methanol, 40% acetone, and 20% water. The caffeine was measured in each eluate by evaporating off the solvent in the presence of water and completing the determination as described in the caffeine method, starting with the addition of sodium phosphate. Further details of a process for the recovery of theobromine and caffeine and their separation from one another are shown in a recent patent granted to Kremers (*9*). This technique for separating the alkaloids was not conveniently incorporated into the analytical procedure for caffeine because of the relatively long time required for quantitative recovery of the caffeine.

Work on the separation of the alkaloids by liquid-liquid extraction provided two alternative methods. One method used benzene to remove the caffeine selectively from a water solution of the two alkaloids. The other method used chloroform to remove the caffeine from a water solution at a high pH. Although both methods will give a quantitative separation of the two alkaloids, the chloroform extraction is preferred. The distribution of

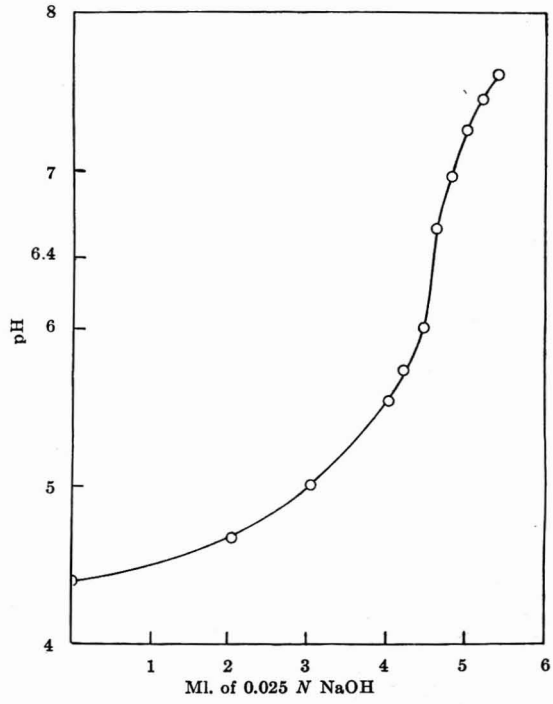

Figure 2. Titration Curve for 20 Mg. of Pure Theobromine

Table II. Separation of Theobromine and Caffeine on Fuller's Earth Column

Theobromine Added	Caffeine Added	Theobromine Found in 75 Ml. of NaOH	Theobromine Recovery	Caffeine in Solvent		Total Caffeine Recovered
				First 100 cc.	Second 100 cc.	
Mg.	*Mg.*	*Mg.*	%	*Mg.*	*Mg.*	%
28.7	9.7	27.8	96.8	8.8	0.3	93.8
28.7	9.7	28.5	99.3	8.2	0.3	87.6
19.1	19.4	19.1	100.0	18.9	0.3	99.0
19.1	19.4	18.8	98.5	19.2	0.6	102.0
9.2	29.1	9.2	100.0	28.2	0.6	99.0
9.2	29.1	9.2	100.0	28.5	1.1	101.7

Table III. Distribution of Caffeine and Theobromine between Water and Chloroform or Benzene[a]

Solvent	Final pH, H_2O	Alkaloid in 100 Ml. of H_2O *Mg.*	Alkaloid in 100 Ml. of Solvent *Mg.*	Total Alkaloid in Solvent %	Distribution Coefficient, Volume Basis, $K = \frac{C\ H_2O}{C\ Solvent}$
			Caffeine		
Chloroform	3.0	1.7	37.8	97.4	0.0450
	6.5	1.7	37.1	95.6	0.0450
	9.7	1.7	37.4	96.4	0.0455
	12.3	1.7	37.1	95.6	0.0450
Benzene	3.1	22.2	16.3	42.0	1.36
	5.6	22.2	16.6	42.8	1.34
	9.0	22.5	16.3	42.0	1.38
	12.3	22.2	16.3	42.0	1.36
			Theobromine		
Chloroform	3.1	27.6	10.9	28.0	2.53
	6.6	27.6	11.6	29.7	2.38
	8.9	28.3	10.9	28.0	2.60
	11.9	39.2	0.0	0.0	....
Benzene	3.1	38.9	0.0	0.0	....
	6.1	38.9	0.0	0.0	....
	8.5	39.2	0.0	0.0	....
	11.9	39.2	0.0	0.0	....

[a] Caffeine added, 38.8 mg.; theobromine added, 39.1 mg.

caffeine in chloroform is more favorable than in benzene (Table III), and the chloroform is not flammable nor so toxic as benzene.

The distribution of theobromine between water and the immiscible solvents is fortunately different from caffeine. No theobromine was found in the benzene extracts of water solutions containing 40 mg. of theobromine per 100 ml. and varying in pH from 3 to 12, nor in the chloroform extract of a water solution of theobromine at pH 12. Theobromine is extracted slowly by chloroform from water solutions of a pH below 10. The effective separation of the alkaloids by chloroform extraction of an alkaline water solution holds for a wide range of concentrations of the two alkaloids. Separations were made with equal effectiveness from solutions in which the ratios of theobromine to caffeine varied from 1 to 20 to 10 to 1.

Trisodium phosphate is used to adjust the pH of the alkaloid solutions because of its high buffering capacity in the range of pH 12. This buffering capacity takes care of the variations in the acidity of different samples, so that the addition of a fixed amount of phosphate gives a uniform pH for practically all samples.

Caffeine is more readily decomposed in strong alkaline solutions than theobromine. The decomposition rate increases rapidly with higher temperatures. It is necessary, therefore, to observe certain precautions in handling alkaline caffeine solutions. If other alkalies than sodium phosphate are used, the pH should not be raised much above 12. The temperature of the solution should not be raised above room temperature while the solution is alkaline. The caffeine should be shaken out of the solution with chloroform within a short time after the pH is raised. Solutions standing overnight at about 25° C. after adding the sodium phosphate lost 3 to 5% of the caffeine present.

The chloroform extraction, using five 30-ml. portions for 150 ml. of water solution, will quantitatively remove the caffeine over a wide range of concentrations. The distribution coefficient or caffeine on a volume basis $\left(K = \frac{C\ H_2O}{C\ CHCl_3}\right)$ is 0.046 for a 1 to 1 volume ratio of solvent to aqueous solution and an initial concentration of 40 mg. of caffeine per 100 ml. of water. The K value is fairly constant for other concentrations and volume ratios. Assuming K to be constant and the caffeine to be contained in 150 ml. of water, 99.8% of the caffeine would be extracted by four shakings with 30 ml. of chloroform and 100% in five shakings.

The acid wash of the chloroform serves two purposes: (1) it removes from the chloroform materials of an alkaline nature which were extracted from the alkaline water solution; (2) it neutralizes any of the alkaline water solution which may be carried into the chloroform layer either by entrainment or in solution in the chloroform. When the acid washing was omitted, significant losses of caffeine were observed during the chloroform distillation.

Measurement of Caffeine. Caffeine is more readily oxidized than theobromine during the Kjeldahl digestion. With the usual reagents and heating facilities, the digestion of caffeine is complete in one hour. This makes the Kjeldahl total nitrogen procedure a more desirable method for measuring caffeine than theobromine. Alternative methods such as the iodometric and turbidimetric procedures have the same limitations when applied to caffeine as described for theobromine.

ACCURACY AND PRECISION OF THEOBROMINE METHOD

The determination of the accuracy of a theobromine method is extremely difficult, (1) because there is no referee method of established accuracy, and (2) because it is difficult to prepare reference samples of known theobromine content. The accuracy of the new theobromine method has been established by comparison with other theobromine methods, the most accurate of which are the methods of Moir-Hinks and Wadsworth; by analysis of samples of known theobromine content; and by the consistency of the results obtained on studies involving a material balance of theobromine during laboratory and plant-scale production operations. The precision of the method has been calculated statistically from analytical data.

Numerous samples of cacao solids and cacao extracts were analyzed by both the proposed method and the Moir-Hinks method The Moir-Hinks procedure as described in the original publication (*12*) was followed.

Comparisons were also made between the new method and the Wadsworth method on samples of English expeller cakes. These samples consisted for the most part of whole cacao beans put

Table IV. Comparison of Theobromine Analyses by New Method and Wadsworth Method

Sample Description	New Method Uncorrected, % as Recd.	Wadsworth Method Corrected, % as Recd.	Deviation, % Theobromine	% Deviation
English expeller cake				
1	2.51	2.73	−0.22	−8.1
2	2.58	2.61	−0.03	−1.1
3	2.44	2.20	+0.24	+10.9
4	1.97	2.00	−0.03	−1.5
5	2.47	2.85	−0.38	−13.3
6	2.42	2.62	−0.20	−7.6
7	2.50	2.92	−0.42	−14.4
8	1.98	2.10	−0.12	−5.7
9	2.34	2.34	0.00	0.0
10	2.12	2.05	+0.07	+3.3
11	2.40	2.24	+0.16	+7.1
12	2.50	2.45	+0.05	+2.0
13	2.34	2.45	−0.11	−7.7
14	2.22	2.49	−0.27	−10.9
15	2.38	2.45	−0.07	−2.8
16	2.24	2.46	−0.22	−8.9
17	2.31	2.34	−0.03	−1.3
18	2.44	2.39	+0.05	+2.1
19	2.76	2.83	−0.07	−2.5
20	2.87	2.78	+0.09	+3.2
21	2.86	2.71	+0.15	+5.5
22	2.61	2.60	+0.01	+0.3
23	2.64	2.75	−0.11	−4.0
24	2.37	2.34	+0.03	+1.3
25	2.34	2.47	−0.13	−5.3
Brazilian expeller cake	2.20	2.32	−0.12	−5.2
Domestic expeller cake	2.21	2.19	+0.02	+0.9
Extracted cacao solids	0.52	0.53	0.01	0.2
Mixed cacao solids 1	1.63	1.79	−0.16	−8.9
Mixed cacao solids 2	1.58	1.55	+0.03	+1.9
Mixed cacao solids 3	1.55	1.50	+0.05	+3.3
Mixed cacao solids 4	1.60	1.60	0.00	0.0

Each value is average of duplicate determinations. Average % deviation for 32 samples = −2.1.

through fat expellers which reduced the fat content to about 12%. The average results of duplicate determinations on 32 samples (Table IV) show the theobromine figures by the new method to be 2.1% below the average value by the Wadsworth method.

One of the best means of testing the accuracy of an analytical method is to analyze samples containing known amounts of the added compound. This method is especially reliable if the compound can be completely and selectively removed from the sample before known amounts of the pure substance are added. This procedure was used for accuracy tests on both the new and the Moir-Hinks method. A sample of essentially theobromine-free cacao waste was prepared as follows:

A sample of cacao solids was exhaustively extracted with lime water. The extract was neutralized and treated with fuller's earth to remove the theobromine, then concentrated under vacuum, and finally evaporated on the extracted residue. Before soluble solids were added, the excess lime was removed from the cacao residue by neutralizing with hydrochloric acid and washing with water. This sample contained only 0.06% theobromine when analyzed by both the Moir-Hinks method and the new method. Two-gram portions of the extracted cacao solids were placed in porcelain dishes and aliquots of a standard theobromine solution containing from 30 to 60 mg. of pure theobromine were added. The water was evaporated slowly on the steam bath with frequent stirring. The recoveries of added theobromine (shown in Table V) averaged 97.1% through the complete procedure of the new method. A standard deviation value of 1.1% was observed on six recovery tests.

The reliability of the new method is shown by the data obtained in a series of theobromine extraction experiments. In this series the starting cacao solids and solutions, as well as the extracted solids and filtrates, were analyzed. Each extraction test required four theobromine determinations. The theobromine accounted for after digestion averaged 99.0% for 70 extraction tests.

Table V. Recovery of Theobromine Added to Extracted Cacao Solids by New Method

Theobromine Added	Theobromine Found		Uncorrected Recovery of Added Theobromine
Mg.	*Mg.*	*G./100 g. cacao solids*	%
0.0	1.3	0.06	..
0.0	1.3	0.06	..
29.3	29.4	1.47	95.9
29.3	29.7	1.49	96.6
39.0	39.4	1.97	97.7
39.0	39.4	1.97	97.7
58.5	58.6	2.93	97.9
58.5	57.8	2.89	96.6
		Av.	97.1

A survey of the results obtained in comparative analyses, recovery experiments, and approximately 10,000 determinations by the new method indicates that for the highest accuracy an empirical correction of about 3% should be added to all values on samples containing soluble cacao materials. The correction for pure solutions of theobromine carried through the entire procedure should be 5%. These corrections are necessary primarily because of losses during the zinc acetate–potassium ferrocyanide clarification step. (A correction of this magnitude should also be applied for this clarification step in the Moir-Hinks method.)

A comparatively high degree of precision is attainable with the new theobromine method. Following the procedures recommended by the American Society for Testing Materials (*1*) and Moran (*13*), the precision of the method has been estimated by statistical calculations (Table VI). The value of $\pm 2\sigma$ has been used in calculating the "limit of uncertainty" (*LU*) value for 95 out of 100 determinations. A correction factor of 0.869 has been applied in these calculations as recommended by the A.S.T.M.

Table VI. Precision of Theobromine Method

Sample	No. of Determinations	Av. % Theobromine Uncorrected, $\overline{X}$	Standard Deviation, σ	Limit of Uncertainty, *LU* 95
Daily control samples of pure theobromine	12	94.9	0.82	1.75
Mixed cacao shell and expeller cake analyzed under routine conditions	12	1.47	0.029	0.062
Mixed cacao shell and expeller cake analyzed under best conditions	11	1.50	0.028	0.059
Expeller cake analyzed under best conditions	6	2.36	0.031	0.070
Pure (U.S.P.) theobromine by direct titration analyzed under best conditions	10	97.5	0.51	1.11
Crude theobromine by direct titration analyzed under best conditions	6	93.2	0.48	1.11

(*1*). The first series of analyses on a standard theobromine sample used in daily control work shows a standard deviation of 0.82%, with an *LU* 95 value of 1.75. The data on two samples of mixed cacao solids and one sample of expeller cake show that the value for *LU* 95 is 0.06 and 0.07% theobromine, respectively. The *LU* 95 values for the direct titration analysis of both pure theobromine (U.S.P. grade) and crude theobromine are 1.11% theobromine.

ACCURACY AND PRECISION OF CAFFEINE METHOD

The application of the caffeine method to cacao samples has not been extensive enough to calculate precision values. However, the method has been used extensively for measuring caffeine in relatively pure solutions and in mixtures with theobromine. Sufficient sample is taken to provide about 150 mg. of caffeine for the nitrogen determination. Under these conditions, the caffeine method has a limit of uncertainty (*LU* 95) value of $\pm 1\%$ caffeine based on 6 determinations on a pure caffeine sample, and six determinations on a mixture of caffeine plus theobromine.

ADVANTAGES OF NEW THEOBROMINE METHOD

The new theobromine method has provided an accurate, easy, and rapid procedure for measuring theobromine in a wide variety of samples. Inexperienced laboratory personnel have been able to use the method with very little training and practice. Equipment and reagents are inexpensive.

Under routine conditions the estimated time required per determination for cacao solids samples ready for analysis is 0.65 hour for the new method, 1.65 hours for the Wadsworth method, and 2.00 hours for the Moir and Hinks method. The time required per determination of liquid samples is estimated to be 0.33 hour for the new method, 2.00 hours for the Wadsworth method, and 1.33 hours for the Moir-Hinks method. The extremely short lapsed time needed for the completion of a number of determinations is also valuable—for example, six determinations on liquid samples can be completed in about 2 hours by the new method, while any of the other available methods would require about 8 hours. This great difference in the time required for analysis has saved many hours in both laboratory experimental work and operation of a theobromine production process.

ACKNOWLEDGMENT

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Improved Polymerization Techniques

Use of Puncture-Sealing Gaskets for Polymerization in Bottles

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A method of conducting emulsion polymerizations in beverage bottles equipped with special puncture-sealing gaskets is discussed. The sealing gasket is described in detail and the advantages realized through its use are pointed out. With this type of closure, materials can be injected against pressures up to 70 pounds per square inch or more by means of a hypodermic syringe. The pressure in the bottle can be determined quickly by a gage equipped with a hypodermic needle. From the pressure measurements, the per cent conversion of monomers to polymer can be determined over a wide range with good accuracy.

BEVERAGE bottles are used extensively in the laboratory as reaction vessels for carrying out emulsion polymerizations of synthetic rubber. When used with ordinary caps, the bottle polymerizations suffer certain disadvantages: Nothing can be added to the system without interrupting the polymerization, and the conversion cannot be determined with any degree of accuracy without coagulating the polymer. The method of following the degree of conversion described by Fryling (*2*) for tube polymerizations by measuring the change in volume of the system is not satisfactory for bottle polymerizations.

Following a suggestion by W. L. Semon, a puncture-sealing cap has been developed consisting of an ordinary crown cap with a center hole covered by a sealing insert and an oil-resistant gasket that can be used to advantage in bottle polymerizations. [Frank and Shepherd of the University of Illinois have developed a similar cap, (*1*).] Using this cap it is possible to add materials during polymerization with a hypodermic syringe and needle and to determine the degree of conversion in the latter stages of the polymerization by pressure measurements through a needle. This method has been used successfully in laboratory synthetic rubber polymerizations for several years.

APPARATUS AND MATERIALS

Bottles. Heavy crown-cap bottles are used. The quart bottles weigh at least 800 grams.

Caps. A $^1/_{32}$ inch hole is drilled in the center of a crown cap which has no gasket (see Figure 1).

Sealing Member. A Butyl rubber sealing member 0.5 inch (1.25 cm.) in diameter is cemented over the hole in the cap with the cloth backing next to the metal (see Figure 1). The sealing members are died out of stock 0.04 inch thick which has been compounded and cured in the recipe given in Table I. This stock swells on contact with butadiene and styrene to seal the opening made by the needle.

Table I. Butyl Rubber Sealant Stock

GR-I	100.0	Captax	0.5
Semireinforcing carbon black	25.0	Stearic acid	1.0
Sulfur	1.0	Zinc oxide	5.0
Tuads	1.0		

Cured 30 minutes at 285° F. with fine cheesecloth backing.

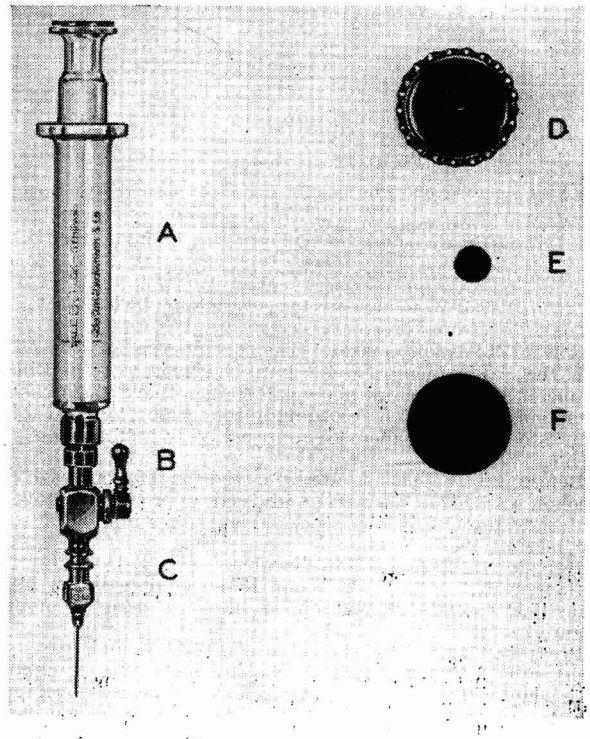

Figure 1. Syringe Assembly and Self-Sealing Caps

A. **Yale B-D Lok-syringe (5-cc. capacity)**
B. **Luer-Lok stopcock No. L/S1**
C. **Yale B-D stainless steel hypodermic needle (20-gage)**
D. **Bottle cap with center hole**
E. **Butyl rubber sealing member**
F. **Solvent-resistant gasket**

Table II. Solvent-Resistant Gasket Stock

Geon number 102	100.0
Hycar OR-15	100.0
P-33 black	50.0
Stearic acid	1.0
Sulfur	2.0
Captax	2.0
Dicapryl phthalate	50.0

Table III. Polymerization Recipe

	Grams
Butadiene	75.0
Styrene	25.0
Dodecyl mercaptan	0.5
S.F. flakes (purified soap flakes)	5.0
Potassium persulfate	0.3
Water	180.0
Temperature, ° C.	50
Conversion, %	75
Polymerization time, hours	12

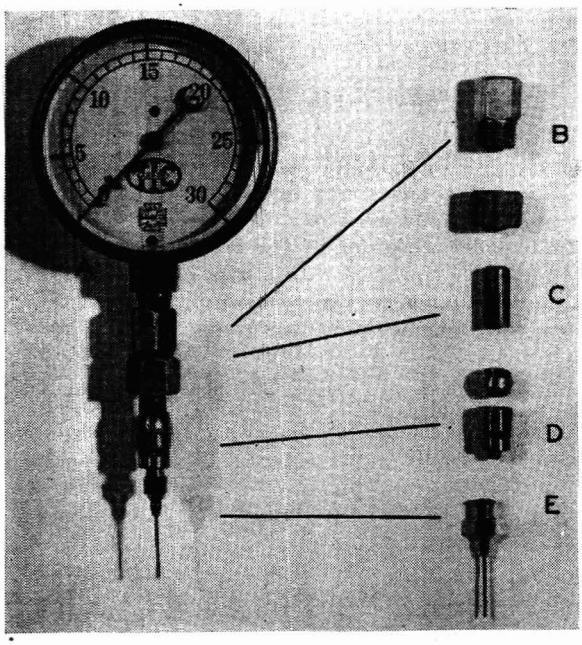

Figure 2. Pressure Gage

A. Gage, assembled for reading pressures through hypodermic needle
B, C, D, E. Component parts of adapter
B. Fitting to reduce from 1/8 inch pipe size to 5/16 inch O.D. copper tubing
C. A 3/4 inch length of 5/16 inch O.D. copper tubing
D. Luer-Lok fitting from syringe as shown in Figure 1
E. Yale B-D stainless steel hypodermic needle (20-gage)

Solvent-Resistant Gasket. The solvent-resistant gasket goes over the sealing member (see Figure 1). This gasket is 1 inch in diameter and is died out of stock 0.06 inch thick which has been compounded and cured according to the recipe given in Table II. This stock is not affected appreciably by butadiene-styrene blends.

Before use, the gaskets are extracted for 0.5 hour with ethyl alcohol, washed with hexane, and dried, in order to remove any materials present in the stock which could inhibit or retard polymerization.

Hypodermic Syringes and Needles. Yale B-D Lok syringes with capacities of 0.5, 1, 2, 5, and 10 cc., equipped with 20-gage Yale B-D rustless hypodermic needles, are used as shown in Figure 1.

Pressure Gages. Pressure gages (0 to 60 pounds per square inch) and (0 to 30 pounds per square inch) ranges, equipped with adapter, Luer-Lok, and hypodermic needle, as shown in Figure 2, are used. The adapter is assembled by soldering the copper tubing to the Luer-Lok, slipping the ring and nut shown in *B* over the tubing, and screwing them together.

PROCEDURE

In Table III a typical butadiene-styrene copolymerization recipe is given.

The following procedure is used in charging a 24-ounce bottle. To a clean bottle are added 175 grams of a soap solution consisting of 5 grams of S.F. flakes dissolved in 170 grams of distilled water. The bottle is then chilled in an ice bath and 10 cc. of a 3% by volume solution of potassium persulfate are added, followed by 25 grams of styrene containing 0.5 gram of dodecyl mercaptan. The chilled bottle is now weighed on a balance sensitive to 0.1 gram, and 75 grams of butadiene are poured in from a thermos bottle equipped with pouring tube. Enough butadiene (4 to 5 grams) should be volatilized to ensure displacement of the air from the bottle. (This should always be done under a good hood.) The bottle, capped with a hand capper, is ready to be placed in a constant-temperature water bath at 50° C. in which a group of these bottles are rotated so that they turn end over end from 25 to 30 times per minute. Though bottles rarely break, the following precautions should be taken: Bottles should be examined for flaws or cracks before use. A bottle should never be taken directly from the ice bath and put into the 50° C. bath or vice versa. A face shield and rubber gloves should be worn when bottles are handled under pressure.

It is frequently desirable or necessary to inject initiator (potassium persulfate solution), soap solution, or monomers into the bottle before the conversion is complete. This is done by drawing the desired amount of material into a graduated syringe, pushing the needle through the gasket, and injecting the contents of the syringe directly into the polymerization emulsion. When materials are injected into bottles at 50° C., a syringe no larger than 5 cc. should be used. The operation is made somewhat easier by using a small valve on the syringe, which is opened after the needle has been inserted. As an additional precaution at high pressures a guard can be used to prevent the plunger from flying out of the syringe.

In determining the pressure, the bottle should be kept in an upright position long enough for the foam and latex to flow away from the cap before the needle is inserted. The gage should always be heated to a temperature equal to or slightly higher than the polymerization bath temperature. If care has been taken in displacing the air when the recipe was charged, the pressure method of determining conversion, after the pressure drop has started, is accurate, giving values of ±2% or better. In order to use this method, one must first determine the pressure-conversion curve for the system being run. Thereafter, the conversion can be determined quickly in the latter stages of polymerization. The pressure-conversion curve for the polymerization recipe given in Table III is shown in Figure 3.

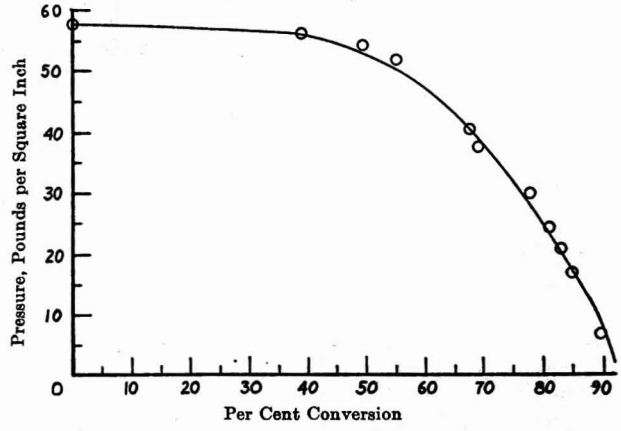

Figure 3. Pressure-Conversion Relation

The method described here for polymerization studies can also be applied to any system where it is necessary to add materials under moderate pressure while a reaction is in progress.

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Improved Polymerization Techniques

Device for Sampling Latex during Polymerization

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A procedure is described for the precise and accurate sampling of latex during the course of an emulsion polymerization without interrupting the reaction. The sampling device, which consists of a hypodermic syringe equipped with a stopcock, and a "stirrup" for stopping the plunger, is used in conjunction with puncture-sealing caps or closures on the polymerization vessels. The technique is simple and rapid and of general applicability. It is particularly useful for following the progress of a polymerization by determination of total solids and for removing samples for special investigations such as mercaptan analysis or gel and intrinsic viscosity studies.

VARIOUS procedures have been employed for following the course of emulsion polymerization reactions conducted in the laboratory. In the tube technique described by Fryling (*1*), the progress of the polymerization was followed by periodic observations of the height of the latex column. Although simple and rapid, this method can be considered only semiquantitative particularly at low conversions where the latex is usually foamy. More refined dilatometric procedures may result in increased precision but become rather laborious. All such methods suffer from at least a theoretical disadvantage in the case of two-monomer systems, in that the reduction in volume of the latex may depend upon the combining ratio of the monomers as well as upon the total hydrocarbon conversion. More serious limitations from the practical point of view, however, are the extremely mild agitation and the low upper limit upon the amount of polymer which can be prepared from each charge in such apparatus.

Screw-capped or crown-capped bottles, with capacities varying from a few ounces to about one quart, have come into rather general use as reactors for emulsion polymerizations. The progress of a given reaction has usually been followed by analysis at appropriate intervals of members of a series of duplicate charges, and conversions have been determined either from the yield of coagulum or from the total solids obtained upon evaporation of a weighed portion of "vented" latex. The apparent conversion calculated from the yield of dried polymer is subject to variations depending upon the method of coagulation, the nature of the coagulum, and the care exercised during washing. Occlusion of coagulant or emulsifying agent, loss of low molecular weight polymer through solubility in the coagulation medium, and mechanical loss of product are possible sources of error. The "total solids" method is capable of high precision, provided that a truly representative sample of latex is evaporated. The analytical sampling of latex containing volatile monomers, such as butadiene, may be complicated by relatively high pressures in the reactor, rapid loss in weight of sample due to evaporation of monomer, and tendency to foam because of the presence of emulsifying agent. The various venting procedures, employed to remove free butadiene from the latex prior to sampling, are tedious and time-consuming. Medalia (*4*) has described a stopcock device for sampling which obviates the difficulties inherent in the venting problem. The necessity of weighing the bottle before and after sampling, however, imposes a limit upon the precision which is attainable and application of the method is restricted to small reactors, such as 4-ounce bottles.

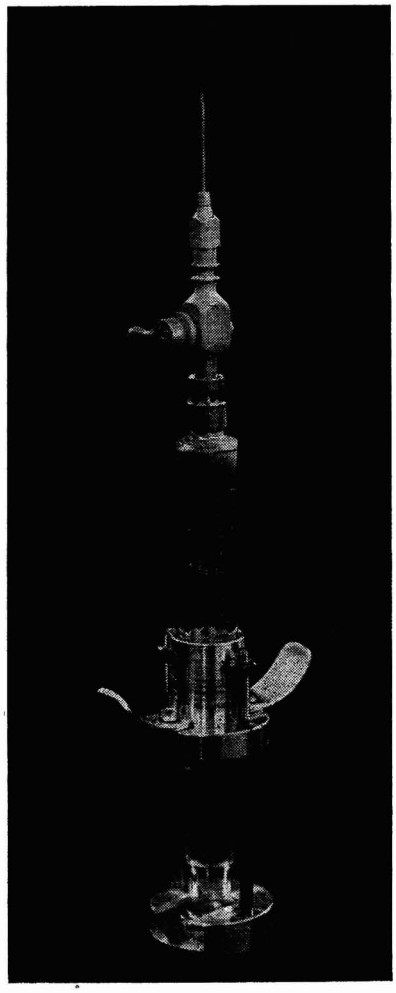

Figure 1. Hypodermic Syringe Sampling Device

A procedure has been developed for the precise and accurate sampling of latex as it is formed in an emulsion polymerization process without interrupting the polymerization. It is possible, therefore, not only to follow the progress of a single reaction throughout its entire course, but to remove samples at any time for special studies such as mercaptan analysis or gel determinations. The technique is simple and rapid and is applicable to reactors of any size.

APPARATUS

The sampling device consists of a hypodermic syringe equipped with a one-way stopcock and a "stirrup" for stopping the plunger when a sample of the desired size has been admitted. It is used in conjunction with puncture-sealing closures such as those described by Harrison and Meincke (*2*). Syringes and fittings are available from Becton, Dickinson and Company, Rutherford, N. J., or their retail outlets. Although the assembly

admits of many variations, an example of a satisfactory combination is tabulated:

Item	B&D Catalog No.
5-ml. syringe with Luer-Lok	5YL
One-way stopcock	L/Sl
Hypodermic needle	LNRH, 20 gage, 1 inch

The stirrup can be made in a few minutes from a strip of thin metal, attached to the syringe by a metal band tightened with a small machine screw, or it can be machined from light metal, as illustrated in Figure 1.

PROCEDURE

The syringe plunger and the stopcock are lubricated lightly with fairly heavy stopcock grease. One lubrication will usually suffice for a considerable number of samples.

In removing samples of latex from bottles, the usual sequence of operations is as follows:

1. Shake the bottle and invert.
2. Insert the hypodermic needle through the bottle cap and open the stopcock.
3. Allow the syringe to fill or, if the pressure in the bottle is low, draw the plunger out until stopped by the stirrup.
4. Close the stopcock and withdraw the needle from the bottle.
5. Wipe latex from the end of the needle and weigh the syringe assembly to the nearest centigram.
6. Eject the latex into a tared dish or into shortstop or coagulant, close the stopcock, and reweigh the syringe assembly.

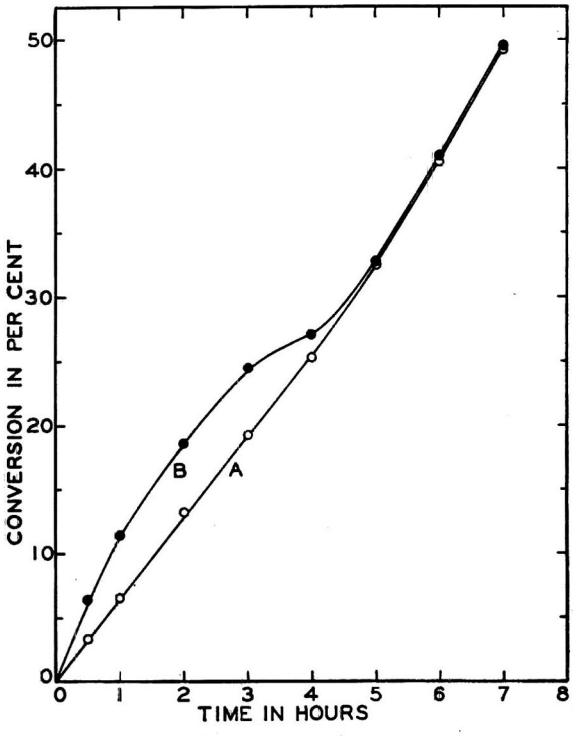

Figure 2. Sampling Deviations in Syringe Sampling Technique

A. Samples withdrawn as rapidly as possible
B. Samples withdrawn 30 seconds after those in series A

For latex at low conversions, because of the instability of most emulsions, sampling must be very rapid if a representative sample is to be assured. In such cases the needle is first inserted with the stopcock open and the plunger of the syringe held down securely with the thumb; the bottle is shaken vigorously and inverted, and the plunger is released. The sample can thus be taken within a few seconds and before appreciable separation of phases takes place. The importance of rapid sampling is emphasized by the data plotted in Figure 2, where the apparent conversions obtained

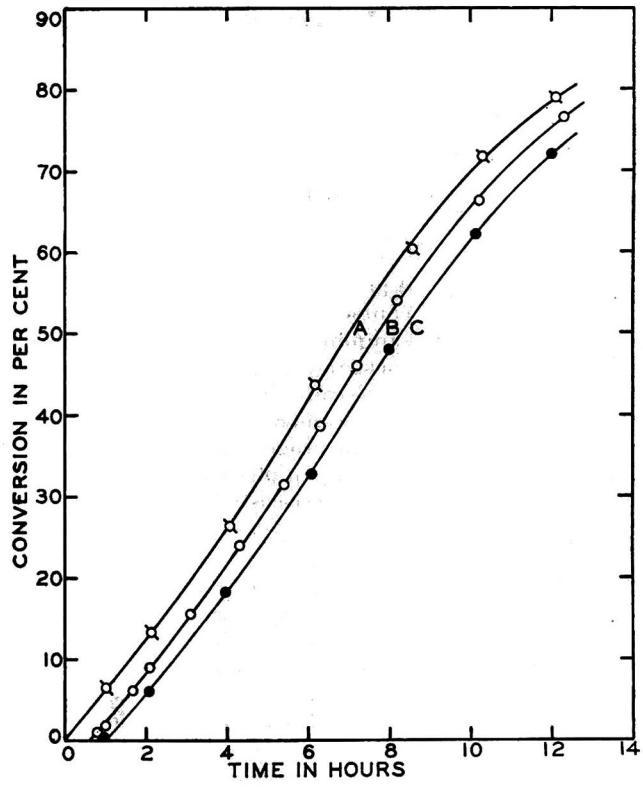

Figure 3. Effect of Oxygen on Polymerization Rates in a Butadiene–Styrene Emulsion System

A. Control; no oxygen
B. 25 ml. of oxygen (atmospheric pressure) per 100 grams of monomers
C. 50 ml. of oxygen (atmospheric pressure) per 100 grams of monomers

by sampling as rapidly as possible are compared with those obtained when the bottle was allowed to remain inverted for an additional 30 seconds before the sample was withdrawn.

In determining total solids, the latex is ejected into a weighed dish containing a suitable shortstop, such as hydroquinone, and evaporated to constant weight on a hot plate or in an oven. It is unnecessary to rinse the syringe between samples, provided the conversions are similar. If such is not the case, the syringe must be rinsed and dried or it may be flushed with latex of suitable solids concentration before it is used again.

The mercaptan content of a synthetic latex can be determined conveniently by amperometric titration with silver nitrate, according to the method of Kolthoff and Harris (*3*). In such an analysis, rapid sampling at low conversions is extremely important because the concentration of mercaptan in the monomer phase is usually high. Improved precision can be obtained in such a case by modifying the sampling procedure. The syringe is weighed, filled with sample, and reweighed; it is then rinsed with water and alcohol in order to transfer the latex and free monomers quantitatively.

DISCUSSION

The utility of the syringe sampling technique has been proved by use over nearly three years by various investigators in the author's laboratories and elsewhere. Hydrocarbon conversions can be determined by the total solids method over most of the range with an accuracy of ±0.5% conversion. Although precision and accuracy decrease slightly at low conversions, the technique is very useful for studying and distinguishing between inhibition and retardation phenomena. For illustration, the data plotted in Figure 3 for a particular butadiene-styrene emulsion system demonstrate unequivocally the inhibiting effect of

oxygen, as evidenced by definite induction periods, with no retardation once normal polymerization has begun.

ACKNOWLEDGMENT

The author is indebted to S. A. Sundet for the experimental data chosen to illustrate the hypodermic syringe sampling technique.

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Chromatographic Separation of Beta-Carotene Stereoisomers as a Function of Developing Solvent

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The quantitative treatment of chromatography as developed by LeRosen has been utilized for studying the separation of β-carotene from its *cis-trans* stereoisomers. The relative efficiency of a number of solvents as developers for this system has been determined. Certain aromatic ethers have been found superior to most of the more common developers for separating the stereoisomers adsorbed on a lime column.

RECENT WORK has shown that the provitamin A activity of carotenoids is influenced to a considerable degree by their stereochemical configuration (*3*, *4*). Since purified carotene extracts may contain up to 44% of the *cis-trans* stereoisomers (*6*), it is essential to include their estimation in any analytical procedure for carotene. Chromatographic adsorption on calcium hydroxide columns will separate the isomers from one another (*10*). However, the column must then be extruded, and each zone separately carved out and transferred to petroleum ether. In a collaborative study of this method of separating the isomers, four laboratories reported results which were in poor agreement and which indicated that the method must be further studied and simplified before it can be recommended for general routine use (*5*).

Chromatographic separation of the isomers would be simplified if it were possible to collect them separately by continuing the development until each zone passes completely through the column. The experiments herein reported were undertaken in order to obtain more information on the role of the developing solvent in increasing the separation of the stereoisomers during chromatographic adsorption analysis. The relative efficiency of the solvents as developers has been determined under standardized reproducible conditions. Quantitative data on the relative eluting strength of the solvents have also been obtained.

MATERIALS AND APPARATUS

Adsorbent. The adsorbent used in this work was Shell Brand lime, chemical hydrate, 325-mesh as recommended by Polgár and Zechmeister (*10*). A 100-pound sack of the fresh material was repacked in tightly closed bottles until used. No difficulty due to change in activity with age was noted. Precautions were always taken to minimize the exposure of the lime to the atmosphere prior to use. Through the courtesy of Polgár, a sample of the lime used in most of his published work was obtained and compared with this material (*10*). The adsorptive properties of the two lime preparations toward the carotene isomers were found to be very similar.

Isomerized-Carotene Solution. This solution was prepared from S.M.A. crystalline β-carotene. Iodine (equivalent to 2% of the weight of the carotene) was added to the carotene dissolved in petroleum ether (87.8° to 98.6° C.). The concentration of the carotene was 53.6 mg. per 100 ml. of solution. Only this concentration of carotene was studied. Solutions were stored in amber-colored flasks in the refrigerator until used. Polgár and Zechmeister have also prepared such iodine-isomerized solutions and chromatographed them on lime columns. Their report gives the relative positions of the isomers on the adsorption columns (*10*). In the present report, the isomers are identified by comparison with the previous work. Similar chromatograms were obtained with solutions stored for periods up to 3 weeks.

Solvents. The petroleum ether (87.8° to 98.6° C.) was dried over sodium and used without further purification. Acetone was treated with silver oxide, dried, and redistilled. Benzene was dried over sodium and redistilled. Ethyl ether was sodium-dried, passed through an alumina column (*2*), and redistilled. Methyl and ethyl alcohols were refluxed over magnesium and redistilled. Carbon tetrachloride was water-washed, dried, and redistilled. Chloroform was shaken with concentrated sulfuric acid, washed, dried, and redistilled. Phenyl ether and cetyl alcohol were recrystallized prior to use. All other solvents were redistilled, prior to use, in a highly efficient all-glass fractionating apparatus (*11*).

Apparatus. The chromatographic tubes (No. 1, 9 mm. in inside diameter × 130 mm.) were described by Zechmeister (*13*). Three No. 1 tubes were used in this work. Each tube was mounted by means of adapter and rubber stopper on a 1-liter suction flask which was connected to a water aspirator. A Zimmerli gage was used for determinations of the pressure at intervals during the chromatographic analysis.

PROCEDURE

Column Characteristics. LeRosen's technique with only slight modifications was used for obtaining the quantitative data presented in this paper (*7*, *8*, *9*). He introduced the following terms to assist in characterizing the systems studied:

T_{50} = time in seconds required for a solvent to penetrate 50 mm. into an initially dry column under vacuum given by a water aspirator

$$S = \frac{\text{length of adsorbent column containing one unit volume of solvent}}{\text{length of tube required to contain same volume of solvent}}$$

(S indicates average packing of column)

$$\%\ \text{volume of adsorbent} = 100\,\frac{(S-1)}{S};$$ gives a measure of percentage of tube volume occupied by adsorbent

V_c = rate of flow of developing solvent through column when a state of constant flow has been reached, mm. per minute

Table I. Characteristics of No. 1 Chromatographic Columns Packed to a Height of 75 Mm. with Shell Brand Lime and Developed with Petroleum Ether (87.8° to 98.6° C.)

Weight of Lime *Grams*	Column Length *Mm./ml.*	Packing Measure *S*	Volume of Adsorbent %	V_c *Mm./min.*	T_{50} *Sec.*	T_{75} *Sec.*
			Column 541			
3.56	16.10	1.75	42.7	18.5	60	121
			Column 536			
3.69	15.85	1.74	42.5	16.8	66	132
			Column 532			
3.85	15.65	1.69	40.9	15.4	67	130

$$R_l \text{ or } R_t = \frac{\text{rate of movement of adsorbate zone (mm. per minute)}}{\text{rate of flow of developing solvent (mm. per minute)}}$$

where R_l is the leading boundary and R_t the trailing boundary of a zone

The columns were packed as uniformly as possible to a height of 75 mm. under the full vacuum of the water aspirator. In packing, the lime was added in small amounts to the column. After each addition, the column was firmly tamped and the surface loosened with a spatula before the next quantity of lime was added. The weight of adsorbent required in each case to pack the column to a height of 75 mm. was noted. The characteristics T_{50}, S V_c, and per cent volume adsorbed were determined for each column, using petroleum ether as the solvent. In addition, T_{75}, the time in seconds required for the lower edge of the advancing solvent to penetrate to the bottom of the initially dry column, was determined. The pressure on the system at T_{50} as well as at intervals during the development of the chromatogram was obtained.

Development of Chromatogram. After petroleum ether had been permitted to flow through the column for about 10 minutes and a uniform rate of flow had been obtained, 0.20 ml. of the isomerized β-carotene solution was added to the column. At the instant that the carotene solution had become adsorbed into the column, the developing solvent (developer) under test was added and the progress of separation of the stereoisomers noted.

A method of plotting the progress of the respective pigment zones through the chromatographic column was employed. The relative positions of the leading and trailing borders of each pigment zone in millimeters' distance from the top of the column was noted at stated intervals during the progressive development of the chromatogram. By plotting these positions against the corresponding volumes of developer absorbed into the column, the borders of each zone were obtained. The graph (chromatograph) so obtained served as a permanent record of the chromatographic analysis. For later reference, the zones were colored so as to reproduce as closely as possible the actual colors of the pigments on the column. Measurements of the trailing border are often difficult to make because of the diffuse nature of this portion of the zone.

Petroleum ether was the nonpolar solvent employed as the carrier for the developers in the present report. For each chromatogram, the developing solvent was dissolved in the petroleum ether, and a chromatograph was prepared as described above. Each developer was studied over a wide range of concentrations. Figure 1 illustrates a series of chromatographs thus obtained, using *p*-cresyl methyl ether as developer. For each concentration of developer, the values of R_l and R_t for each of the three main pigment zones (neo-β B, all-*trans*-β, and neo-β U) were obtained. Several other minor pigment zones were noted. However, as pointed out by Polgár and Zechmeister (*10*) and confirmed in this laboratory, the three main zones account for at least 95% of the pigments in this isomerized mixture. Accordingly, these minor zones were disregarded in the present work.

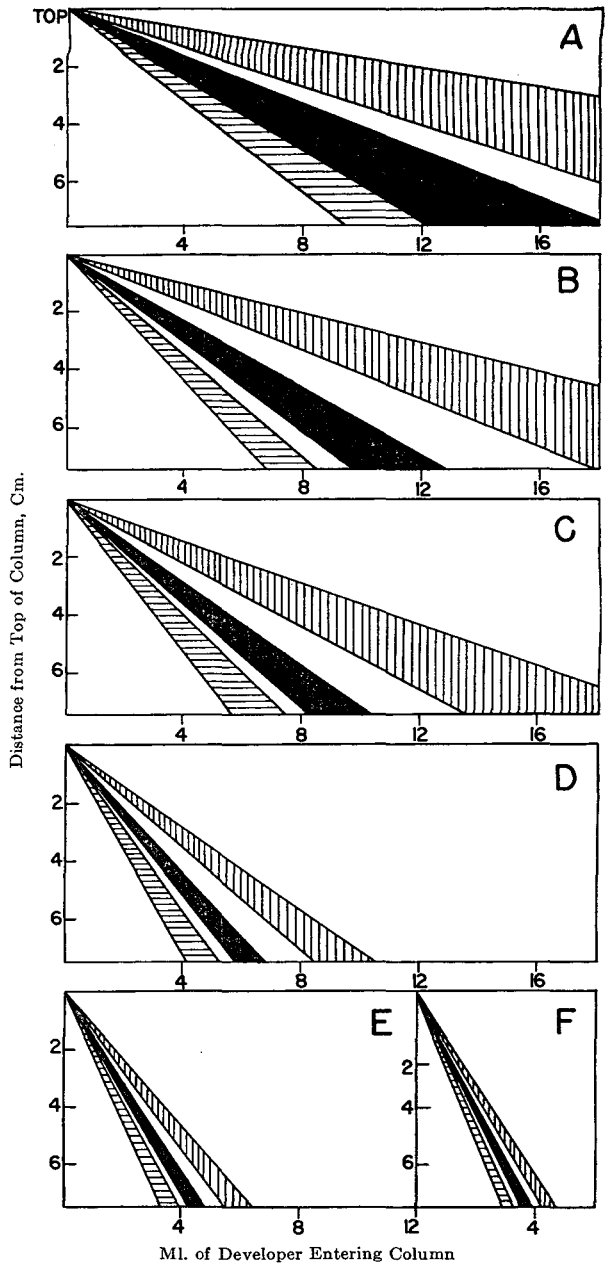

Figure 1. Chromatographs (Diagrammatically Represented) Showing Effect of Concentration of Developer on Resulting Chromatogram

Developer: *p*-cresyl methyl ether dissolved in petroleum ether (87.8–98.6° C.). Concentration of developers: *A*, 0%; *B*, 0.5%; *C*, 1%; *D*, 2.5%; *E*, 5%; *F*, 7.5%. Vertical hatching represents neo-β-carotene U; solid area, all-*trans*; and horizontal hatching, neo-β-carotene B zone

RESULTS

LeRosen has shown that a quantitative treatment of chromatography can be helpful in determining the best conditions for a given operation. He has given evidence that the terms R, S, and V_c are of value in characterizing and standardizing adsorbents as well as in predicting the relative positions of chromatographic zones (*7*, *8*, *9*). In the present work, these terms are employed to define the system studied, and R especially is used to char-

acterize and standardize the properties of the solvents which are used as eluants and developers.

At least two factors are involved in any study of the effect of a group of solvents toward a complex pigment mixture adsorbed upon a chromatographic column. They are (1) the eluting strength of each solvent relative to one another, and (2) the efficiency of the solvents for separating the components of the pigment mixture. In the present study, in which solvents of widely differing strengths as eluants were compared, there was no consistent relationship between these two factors.

Since all the experiments reported here followed the same pattern, the quantitative data can be concisely reported in several summaries. The column characteristics obtained during the preparation and development of over 300 chromatograms are summarized in Table I. Average deviations have been determined for the various quantities. The data are precise to about the following percentage of the values: $S \pm 1\%$, T_{50} and $T_{75} \pm 10\%$, $V_c \pm 10\%$, column length $\pm 2\%$. The average pressure on the system at T_{50} was 4.6 $\pm$ 0.5 cm. of mercury. During the development of the chromatograms the average pressure was 5.2 $\pm$ 0.5 cm. of mercury. These data demonstrate the uniformity obtained with the chromatographic columns. In agreement with others (*1*), the author has found that R is independent of V_c within the limits of this study.

Since R is a measure of the speed with which a pigment zone passes down the column relative to that of the developing solvent, a table of the relative eluting strength of the developing solvents may be constructed by arranging the solvents in the order of increasing values of R for a given concentration of the developer. Such a table has been prepared for all-*trans*-β-carotene, by determining the corresponding R values for 3% solutions of the developers and arranging the developers according to their R values (Table II). Trappe (*12*) has demonstrated that a group of solvents can be arranged in a definite order of diminishing eluting powers, which he calls an eluotropic series, independently of the nature of the adsorbent or of the substance adsorbed.

The relative efficiency of each of the developing solvents for separating the three main stereoisomer zones was then determined.

Table II. Relative Strength of Eluants for β-Carotene Adsorbed on Lime

Eluant	$Rl \times 100$	Eluant	$Rl \times 100$
Carbon tetrachloride	20	Ethyl butyrate	56
n-Amyl ether	22	Butyl acetate	63
Tetrachloroethylene	23	Dioxane	64
Methyl alcohol	25	Anethole	65
Toluene	29	Ethyl acetate	67
Benzene	32	Acetone	68
Ethyl ether	33	Pyridine	72
Chloroform	35	Diacetone alcohol	74
Methylene chloride	38	Cetyl alcohol	77
Ethylene chloride	40	Acetophenone	79
Phenyl ether	42	Octanol	90
p-Cresyl methyl ether	44	Ethyl alcohol	93
s-Tetrachloroethane	45	Phenyl Cellosolve	95
Ethyl laurate	55	Butyl Cellosolve	95
		Methyl Carbitol	100

Eluants used as 3% (by volume) solution dissolved in petroleum ether (87.8–98.6° C.).

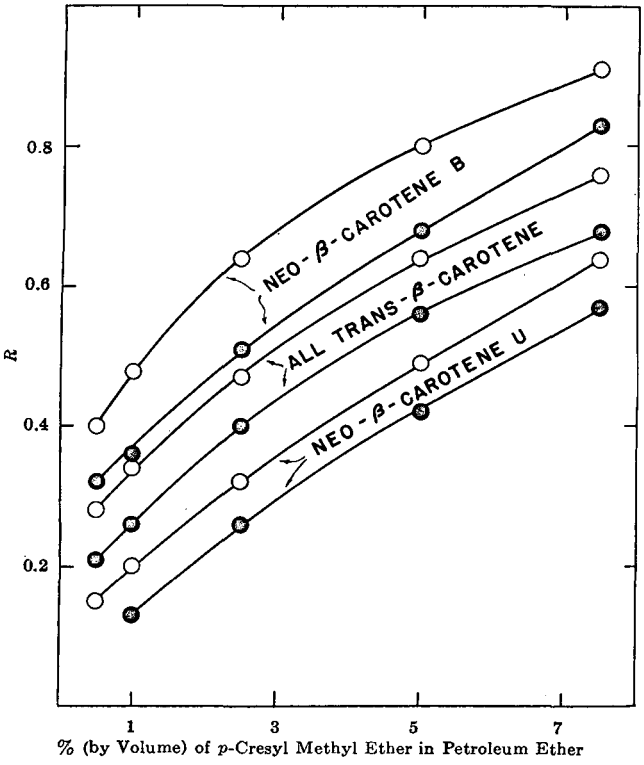

Figure 2. Relationship between R and Concentration of Developer for Neo-β B, All-*trans* β, and Neo-β U

Developer, *p*-cresyl methyl ether in petroleum ether. ●. Rt ○. Rl 87.8° to 98.6° C.

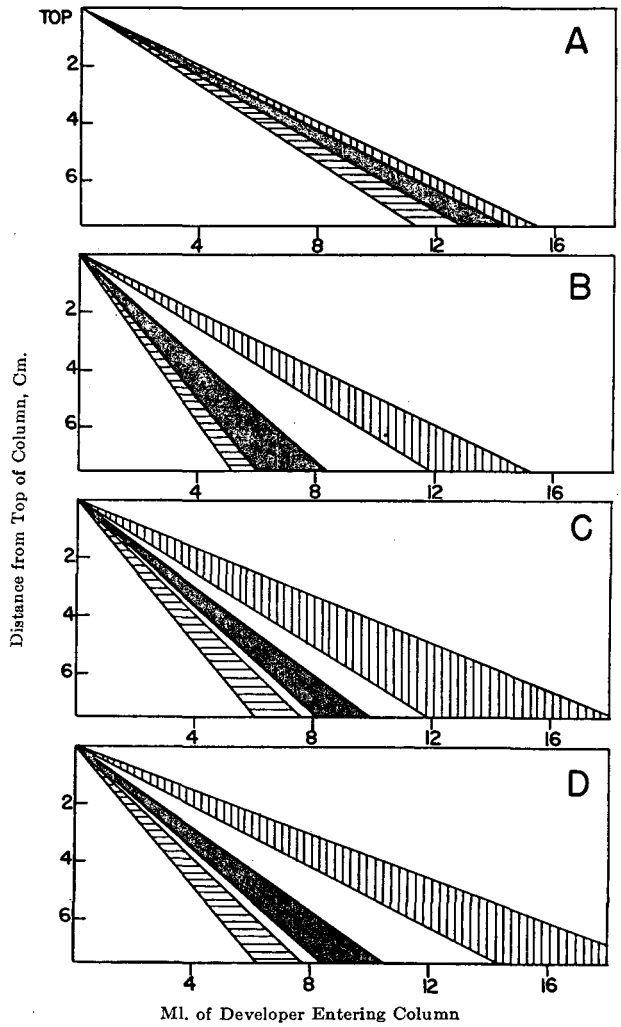

Figure 3. Chromatographs (Diagrammatically Represented) Showing Effect of Developers on Width and Degree of Separation of Zones for Neo-β B, All-*trans*-β, and Neo-β Carotene U

For representation of zones, see Figure 1. Developers: *A*, 0.1% ethyl alcohol; *B*, 1% acetone; *C*, 0.2% butyl acetate; and *D*, 3% methylene chloride, all in petroleum ether

Table III. Influence of Developer on Separating Zones of Isomerized β-Carotene Mixture Adsorbed on Calcium Hydroxide Columns[a]

Developer, %	Neo-β-carotene B		All-*trans*-β-carotene		Neo-β-carotene U	
	$R_{leading}$	$R_{trailing}$	$R_{leading}$[b]	$R_{trailing}$	$R_{leading}$	$R_{trailing}$
Ethyl alcohol (0.5)	43	40	40	38	38	36
Octanol (0.6)	42	40	40	38	38	37
Cetyl alcohol (0.9)	42	40	40	37	37	36
Butyl Cellosolve (0.5)	42	40	40	38	38	35
Phenyl Cellosolve (0.6)	42	40	40	38	38	37
Methyl Carbitol (0.8)	42	40	40	38	38	36
Pyridine (0.6)	52	40	40	30	20	14
Ethyl ether (6.9)	50	40	40	29	22	14
n-Amyl ether (17)	48	40	40	28	16	8
Dioxane (1.1)	48	40	40	33	29	21
Acetone (0.7)	48	40	40	27	19	13
Acetophenone (0.4)	54	44	40	32	23	16
Diacetone (0.6)	48	40	40	33	20	11
Benzene (6.1)	50	40	40	30	22	15
Toluene (7.0)	51	43	40	29	21	16
Tetrachloroethylene (12.2)	48	40	40	21	15	9
s-Tetrachloroethane (1.9)	50	40	40	27	23	13
Carbontetrachloride (23.0)	48	40	40	25	17	11
Chloroform (3.9)	51	40	40	28	21	15
Methylene chloride (3.3)	51	42	40	30	25	17
Ethylene chloride (3.2)	54	42	40	32	25	17
Ethyl acetate (0.4)	54	42	40	32	26	18
Ethyl butyrate (1.0)	52	43	40	32	25	18
Ethyl laurate (0.9)	49	42	40	31	25	15
Butyl acetate (0.6)	50	42	40	33	27	20
Phenyl ether (2.8)	53	44	40	32	26	19
p-Cresyl methyl ether (1.7)	56	44	40	32	25	19
Anethole (0.8)	58	46	40	31	27	21

[a] Values shown are 100 R.
[b] Concentrations of developers in petroleum ether were chosen which gave identical values of R_l for all-*trans* zone.

A plot of the values of R against concentration of developer gave, for each developer, a series of curves similar to those shown in Figure 2. Values of R for both the leading, R_l, and trailing, R_t, borders of each of the three zones (neo-β B, all-*trans*-β, and neo-β U) were thus plotted. By interpolation from these curves, it was possible to obtain the value of R_l and R_t for each zone corresponding to various concentrations of the developer. As a basis for comparison, the concentration of each developer corresponding to the R_t value of 0.40 for the all-*trans*-β zone was obtained from the graphs for each of the developers. Then the R_t and R_l values for all three zones corresponding to this concentration of developer were obtained.

These data for each developer are presented in Table III. The data represent for each solvent the average of at least two separate series of runs. Since the values of R represent relative rates of travel, the difference between R_l and R_t for a particular zone will afford a measure of the width of the zone. The difference between R_t for one zone and R_l for the following zone will measure the interzonal separation. Where these two values are equal, the trailing border of one zone is in actual contact with the leading border of the next following zone. The values of R are dimensionless. When comparisons are made at high values of R, a difference of several units between the values for two zones will represent a smaller relative difference in speed than will a similar difference of several units when measurements are made at lower values of R.

The chromatographs also proved helpful for representing the extent of separation of the zones. When petroleum ether alone was used as the developer, all the bands were wide and diffuse and there was no visible separation between the two lower zones, although there was a slight separation of the upper (neo-β U) zone from the other two (Figure 1,*A*). Of all the developers studied, the alcohols were the most effective in increasing the sharpness of the zones by narrowing down their width. However, at the same time, the bands approached so close together that there was no appreciable separation between them (Figure 3,*A*). The Cellosolves (alcohol-ethers) acted very like the alcohols (Table III). Many of the developers were effective in increasing the interzonal distance between the all-*trans*-β and neo-β-carotene U zones but had no effect on the neo-β-carotene B zone. Acetone, ethyl ether, and benzene were examples of this type of developer (see Table III and Figure 3,*B*). All the esters studied caused a slight separation of the neo-β-B zone (Figure 3,*C*, and Table III). Methylene and ethylene chloride acted like the esters in causing a slight separation between the two lower zones. With these chlorides, the trailing borders were sharp and distinct (Figure 3,*D*). The other chlorides were not effective for separating the neo-β B and all *trans* zones.

Among the best developers found were certain aromatic ethers, such as *p*-cresyl methyl ether. This compound, when used as a developer, caused the trailing borders to become sharp and increased the separation between each two of the three main bands (Figure 1). The separation of the neo-β B zone was sufficient to permit separate elution and collection of each of the three zones. The development of the column and collection of each of the three fractions were accomplished in less than 2 hours. The relative percentage of the pigments found was in agreement with results published by Polgár and Zechmeister by the technique of extruding the chromatographic column (*10*). A carotene extract prepared from dehydrated alfalfa meal was also separated by this developer into three zones. The pigments were identified by mixed chromatograms with the three isomers obtained from the iodine-isomerized carotene solution.

SUMMARY

The relative eluting strengths of a number of solvents for β-carotene and its stereoisomers have been quantitatively determined under standardized reproducible conditions.

The relative efficiency of the solvents as developers for separating β-carotene from its stereoisomers on a lime chromatographic column has also been determined. Anethole and *p*-cresyl methyl ether were found superior to most of the more common developers studied in increasing the degree of separation of the stereoisomers. When small quantities of these ethers were added to the petroleum ether used as the developing solvent, it was possible to collect separately the several stereoisomers by continuing development of the column. This information should be of value for simplifying the analytical procedure for the separation of the carotene stereoisomers from one another.

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RECEIVED June 9, 1947.

Technique for Measuring Reactivity of Gasoline Antioxidants with Air

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An apparatus for measuring the rate and extent of reaction of gasoline antioxidants with air at room temperature and atmospheric air pressure consists of a closed system including a 20-liter reservoir of air, a pump for circulating the air, a tower for countercurrent contact of the antioxidant solution with air, and auxiliary portions such as traps and drying towers. An antioxidant to be tested is dissolved in alcohol or other suitable solvent. Air entering the reactor serves as an air lift to carry the antioxidant solution to the top of the packed reactor column, then separates and enters the bottom of the column for countercurrent contact in the tower with the antioxidant. Once started, the operation of the apparatus is entirely automatic. After periods of contacting, the volume of oxygen absorbed is measured and reaction is continued until no more oxygen is absorbed. Typical results showing the reactivity of *n*-butyl-*p*-aminophenol and *N,N'*-di-*sec*-butyl-*p*-phenylenediamine with air are presented. This apparatus or modifications of it may find application in other studies involving absorption of gases in liquids.

TWO of the more common types of organic compounds used as gasoline antioxidants—aminophenols and phenylenediamines—are reactive with air. In order to gain possible insight into their behavior, the rate and extent of their reaction with air at room temperature and atmospheric air pressure have been determined. Although the results of these studies may be of interest, they are mentioned only briefly and this paper is limited chiefly to the technique developed for these measurements. This technique may be useful in other studies involving the absorption of gases in liquids.

Figure 1 shows the apparatus developed. Essentially, it consists of a closed system including a 20-liter reservoir of air, a pump for circulating the air, a tower for countercurrent contact of the inhibitor solution and air, and auxiliary portions such as traps, drying towers, etc.

The individual units in the system are calibrated for volume before assembly by being filled completely with water, and allowance is made for the volume of drying reagents, inhibitor solution, etc., to be added. The assembly is made completely airtight.

In a test, the antioxidant dissolved in alcohol or other solvent is introduced into the reactor by opening the system at trap *D* and the system is again closed. Then the air pump, *A*, is started. This is a diaphragm pump which circulates air into the 20-liter bottle, *B*, and from there through drying tower *C* containing magnesium perchlorate. The air then passes through trap *D* and into the air lift at *E*, where the inhibitor solution is picked up and carried to reservoir *F*. The air rate is adjusted by regulating screw clamp *L* on the by-pass line to give the most efficient operation of the air lift. From *F* the inhibitor solution flows by gravity over to the top of the reactor column, *G*, which is packed with glass helices. The air separates in *F* and goes to the bottom of *G*, coming up through the column and thus undergoing countercurrent contact with the inhibitor solution coming down. The air leaves *G* via dry ice trap *H*, which condenses the solvent, and then passes through tower *I* filled with silica gel to absorb any remaining solvent vapors. It continues through the bulb with manometer *J* attached and thence to the suction side of pump *A*.

The only point at which the system is open to the atmosphere during an experiment is via the water seal into leveling bottle *K*. This bottle is used to adjust the pressure in the system to atmospheric prior to making a reading. *K* is weighed initially and at about 50-hour intervals and the loss in weight is used as the measurement of the volume of oxygen absorbed from the system and replaced by water entering *B*. Air volumes from all measurements are corrected to standard temperature and pressure and a dry basis—that is, water-free and solvent-free. The circulation is continued, with volume measurements at desired intervals, until no further oxygen is absorbed.

This technique provides continuous, automatic reaction in a closed system without danger of contamination from outside sources. The air to be reacted with the inhibitor is also made to serve as an air lift to carry the inhibitor solution to the top of the packed reactor tower. The air in the 20-liter bottle is renewed as required, so that the oxygen content is never depleted below 15%. Weighing the small bottle of water gives an accurate measurement of the volume of oxygen absorbed (±10 ml.). The size of this bottle can be varied in accordance with the amount of oxygen being consumed to provide maximum accuracy in weighing. The bottle used by the author was of 3-liter capacity.

The dimension of most parts of the apparatus is not critical. However, if the tube leading from air lift *E* up to reservoir *F* is too large, the air will by-pass the liquid in the tube and the lift will not function properly. The author used a tube 5 mm. in outside diameter. In the apparatus used by the author *F* was of 125-ml. capacity. The reactor tower was 25 mm. in outside diameter and had 42.5 cm. (17 inches) of packed section.

In addition to the diaphragm pump, a double bellows pump operating out of phase, with suitable check valves, was found suitable for air circulation. The system described includes a 20-liter bottle to serve as a large air supply to decrease dilution with residual nitrogen. If a pure gas were being employed, a much smaller gas supply vessel could be

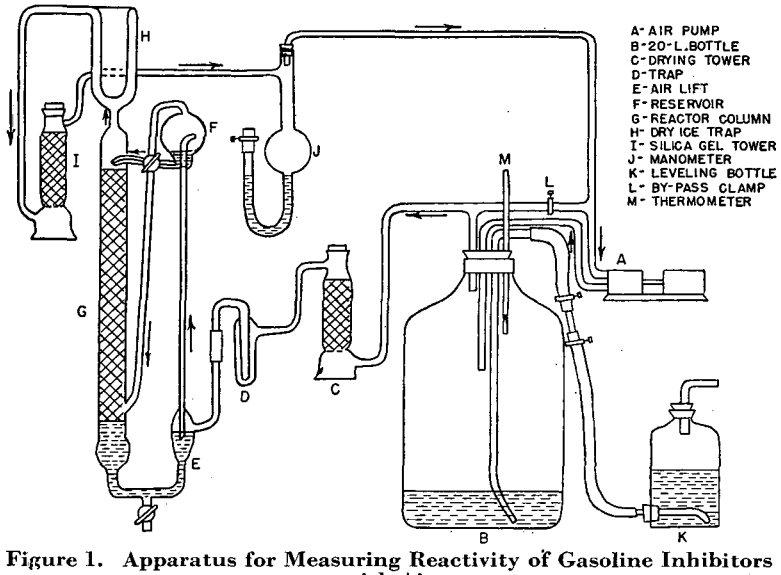

Figure 1. Apparatus for Measuring Reactivity of Gasoline Inhibitors with Air

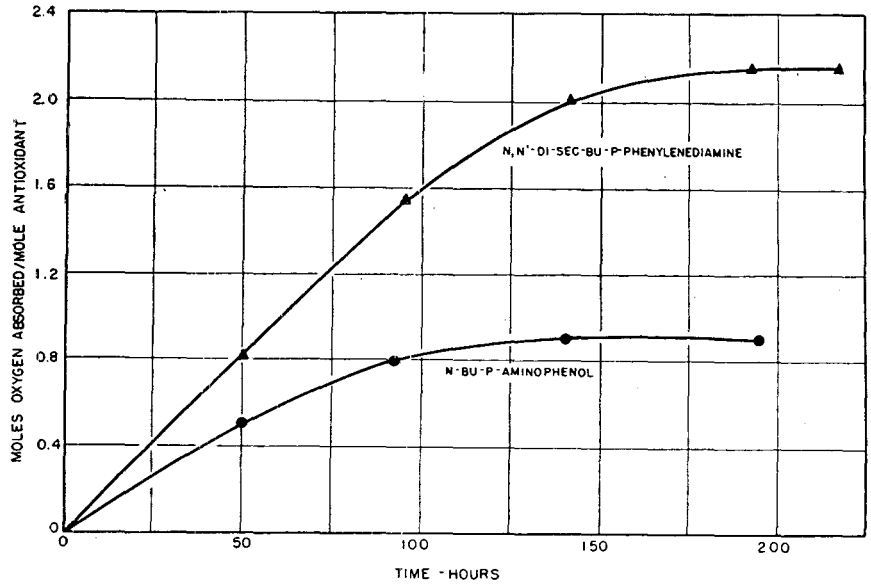

Figure 2. Reactivity of *N*-Butyl-*p*-aminophenol and *N,N'*-di-*sec*-butyl-*p*-phenylenediamine with Air

used and volume measurement thereby simplified. In order to correct the air volumes to a dry basis, the entire system is considered in three sections. The main section including the large bottle is assumed to be saturated with water vapor and is corrected accordingly. The two drying bottles are assumed to be free of water or solvent. The section which includes the vapor space in the reactor column and the dry ice trap, is corrected for the vapor pressure of the inhibitor solvent. The dry ice trap is allowed to warm to room temperature, so that the whole system is in equilibrium before a volume measurement is made. To clean the apparatus, spent inhibitor solution and washings with fresh solvent are withdrawn through the stopcock at the bottom of the absorber tower.

Figure 2 shows typical results from measurements of reactivity with oxygen for *n*-butyl-*p*-aminophenol and *N,N'*-di-*sec*-butyl-*p*-phenylenediamine. The former absorbed a total of about 1 mole of oxygen per mole and the latter absorbed about 2 moles of oxygen.

In each case, 13.5 grams of the antioxidant were dissolved in enough absolute ethyl alcohol to make 90 ml. of solution. At the end of 141 hours' reaction time, 1639 ml. of oxygen (0.89 mole per mole of antioxidant) had been absorbed by *n*-butyl-*p*-aminophenol and after 193 hours *N,N'*-di-*sec*-butyl-*p*-phenylenediamine had absorbed 2935 ml. of oxygen (2.15 moles per mole of antioxidant). There was no further oxygen absorption. The size of sample chosen was such as to give volumes of oxygen absorbed convenient to measure.

An aqueous solution 1.2 molar in pyrogallol and 3.6 molar in sodium hydroxide was found to absorb about 1.5 moles of oxygen per mole of pyrogallol in 85 minutes, after which little further absorption occurred.

ACKNOWLEDGMENTS

The author wishes to express his gratefulness to H. G. M. Fischer for his interest in and guidance of this work.

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Measurement of Enzymatic Activity on Limit Dextrin

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A method is presented whereby the limit dextrinase activity of an enzymatic agent may be expressed quantitatively in terms of milligrams of fermentable sugar produced from a standard limit dextrin solution by 1 gram of the enzymatic material in 1 hour at 30° C. The method presented may be used also in the determination of the effect of variables such as temperature, pH, and reaction time on limit dextrinase activity. This method is reproducible to the extent of approximately ±12% of the average value.

THE efficacy of enzyme preparations in the conversion of diastatically produced limit dextrin to fermentable sugar is of great technical importance, particularly when rapid and complete conversion of starch to fermentable sugar is desired. It has been shown that cornstarch may be 65 to 75% converted to fermentable sugars by malt enzymes in 15 minutes (*1*). However, at the end of this period the reaction rate greatly decreases, and the amylase-resistant limit dextrin remaining is only very slowly converted to fermentable sugar.

In the latter stage of saccharification, there is evidence that enzymes other than α- and β-amylase are in action. Kerr and coworkers have shown that α-glucosidase has a saccharifying effect on limit dextrin (*3*, *4*). Kneen has found evidence that a saccharifying enzyme other than α- and β-amylase is present in certain bacterial and fungal preparations and cereal malts (*5*).

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Despite the importance of limit dextrinase activity (where limit dextrinase activity is defined as the power of an enzymatic preparation to saccharify limit dextrin produced by the action of barley malt diastase on cornstarch), no method exists for expressing the limit dextrinase activity on a quantitative basis.

The usual methods of evaluating a starch-saccharifying enzymatic agent in terms of its amylase action are inadequate to express the limit dextrinase activity, since such methods are based on the preliminary conversion of starch. Methods based on rate and degree of fermentation of converted starch or grain may give an indication of the presence of an enzyme with limit dextrinase activity, but under the experimental conditions employed it is difficult to differentiate this action from the action of the amylases. In addition, complete fermentation studies are tedious, time-consuming, and difficult to run in large numbers; numerous variables also play an important role.

Evaluation of enzymatic preparations on the basis of their

limit dextrinase activity and the determination of the effect of various reaction conditions on the activity require a method that is rapid and dependable, that will enable the simultaneous determination of a number of samples, and that will permit variation in the reaction conditions.

The proposed method described herein was devised as a means of evaluating enzymatic preparations on the basis of their power to convert diastatically produced limit dextrin to fermentable sugars under standard conditions of time, temperature, pH, and substrate concentration. The method may be modified in order that the effects of such variables as pH, temperature, time, and presence of various foreign substances may also be determined.

DETERMINATION OF FERMENTABLE SUGARS PRODUCED BY LIMIT DEXTRINASE ACTIVITY

Several methods for determining the degree of conversion of limit dextrin to fermentable sugar were investigated. A method widely used to measure conversion in malt hydrolysis of starch is the determination of reducing power (usually expressed in terms of maltose). However, as is well recognized, such a determination lacks quantitative significance, because of the variety of reaction products of different reducing powers that are produced (*8*, *14*).

Another method, based on complete, rapid removal of fermentable sugar by yeast, was introduced by Hiller and associates (*2*) and improved and developed by Somogyi (*12*, *13*, *15*). It has been used successfully for the estimation of fermentable sugars in such substances as biological fluids and wood hydrolyzates. The usual procedure is the measurement of the difference in reducing powers before and after yeast sorption. It was found by Saeman and co-workers that the fermentability of acid-hydrolyzed wood sugar worts as determined by the rapid yeast sorption method was essentially the same as when determined by the conventional fermentation method (*9*).

Limit dextrin systems after enzymatic saccharification contain fermentable sugars of different reducing powers in addition to nonfermentable substances of different reducing powers; therefore, it is necessary to acid-hydrolyze aliquot portions of the blank (sample in which enzyme action is destroyed before addition of limit dextrin) and the enzyme-treated sample, so that the difference in reducing powers correctly represents the amount of fermentable sugars sorbed, in terms of glucose.

Method Adopted for Determination of Fermentable Sugars

The method adopted for determination of fermentable sugar produced by limit dextrinase activity of enzymatic preparations is based on the difference in reducing power (after acid hydrolysis) in the blank (enzyme destroyed before limit dextrin added) and the enzyme-treated sample after complete, rapid removal of fermentable sugar from a dilute solution by a large quantity of baker's yeast. A 2.5-hour treatment of the dilute solution of fermentable sugars at pH 4.8 and at 30° C. with fresh baker's yeast (15 grams of yeast to 100 ml. of liquid treated) has been selected. These conditions have been shown by Stark and Somogyi (*15*) to result in complete removal of maltose. If the fermentable sugar is composed only of glucose, the amount of yeast may be reduced to 5 grams per 100 ml. of solution and the fermentation time cut to 1 hour.

Since unwashed yeast contains a variable amount of reducing substance, Somogyi's method (*12*) of repeatedly washing and centrifuging the yeast until the wash water is clear has been adopted. Some dilution of the sugar solution occurs when the washed yeast is added, but this may be corrected for by determining the reducing power of the blank before and after yeast sorption; in each case the reducing power is determined after acid hydrolysis.

Reducing power before and after removal of fermentable sugars by yeast is determined by the Shaffer-Hartmann sugar analysis micromethod (*11*) as described by Stiles, Peterson, and Fred (*16*).

The proposed method for the determination of limit dextrinase activity (in terms of the milligrams of fermentable sugar produced from limit dextrin by 1 gram of enzyme) is based on the fact that conversion of a standard solution of limit dextrin is proportional to the quantity of enzyme employed up to a certain degree of conversion. This degree of conversion must be determined experimentally for the enzyme under consideration. Obviously, for the method described here, it is essential that the amount of enzyme employed produce a degree of conversion that is within the range of linear proportionality between limit dextrin conversion and quantity of enzyme employed.

PROPOSED METHOD FOR DETERMINATION OF LIMIT DEXTRINASE ACTIVITY

Materials and Reagents Required. Preparation of Diastatically Produced Limit Dextrin. Malt extract for use as "premalt" and as the saccharifying agent is prepared by extracting 550 grams of finely ground barley malt with 2 liters of water for 1 hour with frequent shaking, followed by centrifuging to separate the extract from the solids.

To 12 liters of water in a carboy is added a water dispersion containing 1.8 kg. of cornstarch. The temperature is raised to 68° C. and 320 ml. of malt extract, previously held at 68° C. for 2 to 3 minutes, are added as a premalt. The starch dispersion is agitated at 80° C. for 2 hours and then pressure-cooked at 60 pounds per square inch for 7.5 minutes (or 1 hour at 15 to 20 pounds per square inch). The dispersion is then cooled to 58° C. and adjusted to pH 5.5, and 1280 ml. of malt extract are added. After a 45-minute hydrolysis period at 55° to 58° C., the hydrolyzate is autoclaved for 1 hour at approximately 16 pounds per square inch to destroy enzyme action. After cooling to 30° C., baker's yeast (123 grams) is added, and the volume is adjusted to 16 liters and held at 30° C. for 44 hours. To remove solids, the mass is centrifuged. This may be done rapidly and continuously by means of a supercentrifuge.

After removal of solids, the volume is reduced to approximately 1 liter by evaporation under reduced pressure. The concentrate is then filtered and the filtrate is added to two to four times its volume of methyl alcohol, stirred, and allowed to stand overnight. The gummy residue is removed from the main bulk of the liquid and purified by redissolving in a small quantity of water, filtering, and reprecipitating with methyl alcohol. Finally, the gummy mass is repeatedly washed with methyl alcohol and then dried in a vacuum oven at 50° C. The dried limit dextrin is then finely powdered (using mortar and pestle) and passed through a fine screen.

Buffer Solution (pH 4.8). Dibasic sodium phosphate (35.32 grams of $Na_2HPO_4.12H_2O$) plus 9.73 grams of citric acid are made to 1 liter. The pH should be checked and if necessary adjusted to 4.8 by addition of the proper constituent. In an extended investigation, it is advisable to sterilize a number of approximately 100-ml. portions of the buffer solution and store until needed.

Sulfuric Acid, 1.50 *N*.

Sodium Hydroxide, 1.50 *N*.

Buffered Limit Dextrin Solution. A solution of 0.90 gram of limit dextrin per 100 ml., containing 20% by volume of the buffer solution, is prepared fresh each day.

Reagents for Determination of Reducing Sugar. The method for preparation of the alkaline copper sugar reagent and thiosulfate is described by Stiles, Peterson, and Fred (*16*).

Washed Yeast. Baker's yeast is repeatedly suspended in fresh portions of water and centrifuged until the wash water becomes clear. After the final wash water has been decanted, the yeast is placed on absorbent paper and pressed to remove excess moisture.

Preparation of Enzyme Extract. If a dry enzymatic preparation is to be tested, it is first ground thoroughly and then 0.900 gram is extracted with 20 ml. of water (accurately pipetted) for 1 hour at 30° C. in a tightly stoppered flask; continuous agitation is maintained. After centrifuging and decanting, the decanted extract is ready to be diluted as specified in the procedure.

If the enzymatic agent is in liquid form, extraction is unnecessary and the material is simply centrifuged or filtered through glass wool to remove solids.

Procedure for Determination of Limit Dextrinase Activity. The method depends on utilizing a concentration of enzyme in the range in which limit dextrin conversion is linear with respect to enzyme concentration. Therefore it is necessary first to standardize the particular type of enzymatic agent being tested

by determining limit dextrin conversions produced with a series of different amounts of the enzymatic agent. The standardization is carried out by adding 5-ml. portions of diluted enzyme extract (the stock enzyme extract prepared as previously described is diluted to give a series of enzyme extracts containing varying quantities of the stock extract). By plotting the degree of conversion of limit dextrin against the quantity of enzyme employed, the range of linear proportionality is evident.

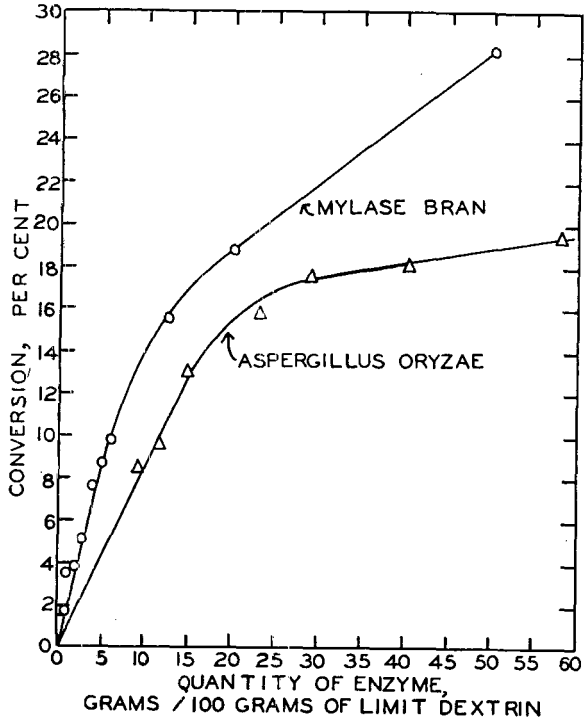

Figure 1. Comparison of Effect of Quantity of *Aspergillus oryzae* and Mylase Bran on Conversion of Limit Dextrin to Fermentable Sugar

Hydrolysis time, 1 hour; temperature, 30° C.; pH, 4.8

The desired number of 50-ml. volumetric flasks, each containing a 20-ml. portion (accurately pipetted) of limit dextrin solution (containing 0.18 gram of limit dextrin) are placed in a water bath at 30° C. and the contents are allowed to come to this temperature. Five milliliters of enzyme extract of the proper dilution, based on the standardization, are added to each flask to give an enzyme concentration within the range of linear proportionality between conversion and quantity of enzyme. The contents of each flask are mixed, the reaction is allowed to proceed for 60 minutes, and then the enzymatic activity is destroyed by the addition of 5 ml. of 1.50 *N* sodium hydroxide. After 30 minutes, the mixture is adjusted to pH 4.8 with 1.50 *N* sulfuric acid, using methyl red indicator. If the enzymatic agent is buffered, the pH of the converting mixture should be checked. It is necessary that the conversion be carried out at the specified pH level of 4.8.

It is desirable to determine limit dextrinase action using at least three different enzyme concentrations. Within the linear proportionality range, the higher the conversion obtained, the less the relative error in the determination. Because of the high error in the low conversion range, conversions below the 3% level should be avoided. Reproducibility of results at varying conversion levels are discussed in the section on application of the method.

A blank is run in a similar manner except in the order of addition.

Five milliliters of the enzyme extract are placed in a 50-ml. volumetric flask containing 5 ml. of 1.5 *N* sodium hydroxide. After 30 minutes, 20 ml. of the standard limit dextrin solution are added and the pH is adjusted to 4.8 by the addition of 1.5 *N* sulfuric acid using methyl red indicator. From this point on, the blank is treated exactly as described for the samples. (If the enzymatic agent contains no fermentable substance or substance yielding reducing power after acid hydrolysis, the blank may be run in exactly the same manner as the samples, except that no enzyme extract is added.)

After each of the samples and the blank have been made to volume, a 20-ml. portion from each is placed in 40-ml. graduated centrifuge tubes. From the blank a second portion of 10 ml. is removed and added to 10 ml. of 1.38 *N* hydrochloric acid. (It is convenient to use 8-inch test tubes for the sample-acid mixture.)

To each of the 20-ml. portions previously placed in centrifuge tubes are added 3 grams (moist weight) of washed fresh baker's yeast. After being shaken thoroughly to disperse the yeast, each tube is held for 2.5 hours at 30° C. (The tubes are shaken several times during the 2.5-hour period.) Each tube is then centrifuged, the liquid portion is decanted, and 10 ml. are added (by accurate pipet) to 10 ml. of 1.38 *N* hydrochloric acid. All the samples which have been added to hydrochloric acid are then hydrolyzed in a boiling water bath for 2.5 hours, cooled, neutralized to the phenolphthalein end point with sodium hydroxide (1 to 2 *N*), and made to a volume of 100 ml. Triplicate 5-ml. portions from each sample are added to 5-ml. portions of sugar reagent and reducing sugar is determined as described in the standard method (*16*).

STANDARDIZATION OF SUGAR REAGENT. In the accurate determination of reducing sugar, it is necessary to construct a a curve relating thiosulfate titration values with quantity of pure dextrose. It is suggested that a curve be drawn (on a large piece of graph paper) relating a series of 5 to 50 mg. % samples of dextrose to thiosulfate titration. It is convenient to employ concentrations of dextrose at intervals of 5 mg. %. National Bureau of Standards dextrose should be used. The results should give a straight line, or nearly so. (The curve does not pass through the origin.) Intermediate values are obtained from the curve by interpolation.

MODIFICATION OF STANDARD METHOD TO PERMIT DETERMINATION OF VARIABLES ON LIMIT DEXTRINASE ACTIVITY

By slight modification of the standard method given, the effect of any variable on limit dextrinase activity may be studied. For example, the enzymatic preparation may be added to limit dextrin solution buffered at any desired pH level, or temperature or reaction time may be varied. After destruction of the action of the enzyme system, the procedure is the same as in the standard method. However, if any substance is added that yields reducing power on hydrolysis, proper correction must be made.

It is desirable during the yeast sorption step that the amount of glucose in solution not exceed 400 mg. %; during the acid hydrolysis step, the limit dextrin concentration should also be low (less than 1%), so that hydrolysis to glucose may proceed to as near completion as possible. In the determination of reducing power as glucose, the samples must be so diluted that the 5-ml. sample taken for analysis does not exceed 2.2 mg.

In determining the effect of variables on limit dextrinase activity, it is convenient to express the action in terms of per cent conversion to fermentable sugar.

Calculations.

B_1 = mg. of glucose in a 5-ml. sample of the blank before yeast sorption. (Sample removed after acid hydrolysis and final dilution)

B_2 = mg. of glucose in a 5-ml. sample of the blank after yeast sorption. (Sample removed after acid hydrolysis and final dilution)

G_2 = mg. of glucose in a 5-ml. sample of the enzyme-treated sample after yeast sorption. (Sample removed after acid hydrolysis and final dilution)

E = grams of enzyme preparation extracted per 5 ml. of enzyme extract added to the 20-ml. portion of limit dextrin

$$\frac{(B_2 - G_2)}{B_2} 100 = \% \text{ conversion}$$

Defining limit dextrinase units as the milligrams of fermentable sugar produced from limit dextrin by 1 gram of enzyme preparation in 1 hour at 30° C.,

$$\frac{(B_2 - G_2)\ 100}{E\left(\frac{B_2}{B_1}\right)} = \text{L.D. units (on "as is" basis)}$$

The factor $\left(\frac{B_2}{B_1}\right)$ is required to correct the enzyme quantity for dilution introduced by the use of washed yeast.

This value multiplied by 100 and divided by the per cent dry matter of the enzyme preparation gives the limit dextrinase units on a dry basis.

If the enzymatic agent tested was originally in liquid form, the limit dextrinase activity may be expressed in terms of milligrams of fermentable sugar produced by 1 ml. of liquid enzyme preparation, or in terms of the dry weight of the enzymatic preparation.

APPLICATION OF METHOD

The effect of the enzyme–limit dextrin ratio on conversion of limit dextrin was tested over a wide range for Mylase bran (Wallerstein) and a 48-hour-submerged culture of *Aspergillus oryzae*. The data obtained are shown graphically in Figures 1 and 2. In order to indicate the range of conversion values obtained for given quantities of Mylase bran, the results of repeated tests by two operators are shown in Figure 2. In the conversion range of 3.0 to 8.6%, Mylase bran gave an average limit dextrinase value of 2060 with a standard deviation of 330. Over the range 7.1 to 8.6% conversion, the standard deviation was 188; over the range 4.7 to 7.2% conversion, the standard deviation was 220, while in the range 3.0 to 4.6% conversion, the standard deviation was 790. Obviously, the higher the conversion (within the limits of linear proportionality) the greater the accuracy of the method.

It may be observed from both Figures 1 and 2 that under the conditions of the experiment, the relationship between the quantity of enzyme preparation used and the conversion of limit dextrin to fermentable sugar is linear up to approximately 9% conversion for Mylase bran and 12% conversion for *Aspergillus oryzae*. In the determination of limit dextrinase units for various preparations of Mylase bran or submerged culture of *A. oryzae* the quantity of enzyme used must be so regulated that the 9 or 12% conversion is not exceeded. Further, in the determination of limit dextrinase activity of other enzyme preparations, the range of linear proportionality of enzyme quantity per cent to limit dextrin and the conversion of the limit dextrin must be established; it is necessary when determining limit dextrinase activity in limit dextrinase units that conversion be held within this limit by proper regulation of the enzyme quantity.

Table I. Limit Dextrinase Activity of Enzymatic Preparations

Enzyme Preparation	Enzyme, Grams/ 100 Grams of Limit Dextrin	β-Amylase, Maltose Equivalents/ Gram of Limit Dextrin[a]	α-Amylase, Units/Gram of Limit Dextrin[b]	Conversion of Limit Dextrin, %	L.D. Units
Aspergillus oryzae (535) (submerged culture)	14.6	Negligible	171.0	13.2	1000
	11.6		137.0	9.6	890
	9.1		107.0	8.9	1060
Mylase bran	4.0		32.0	8.0	1970
	3.1		25.0	6.1	1880
	2.5	Negligible	20.0	5.5	2120
	1.5		12.0	3.2	2080
Barley malt					
A	50.0	1.0	15.5	1.4	30
B	50.0	1.2	30.0	2.7	60
C	50.0	1.5	17.5	3.4	70
D	50.0	3.0	32.5	7.9	160

[a] β-Amylase maltose equivalents are defined as grams of reducing substance expressed as grams of maltose produced from 100 grams of malt acting on soluble starch under specified experimental conditions. Value obtained is corrected for action of α-amylase. [Olson, Evans, and Dickson (7) modification of Kneen and Sandstedt method (6) employed.]
[b] α-Amylase units determined by method of Sandstedt, Kneen, and Blish (10).

The limit dextrinase activity of Mylase bran, *A. oryzae* (48-hour-submerged culture), and four samples of barley malt are tabulated in Table I. For purposes of comparison, the α- and β-amylase contents of the enzyme preparations in units added per gram of limit dextrin are given.

The data presented in Table I show that, under the experimental conditions employed, the degree of conversion of limit dextrin by an enzymatic agent is not related to the α-amylase units in the enzyme preparation. For example, Mylase bran containing 12 units of α-amylase to 1 gram of limit dextrin resulted in a higher conversion of limit dextrin than did barley malt containing 15 to 30 units of α-amylase plus a relatively high quantity of β-amylase; *A. oryzae* containing 107 units of α-amylase resulted in practically the same conversion of limit dextrin as Mylase bran containing 32 units of α-amylase.

The β-amylase may have an effect on the conversion, but the effect is not great as is reflected in the low limit dextrinase units of the various barley malts tested. It appears then that α- and β-amylase have little effect on the limit dextrinase activity.

The relatively high limit dextrinase activity of mold-enzymatic agents (Mylase bran and *A. oryzae*) as compared with barley malt may be an important factor in explaining the higher yields, and possibly higher rate of production, of alcohol obtained when mold preparations are used (alone, or as a supplement to malt) as conversion agents of starch.

Further experimental work is necessary to establish the importance of the limit dextrinase activity. However, it appears that a knowledge of limit dextrinase activity in conjunction with a knowledge of α- and β-amylase content may be of worth in evaluating enzymatic agents for rapid and complete conversion of starch.

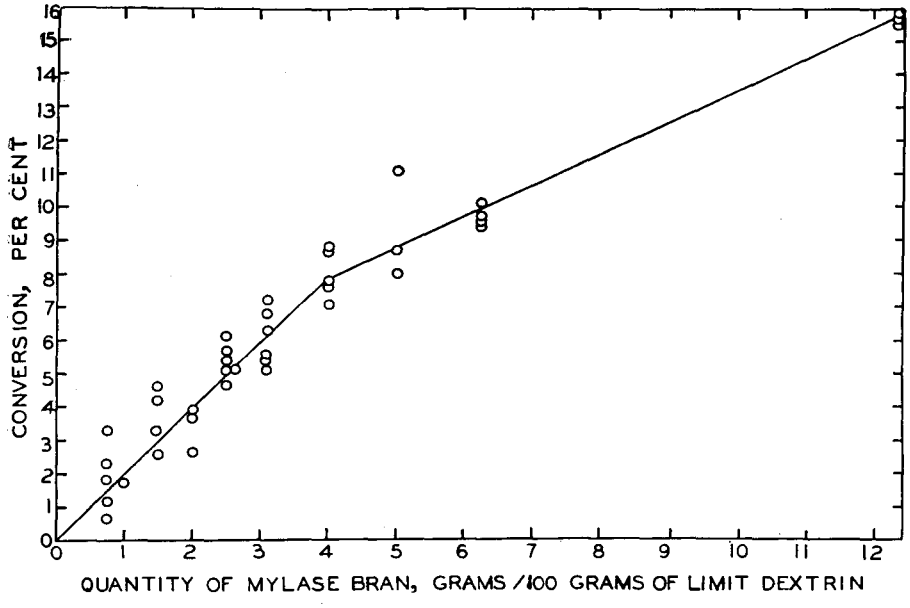

Figure 2. Effect of Quantity of Mylase Bran on Conversion of Limit Dextrin to Fermentable Sugar

Hydrolysis time, 1 hour; temperature, 30° C.; pH, 4.8.

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Determination of Hydrogen Chloride in Presence of Chlorine

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A rapid method for determination of hydrogen chloride in the presence of chlorine is described. Chlorine can be almost completely boiled out of solutions as dilute as 0.008 *N* in hydrochloric acid in 15 minutes, and the hydrochloric acid can then be titrated directly with alkali. The blank is constant down to this concentration and is very small (0.0007 equivalent per liter). The system hydrogen chloride–chlorine-water is discussed. Evidence is presented which supports the mechanism of hydrolysis of chlorine recently postulated by Morris—the reaction of chlorine molecules with hydroxyl ions.

IN A series of chlorination studies the progress of the reaction was followed by observing the amount of hydrogen chloride formed. In practice the mixture of hydrogen chloride and the excess chlorine from the reaction vessel was passed through gas-scrubbing bottles containing water, in which the hydrogen chloride was completely absorbed, and the chlorine, after saturating the solution, was allowed to escape to a hood vent. The scrub water was then made up to volume with an equal volume of water (used to rinse the scrubbers). Accordingly, the solutions to be analyzed consisted of aqueous solutions of hydrogen chloride half-saturated with chlorine. (This is only approximately true, since the solubility of chlorine varies considerably with the hydrogen chloride concentration.)

The problem of analysis of such solutions suggests separate analyses for both elementary chlorine and total chloride, the difference between which would give the amount of hydrochloric acid present. Such a procedure, however, has the following disadvantages: In the case of small amounts of hydrogen chloride, it involves the small difference of two relatively large values and therefore decreases the accuracy; error can readily arise through loss of chlorine from the solution during sampling; and the procedure is unnecessarily laborious contrasted with the method described below.

A study was made therefore to find a more suitable method. As a result a procedure was devised by which the hydrogen chloride could be determined by a single titration. It consisted, briefly, of boiling out the chlorine under a reflux, sleeve-type condenser and titrating the hydrochloric acid with alkali. The procedure entailed a small blank arising from a small amount of hydrolysis of chlorine. The following experimental data demonstrate the applicability of the procedure to such analyses.

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EXPERIMENTAL

One liter of 2 *N* hydrochloric acid was made up; 500 ml. were used to make up 1 liter of 1 *N* solution, 250 ml. were transferred to a 500-ml. volumetric flask for saturation with chlorine, and the remaining 250 ml. were used for analysis and rinsing of pipets. With appropriate dilutions, this procedure was repeated in preparing two series of solutions of hydrochloric acid having the concentrations shown in Table I, and differing only in that one contained chlorine and the other was chlorine-free.

Saturation of the acid solution was accomplished by leading a fairly rapid stream of chlorine gas, which had first been washed by passing through a scrubber filled with water, into the solution by means of a glass tube drawn to a capillary tip. After saturation, the tube was rinsed off into the flask and sufficient water was added to make the volume up to 500 ml. Accurately calibrated volumetric flasks were chosen to ensure that the normality of this solution was exactly that of the liter of the reference solution made from the same stock solution.

The sample was prepared for analysis by pipetting appropriate 10-, 25-, or 50-ml. samples of the chlorine-containing solutions into dry, 125-ml., ground-glass-jointed Erlenmeyer flasks, fitting these with reflux condensers, adding a few boiling chips, and boiling the solutions for 15

Table I. Effect of Acidity on Blank of Boiled Reference Hydrogen Chloride Solutions

NaOH Normality	Chlorine-Free Solutions Sample	Av. titer	Normality N_1	Chlorine-Containing Solutions Sample	Av. titer	Normality N_2	Blank $(N_2 - N_1)$
	Ml.	*Ml.*		*Ml.*	*Ml.*		
0.3392	10	29.96	1.0162	10	29.99	1.0171	0.0009
	25	37.50	0.5088	10	15.01	0.5094	0.0006
	25	18.79	0.2549	25	18.83	0.2555	0.0006
	25	9.38	0.1273	25	9.44	0.1281	0.0008
	25	4.70	0.0638	25	4.75	0.0645	0.0007
0.03392	25	24.04	0.03262	25	24.47	0.03320	0.0006
	50	24.19	0.01641	50	25.05	0.01699	0.0006
	50	11.88	0.00806	50	12.91	0.00876	0.0007
	50	0.00	0.0000	50	4.00	0.00271	0.0027

minutes, in which time all free chlorine disappeared, with the exception of the solution containing no hydrogen chloride.

It was found that the ordinary type of reflux condenser necessitated a much longer boiling time to boil off the chlorine, owing to the slow diffusion of chlorine up the tube and its tendency to redissolve in the condensate. The condensers shown in Figure 1 are better adapted to this procedure because of the proximity of the condensing surface to the point of escape of the chlorine gas.

The samples were cooled and titrated with standard sodium hydroxide to the phenolphthalein end point. Titrations were made on the corresponding chlorine-free solutions, and the differences were taken as blanks. The results are shown in Table I.

The blank apparently has a constant value of 0.0007 mole of hydrogen chloride per liter over the range from 1 *N* to 0.008 *N*. The irregular variations of the blank with respect to normality of hydrochloric acid can be attributed to experimental error. At concentrations of hydrochloric acid below 0.008 *N*, 15 minutes' boiling gave a larger blank, which reached its maximum value, 0.0027 mole per liter, for zero concentration of hydrogen chloride. Experiment showed that prolonged boiling did not further reduce the blank for solutions in the range of 1.0 *N* to 0.008 *N*; hence the value of the blank represents a definite minimum. Thirteen hours' boiling of the hydrogen chloride–free solution, however, reduced the blank from 0.0027 to 0.0014, showing that progressively longer times are required to reach a constant blank at concentrations below 0.008 *N*. It is suggested that enough standard hydrochloric acid be added to such solutions to make the over-all concentration over 0.10 *N* before boiling out the chlorine.

DISCUSSION

The system hydrogen chloride–chlorine-water is a ternary system of considerable theoretical interest; only a small region, of which this is a part, has been studied. Information on the binary system hydrogen chloride–chlorine is practically nonexistent with the exception of liquid phase–solid phase equilibrium data (*5*).

The particular conditions that exist in an aqueous solution of chlorine and hydrogen chloride are complicated by various reactions which chlorine undergoes with water. Recent studies by Morris (*3*) show the main reaction to be

$$Cl_2 + OH^- \rightleftarrows HOCl + Cl^- \quad (1)$$

rather than the previously accepted mechanism proposed by Jakowkin (*1*);

$$Cl_2 + H_2O \rightleftarrows HOCl + H^+ + Cl^- \quad (2)$$

Subsequent reactions follow the primary hydrolysis, such as

$$3HOCl \rightleftarrows HClO_3 + 2HCl \quad (3)$$

and a photochemical one,

$$HClO + (h\nu) \rightarrow HCl + O \quad (4)$$

An added complication of importance lies in the fact that the solubility of chlorine varies not only with temperature but with the concentration of hydrochloric acid present (*2*).

Early attempts were made to determine the amount of hydrogen chloride in the chlorine-saturated scrub waters gravimetrically without first boiling out the chlorine. The blanks were found to vary widely, and a determination of the blank with varying acidity showed it to have a maximum value for zero concentration of hydrochloric acid, decrease with increasing acidity to a value about one half this maximum value at an acidity of 0.06 *N*, increase again to reach a smaller maximum at an acidity of 0.25 *N*, then slowly decrease with further increase in acidity. The similarity of the trend of this gravimetric blank to the solubility curve for chlorine in hydrochloric acid solutions was noted and calculations showed that the blank was closely equivalent to the total amount of chlorine in solution up to the second maximum. However, the solubility of chlorine continues to increase linearly after the minimum instead of reaching a second maximum and decreasing as does the blank, and it is at this second maximum that the values begin to disagree. This was attributed to the following cause:

At these concentrations of hydrochloric acid the chlorine is mostly undissociated, and since the gravimetric method involves only the hydrolytic products, reaction of the chlorine with hydroxyl ion must occur before precipitation of silver chloride can occur. In acid solution, however, the hydroxyl-ion concentration can be so low that the usually rapid reaction can be slow and chlorine is lost as gaseous chlorine in spite of the presence of sufficient silver nitrate to react with the total amount of chlorine, should it all undergo hydrolysis. If the mechanism were that proposed by Jakowkin, this would not be the case, since the concentration of water molecules is so large as to be independent of the acidity. The gravimetric blank was abandoned in favor of the volumetric boiled blank because of the former's obvious disadvantages of large values, difficulty in checking at high acid concentrations due to loss of chlorine, and its variation with acidity in comparison with the small, constant value of the volumetric blank.

Richardson (*4*) in a study of the distillation of chlorine water showed that chlorine water contained two easily volatilized substances, chlorine and hypochlorous acid, and a nonvolatile component, hydrochloric acid. The chlorine and the hypochlorous acid were found to differ sufficiently in volatility (the former being a gas and the latter a vapor at room temperature and pressure) to be separated by aspiration of the cold solution. Complete removal of the chlorine by this method, however, involved periods which were too long to be practical. Boiling of chlorine water without reflux was found to eliminate a mixture of chlorine and hypochlorous acid, leaving some hydrogen chloride of hydrolysis in the undistilled residue. Refluxing under a condenser was shown to prevent escape of hypochlorous acid, yet allow chlorine to escape slowly. This method of expelling chlorine was used in the procedure described above and found to be complete in 15 minutes, provided the acidity was at least 0.008 *N*.

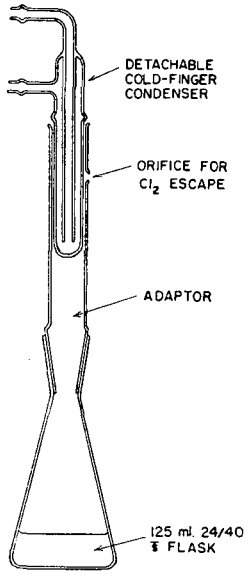

Figure 1. Recommended Boiling Apparatus

Considering the mechanism proposed by Morris (Equation 1) in relation to the slower elimination of chlorine from solutions less than 0.008 *N* in hydrochloric acid, it may be seen that the equilibrium of these solutions, in which the chlorine has been partly hydrolyzed, must be displaced before elimination of the chlorine can occur. This displacement should be effected by either addition of chloride ion or reduction of hydroxyl-ion concentration. Sodium chloride was added and found to decrease the time required to expel chlorine from these solutions. The addition of sulfuric acid was equally effective, whereas the addition of hydrochloric acid was more effective than either.

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Determination of Cyclopropane by Selective Absorption

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Cyclopropane can be determined in gases containing both olefins and paraffins by absorption of the olefins in acid mercuric sulfate reagent followed by absorption of the cyclopropane in 87% sulfuric acid. Ethylene can be distinguished from other olefins by a second analysis in which the order of absorbents is reversed, and cyclopropane plus olefins other than ethylene is absorbed in 87% sulfuric acid and then ethylene is absorbed in acid mercuric sulfate reagent.

CYCLOPROPANE is occasionally encountered as a component of hydrocarbon gases and a simple and direct method, using conventional gas analysis apparatus, for its determination in the presence of other hydrocarbon gases would be desirable. Mixtures of propylene and cyclopropane have been analyzed by absorption of the propylene in 3% aqueous potassium permanganate solution, and the cyclopropane has been determined by difference after the necessary corrections have been made for solubility of cyclopropane in this reagent (*1*, *5*, *7*). Such mixtures have also been analyzed by a micromethod, using saturated aqueous silver nitrate solution for removal of propylene (*6*). A method for the determination of cyclopropane in the presence of both propylene and inerts, described by Corner and Pease (*2*), is based on the selective hydrogenation of propylene over a nickel-kieselguhr catalyst partially poisoned with mercury, followed by hydrogenation of cyclopropane over a nonpoisoned catalyst. Although this method is apparently capable of yielding data of a high order of accuracy, difficulty might be experienced in securing or preparing catalysts of the required activity level.

During a study of the behavior of cyclopropane toward absorption reagents used in this laboratory for the determination of olefinic gases, it was found that cyclopropane is not absorbed by acid mercuric sulfate reagent (*4*), but is rapidly absorbed by 87% sulfuric acid, which is used for the selective absorption of propylene in the presence of ethylene. These observations suggested a simple means for the determination of cyclopropane in gases containing both saturated and unsaturated hydrocarbons. This paper presents the results of an investigation of this method of analysis.

APPARATUS AND REAGENTS

The apparatus used for the tests described consisted of a 100-ml. gas buret graduated in 0.2-ml. divisions and three gas pipets which communicated to the buret through an all-glass manifold. Mercury was used as a displacement liquid in the buret, and the gas was saturated with water vapor before each volume measurement by maintaining a film of water upon the wall of the buret. The pipets each contained approximately 180 ml. of reagent and were packed with vertical tubes to provide contact with a large film of reagent. Two of the pipets containing 87% sulfuric acid and one containing acid mercuric sulfate reagent were attached to the manifold so that the latter was located between the sulfuric acid pipets.

The 87% sulfuric acid was prepared by dilution of C.P. concentrated acid, and is used in this laboratory for the selective absorption of propylene in the presence of ethylene. The acid mercuric sulfate reagent was prepared according to Francis and Lukasiewicz (*4*) and modified by saturation with magnesium sulfate ($MgSO_4.7H_2O$), as suggested by Davidson and Anderson (*3*).

PROCEDURE

Two methods of analysis are presented. The first procedure concerns samples containing propylene, cyclopropane, and inert gases; the second, samples which contain ethylene as well.

For most accurate analyses, each reagent should be saturated with the residual gas that will be present in the mixture after absorption of the gas reactive with that particular reagent. For this purpose, measure approximately 50 ml. of sample into the gas buret and pass through the reagents in the manner described for analysis of the sample, but make no effort to read volumes accurately or to record data. If a series of samples of similar composition is being analyzed, the presaturation need be carried out only for the first sample, as each analysis can serve as a presaturation for the one following.

A. Samples Containing Propylene, Cyclopropane, and Inerts. Measure a 100-ml. portion of the gas sample into the gas buret. To determine the propylene content, pass the gas into the acid mercuric sulfate reagent five times and record the residual gas volume. Repeat in groups of five passes until a constant volume or a constant absorption per group of passes is obtained. Samples containing high concentrations of cyclopropane will give a small constant coabsorption of cyclopropane in this reagent, for which appropriate correction must be made. To determine the cyclopropane content, pass the residual gas into the second sulfuric acid pipet in groups of five passes until a constant residual volume is obtained.

B. Samples Containing Ethylene, Propylene, Cyclopropane, and Inerts. Measure a 100-ml. portion of the gas sample into the gas buret and treat as described in Procedure A. In this case the absorption in acid mercuric sulfate reagent is a measure of propylene plus ethylene. If ethylene and propylene values are desired, measure another 100-ml. portion of sample into the buret and pass into the first 87% sulfuric acid pipet to determine propylene plus cyclopropane and then pass the residual gas into the acid mercuric sulfate reagent to determine the ethylene content. Samples containing high concentrations of ethylene give a small, constant coabsorption of ethylene in the 87% sulfuric acid, for which appropriate correction must be made. The propylene content is calculated as the difference between the propylene plus ethylene value found in the first analysis and the ethylene value found in the second analysis.

EXPERIMENTAL

A series of hydrocarbon mixtures was prepared from the pure components to contain various concentrations of ethylene, propylene, cyclopropane, and propane. The pure components used

Table I. Analysis of Hydrocarbon Mixtures

Blend No.	Method	Component, Mole Per Cent: Cyclopropane Found	Cyclopropane Calcd.	Propylene Found	Propylene Calcd.	Ethylene Found	Ethylene Calcd.	Propane Found[a]	Propane Calcd.
1	B	13.9	14.0	23.3	23.3	28.4	28.7	34.3	34.0
2	B	11.2	11.2	11.3	11.2	11.3	11.2	66.2	66.5
3	B	3.7	3.4	−0.1	0.0	50.4	50.4	46.0	46.3
4	B	33.3	33.3	33.3	33.3	0.0	0.0	33.4	33.4
5	B	3.1	3.4	47.2	47.0	0.0	0.0	49.7	49.7
6	A	46.8	46.9	3.1	3.3	...	0.0	50.1	49.8
7	A	0.0	0.0	33.4	33.4	...	0.0	66.6	66.6
8	A	75.0	75.0	9.7	10.0	...	0.0	15.3	15.0
9	A	93.6	93.4	3.2	3.3	...	0.0	3.2	3.3

[a] By difference.

were analyzed mass-spectrometrically and found to contain not more than 0.5% of impurities. The results from the analysis of these mixtures, using the two procedures described above, are presented in Table I, which shows that the method yields accurate values over a wide range of concentrations.

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Selenium as Catalyst in Kjeldahl Digestions

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In the conversion of organic to ammoniacal nitrogen by the Kjeldahl method, the use of selenium to catalyze the oxidation results in loss of nitrogen during prolonged heating following clearance of the digest. Comparatively better recovery of nitrogen is obtained when a combination of selenium and mercuric oxide is used as catalyst; this is due to the formation of mercury-ammonium complexes which resist oxidation to a greater degree than do free ammonium salts. The period of afterboil necessary to obtain theoretical yields of nitrogen must be accurately standardized for each type of nitrogenous organic matter and selenium cannot be recommended as a general reagent for Kjeldahl determinations.

THE catalytic effect of small amounts of selenium, selenium oxychloride, selinites, or selenates in the oxidative digestion of nitrogenous organic matter by the Kjeldahl method is well known (*5, 13, 17, 20, 28, 32*). A combination of mercuric oxide and selenium acts much more effectively than either of them singly in accelerating the decomposition of proteins (*1, 4, 22, 25, 33, 35*). In a study of the mechanism of selenium catalysis, Sreenivasan and Sadasivan (*31, 32*) have shown that selenium acts as an efficient carrier of oxygen when heated with sulfuric acid and reducing organic matter by means of rapid, reversible reactions involving the formation of selenic and selenious acids in the presence and absence, respectively, of mercuric oxide; selenic acid is comparatively unstable, being easily decomposed into, and having therefore a greater oxidizing capacity than, the lower (selenious) acid. This explains the synergetic action of selenium and mercuric oxide in catalyzing the oxidation.

Although the use of selenium in Kjeldahl digestions is extensive, scattered references in the literature question the advisability of its use.

Sandstedt (*27*) reported, soon after the original observation of Lauro on the effect of selenium (*20*), that prolonged digestion with selenium catalysts resulted in low values for total nitrogen. Similar losses of nitrogen were noted by Davis and Wise (*14*), Osborn and Krasnitz (*22*), Snider and Coleman (*29*), Dalrymple and King (*13*), and others. Illarionov and Ssolovjeva (*16*) observed that the catalytic action of selenium was proportional to the quantity added, while Bradstreet (*9*) stated that an increase in the amount of selenium used gave low results. Miller and Houghton (*21*) failed to obtain quantitative values for the nitrogen contents of lysine and tyrosine using selenium as catalyst. Van Slyke *et al.* (*34*) found that none of the catalysts recommended in the literature for the Kjeldahl procedure, including selenium, yielded more than 90% of the total nitrogen of tryptophane and lysine. In experiments on the use of oxidizing agents like peroxides, permanganate, and dichromate in Kjeldahl digestions, Sreenivasan (*30*) also observed that the use of these catalysts, in more than small quantities, resulted in loss of nitrogen. Wicks and Firminger (*36*), Kaye and Weiner (*18*), and Jonnard (*17*), using perchloric acid as an aid to hasten digestion (*23*), reported low results for nitrogen. Some of the discrepancies in the values obtained for total nitrogen by using modifications of the Kjeldahl method have been attributed to the formation of free nitrogen (*24*) and to undecomposed amines (*15*).

On the other hand, provided an adequate period of afterboil following clearance of the digest is given, correct results are obtained (*2, 6, 10, 26*) and the erratic results sometimes obtained with selenium compounds may frequently be due to insufficient digestion (*3, 25, 28*). Clark (*11*) was of the opinion that many compounds, although requiring a very long digestion period, will nevertheless yield their nitrogen quantitatively to one or the other of the modified Kjeldahl procedures. According to Miller and Houghton (*21*) and Jonnard (*17*), the catalyst and the period of digestion are not necessarily identical for all compounds of the same general class and the low values obtained with selenium are due to conditions of heating during digestion and distillation.

In view of the divergent observations of earlier workers, a reinvestigation of the catalytic action of selenium on Kjeldahl digestion suggested itself and this paper presents the results of a comparative study of the influence of the duration of digestion, with and without selenium or selenium and mercuric oxide, on the extent of recovery of nitrogen by the Kjeldahl method.

EXPERIMENTAL

Procedure. All determinations were carried out with the conventional Kjeldahl apparatus in the usual manner, using as source of heat gas burners of more or less uniform heating power. Chemicals were of analytical grade and were specially purified where required. Digestions were carried out with substances in amounts such that the nitrogen content of each sample was about 10 mg. together with 20 ml. of sulfuric acid, 6 grams of potassium sulfate, and 0.20 gram of crystalline copper sulfate, the last two referred to as salt mixture below. The quantities of elemental selenium and mercuric oxide, where employed, amounted to 50 mg. and 0.50 gram, respectively; in the latter case, the mercury-ammonium complexes were destroyed during distillation by addition of 0.50 gram of sodium thiosulfate. All results represent the average of at least two determinations except where duplicates were not in good agreement, when three or four determinations were carried out. The conditions of digestion and distillation were carefully checked, using standard solutions of ammonium sulfate which, in several trials and, by employing the Kjeldahl procedure without selenium and for varying periods of digestion up to 3 hours, gave results which were in error by not more than 0.4%.

Digestions with Casein. In Table I are presented the results obtained using casein.

Table I. Comparative Nitrogen Recovery with Casein

(Theory, 15.65% N)

Catalyst	Time of Clearance	Nitrogen Recovery: At clearance	Minutes after Clearance: 15	30	60	180	360
	Min.	%	%	%	%	%	%
Salt mixture[a]	40	87.5	94.9	99.0	100.3	99.4	99.5
Salt mixture + selenium	10	94.4	99.8	99.2	97.8	95.4	95.6
Salt mixture + selenium + mercuric oxide	11	97.8	100.2	99.6	99.2	98.7	97.8

[a] K_2SO_4 + $CuSO_4.5H_2O$.

Conversion to ammonium sulfate is not complete at clearance, although digestion at this stage has proceeded farthest with selenium and mercuric oxide and somewhat less with selenium alone as compared to the usual salt mixture, in spite of the fact that, in these cases, the solution became clear within a very short time. Correspondingly, the period of afterboil necessary for completeness of digestion is less where selenium is present. But the most significant observation relates to the fact that while, with salt mixture, prolonged digestion up to 6 hours does not make any appreciable change in recovery of nitrogen, use of selenium results in definite loss of nitrogen. Since nearly theoretical recovery of nitrogen is obtained before any loss becomes noticeable, it may be presumed that this loss does not take place or is, at any rate, negligible till oxidation to ammonium sulfate is complete. The rate and extent of loss of nitrogen, once oxidation to ammonium sulfate is complete, are much less with selenium and mercuric oxide than with selenium alone, notwithstanding the fact that the former combination is more powerful in catalyzing the oxidation of complex nitrogenous materials. In their study of the relative catalytic speeds of mercuric oxide, selenium, and selenium plus mercuric oxide, using a variety of organic substances for digestion, Osborn and Krasnitz (*22*) reported that, with extended periods of boiling, the danger of loss of nitrogen increased in the above order. These authors also observed that nitrogen loss could be prevented by using larger quantities of acid for digestion.

Digestions with Ammonium Sulfate. The observation that, on continued digestion with selenium, acid, and salt mixture, the ammonium sulfate formed in the oxidative decomposition of nitrogenous organic matter yields lowered values for nitrogen was further confirmed in experiments with pure ammonium sulfate reported in Table II.

Table II. Recovery of Nitrogen Using Ammonium Sulfate

(Theory, 21.20% N)

Catalyst[a]	Time of Digestion: 10 min.	20 min.	30 min.	60 min.	120 min.	180 min.
	Nitrogen Recovery: %	%	%	%	%	%
Salt mixture + selenium	99.7	99.2	98.2	97.4	95.6	94.5
Salt mixture + selenium + mercuric oxide	99.6	100.1	99.3	98.1	98.4	98.6

[a] With salt mixture alone, digested up to 3 hours, recoveries from 99.6 to 100% were obtained.

As before (Table I), the recoveries obtained with selenium and mercuric oxide are better than with selenium alone (cf. *22*). Obviously, this is due to the formation of mercury-ammonium complexes which, at any rate in the initial stages, resist oxidation to a greater degree than do free ammonium salts. This assumption may be held justifiable when it is realized that, in the subsequent distillation of ammonia from the digest through boiling with strong alkali, it becomes necessary to destroy these mercury-ammonium complexes by addition of sodium sulfide or thiosulfate. They do not, however, resist continuous heating in the acid digest, as may be inferred from the low results obtained for nitrogen on prolonged heating (Tables I and II).

Attempts were made to study the nature of oxidation of ammonium sulfate by selenium and sulfuric acid but as no nitrite, nitrate, or the various oxides of nitrogen could be detected at any stage during digestion, it is presumed that nitrogen is lost in the elemental state, either through decomposition of oxides of nitrogen or, as is more likely, through the interaction of nitrites and nitrates formed as intermediates.

Digestion of Ring-Type Nitrogen Compounds. Several cyclic nitrogen compounds are extremely resistant to oxidation by the Kjeldahl method (*8*, *19*, *34*). However, several workers (*7*, *11*, *28*) were able to analyze this type of compound apparently successfully by using mercuric oxide alone or a mixture of mercuric oxide and selenium as catalysts. In Table III are given the results obtained with two typically refractory compounds, nicotinic acid and quinoline, digested for varying periods of time using ordinary salt mixture as such, with selenium and with selenium and mercuric oxide as catalysts.

Digestion even for 6 hours did not result in complete oxidation to ammonium sulfate by any of the procedures, although there was, in all cases, a steady increase with time in the values for nitrogen. Since loss of nitrogen commences only after oxidation to ammonium sulfate is complete, this complication had not arisen during the period these digestions were carried out. Digestion could not be prolonged beyond 6 hours after clearance, as there was excessive bumping which could not be overcome.

DISCUSSION

It would appear that selenium, either as such or with mercuric oxide, cannot unreservedly be recommended as a general reagent for Kjeldahl determinations in spite of its undoubted catalytic effect on the acceleration of the time of digestion. While a period of afterboil following clearance of the digest is, as is generally recognized, always necessary for complete oxidation of organic to ammoniacal nitrogen, with selenium catalysts, this time for maximum yield of nitrogen would have to be rather accurately determined and controlled for each type of protein or other nitrogenous material; this procedure is not always practicable.

The loss of nitrogen on continued digestion of ammonium sulfate is perhaps a general phenomenon with all oxidizing agents used as catalysts (*7*, *18*, *24*, *30*, *36*) and may occur measurably even with sulfuric acid and salt mixture. Although this was not fully ascertained, yet it was observed that, in several experiments with ammonium sulfate digested for over 6 hours in the usual manner, recovery of nitrogen was somewhat erratic and ranged only from 98.6 to 99.4%. That the discrepancies were not due to conditions of distillation which, with adequate precautions, were always rigidly reproduced, could be stated from the fact that more or less theoretical yields of nitrogen were always secured with shorter periods of heating.

The good results obtained with refractory ring-type compounds may be

Table III. Nitrogen Recovery from Ring Compounds

Compound	Catalyst	Time of Clearance	Nitrogen Recovery: At clearance	Minutes after Clearance: 30	60	180	360
		Min.	%	%	%	%	%
Nicotinic acid (theory, 11.37% N)	Salt mixture	Within 10	2.4	37.6	48.8	57.5	69.5
	Salt mixture + selenium	Within 10	3.0	44.4	70.9	75.0	91.0
	Salt mixture + selenium + mercuric oxide	Within 10	8.2	69.1	85.2	93.4	98.2
Quinoline (theory, 10.85% N)	Salt mixture	Within 10	13.9	54.4	68.3	76.9	..
	Salt mixture + selenium	Within 10	29.6	60.2	71.0	81.6	..
	Salt mixture + selenium + mercuric oxide	Within 10	38.4	75.1	86.7	94.2	99.4

explained by the fact that considerably longer hours of digestion are required for these (*28*) and that loss of nitrogen does not commence appreciably until oxidation of organic to ammoniacal nitrogen is complete. The catalytic effect of selenium is, as shown by Sreenivasan and Sadasivan (*32*), most pronounced during the initial stages of decomposition of reducing organic matter and as, with ring compounds, oxidation is extremely slow from the commencement, the chances of better recovery of nitrogen on prolonged digestion are obviously more than with proteinaceous materials.

In view of the general ease with which conditions of digestion in semimicro and microprocedures can be controlled (*12*), it is possible that, as recently reported (*7, 11, 12, 17, 18, 21*), these may yield more concordant results for nitrogen determinations by the Kjeldahl method and its modifications. It is desirable that further extended trials by the micromethod, using various types of compounds, be carried out with a view to verifying whether the period of afterboil following clearance that is necessary for completeness of conversion to ammoniacal nitrogen is not so rigid a factor in determining the accuracy of the results obtained as in the macromethods.

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Determination of Gaseous Hydrocarbons

Combustion over Precipitated Copper Oxide Containing 1% Iron Oxide

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A method is described for the rapid and quantitative determination of gaseous paraffins by combustion at 700° C. over precipitated copper oxide containing 1% iron oxide. This method is an adaptation of that proposed by Brückner and Schick (*1*) and has great advantages over commonly employed methods that use copper oxide wire which requires a temperature of around 900° C., or combustion over a heated filament or catalyst which requires the addition of oxygen. Formation of explosive mixtures and the hazard created thereby are eliminated.

IN THE analysis of gases containing only nitrogen and paraffinic hydrocarbons, the paraffin content is usually determined by combustion analysis. Methods most commonly used for the combustion (*4, 7*) require admixture of a portion of sample with a suitable excess of oxygen and passage of this mixture over a heated catalyst or filament to promote combustion. Such methods necessitate a source of oxygen of accurately known purity and errors may be introduced by contamination or inaccurate measurement of the oxygen used. In addition, it is difficult completely to avoid formation of explosive mixtures and the hazards which arise therefrom. These disadvantages can be eliminated by the use of a combustion agent having readily available combined oxygen. It is also desirable that this agent display sufficiently high reactivity to permit combustion at a moderate temperature, so that special apparatus and techniques are unnecessary.

Copper oxide has often been used as such a combustion agent, but according to Lunge (*5*) combustion of methane is not rapid even at 950° C. and numerous passes over the oxide are required for complete combustion. At this temperature dissociation of copper oxide (*2, 6*) may introduce appreciable error if the oxygen formed is not removed prior to measurement of the residual gas volume. Campbell and Gray (*3*) have reported that copper oxide displays improved activity when impregnated with small amounts of cuprous chloride or other metallic oxides. Brückner and Schick (*1*) found that copper oxide prepared by

precipitation, and containing 1% iron oxide, is capable of rapid and complete combustion of hydrogen at 220° C. and of methane at 600° C. This combustion agent has been further tested by the authors and has proved to be much superior to commercial copper oxide for the combustion of paraffins.

APPARATUS AND MATERIALS

The precipitated copper oxide–iron oxide mixture (hereafter referred to simply as precipitated copper oxide), according to Brückner and Schick, is prepared as follows:

Dissolve in 3 liters of distilled water appropriate weights of cupric nitrate and ferric nitrate to produce 100 grams of 99 to 1 copper oxide–iron oxide, and add 30% potassium hydroxide solution in slight excess. Boil the resulting mixture for 20 minutes to convert the precipitated copper hydroxide to copper oxide. Then cool, wash the precipitate several times by decantation with water, and filter to the consistency of a heavy paste. Press pellets 1 mm. in diameter and 6 mm. long from the paste, dry in a stream of air at 100° C., and remove the last traces of water by heating to 400° C. Reduce the dried pellets completely with hydrogen and reoxidize with air at 400° C.

The authors found it more convenient to prepare a hard filter cake of the precipitated oxides, crush it into small pieces, and dry it at 400° C. The dry particles were then reduced with hydrogen and reoxidized in a stream of air for 3 to 4 hours at 400° C. During the reduction and subsequent reoxidation considerable shrinkage of the oxide particles took place. Following reduction and reoxidation the material was screened to obtain granules of the desired size and then packed into the combustion tubes. The combustion tubes used consisted of U-tubes about 120 mm. in length and 3.5 to 4.0 mm. in inside diameter, and were fitted with spherical joints to permit convenient attachment to the all-glass manifold. These combustion tubes held from 4 to 7 grams of the precipitated copper oxide, depending to some extent on the mesh size of the material used.

Since ordinary Pyrex softens excessively at 700° C., it is necessary to use combustion tubes of fused silica or of special heat-resistant glass, such as Corning No. 172, at that temperature. Both types of combustion tubes were used and found satisfactory, but the heat-resistant glass is more easily worked and is less expensive. The copper oxide packing was retained in these tubes by plugs of loosely rolled sheet asbestos.

EXPERIMENTAL

Combustion of Hydrogen. Preliminary tests confirmed the findings of Brückner and Schick that hydrogen can be burned rapidly and quantitatively by passage over the precipitated copper oxide at 220° C. This high activity, however, proved to be of no special advantage for the selective combustion of hydrogen, for at 220° C. hydrocarbons are oxidized by the precipitated copper oxide at approximately the same rate as with commercial copper oxide wire at 270° C., the temperature used in this laboratory for the selective combustion of hydrogen.

Combustion of Hydrocarbons. Samples of natural gas were passed over the precipitated copper oxide at 600° and 700° C. Slightly lower hydrocarbon values were found at the lower temperature and the combustion rate was somewhat slower. It was disclosed by mass spectrometric analysis of the residues that small amounts of unburned methane remained from the combustions at 600° C. No methane was found in the residues from combustions carried out at 700° C.

In order to establish clearly the difference between the precipitated copper oxide and copper oxide wire, with regard to activity in effecting combustion of hydrocarbons, a comparative test was made. The copper oxide wire used for this test was given the same preliminary treatment as was the precipitated oxide—i.e., it was reduced with hydrogen and reoxidized with air at 400° C. before being packed into the combustion tube. Because the true composition of the natural gas used above was not known, a sample of methane of high purity was prepared by careful fractionation of the natural gas. Methane prepared in this manner was found to have a purity of 99.8+% by mass spectrometric analysis. Samples of the methane were then passed over the two combustion agents and the rates at which it burned were noted. As can be seen from Figure 1, at least 18 complete cycle passes were required for complete combustion by the copper oxide wire while only 3 to 6 passes over the precipitated oxide were necessary.

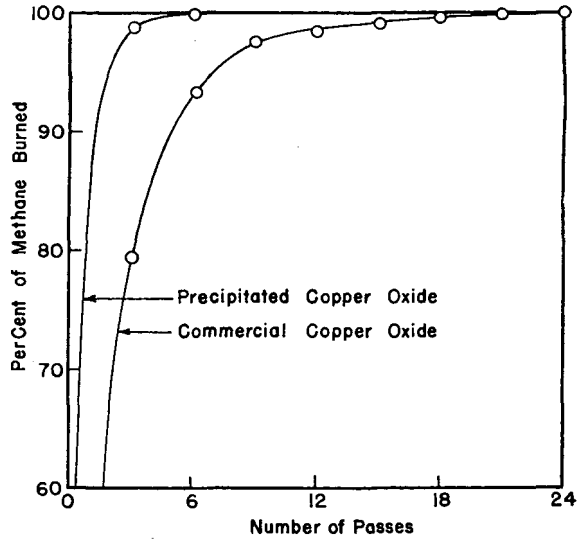

Figure 1. Combustion of Methane over Copper Oxide at 700° C.

The pure methane was then analyzed by combustion over precipitated copper oxide at 700° C. and by two other combustion methods: (1) by passage over an incandescent platinum filament in a slow combustion pipet, and (2) by passage over platinized silica gel catalyst at 500° C. The results, shown in Table I, indicate that combustion over the precipitated copper oxide gives at least as good accuracy and precision as are obtained by the other methods tested. The time required for an analysis was about the same for each method. To determine the applicability of this method to the analysis of hydrocarbons other than methane, samples of purified propane, isobutane, and *n*-butane were also analyzed by combustion over the precipitated copper oxide. No difficulty was encountered in obtaining rapid and complete combustion of these hydrocarbons and the results, shown in Table II, indicate the method is reliable. In the analysis of pure hydrocarbons by combustion it is necessary to add an accurately measured volume of a carrier gas to permit the determination of the amount of carbon dioxide formed and the residual gas volume. Nitrogen was used for this purpose in the analyses summarized by Tables I and II.

Oxidation of Copper Oxide. Brückner and Schick reported that the precipitated copper oxide they prepared could be oxidized more completely than could the commercially available wire form. They found the combined oxygen content of the precipitated oxide to be between 95 and 100% of the theoretical value when oxidized at 450° to 550° C., while under the optimum conditions only about 40% of the theoretical value could be obtained for the copper oxide wire. The authors have studied the effect of several variables on the rate at which the reduced precipitated copper oxide is oxidized in an effort to arrive at a rapid and convenient method for regenerating the oxide.

A sample of precipitated copper oxide was reduced and reoxidized at 400° C. and screened to retain three fractions, having

Table I. Determination of Methane by Various Combustion Methods

Combustion Method	Sample Volume	Volume of CO_2 Formed	Volume of O_2 Used	Total Volume Contraction	Per Cent Methane Found: By volume of CO_2 formed[a]	By volume of unburned residue[b]	By volume contraction and O_2 used[c]
	Ml.	*Ml.*	*Ml.*	*Ml.*			
Platinized silica gel	40.2	40.2	80.2	80.9	100.0	99.5	101.0
	40.2	40.0	80.5	80.7	99.5	99.3	100.5
	40.3	40.1	80.9	80.8	99.5	98.7	100.3
	39.9	40.1	81.0	80.7	100.5	99.8	101.1
	40.7	40.5	80.6	80.3	99.5	99.6	98.4
	40.1	40.1	80.8	80.3	100.0	99.6	99.8
Av.	...	...	...		99.75	99.4	100.2
Pptd. copper oxide	40.3	40.6	...	...	101.0	100.0	...
	40.9	40.8	...	...	99.8	100.0	...
	40.6	40.4	...	...	99.5	99.3	...
	40.1	39.9	...	...	99.5	100.0	...
	39.9	39.7	...	...	99.5	100.0	...
	40.3	40.0	...	...	99.3	100.0	...
Av.	...	...	...	...	99.8	99.9	...
Slow combustion pipet	40.7	40.8	82.4	82.1	100.2	99.8	100.5
	40.5	40.4	81.4	81.4	99.8	99.8	100.5
	40.3	40.3	81.2	81.1	100.0	100.5	100.5
Av.	...	...	...	...	100.0	100.0	100.5

[a] % methane $= \frac{C}{S} \times 100.0$ (for methane C should equal S).

[b] % methane $= \frac{S - N}{S} \times 100.0$.

[c] % methane $= \frac{1/4(3TC - O)}{S} \times 100.0$.

where: C = volume of carbon dioxide formed on combustion
S = initial sample volume
N = volume of unburned residue
TC = volume contraction on combustion
O = volume of oxygen consumed

Table II. Determination of Gaseous Paraffins by Combustion over Precipitated Copper Oxide at 700° C.

Material Analyzed	Volume of Sample	Unburned Residue	Volume of CO_2 Formed	Paraffins Found[a]
	Ml.	*Ml.*	*Ml.*	%
Methane	40.3	0.0	40.6	100.0
	40.9	0.0	40.8	100.0
	40.6	0.3	40.4	99.3
	40.1	0.0	39.9	100.0
	39.9	0.0	39.7	100.0
	40.3	0.0	40.0	100.0
Propane	25.9	0.0	78.2	100.0
	28.9	0.0	86.7	100.0
	28.5	0.05	85.5	99.8
n-Butane	23.3	0.05	95.3	99.8
	21.3	0.0	88.0	100.0
	19.1	0.0	78.6	100.0
Isobutane	21.0	0.0	84.5	100.0
	19.8	0.05	80.4	100.2
	24.2	0.0	97.9	100.0

[a] See footnote[b] of Table I for method of calculation.

respective particle diameters of 0.5 to 1.0, 1.0 to 2.0, and 2.0 to 3.0 mm. Portions of each of these fractions were packed into combustion tubes and alternately reduced completely with hydrogen and then reoxidized with air and/or oxygen at various temperatures and for various lengths of time. Oxygen contents between 19 and 73% of theoretical were observed gravimetrically. Oxidation temperatures above 700° C. were not investigated, since it was thought desirable to avoid the expense and inconvenience of combustion tubes made from fused silica. Oxidation periods in excess of 3 hours were not investigated, because such prolonged periods of oxidation would be impractical for routine use in most laboratories. The most favorable temperature range for reoxidation of the reduced oxide appeared to be 400° to 500° C. However, by drawing air through the combustion tube while the furnace was being heated to operating temperature (700° C.), which required only 15 minutes, an oxygen content of about 55% of the theoretical was attained. Probably the shifting of particles which occurs while the oxide is being heated results in greater surface exposure to the oxidizing gas. At temperatures above 600° C., oxidation proceeds at a diminished rate. Oxidation with oxygen proceeds somewhat more rapidly than with air but closer control of the flow rate is necessary to avoid too rapid oxidation, which may result in overheating and sintering of the copper oxide.

Copper oxide with average particle diameters of 0.5 to 1.0 mm. appears to be superior to the coarser fractions tested, inasmuch as it can be packed more solidly into the combustion tubes, presents a larger surface area, and reduces the likelihood of channeling of the gas stream. In the above tests it was noted that the more finely divided oxide generally was oxidized more rapidly than the coarser material. All these factors tend to promote increased activity, both in the combustion of hydrocarbon gases and in the regenerative oxidation of the combustion agent. Appreciably smaller particles impede the flow of gas through the tube and may be swept from the combustion tube past the retaining plugs of sheet asbestos.

During the combustion of *n*-butane, it was observed after several portions had been burned without reoxidation of the copper oxide that the combustion rate gradually became low and when the copper oxide became highly reduced, olefinic hydrocarbons were found in the combustion residues. Apparently this was due to cracking of the butane in the absence of sufficient available oxygen for complete combustion. Since the oxygen available in the copper oxide is fairly rapidly depleted, care must be exercised to maintain the copper oxide in a well-oxidized state; otherwise slow and incomplete combustions are likely to result. Reoxidation of the precipitated oxide after combustion of about 50 ml. of butane or proportionately greater volumes of the lower hydrocarbons was found satisfactory.

SUMMARY AND CONCLUSIONS

Precipitated copper oxide containing 1% iron oxide is a satisfactory combustion agent for the determination of gaseous paraffins and offers several important advantages over other existing methods. Its use not only eliminates the need for supply of oxygen of accurately known purity but also avoids errors arising from incorrect measurement of oxygen volume or contamination of the oxygen supply, and eliminates any explosion hazard from formation of an explosive mixture of hydrocarbon gases and oxygen. Results obtained by its use are equal or superior in accuracy and precision to those obtained by other combustion methods tested.

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Analysis of Water-Soluble Chlorohydrins and Other Organic Chlorides

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A method of quantitatively determining chlorohydrins in mixtures with other organic chlorides and inorganic chlorides is presented. The chlorides are selectively hydrolyzed with alkalies and the resulting alkali chloride is volumetrically determined by titrating with silver nitrate by the Mohr method (2).

THE method described below provides means of differentiating the chlorine combined in the chlorohydrins from the chlorine in such other compounds as inorganic chlorides, organic dichlorides, and dichloro ethers.

A survey of the literature revealed two methods of replacing the chlorine of aliphatic chlorohydrins with the OH group. Francis (*1*) states that chlorohydrins are quantitatively converted to glycols by heating with a solution of sodium bicarbonate. Uhrig (*3*) used hydrolysis with sodium hydroxide to determine chlorohydrins.

Experimental work using sodium bicarbonate as hydrolyzing agent showed that chlorohydrins were selectively hydrolyzed with this agent to the glycol and sodium chloride. Other aliphatic chlorides were unaffected. On the other hand, sodium hydroxide not only hydrolyzed the chlorohydrins to glycols and sodium chloride but also hydrolyzed the small amounts of other aliphatic chlorides which were dissolved in the chlorohydrins.

It is possible by these means to determine the inorganic chloride present in the sample, and then by using two hydrolyses to determine the chlorohydrin chloride and the other aliphatic chlorides such as ethylene dichloride, propylene dichloride, and the respective dichloro ethers.

REAGENTS

0.1 *N* silver nitrate, standardized; dilute sodium hydroxide, 4%; dilute sulfuric acid, 5%; 5% aqueous sodium chromate; C.P. solid sodium bicarbonate; and phenolphthalein indicator.

APPARATUS

Gas or electric hot plate; condenser rack. Reflux water-cooled condensers with standard-taper ground-glass joints, approximately 16-inch (40-cm.) jacket; 250-ml. flasks with standard-taper ground-glass joints to fit condensers; 250-ml. wide-mouthed Erlenmeyer flasks; 50-ml. burets; 100-ml. volumetric flask; and 10-ml. pipet.

PROCEDURE

Accurately weigh to ±1 mg. a sample of such size that when it is diluted to 100 ml., 5 ml. will contain approximately 0.25 gram of chlorohydrin. Transfer to a 100-ml. volumetric flask and make up to the mark with distilled water. Use aliquots for the following tests.

Inorganic Chloride. Pipet a 5-ml. aliquot into a 250-ml. Erlenmeyer flask containing approximately 50 ml. of distilled water, and add several drops of phenolphthalein indicator. If pink, neutralize the contents of the flask with dilute sulfuric acid until the pink color is just destroyed; if colorless, add dilute sodium hydroxide until faintly pink, then destroy pink color with 1 or 2 drops of dilute sulfuric acid. Add 1 ml. of the 5% sodium chromate and titrate the mixture with 0.1 *N* silver nitrate until the first permanent appearance of the red precipitate of silver chromate. Each milliliter of 0.1 *N* silver nitrate is equivalent to 0.003546 gram of chlorine.

$$\frac{\text{Ml. of 0.1 } N \text{ silver nitrate} \times 0.3546 \times 20}{W} = \% \text{ chlorine}$$

when W = weight of sample which was diluted to 100 ml. Record per cent chlorine as A.

Chlorohydrin Chloride. Pipet a 5-ml. aliquot into a 250-ml. Erlenmeyer flask with 24/40 standard-taper ground-glass joint, add approximately 50 ml. of water and 2 grams of C.P. sodium bicarbonate, attach to reflux condenser, and reflux at boiling for 30 minutes. Remove the flask from the hot plate and cool the contents to room temperature. Add several drops of phenolphthalein indicator, neutralize the contents of the flask, and titrate with 0.1 *N* silver nitrate as described above for inorganic chloride. Calculate and record per cent chlorine as B.

$$B - A = \% \text{ Cl combined in chlorohydrin}$$

Organic Chlorides. Pipet a 5-ml. aliquot into a 250-ml. Erlenmeyer flask with 24/40 standard-taper ground-glass joint, add approximately 50 ml. of distilled water and 10 ml. of dilute sodium hydroxide, attach to the reflux condenser, and reflux for 1 hour. Remove the flask from the hot plate, cool to room temperature, neutralize to phenolphthalein, and titrate as described above for inorganic chloride. Calculate and record per cent chlorine as C.

$$C - B - A = \% \text{ Cl present in organic materials other than chlorohydrins.}$$

RESULTS AND DISCUSSION

Aqueous mixtures of inorganic chloride, ethylene chlorohydrin, propylene chlorohydrin, dichloroethyl ether, dichloroisopropyl ether, ethylene dichloride, and propylene dichloride were made which contained these components in varying amounts. Table I gives the exact composition of the mixtures and lists the analytical results obtained in the laboratory and the theoretical values calculated from per cent chlorine in the various compounds.

This method has been found applicable for analysis of chlorohydrins in aqueous solutions and in water-soluble solvents—i.e., alcohols, glycols, etc.—in concentrations varying from 2 to 100% chlorohydrin. The total grams of chlorohydrin present in the

Table I. Composition of Mixtures

	I %	II %	III %	IV %	V %	VI %	VII %
Ethylene chlorohydrin	5.3	5.3	5.3	5.3	30.2	68.0	5.3
Propylene chlorohydrin	2.6	2.6	2.6	2.6	14.2	32.0	2.6
Ethylene dichloride	0	0	0.7	0.5	0	0	0.5
Propylene dichloride	0	0	0.3	0.2	0	0	0.2
Dichloroethylene	0	1.0	0	0.2	0	0	0.2
Dichloroisopropyl-ethylene	0	0	0	0.1	0	0	0.1
Hydrochloric acid	0	0	0	0	0	0	3.48
Water	92.1	91.1	91.1	91.1	55.6	0	87.62
Chlorine from inorganic compounds							
Theory	0	0	0	0	0	0	3.39
Found	0	0	0	0	0	0	3.34
Chlorine from chlorohydrin							
Theory	3.32	3.32	3.32	3.32	18.62	42.00	3.32
Found	3.28	3.30	3.35	3.31	18.49	42.06	3.31
Chlorine from aliphatic chlorides							
Theory	0	0.50	0.69	0.63	0	0	0.63
Found	0	0.49	0.70	0.61	0	0	0.64

hydrolysis mixture must not exceed approximately 0.25 gram (calculated as ethylene chlorohydrin). This sample size is limited by the amount of chloride which can conveniently be titrated with 0.1 *N* silver nitrate.

It is not feasible to analyze chlorohydrins dissolved in water-insoluble solvents by this method. The presence of excess water-insoluble solvent prevents the reaction with the aqueous alkalies and yields low results. For such solutions the solvent must be removed by some method such as distillation, or the chlorohydrin extracted with water.

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Fluorine Content of Certain Vegetation in a Western Pennsylvania Area

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In the Pittsburgh area unexpectedly large amounts of fluorine are to be found in the foliage of trees and in grass, alfalfa, and other vegetation. There seems to be a marked tendency towards increased concentrations of fluorine as the growing season progresses. As leaves grow old they have a higher fluorine content. It is also possible that fluorine accumulates on the surface of leaves as fluorine-bearing dusts, because of long exposure to the atmosphere. Foliage samples taken in the autumn may show higher fluorine contents because of increase in atmospheric fluorine occasioned by increase of coal smoke in the air during the early autumn, but this is probably a minor factor. The presence of fluorine in coal would indicate that fluorine might well have been a normal constituent of the ligneous or organic material from which coal was derived. There appear to be two sources from which plants may absorb fluorine—soil and atmosphere. The presence of fluorine in soils is well recognized and coal smoke is an important source of air-borne fluorine.

IN RECENT years there has been manifested an increasing interest in the compounds of fluorine as important industrial materials. This commercial and industrial interest in fluorine compounds has been paralleled by a widespread study of the occurrence of fluorides in municipal and domestic waters. This latter phase of the problem found its inception in a publication by the senior author (*1*). A rather complete study of the problem of fluorine in potable waters is contained in a publication of the American Geographical Society (*3*). Interest in the fluoride content of waters has led to studies of the occurrence of fluorine compounds in various forms of plant and vegetable life. In the present paper are presented analytical data covering the presence of fluorine in tree leaves and other forms of vegetation, chiefly in the Pittsburgh district.

These data are reported as parts per million based on the dry weight of samples. In brief, the analytical procedure was as follows:

A portion of each sample of leaves was oven-dried to constant weight at a temperature of 105° C. and the percentage of moisture in the sample was calculated. The sample for fluorine analysis was weighed from the undried portion of the sample, since it was felt that some fluorine compounds might be lost in the drying process. The dry weight of the sample actually used was calculated. The sample was covered with lime water and taken to dryness on a hot plate, with maintenance of caustic alkalinity by lime additions throughout the operation. The dried alkaline residue was ignited at a temperature of 500° to 550° C. The entire ash was used for the determination of fluorine. The fluorine was isolated by the well-known Willard-Winter distillation process (*4*) and its concentration was measured by thorium nitrate titration, using sodium alizarin sulfonate indicator.

Most of the samples here reported were taken in an area northeast of Pittsburgh; all locations mentioned are in Pennsylvania except as indicated. Table I lists fluorine contents of various samples of tree leaves. Fluorine as used in this paper should be construed as meaning fluorine combined as fluoride—i.e., fluorine as hydrofluoric acid, calcium fluoride, sodium fluoride, etc.—in no case do the authors mean elemental fluorine as such. In Table I each tree which was sampled is numbered. Letters in parentheses indicate separate samples from the same tree.

The condition of the foliage was normal. The data give clear evidence that fluorine is a normal constituent of vegetation in western Pennsylvania. It seems obvious that fluorine, at least to the extent herein reported, may be present in vegetation without showing any evidence of attack or deterioration. In further evidence that no deterioration had occurred is the fact that during the years covered by the taking of samples, all the fruit trees sampled bore normal crops. Most of the trees sampled have never been sprayed with fluorine-bearing insecticides, but some trees had been sprayed with fluorine-bearing material in years previous to the years of sampling covered by the data in the tables.

All trees and vines sampled at each location were located on the same plot of ground, and care was taken to obtain a representative and average sample of the foliage. All the leaves sampled were in good condition and had the normal appearance of leaves for the season of sampling.

One interesting aspect revealed by the data in Table I is the apparent increase in fluorine content of leaves as they grow older. In connection with this it is interesting to note the data on the fluorine content of grass from the Aluminum Research Laboratories' lawn at New Kensington, as given in Table II. The lawn was mowed weekly and cuttings were not removed but served to mulch the soil. Fresh cuttings were taken as samples on the dates given in the table.

Table I. Fluorine Content of Tree Leaves

No.	Type of Tree	Location	Date of Sampling	P.P.M. Fluorine (Dry Weight Basis)	Type of Location[a]
1	Cedar	New Kensington	Oct. 1943	64	1
2	Oak	New Kensington	Oct. 1943	62	1
3	Black oak	Freeport	Dec. 1943	51	2
4	Oak	Ingleside	Nov. 1943	157	3
5	Oak	(A) New Kensington	June 1944	21	4
		(B) New Kensington	Aug. 1944	33	4
		(C) New Kensington	Oct. 1944	77	4
6	Pin oak	(A) New Kensington	Nov. 1944	129	4
		(B) New Kensington	May 1945	43	4
		(C) New Kensington	Aug. 1945	53	4
		(D) New Kensington	Oct. 1945	104	4
7	Sycamore	New Kensington	Oct. 1943	147	4
8	Norway pine	Freeport	Dec. 1943	67	2
9	White pine	Freeport	Dec. 1943	58	2
10	Gray pine	Freeport	Dec. 1943	106	2
11	Pine	Ingleside	Nov. 1943	17	3
12	Sycamore	Ingleside	Nov. 1943	112	3
13	Sycamore	(A) New Kensington	June 1944	35	4
		(B) New Kensington	Aug. 1944	53	4
		(C) New Kensington	Oct. 1944	126	4
14	Willow	Ingleside	Nov. 1943	31	3
15	Aspen	(A) New Kensington	June 1944	45	4
		(B) New Kensington	Aug. 1944	47	4
		(C) New Kensington	Oct. 1944	116	4
16	Elm	(A) New Kensington	June 1944	19	4
		(B) New Kensington	Aug. 1944	6	4
		(C) New Kensington	Oct. 1944	19	4
17	Locust	(A) New Kensington	June 1944	53	1
		(B) New Kensington	Aug. 1944	76	1
		(C) New Kensington	Oct. 1944	104	1
18	Wild cherry	(A) New Kensington	June 1944	37	1
		(B) New Kensington	Aug. 1944	77	1
		(C) New Kensington	Oct. 1944	177	1
19	European beech	(A) New Kensington	Nov. 1944	226	4
		(B) New Kensington	May 1945	44	4
		(C) New Kensington	Aug. 1945	46	4
		(D) New Kensington	Oct. 1945	88	4
20	Mulberry	(A) Oakmont	Aug. 1944	47	5
		(B) Oakmont	Oct. 1944	66	5
21	Oriental cherry	(A) New Kensington	Nov. 1944	142	4
		(B) New Kensington	May 1945	33	4
		(C) New Kensington	Aug. 1945	71	4
		(D) New Kensington	Oct. 1945	119	4
22	Plane	(A) New Kensington	Nov. 1944	161	4
		(B) New Kensington	May 1945	50	4
		(C) New Kensington	Aug. 1945	61	4
		(D) New Kensington	Oct. 1945	157	4
23	Rhododendron	(A) New Kensington	Nov. 1944	48	4
		(B) New Kensington	May 1945	32	4
		(C) New Kensington	Aug. 1945	35	4
		(D) New Kensington	Oct. 1945	105	4
24	Russian olive	(A) New Kensington	Nov. 1944	139	4
		(B) New Kensington	May 1945	76	4
		(C) New Kensington	Aug. 1945	75	4
		(D) New Kensington	Oct. 1945	120	4
25	Silver poplar	(A) New Kensington	Nov. 1944	162	4
		(B) New Kensington	May 1945	51	4
		(C) New Kensington	Aug. 1945	107	4
		(D) New Kensington	Oct. 1945	208	4
26	Sweet gum	(A) New Kensington	Nov. 1944	73	4
		(B) New Kensington	May 1945	36	4
		(C) New Kensington	Aug. 1945	35	4
		(D) New Kensington	Oct. 1945	58	4
27	Wild crabapple	(A) New Kensington	Nov. 1944	152	4
		(B) New Kensington	May 1945	42	4
		(C) New Kensington	Aug. 1945	100	4
		(D) New Kensington	Oct. 1945	123	4
28	Concord grape	(A) Oakmont	June 1946	9	5
		(B) Oakmont	Aug. 1946	20	5
		(C) Oakmont	Oct. 1946	81	5
29	Early red apple	(A) Oakmont	June 1946	25	5
		(B) Oakmont	Aug. 1946	64	5
		(C) Oakmont	Oct. 1946	112	5
30	Golden Delicious apple	(A) Oakmont	June 1946	13	5
		(B) Oakmont	Aug. 1946	50	5
		(C) Oakmont	Oct. 1946	112	5
31	Plum	(A) Oakmont	June 1946	14	5
		(B) Oakmont	Aug. 1946	59	5
		(C) Oakmont	Oct. 1946	105	5
32	Maple	(A) Oakmont	June 1946	22	5
		(B) Oakmont	Aug. 1946	43	5
		(C) Oakmont	Oct. 1946	55	5
33	Red winter apple	(A) Oakmont	June 1946	13	5
		(B) Oakmont	Aug. 1946	54	5
		(C) Oakmont	Oct. 1946	154	5
34	Summer apple	(A) Rosedale	June 1946	24	5
		(B) Rosedale	Oct. 1946	177	5
35	Pear	(A) Rosedale	June 1946	18	5
		(B) Rosedale	Oct. 1946	117	5
36	Damson plum	Rosedale	June 1946	20	5
37	Sour cherry	(A) Rosedale	June 1946	17	5
		(B) Rosedale	Oct. 1946	120	5
38	Green gage plum	(A) Rosedale	June 1946	24	5
		(B) Rosedale	Oct. 1946	133	5
39	Damson plum	(A) Rosedale	June 1946	18	5
		(B) Rosedale	Oct. 1946	108	5
40	Winesap apple	(A) Freeport	June 1946	33	3
		(B) Freeport	Oct. 1946	152	3
41	Italian prune	(A) Freeport	June 1946	29	3
		(B) Freeport	Oct. 1946	269	3
42	Yellow plum	(A) Freeport	June 1946	22	3
		(B) Freeport	Oct. 1946	124	3
43	Sweet cherry	(A) Freeport	June 1946	33	2
		(B) Freeport	Oct. 1946	115	2
44	Kieffer pear	(A) Freeport	June 1946	15	2
		(B) Freeport	Oct. 1946	177	2
45	Red grape	(A) Freeport	June 1946	14	2
		(B) Freeport	Oct. 1946	71	2
46	Apricot	(A) Freeport	June 1946	30	2
		(B) Freeport	Oct. 1946	233	2
47	Sour blue plum	(A) Freeport	June 1946	32	2
		(B) Freeport	Oct. 1946	126	2
48	Peach	(A) Freeport	June 1946	10	2
		(B) Freeport	Oct. 1946	38	2
49	Scotch mountain pine, new growth only	New Kensington	June 1946	13	1

[a] Type of location (all sampling sites in Allegheny River Valley within 10 to 30 miles of Pittsburgh).

1. Residential section of industrial community.
2. Rural, remote from industry or railroad.
3. Rural, near Allegheny River and railroad.
4. Aluminum Research Laboratories grounds, located in residential section.
5. Residential section of community, much less industrialized than No. 1.

Table II. Fluorine Content of Lawn Grass

Date of Sampling	P.P.M. Fluorine (Dry Weight Basis)
Nov. 1944	45
May 1945	16
Aug. 1945	37
Oct. 1945	53

Table III. Fluorine Content of Alfalfa

Date of Sampling	Description	P.P.M. Fluorine (Dry Weight Basis)
June 1944	1st cutting, hay	15
July 1944	2nd cutting, hay	21
Aug. 1944	3rd cutting, hay	27
Oct. 1944	Green	36

Table IV. Fluorine Content of Threshed Buckwheat, Oats, and Oat Straw

Sample	Date of Sampling	P.P.M. Fluorine (Dry Weight Basis)
Buckwheat	July 1944	2
Oats	July 1944	3
Oat straw	July 1944	11

The seasonal increase in the average fluorine content of leaves, or possibly its increase during the life of leaves, was further investigated by analyses made on samples of alfalfa taken from a farm near New Kensington, Pa. (Table III).

In July 1944, samples of threshed buckwheat, oats, and oat straw were obtained from the same farm from which the alfalfa samples were obtained. These samples were analyzed for fluorine content with the results given in Table IV.

Regardless of whether the fluorine found when leaves are analyzed is derived from surface accumulation or from the structure of the leaf itself, the source of the fluorine is of some interest. In the field where the alfalfa covered by Table III was grown, samples of soil to a depth of 6 inches were taken from the periphery of a 25-foot circle. The samples were carefully mixed to make a composite sample and this composite sample was analyzed for fluorine. The analysis revealed that the soil contained 730 p.p.m. of fluorine.

Table V. Effect of Fluoride Addition

Plot	P.P.M. of Fluorine Found June	July	Sept.
1	45	54	97
2	3035	242	118
3	756	64	63
4	154	55	77

To supplement the data presented in Table II, certain additional work was carried out in 1946 to ascertain whether the addition of mineral fluorides to the soil would affect the fluorine content of the grass. Grass from each of four 10 × 10 foot plots on the lawn of Aluminum Research Laboratories was completely mowed, sampled, and analyzed for fluorine content. The plots were adjacent to each other, but sufficiently separated to avoid contamination of any plot by fluoride material from other plots. The plots were treated by scattering fluorine-bearing material as indicated:

Plot 1, no fluorine-bearing material added
Plot 2, 2 pounds of cryolite applied to area
Plot 3, 2 pounds of fluorspar applied to area
Plot 4, 2 pounds of superphosphate (1.66% F) applied to area

The high fluorine contents shown for plots 2, 3, and 4 for the month of June were probably caused in large part by the dusting of the grass blades and the retention of the dust in the intercepts of grass blades. Since the average of the treated plots for September is less than the figure for the untreated plot, the addition of mineral fluorides to the soil did not appreciably affect the fluorine content of grass. It would appear that a considerable portion of the increased fluorine in the vegetation in Table II must be derived from the atmosphere.

Since it appears that the main source of the fluorine found in these tests is the atmosphere, it should be pointed out that an important source of air-borne fluorine is coal smoke. Analysis of a sample of western Pennsylvania coal revealed the presence of 85 p.p.m. of fluorine. A sample of southern Illinois coal analyzed in parallel with the Pennsylvania sample showed a fluorine content of 167 p.p.m. The occurrence of fluorine in coal has been noted by several authors, among whom was Crossley (*2*).

A. W. Petrey of the Aluminum Company of America Works at Vancouver, Wash., has furnished the authors with fluorine determinations on six samples of coal, taken in Vancouver, Wash. These data show that fluorine is found in coal of the Rocky Mountain area as well as in coal from Pennsylvania and Illinois and from English sources.

Table VI. Fluorine Content of Western Coals

Location of Mine	Size	P.P.M. of Fluorine
Utah	Pea	195
Utah	Stoker	195
Unknown	Nut	240
Utah	Nut	145
Utah	Stoker	295
Unknown	Stoker	195

Table VII. Fluorine Content of Rhododendron and Alfalfa

Sample	P.P.M. of Fluorine (Dry Weight Basis)
Rhododendron stalks	24
Rhododendron leaves	65
Alfalfa stems	13
Alfalfa leaves	46

To ascertain if there seemed to be any localization of fluorine in vegetation, samples of rhododendron and alfalfa taken near New Kensington, Pa., were analyzed for fluorine content. Table VII clearly indicates a concentration of fluorine in the leaves of the two plants studied.

Some data are available which indicate that fluorine is found in wood itself in addition to the leaves. Petrey also reports fluorine determinations on samples of sawdust derived from various woods in the state of Washington.

It is hoped that this paper will stimulate investigations of the fluorine content of similar materials in other geographical areas to furnish data as to the influence of various factors on the fluorine content of vegetation growing in industrial, residential, and rural areas.

Table VIII. Fluorine Content of Sawdust

Sample No.	Variety of Wood	P.P.M. of Fluorine (Dry Weight Basis)
1	Douglas fir	3.6
2	Fir chips	2.0
3	Fir	3.0
4	Spruce	2.7

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Spark Technique in Spectrographic Analysis of Slags

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A method is described for the quantitative spectrographic analysis of open hearth slag samples for lime-silica ratio, which gives sufficient accuracy for open hearth control. Calibration curves have been prepared for a high voltage condensed spark excitation of the samples.

IN OPEN hearth steel production it is necessary to keep the lime-silica ratio (or V ratio as it is called) sufficiently high to hold the sulfur in the slag and prevent phosphorus reversion from the slag to the molten steel bath. The method of determining this ratio must be rapid and reasonably accurate if it is to be of value during the working of a heat. The chemical determination of lime and silica is long and involved. A rapid method of determining the ratio by the hydrolysis of a sample of slag in water and the subsequent measuring of the pH of the aqueous suspension has been used, but small amounts of unreacted lime in the slag sample cause the results to be in error. Another widely used method consists of a visual examination of a slag pancake. The spectrograph is the logical instrument to be used when quantitative results in large numbers and in a reasonable length of time are desired.

Both the alternating and direct current arcs have been used as excitation sources by other investigators, but to the authors' knowledge, a low inductance condensed spark as an excitation source has not been employed previously for slag analysis. Analytical curves made with the direct current arc are unstable and have a tendency to shift from day to day, thus necessitating the running of numerous standard samples. This phenomenon is attributed to the difficulty of duplicating the high temperature. In the arc there is fractional distillation. This source of inaccuracy can be more or less eliminated if the sample is arced sufficiently long to accomplish complete volatilization, but the procedure requires a great deal of time.

Stable analytical curves are necessary for production work. When dozens of standard samples have to be run daily because of curve shifting, there is a tremendous waste of effort. Stability of analytical curves is dependent upon many factors, the most important of which are (1) extremely high vapor temperature, (2) matched spectral line pairs, (3) coolness of electrodes, (4) tight, clean electrical connections, and (5) clean insulating surfaces. In spark technique the vapor temperature is at least double that of the arc, although much less sample is vaporized and the surface of the electrodes being sparked remains reasonably cool. The other factors may be equal.

EQUIPMENT AND PROCEDURE

The source of excitation consists of a high-voltage commercial spark unit of 2 kv.-amp. with an added inductance of 0.045 millihenry and a capacitance of 0.021 microfarad. This unit has a Fuessner synchronous rotating auxiliary gap. These electrical conditions provide a short-period condensed spark with a low power factor.

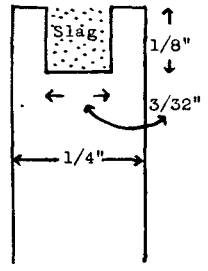
The spectrograph is a 1.5-meter instrument with a 24,000 lines per inch (2.5 cm.) grating, providing a uniform dispersion of 7 Å. per millimeter. The camera holds a 100-foot roll of film. Spectrum analysis No. 1 film is used.

Slag is a nonconductor and it is necessary to provide a means whereby the slag is introduced into the spark discharge. A cupped graphite electrode filled with powdered slag was tried, but the spark blew away all the slag in the first second or two. The time of blow-away had to be lengthened sufficiently to give a good spectrogram. The blow-away itself seemed desirable, since fresh sample was being sparked at all times, this being the acme in point 3 of the authors' stable analytical curve qualifications.

Finally it was found that a weak solution of Ethocel (an ethylcellulose ether) in butyl acetate would cement the slag particles together sufficiently to lengthen the blow-away time to 10 to 15 seconds. Presumably, other organic cementing compounds could also be used.

The complete technique for sample preparation is as follows:

A graphite electrode 0.25 inch in diameter is cut flat at one end, and a hole $^3/_{32}$ inch in diameter and 0.125 inch deep is then cut through the flat surface. The shaping is done on a motor-driven cutter and requires about 10 seconds. This electrode is then shoved cup-side down into a small pile of powdered slag (ground to pass through an 80-mesh sieve) with sufficient force to pack the slag tightly. The electrode is withdrawn and a drop or two of 2% solution of Ethocel in butyl acetate is placed on the slag surface with an eye dropper. The solution is rapidly absorbed. The loaded electrode is then dried. A stream of air from a blower of the hair-dryer type will evaporate the solvent in 2 minutes or less, or the electrode can be heated in any convenient manner, such as holding it next to a hot Nichrome heating element, which will reduce the drying time to 5 to 10 seconds.



Cross Section of Cupped and Loaded Graphite Electrode

Exposure Conditions. The dried, loaded graphite is used as the lower electrode and a graphite rod 0.25 inch in diameter with a 120° cone-shaped tip is used as the upper electrode. The normal sparking time is 10 seconds, at which time the cupped graphite electrode is empty or almost empty. No prespark period is used.

Development and Photometry. The film is developed for 2 minutes in Eastman D-19 developer, placed in a 2.5% acetic acid stop bath for 10 seconds, and fixed in Kodak rapid liquid fixer for 45 seconds. After washing for 1 minute, the film is dried by infrared radiation in a stream of warm air.

The film is stretched taut in a film holder and the whole is placed in a commercial microphotometer in order to measure the density of the lines on the film.

WORKING CURVES

Two spectral line pairs are used: 3905.53 Å. Si with 4302.53 Å. Ca and 3905.53 Å. Si with 4318.65 Å. Ca. The first line pair is used for the range of lime-silica ratios from 0.6 to 3.0 and the second for the range of ratios 2.5 to 8.5. There is little choice in line pairs because of the simplicity of the spectra of calcium and silicon.

A film calibration curve is prepared with a rotating logarithmic sector, or by any other convenient method, for the 3900 to 4300 Å. region of the spectrum.

The analytical curves are prepared by plotting the log relative intensities against the log of ratios of per cent lime to per cent silica. Thus, the lime-silica ratio is taken directly from the analytical curve, using the film calibration curve. The actual

percentages of lime and silica remain unknown, and only the ratio of these percentages is used.

ACCURACY AND SPEED

The general reproducibility of results is approximately 10% of the lime-silica ratio value. Table I shows a comparison of results obtained on eleven slags run spectrographically and chemically. The samples were run in quadruplicate spectrographically and all results tabulated. Ordinarily, samples are run in duplicate.

Occasionally, slags are found where repeated checks give variations of 1.0 or even 1.5 in the ratio. These same slags, after once having been powdered, have a tendency to cake upon long standing. Their true lime-silica ratio is generally over 3.0. Both the caking and erratic results, it is believed, can be accounted for by the presence of relatively large amounts of free-unreacted lime. Free lime causes the spectrographic result to run high. To date, attempts to overcome this difficulty have been unsuccessful.

Table I. Comparison of Lime-Silica Ratios

Slag	Chemical	Spectrographic	
		Individual	Average
A	0.67	0.70, 0.68, 0.63, 0.68	0.67
B	0.76	0.69, 0.74, 0.61, 0.70	0.68
C	1.85	2.0, 1.9, 1.7, 1.9	1.9
D	2.04	1.7, 2.0, 2.1, lost[a]	1.9
E	2.27	Lost[a], 2.3, 2.3, 2.2	2.3
F	2.91	3.2, 2.9, 2.9, 3.0	3.0
G	3.70	3.4, 3.9, 3.6, 3.6	3.6
H	4.70	4.6, 4.8, 4.5, 5.1	4.8
I	6.00	6.6, 5.9, 5.8, 6.2	6.1
J	8.43	8.1, 8.8, 9.2, 7.8	8.5

[a] One sample of D and one sample of E were accidentally double-exposed, their spectrograms being superimposed.

If there are no interruptions, a lime-silica ratio can be determined within 15 to 20 minutes of the time of delivery of the powdered sample to the spectrographic laboratory.

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Spectrographic Determination of Beryllium in Biological Material and in Air

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A spectrographic method for the determination of beryllium in biological material and in the air has been developed, which is capable of detecting as little as 0.25 microgram of beryllium. Details are given for the analysis of samples of freshly voided urine without ashing and for a wet-ashing procedure which is generally applicable. Methods for collecting and analyzing air samples are also given. The most satisfactory procedure consists in ashing the samples with sulfuric, nitric, and perchloric acids, isolating the beryllium as the phosphate, and dissolving the phosphate precipitate in an acid spectroscopic buffer solution. Aliquot portions (0.2 ml.) of the latter solutions are used to impregnate graphite rods which are used as the lower positive electrodes of direct current arcs. Densitometry involves the plotting of H and D curves obtained for the stepped line of beryllium at 2348.6 Å. and that of the internal standard, thallium at 2379.7 Å., and obtaining the separation of the two curves at a constant blackening (T = 0.50). This separation is then evaluated from a working chart prepared beforehand from known amounts of beryllium which are added to the spectroscopic buffer solution.

BERYLLIUM has been determined in alloys, ores, and rocks colorimetrically (*5*, *9*, *11*, *16*), fluorometrically (*6*, *15*, *17*, *18*), and spectrographically (*4*, *8*, *10*, *12*, *13*). Hyslop and coworkers (*7*) found that most of these methods lack sensitivity when applied to biological material, and that it was necessary to evolve special methods for the determination of beryllium in animal tissues. As a result, they investigated three possible tests (*7*), but found that none of these was uniformly applicable. The principal difficulty in the use of chemical methods is their lack of specificity and the consequent need for removing interfering ions (*6*, *14*). Since many interfering ions (iron, calcium, magnesium, and phosphate) occur regularly in biological material, the difficulty of isolating minute amounts of beryllium therefrom without appreciable loss is very serious. Accordingly, it has seemed desirable to investigate the applicability of spectrographic procedures as a means of studying the behavior of beryllium in the animal organism.

The results of preliminary spectrographic examinations of solutions of pure beryllium sulfate were so promising from the aspects of specificity and sensitivity as to justify further investigation of the entire analytical procedure. It was found early that the spectrographic techniques themselves offered no difficulties and that the most important factor in the reliable estimation of minute quantities of beryllium related to the preparation of samples. The latter involved the destruction of organic matter and the isolation and concentration of beryllium without loss, points which have not been studied by others. Procedures for both preparation and analysis of samples have been developed which are applicable to a wide variety of materials, including those which are usually encountered in physiological and environmental investigations.

SPECIAL REAGENTS

Calcium Phosphate Solution. Two and one-half grams of calcium carbonate (calcite) are dissolved in just sufficient concentrated hydrochloric acid (specific gravity 1.19) to effect solution (about 7 ml.). Diammonium phosphate (2.5 grams) is added and the solution is made up to 100 ml. with distilled water. If a precipitate forms, it is resolved by adding hydrochloric acid drop by drop.

Spectroscopic Buffer Solution. Any ionizable salt or salt mixture may be used. The following solution was used because of its availability in connection with other spectrographic work: 125 ml. of a stock solution (*1*), designed to simulate urine in its salt content (sodium chloride, 170.5 grams; potassium chloride, 63.5 grams; calcium chloride hexahydrate 31.5 grams; magnesium chloride hexahydrate, 20 grams; and sodium dihydrogen phosphate monohydrate, 37.5 grams, dissolved in and diluted to 1000 ml. with 10% by volume hydrochloric acid) are mixed with

50 ml. of hydrochloric acid, specific gravity 1.19, and 5 ml. of thallic nitrate solution (1 ml. = 10 mg. of thallium), and the whole is diluted to 500 ml.

PREPARATION OF SAMPLES

Urine (Freshly Voided). Forty milliliters of urine are placed in a 50-ml. centrifuge tube having a round bottom, 0.5 ml. of the calcium phosphate solution is added, and then small drops of concentrated ammonia (specific gravity 0.900) are added until the solution is just alkaline to phenol red. The solution is centrifuged for 3 minutes at 2500 to 3000 r.p.m. and the supernatant liquid is decanted and discarded. The precipitate is dissolved in 0.5 ml. of hydrochloric acid and a little distilled water and the solution is transferred quantitatively to a graduated 15-ml. conical centrifuge tube. One milliliter of the spectroscopic buffer solution is added and the volume is adjusted to 1 ml. by evaporating the solution in an oil or glycerol bath at about 120° to 140° C.

Animal Tissues. Ten grams or more of tissue or blood (or 50 ml. of urine) are placed in a 250-ml. Pyrex beaker, 5 ml. of concentrated sulfuric acid and 20 ml. of concentrated nitric acid are added, and the mixture is heated until charring occurs. The char is destroyed by the addition of a small amount of nitric acid and heat, and the process is repeated until no charring occurs when the mixture is heated until fumes of sulfur trioxide are evolved. The digestion and oxidation may be hastened if, toward the end, 5 ml. of perchloric acid (70 to 72%) are added. After oxidation is complete, all but a trace of the sulfuric acid is evaporated off. On cooling, the solution is transferred to a 50-ml. centrifuge tube and dealt with exactly as was the sample of freshly voided urine.

In digesting samples of bone or other samples where large amounts of calcium are thrown down as the sulfate, the calcium sulfate is removed by filtration or centrifuging before the beryllium is isolated as the phosphate. Because of the large amount of ammonium sulfate which is formed, "salting out" may occur before a final volume of 1 ml. can be reached. When this occurs, 2 ml. of spectroscopic buffer are used, and the final volume is adjusted to 2 ml.

ALTERNATE METHOD FOR BLOOD WITH IRON REMOVAL

After 10 grams or more of blood have been digested according to the foregoing procedure, the beryllium is isolated as the phosphate by the method given for dealing with freshly voided urine. The phosphate precipitate is then dissolved in hydrochloric acid and the solution is transferred to a separatory funnel. The volume of the solution is adjusted to 20 ml. with distilled water and iron is removed by the following procedure.

Four milliliters of a 6% by weight cupferron solution are added and the mixture is shaken with successive 10-ml. portions of chloroform until the last portion of chloroform added is found to be colorless. The chloroform layers are discarded while the aqueous phase is provided with 1 or more ml. of spectroscopic buffer solution and is evaporated in the oil or glycerol bath to the volume of spectroscopic buffer solution which had been added.

DEPOSITED DUST OR SAMPLES OF DUST OBTAINED FROM AIR

Samples may be collected from the air by electrostatic precipitation, by filtration on paper, or by means of an impinger.

Samples Obtained by Electrostatic Precipitation. The tubes are rinsed and policed down with 5% by volume nitric acid, and the rinse solution is caught in a 250-ml. Pyrex beaker. The rinse solution is concentrated to a small volume and then transferred to a graduated 15-ml. conical centrifuge tube. Spectroscopic buffer solution is next added and the mixture is evaporated in the oil bath to the volume of buffer solution which wasa dded. The amount of buffer solution to be added is determined by experience and on the basis of the expected concentration of beryllium.

Samples on Filter Paper. The filter paper is digested as are animal tissues, and the beryllium is isolated as for urine (freshly voided). Again, the quantity of spectroscopic buffer employed is determined by experience and by anticipation of the quantities of beryllium available.

Samples Obtained by Impinger. The liquid (water or alcohol) is transferred from the impinger to a 250-ml. Pyrex beaker, and the procedure prescribed for samples obtained by electrostatic precipitation is followed.

SPECTROGRAPHIC TECHNIQUE

Graphite electrodes (0.78 cm. in diameter and 3.75 cm. in length, prepared to have a crater 3 mm. in diameter by 10 mm. in depth) are impregnated with 0.2-ml. portions of the solutions which have resulted from the preparatory procedures. Duplicate rods are used for each solution. The impregnated rods are dried for 30 minutes at 100° to 110° C. and then are used as (lower) positive electrodes of the arc. The (upper) negative electrodes are made up of fresh 5.00- to 7.5-cm. lengths of rod, one end of which has been turned to a fine point in a pencil sharpener. Excitation is accomplished by direct current from a 110-volt direct current power line, the arc being operated at 10 amperes. Each exposure continues for 2 minutes, and the spectra are photographed on Eastman No. 33 plates. Each spectrum is "stepped" by means of a stepped sector (factor 2) which is rotated before the slit (0.03 mm. wide and 15 mm. long) of a Bausch & Lomb large Littrow quartz spectrograph. After the plates have been developed in D-19 developer, fixed, washed, and dried, the H and D curves are obtained for the beryllium line at 2348.6 Å. and the thallium line at 2379.7 Å., by measurement with a nonrecording

Table I. Beryllium Recovered from Urine by Direct Method of Analysis (No Ashing)

(40-ml. samples)

Be Added, γ	Be Found, γ
0, 0	0, 0
0.5, 0.5	0.65, 0.60
1.0, 1.0	1.10, 1.05
2.0, 2.0	2.0, 2.0
4.0, 4.0	3.5, 3.5
8.0, 8.0	8.8, 9.0

Table II. Beryllium Recovered Following Addition to Biological Material

(Materials ashed by wet method)

Material	Be Added, γ	Be Found, γ
50 ml. of urine	0, 0	0, 0
	0.5, 0.5	0.4, 0.4
	1.0, 1.0	1.0, 0.9
	2.0, 2.0	1.9, 2.0
	4.0, 4.0, 4.0	3.7, 3.7, 4.4
10 grams of liver	0, 0	0, 0[a]
	0.5, 0.5	0.8, 0.6[a]
	10.0, 10.0	11.5, 0.0[a]
	0,0	0,0
10 grams of blood	0.5, 0.5, 0.5, 0.5	0.65, 0.60, 0.60, 0.5
	1.5, 1.5	1.6, 1.6

[a] Iron not removed.

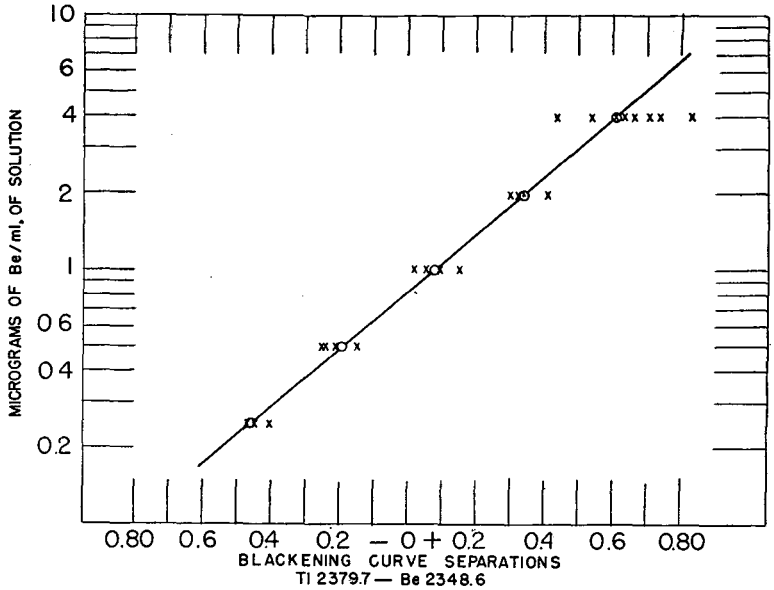

Figure 1. Working Curve for Spectrographic Determination of Beryllium

densitometer (*2*). The average separation between the two H and D curves at a constant blackening value (at $T = 0.50$) is then read from a calibration curve (Figure 1) prepared by adding known amounts of beryllium to 40 ml. of distilled water and proceeding according to the method given for the preparation of freshly voided samples of urine (*2*).

RESULTS

The extent of the recovery of known amounts of beryllium added to freshly voided urine and handled without ashing is shown in Table I. The recovery achieved following the use of wet-ashing techniques is given in Tables II and IV.

DISCUSSION

The most accurate analytical range of the working curve is that which lies between 0.25 and 2 micrograms per ml. of solution. Larger quantities of beryllium may be determined by diluting the prepared sample with suitable quantities of the spectroscopic buffer. Thus if the quantity to be read from the curve exceeds 2 micrograms, it is advisable to repeat the analysis following dilution with spectroscopic buffer solution. The inaccuracy above 2 micrograms per ml. is due to the varying degrees of self-reversal shown by the beryllium line at 2348.6 Å. under the conditions of excitation. This effect is illustrated in Figure 1, in which each cross on the figure represents the average separation value obtained for a set of six spectra on a single plate. The straight line passing through the circles is the working curve, and at each concentration the circle gives the mean separation value obtained from the total number of spectra taken. Although data for 8 micrograms per ml. are not shown, the few plates taken at this concentration show even wider variations in blackening curve separation values than do those representing 4 micrograms per ml. The amount of self-reversal obtained depends upon the concentration of the beryllium vapor in the arc. This concentration of vapor varies within limits set by the absolute amount of beryllium present and the width of the gap between the electrodes each time the arc is struck. Since for the authors' purposes the width of this gap, approximately 5 mm., was judged visually, variations up to 50% may well have occurred. It is very likely that more consistent blackening curve separation values for concentrations exceeding 2 micrograms per ml. can be obtained by maintenance of a constant gap between the electrodes. This can be done by projecting an image of the arc upon a screen, and by constantly adjusting the electrodes so as to maintain their images at a fixed distance apart as indicated by marks upon the screen.

Experience with spectrographic methods of analysis has shown that the method of densitometry based on the data of blackening curve separations (*2*) is the most practical when biological material is handled. Except for extremely low concentrations of some metals, there is no need for calibration of plates or corrections of the background (*3*), while there is added advantage in the fact that the procedure frequently permits the derivation of a single calibration curve for the entire workable range. This is not possible when the intensity ratio method is employed; it is necessary, usually, to derive several working charts to cover the analytical range even if stepped spectra are used. The accuracy of the intensity ratio method is also dependent upon the need for continual calibration of plates and to some degree upon corrections of background.

Direct ashing of biological material in either silica or platinum dishes, at temperatures as low as 500° C., results in significant losses of beryllium. Such losses are illustrated in Table III.

The method for the determination of beryllium in urine has been designed to handle samples of freshly voided urine without ashing. Aged urine samples, in which deposits of phosphate have formed, may also be handled by this technique, but only after the deposit has been dissolved by the addition of acid. The actual volume of the urine which is subjected to analysis in these instances will be less than 40 ml., and the dilution factor may affect the sensitivity of detection. Since wet ashing permits the use of larger aliquots, it is preferred for handling aged samples of urine.

Certain of the beryllium compounds which may be encountered in the air are very inert and can be dissolved only after the employment of a fusion technique. Such a procedure is time-consuming and tedious, and, in view of the data of Table III, probably unsatisfactory. However, it was found possible to deal with such materials satisfactorily by dispersing sediment or particulate matter in the final solution just before impregnating the rod. This can be done by blowing into the solution through the pipet which is used to impregnate the electrodes. This procedure was tested with a suspension of beryllium oxide, 3 ml. of which, corresponding to 15 micrograms of beryllium as the oxide, were pipetted upon 12.5-cm. Whatman No. 40 filter papers in triplicate. The papers were digested and the beryllium was isolated in 15 ml. of spectroscopic buffer solution, 15, 15, and 16 micrograms of beryllium, respectively, being recovered.

The beryllium and thallium lines used in this work may be masked by iron lines if the instrument employed does not have adequate dispersion or resolution. In spectra taken with the Bausch & Lomb quartz large Littrow spectrograph, no difficulty was encountered in identifying the beryllium line in the presence of large amounts of iron. The densitometry of weak beryllium lines, however, was affected, in that the presence of large amounts of iron apparently increased the intensity of the beryllium line to a greater extent than that of the internal standard.

The effect of iron is illustrated in Table IV, in which it may be seen that the recoveries of small quantities of beryllium are in closer agreement with the added quantities when iron has been removed according to the procedure of the alternate method. The error of analysis in this range in the presence of iron, while large on a percentage basis, is not, however, too significant when it is examined on the basis of the absolute amounts of beryllium involved. When the quantities of beryllium are above 2 micrograms per ml. of solution, iron offers no difficulties, since dilution with the spectroscopic buffer solution reduces the iron concentration to a level at which it has very little effect upon the intensities of the beryllium and thallium lines. No effect has been noted when the iron concentration in the final solution does not exceed 1 mg. per ml. In the authors' hands, iron was removed only from samples of blood and large samples of other tissue when it was expected that the beryllium would have to be concentrated in a volume of 1 ml. This procedure was followed rather than that of correcting for the iron by the derivation of a separate calibration curve from a standard solution containing a fixed amount of iron. The use of such a correction procedure was discarded when it was found that the iron had to be removed in order to maintain adequate control of its effects within the

Table III. Loss of Beryllium Associated with Dry Ashing at 500° C.

Type of Dish	Material	Be Added, γ	Be Recovered, γ	Average Recovery, %
Glazed silica	Urine (100 ml.)	1, 1	0.9, 0.4	65
Glazed silica	Urine (100 ml.)	2, 2, 2	0.4, 1.2, 1.4	50
Glazed silica	Urine (100 ml.)	4, 4	3.0, 2.2	65
Glazed silica	Urine (100 ml.)	16, 16	9.6, 2.4	38
Platinum	Urine (60 ml.)	4	0.5	12.5
Platinum	Urine (60 ml.)	8	3.2	40

Table IV. Recovery of Beryllium in Presence or Absence of Iron

(Material = 10 grams of blood except as indicated)

Be Added, γ	Micrograms of Be Recovered: Fe present	Fe removed
0, 0	0, 0	0, 0
0.5	1.4, 0.90, 0.8, 0.70	0.65, 0.60, 0.60, 0.50
0.5 (10 grams liver)	0.8, 0.6	0.5
1.5	1.7, 1.9	1.60, 1.60

arc. Moreover, contrary to expectations, the presence of iron did not increase the sensitivity of detection of beryllium, since the lower limit of detectability (0.04 microgram on the arc) remained unchanged in its absence.

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Determining Small Amounts of Calcium in Plant Materials

A Colorimetric Method

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A rapid photocolorimetric method for the determination of small amounts (0.4 to 1.1 mg.) of calcium in plant materials is based on the measurement of color diminution that accompanies precipitation of calcium chloranilate from intensely colored chloranilic acid solutions. Factors affecting transmittance of chloranilic acid solutions and tolerable interfering cation concentrations are reported. Iron, aluminum, barium, strontium, sodium, potassium, and manganese do not interfere in amounts normally encountered in plants. The most serious interference arises from occlusion of magnesium. The minimization of this error is discussed. The colorimetric method compares favorably in precision and accuracy as a routine procedure with an official volumetric macroprocedure.

CALCIUM is important in the nutrition of practically all green plants. At present, it is determined almost exclusively by gravimetric and volumetric procedures after precipitation as calcium oxalate. Yoe (*8*) describes two methods for the colorimetric determination of calcium—the alizarin and the oleate procedures—neither of which, however, is well known or suited for rapid routine determinations.

Barreto (*2*) has recently noted the insolubility of the calcium salt of chloranilic acid and has developed gravimetric and colorimetric procedures for the determination of calcium in soil extracts based on this insolubility. The gravimetric procedure would appear to have no practical advantage over present oxalate methods. The colorimetric procedure, however, might have considerable value. Unfortunately, Barreto gives no clue as to the precision or accuracy of either procedure.

The colorimetric procedure is dependent upon the color diminution that accompanies the precipitation of calcium chloranilate from intensely colored solutions of chloranilic acid. The procedure given is essentially as follows:

An acid extract of soil is evaporated to dryness, redissolved in distilled water, alkalinized with ammonia, made up to 200-ml. volume, and filtered. One hundred milliliters of the filtrate are concentrated to 30 ml. To the concentrate, 100 ml. of 0.15% chloranilic acid are added. The solution is then slightly acidulated with hydrochloric acid and brought up to 200-ml. volume by the addition of distilled water. After 2 hours of standing it is filtered. The filtrate is compared with equally acidified chloranilic acid standard solutions.

The present study was made to determine the effect of hydrogen-ion concentration and temperature on color intensity, time required for maximum color diminution, and sensitivity to small amounts of calcium and interfering cations with the object of developing a photocolorimetric method for the rapid determination of calcium in plant materials.

PROPERTIES AND REACTIONS OF CHLORANILIC ACID

Chloranilic acid (2,5-dichloro-3,6-dihydroxyquinone) has a molecular weight of 208.99 and is a strong dibasic organic acid. It dissolves sparingly in water to give intensely colored solutions very similar in color to that of potassium permanganate. The calcium salt is reddish-purple, crystalline, and easily filterable.

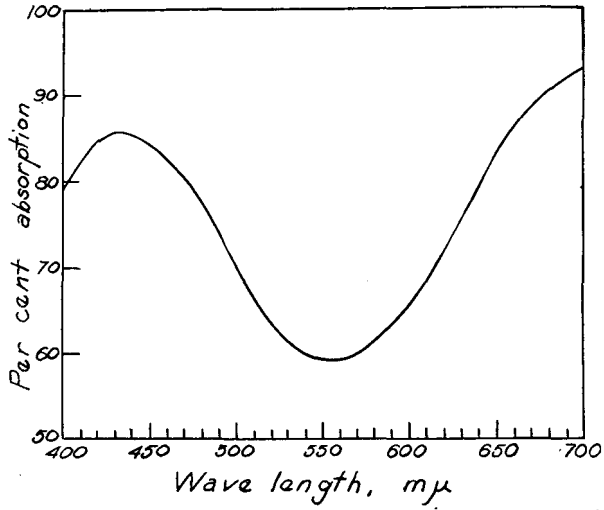

Figure 1. Spectral Transmittance Characteristics of Chloranilic Acid

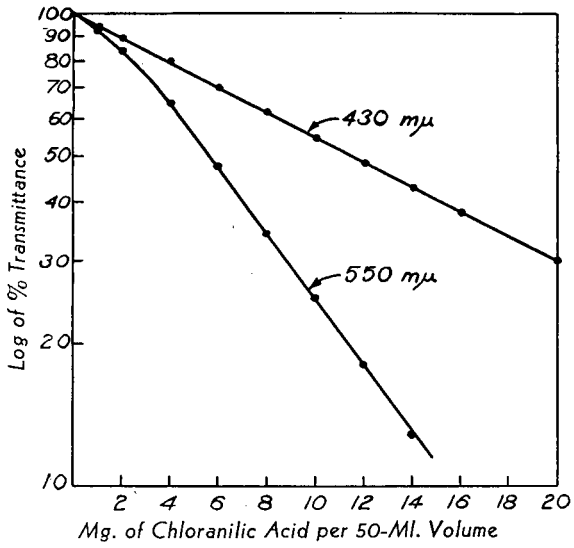

Figure 2. Relation of Chloranilic Acid Concentration to Transmittance and Adherence to Beer's Law

Chloranilic acid was first synthesized and named by Erdman (*7*) over 100 years ago. Erdman reported that solutions of potassium chloranilate formed precipitates of variable solubility with barium, lead, cupric, mercurous, and silver salts. Ferric chloride solutions gave a dark turbidity. With ferrous, nickel, cobalt, and mercuric salts no precipitates were observed. In 1907, Jackson and MacLaurin (*5*) listed cadmium, zinc, magnesium, and strontium as reacting with ammoniacal potassium chloranilate solutions, but did not investigate the solubility of the chloranilate of calcium, the commonest alkaline earth metal.

EXPERIMENTAL

Spectrum Transmittance Characteristics. A 5-ml. aliquot of 0.1% chloranilic acid was transferred to a 50-ml. volumetric flask and made up to volume with distilled water. The transmittance spectrum was determined on a Coleman Model 11 Universal spectrophotometer against a water blank between wave lengths of 400 and 700 mμ. The results given in Figure 1 indicate points of maximum and minimum light absorption at 430 and 550 mμ, respectively, with continually increasing light absorption at higher wave lengths.

Adherence to Beer's Law. The precipitation of calcium chloranilate from chloranilic acid solutions is accompanied by a diminution in color. This amounts essentially to a dilution of chloranilic acid. In order to determine whether chloranilic acid follows Beer's law at different concentrations, varying amounts of 0.1% chloranilic acid were measured out into 50-ml. volumetric flasks and made up to volume with distilled water. The transmittance of these solutions was measured at wave lengths of 430 and 550 mμ, respectively. The results plotted in Figure 2 indicate adherence to Beer's law at all concentrations up to 20 mg. (400 p.p.m.) at 430 mμ. The transmittance at 550 mμ followed Beer's law between concentrations of 4 and 14 mg. (80 to 280 p.p.m.) of chloranilic acid. The slope of the segment of the transmittance concentration line adhering to Beer's law at 550 mμ is considerably greater than at 430 mμ. Since this increases the accuracy of reading small concentration differences from calibration graphs, all subsequent studies were made at a wave length of 550 mμ.

Effect of Hydrogen-Ion Concentration on Transmittance of Chloranilic Acid Solutions. Five-milliliter aliquots of 0.1% chloranilic acid were transferred to 50-ml. volumetric flasks, varying amounts of 0.1 *N* hydrochloric acid and 0.1 *N* sodium hydroxide were added, and the solution was made up to volume. The effect of pH change on transmittance is given in Figure 3. These data indicate that considerable disturbance in transmittance of chloranilic acid solutions accompanies any induced changes in hydrogen-ion concentration. Control of hydrogen-ion concentration within narrow limits is, therefore, very important.

Time Required for Maximum Color Diminution of Chloranilic Acid Solutions Containing Calcium. Aliquots of a calcium chloride solution containing 0.5 and 1.0 mg. of calcium were measured out into a number of 50-ml. volumetric flasks. Ten milliliters of 0.1% chloranilic acid were added and sufficient distilled water for a total volume of 30 ml. in each flask. The 0.5-, 1-, 2-, 3-, and 4-hour samples were gently agitated at frequent intervals. A set containing similar amounts of calcium had been started the previous evening and allowed to stand overnight. At the expiration of each specific time interval, the flasks were made up to 50 ml. with distilled water and filtered. The results are given in Table I. Maximum color diminution and calcium recovery were achieved in 3 hours.

Effect of Acetic Acid on Calcium Recovery. In the determination of calcium in plant materials, the ash is usually taken up with dilute hydrochloric acid. As shown previously, the transmittance of chloranilic acid is affected by the presence of small amounts of dilute hydrochloric acid. Preliminary studies, not reported herein, indicate that calcium chloranilate has marked solubility in dilute hydrochloric acid. On the other hand, acetic acid is weakly dissociated. It was considered possible, therefore, that its effects in dilute solution on transmittance and the solubility of calcium chloranilate might not be great.

Table I. Calcium Recovery at Various Time Intervals

(Overnight precipitation = 100%)

Calcium, Mg.	Per Cent Calcium Recovery				
	30 min.	1 hour	2 hour	3 hour	4 hour
0.5	88.0	90.0	94.0	100.0	100.0
1.0	97.5	96.0	99.0	101.0	100.0

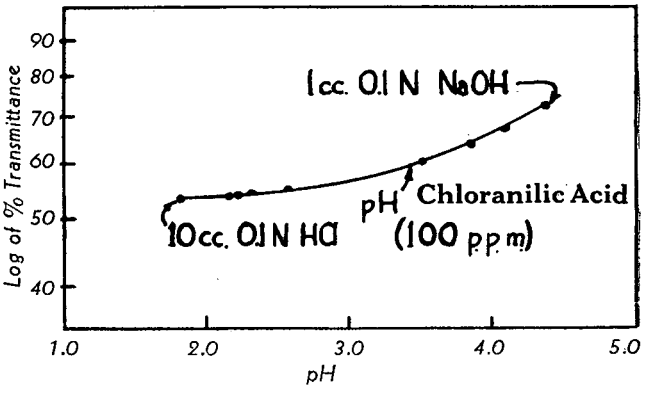

Figure 3. Effect of pH on Transmittance of Chloranilic Acid Solutions

Aliquots of calcium chloride containing 0.5 and 1.0 mg. of calcium were added to a number of 50-ml. volumetric flasks. One, 2, and 5 ml. of 0.1 *N* acetic acid were then added to various flasks. At the same time a series containing 0, 0.5, and 1.0 mg. of calcium but with no acetic acid was started. Ten milliliters of 0.1% chloranilic acid were added to each flask and the contents were adjusted to 30 ml. with distilled water. The flasks were gently agitated at frequent intervals and allowed to stand for 3 hours, after which they were made up to 50-ml. volume and filtered and their transmittance was read. A calibration graph employing the knowns to which no acetic acid had been added was drawn and the recovery of calcium at two calcium and three acetic acid additions was determined. The results are given in

Table II. Effect of Acetic Acid Additions on Recovery of Known Quantities of Calcium

	0.1 *N* Acetic Acid per 50-Ml. Volume					
	1 Ml.		2 Ml.		5 Ml.	
Calcium	Ca	Recovery	Ca	Recovery	Ca	Recovery
Mg.	*Mg.*	%	*Mg.*	%	*Mg.*	%
0.5	0.49	98	0.47	94	0.47	94
1.0	1.00	100	1.00	100	0.996	99.6

Table II. At low calcium levels (0.5 mg.) the recovery is slightly low with all acetic acid additions. However, as much as 1 ml. of 0.1 *N* acetic acid may be present without serious error in calcium recovery. With 1.0 mg. of calcium, almost complete recovery at all levels of 0.1 *N* acetic acid was secured. The pH of the filtrates was determined by the glass electrode method and this indicated virtually no effect on pH at the 1-ml. acetic acid level. It was concluded that 0.1 *N* acetic acid might be used to take up the ash after its initial solution with dilute hydrochloric acid with no deleterious effects on the calcium determination, provided the calcium in the ash was completely dissolved.

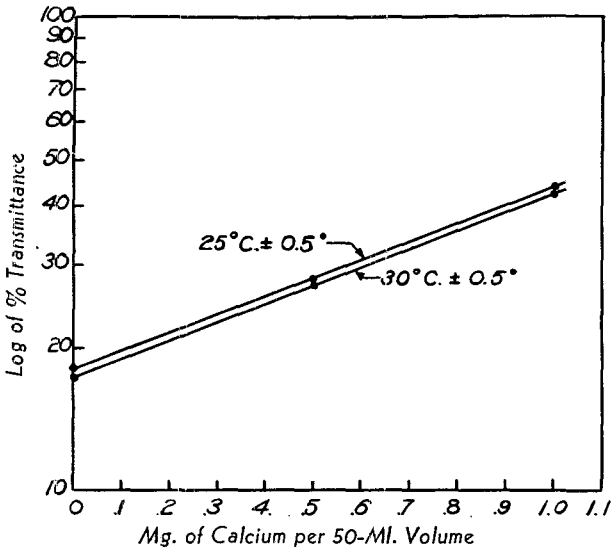

Figure 4. Relation of Precipitation Temperatures to Transmittance of Chloranilic Acid at Varying Calcium Concentrations

Effect of Temperature on Transmittance of Chloranilic Acid Solutions Containing Calcium. Ten milliliters of 0.1% chloranilic acid were added to a series of 50-ml. volumetric flasks containing 0, 0.5, and 1.0 mg. of calcium. The contents were adjusted to 30 ml. with distilled water and the flasks placed in thermostatically controlled water baths at 25° and 30° C. for 3 hours. At the expiration of this time, they were made up to volume with distilled water of the same temperature, thoroughly mixed, and filtered. The filtrates were transferred to the test tubes used in the spectrophotometer. These were placed in a beaker of distilled water and returned to the baths to come to their original temperature. The outsides of the tubes were then carefully dried and the transmittance was determined. The results are given in Figure 4. Transmittance decreases with increasing temperature and increases with decreasing temperature. Although these differences are slight, it is obvious that a permanent calibration line cannot be used in the determination of calcium. The effect of varying temperature, however, can be disregarded if a series of knowns is included along with each series of unknowns.

Stability of Chloranilic Acid Solutions. Even with rigid temperature control, a permanent calibration curve is impractical, since the transmission of light by chloranilic acid solutions increases with age. This drift, however, in no way impaired the value of stock solutions held as long as 60 days (Figure 5). It does, however, make imperative the determination of new calibration lines with each series of unknowns.

The chloranilic acid stock solution was first stored in a clear Pyrex container. As soon as the drift became apparent, a portion of this solution was transferred to an amber bottle to determine whether light was responsible for the drift. After 6 weeks the solution in the amber bottle was compared to that stored in clear glass. The transmittances were identical, indicating that a steady upward drift in the transmittance of chloranilic acid solutions takes place under storage, regardless of whether storage is in clear or amber glass. The time required for stability to be achieved is unknown. After 2 months there was no evidence of mold or bacterial growth. Chloranilic acid solutions are apparently very stable biologically.

INTERFERING CATIONS

Interference as shown in Table III is affected in three different ways—through formation of chloranilic complexes with foreign cations, through precipitation of foreign cations along with calcium, and through occlusion of foreign cations by calcium chloranilate.

Complex-Forming Cations. Ferric iron and aluminum interfere through the formation of soluble complexes with chloranilic acid solutions. Depending upon the concentrations of ferric iron and aluminum, calcium recovery is either low or completely prevented.

Ferric iron alters the color and causes a shift in the maximum and minimum spectral transmittance characteristics of chloranilic acid. Concentrations as low as 0.02 mg. of ferric ion per 50-ml. volume impart a characteristic dark color to chloranilic acid. Precipitates are formed with high concentrations of ferric iron. Aluminum chloranilate complexes do not affect the basic color or spectral transmittance characteristics of chloranilic acid.

Table III. Influence of Cations on Calcium Recovery

		10 Ml. of 0.1% Chloranilic Acid per 50-Ml. Volume		15 Ml. of 0.1% Chloranilic Acid per 50-Ml. Volume		
		Calcium Added				
		0.5 mg.	1.0 mg.	0.5 mg.	1.0 mg.	2.0 mg.
		Calcium Recovered				
Cation	Mg. per 50-Ml. Volume	%	%	%	%	%
Aluminum	0.02	100	...	...	...	...
	0.05	98	...	...	...	...
	0.10	67	...	...	...	...
	0.50	0	...	...	...	...
Barium	0.10	100	...	...	...	...
	0.50	100	...	...	...	...
	1.00	102	...	...	...	...
Copper	0.03	100	...	...	...	...
	0.05	102	...	...	...	...
	0.10	104	...	...	...	...
Iron (Fe^{+++})	0.01	98	...	...	...	...
	0.02	88	...	...	...	...
Magnesium	0.10	102	101	104	...	...
	0.20	...	...	112	...	...
	0.50	110	102	126	106	102
	1.00	115	104	128	117	104
	2.00	...	...	...	...	105
Manganese (Mn^{++})	0.01	100	...	...	...	...
	0.05	102	...	...	...	...
	0.10	103	...	...	...	...
	0.20	106	...	...	...	...
Potassium	0.50	102	100	...	...	...
	1.00	104	100	...	...	...
	2.00	104	99	...	...	...
Sodium	0.50	102	101	...	...	...
	1.00	104	100	...	...	...
	2.00	104	100	...	...	...
Strontium	0.05	101	...	...	...	...
	0.10	104	...	...	...	...
	0.50	110	...	...	...	...

Table IV. Comparison of Calcium Content of Plant Materials by Colorimetric and Volumetric Methods

No.	Sample	Dominant Ash Color	Colorimetric Chloranilic Acid Method, % Ca	Volumetric A.O.A.C. Method, % Ca	Difference
1	Corn leaves	White	1.03	0.95	+0.08
2	Corn leaves	Light gray	1.24	1.20	+0.04
4	Alfalfa hay	White	1.81	1.72	+0.09
7	Sweet clover hay	Light gray	1.12	1.03	+0.09
9	Beech leaves	Light gray	1.17	1.13	+0.04
11	Black walnut leaves	Almost white	4.29	4.23	+0.06
12	Pasture clippings	Light gray	0.72	0.66	+0.06
13	Pasture clippings	Light gray	1.14	1.08	+0.06
14	Pasture clippings	Light gray	0.82	0.79	+0.03
15	Pasture clippings	Light gray	0.91	0.87	+0.04
16	Pasture clippings	Light gray	1.20	1.13	+0.07
17	Pasture clippings	Light gray	0.67	0.63	+0.04
19	Black locust leaves	White	2.61	2.51	+0.10
21	Short-leaf pine needles	Light gray	0.41	0.43	−0.02
23	Sycamore leaves	White	3.97	3.84	+0.13
24	Soybean hay	White	1.58	1.48	+0.10
25	Timothy hay	Light gray	0.28	0.26	+0.02
	Mean		1.47	1.41	+0.06
	Standard error mean		0.193	0.189	
3	Oat hay	Light brown	0.39	0.37	+0.02
6	Cabbage	Light brown	0.94	0.86	+0.08
8	Tamarack needles	Light brown	0.85	0.83	+0.02
10	Pin oak leaves	Dark brown	1.57	1.55	+0.02
20	Red spruce needles	Dark brown	0.53	0.51	+0.02
22	Rhododendron leaves	Dark brown	1.28	1.23	+0.05
	Mean		0.93	0.89	+0.04
	Standard error mean		0.123	0.121	
26	Carrot roots	Very faint green	0.39	0.32	+0.07
18	Buckwheat hay	Faint green	1.83	1.74	+0.09
5	Lettuce	Very green	1.67	1.51	+0.16
	Mean		1.30	1.19	+0.11
	Standard error mean		0.288	0.280	
	Mean of 26 samples		1.32	1.26	+0.06
	Standard error mean		0.135	0.133	

A study of Beeson's (*3*) compilation of the mineral composition of crops grown on different soils indicates that iron occasionally may be present in sufficient quantities in plant materials to prevent complete calcium recovery by the colorimetric method. However, if ferric iron does not exceed 0.01 mg. per aliquot volume this is not serious. The characteristic color of the ferric chloranilate complex should enable an analyst readily to distinguish samples where ferric iron exceeds this concentration.

Aluminum is usually present in very low amounts in plants, in fact, much lower than iron. In rare cases it may interfere if present in excess of 0.05 mg. per aliquot volume, in which case calcium recovery is low.

Cations Forming Precipitates. Copper, manganese, barium, and strontium form precipitates with chloranilic acid, thereby reducing its residual concentration without affecting its basic color or spectral transmittance characteristics. The copper content (*3*) of plants is normally very low. Rarely will it be high enough to exceed 0.05 mg. per aliquot volume, the tolerable limit. Samples contaminated by copper-containing spray residues should be avoided.

The manganese content of plants (*3*) is usually low. Occasionally samples will contain manganese in excess of 0.01 mg. per aliquot volume, the tolerable limit. Fortunately, such samples, particularly if the ash is gray or white, are easily detected by the green tints of the ash which turns pink on solution in dilute hydrochloric acid. Several samples of this type were encountered. The seriousness of manganese interference can be judged from the calcium contents reported for such samples (Table IV).

Barium and strontium are reported as giving chloranilate precipitates (*2*, *5*). With the method described below, they gave no interference at concentration levels several times in excess of any ever reported in plants (*3*, *4*).

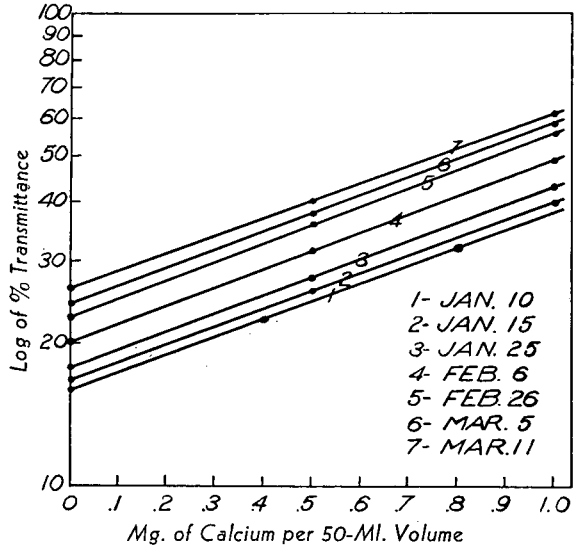

Figure 5. Upward Drift in Transmittance of a Chloranilic Acid Solution at Varying Calcium Concentrations with Increasing Age

Errors Due to Coprecipitation. Sodium, potassium, and magnesium in the concentrations employed did not form precipitates. Evidence indicates that these cations are occluded by calcium chloranilate. With sodium and potassium this is not serious in the amounts normally occurring in plants. Moreover, the errors which seem to result from sodium and potassium occlusion apparently are not additive.

Magnesium occlusion under certain circumstances can be serious. A study of the data in Table III indicates that magnesium occlusion is most serious in solutions containing large excesses of chloranilic acid. Magnesium occlusion appears to follow the rule discussed by Kolthoff and Sandell (*6*) that occlusion of foreign cations is greatest when the solution contains an excess of lattice anions—e.g., chloranilic acid—during the precipitation. The small ionic radii of magnesium and its chemical similarity with calcium perhaps facilitate mixed crystal formation. All attempts to eliminate or minimize magnesium occlusion at low calcium concentrations were ineffective. The magnitude of magnesium occlusion can be reduced only by selecting aliquots which contain sufficient calcium to precipitate the major portion of the chloranilic acid.

The seriousness of magnesium occlusion must be viewed in light of the ratios of magnesium to calcium as they normally occur in plants and the accuracy desired in the calcium determination. Magnesium is usually less abundant percentagewise than calcium in the vegetative parts of plants. Occasionally this ratio is 1 to 1. With the exception of seeds, seldom is this ratio greater than 1 to 1. Thus, if the magnesium-calcium percentage ratio is 1 to 1 and the calcium content is low, apparent calcium recovery may be high by 10%. On the other hand, through the use of larger aliquots of the unknown, quantities of calcium approaching 1 mg. may be obtained, and the error due to occlusion considerably reduced.

If the ratio of magnesium to calcium in plant materials were constant, a factor could be employed to correct for magnesium occlusion. Unfortunately, this is not the case. In spite of this error, calcium in plant material can be determined with sufficient

accuracy by the colorimetric procedure to warrant its use in numerous investigations.

CONCENTRATION OF CHLORANILIC ACID AND CALCIUM RECOVERY

Assuming that chloranilic acid acts as a dibasic acid, 1 mg. of chloranilic acid in solution will theoretically combine with 0.183 mg. of calcium. Thus, 10 ml. of 0.1% chloranilic acid, disregarding the kinetics of the reaction, would theoretically precipitate 1.83 mg. of calcium. However, in Figure 2 it was noted, using a wave length of 550 mμ, that at concentrations of 4 mg. or less of chloranilic acid per 50-ml. volume, that transmittance deviated from Beer's law. Therefore, only 6 out of the 10 ml. of chloranilic acid will remain for the precipitation of theoretically 1.1 mg. of calcium. This corresponds with the consistent upper limit for linear calcium recovery as determined experimentally.

The question of the lower limit of consistent linear calcium recovery is dependent upon the solubility of calcium chloranilate in dilute solutions of chloranilic acid at various temperatures. Unlike calcium oxalate, at high temperatures calcium chloranilate is very soluble. At room temperatures (21° to 30° C.) it was found that 0.4 mg. of calcium could be quantitatively recovered. At precipitation temperatures of 5° C. as little as 0.1 mg. calcium is quantitatively recovered.

The upper range of calcium recovery could be extended considerably by the use of larger amounts of chloranilic acid. However, from the data given in Table III it is apparent that large errors at low calcium concentration due to magnesium occlusion are apt to be encountered.

OUTLINE OF METHOD

Apparatus and Reagents. Coleman Model 11 Universal spectrophotometer with filter PC-4.

Automatic 10-ml. pipet and 1-, 2-, 3-, 4-, 5-, 10-, and 15-ml. pipets.

Chloranilic Acid (0.1% solution). Add 1 gram to 500 ml. of distilled water, heat to about 50° C. to hasten solution, transfer to a 1000-ml. volumetric flask, and bring almost to volume with distilled water. After cooling to room temperature, adjust to volume and filter. Storage in clear or amber glass is optional.

Acetic Acid Solution (approximately 0.1 *N*). Dilute 6 ml. of 99.5% glacial acetic acid to 1000 ml. with distilled water.

Hydrochloric Acid (1 to 3). Dilute 25 ml. of concentrated hydrochloric acid to 100 ml. with distilled water.

Calcium Chloride Solution. Dissolve 3.6684 grams of calcium chloride dihydrate, reagent grade, in 1 liter. This solution contains 1 mg. of calcium per ml. It is desirable to analyze this solution for calcium by a standard procedure to determine its actual calcium content. Dilute 100 ml. of the above solution to 1 liter. This solution contains 0.1 mg. of calcium per ml. Use this solution for knowns.

Recommended Procedure. Weigh out 1-gram samples of thoroughly mixed, finely ground plant material, transfer to 50-ml. Pyrex beakers, and ash in an electric muffle furnace at 450° C. Moisten the ash with a few drops of distilled water, dissolve in 5 ml. of dilute (1 to 3) hydrochloric acid, and evaporate to dryness on a steam chest or water bath to dehydrate the silica and expel excess hydrochloric acid. Take up the residues in 5 ml. of 0.1 *N* acetic acid, warm for a few minutes, and allow to cool. Then loosen the residues, break them up with a rubber policeman, transfer to a 100-ml. volumetric flask, make up to volume with distilled water, mix, and filter through a No. 589 S and S Blue Ribbon filter paper into dry Erlenmeyer flasks. Preliminary trials indicated that complete solution of the calcium in ash was effected by this procedure. The filtrates show mold growth after a few days. The determination of calcium, therefore, should not be delayed.

Measure out aliquots of the filtrates containing 0.4 to 1.1 mg. of calcium into 50-ml. volumetric flasks. The following are suggested aliquot volumes for varying calcium ranges:

15 ml., 0.2 to 0.4% Ca	3 ml., 2.2 to 3.7% Ca
10 ml., 0.4 to 1.1% Ca	2 ml., 3.7 to 5.5% Ca
5 ml., 1.1 to 2.2% Ca	

Adjust volume to 20 ml. with distilled water, add 10 ml. of 0.1% chloranilic acid by means of an automatic pipet, mix by rotation of the flask, and allow to stand overnight or for 3 hours with frequent agitation at room temperatures not exceeding 30° C. (When laboratory temperatures exceed 30° C., the following procedure has proved satisfactory: The knowns and unknowns are set in a refrigerator. At the expiration of the precipitation interval the solution in flasks are made up to volume with distilled water previously cooled to the same temperature. They are then mixed and filtered while cool. The filtrates are allowed to come to room temperature and read in the photocolorimeter.) A series of knowns containing 0, 0.5 and 1.0 mg. of calcium should be included with each series of unknowns. After 15 to 30 minutes, examine the unknowns. If the calcium content of any unknown greatly exceeds 1.0 mg. of calcium, using the known for comparison, a smaller aliquot of unknown should be used. At the expiration of 3 or more hours, bring the contents of the flasks up to 50-ml. volume with distilled water, mix, and filter through a No. 589 S and S Blue Ribbon filter paper into dry flasks. Transfer the filtrates to a balanced set of tubes and read against a water blank at a wave length of 550 mμ. Using the knowns, construct a calibration graph and determine the milligrams of calcium in the unknowns. Calculate the results:

$$\% \text{ calcium} = \frac{\text{mg. of calcium in unknown}}{\text{aliquot volume used} \times 0.1}$$

COMPARISON OF COLORIMETRIC METHOD WITH STANDARD VOLUMETRIC PROCEDURE

Twenty-six plant samples were analyzed for calcium by the volumetric macro procedure of the Association of Official Agricultural Chemists (*1*) and the colorimetric chloranilic acid procedure. Many of the forest tree samples, because of growth on highly acid soils, could be expected to have greater than average iron, aluminum, and manganese contents. The samples are grouped accordingly by their dominant ash color. Plant ash high in iron is apt to be brown, whereas manganese imparts to white ash a greenish color which turns pink on the addition of hydrochloric acid. The results of the analysis are given in Table IV.

In spite of varied origin, no sample contained sufficient iron to interfere. The general level of accuracy for samples giving light-colored ash is of the same order as those giving brown to dark brown ash. Samples containing sufficient manganese to impart a greenish tint to the ash give greater than average differences between the colorimetric and volumetric procedures in the light of their mean calcium contents.

With the exception of sample 21, the colorimetric procedure consistently gave calcium contents somewhat higher than those obtained with the volumetric procedure. This is not due to lower precision in view of the nearly identical standard errors of the means for the two methods. The writer is of the opinion that occlusion is primarily responsible for the higher results secured by the colorimetric procedure.

The mean for all 26 samples analyzed for calcium by the colorimetric method averages 4.8% higher than the volumetric method. Thus a hay sample containing 1% calcium by the volumetric procedure would have 20 pounds of calcium per ton, and by the colorimetric method almost 21 pounds. This is within the desired range of accuracy for a routine method.

ACKNOWLEDGMENT

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Microbiological Assay for Riboflavin

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A microbiological method for the determination of riboflavin is described. The organism used is *Leuconostoc mesenteroides* 10,100. Its response to riboflavin has permitted the development of an assay method which is sensitive to 0.0001 microgram of the vitamin per ml. With the growth medium used, good agreement is obtained among riboflavin values of samples assayed at different levels as well as good recoveries of added riboflavin. Determinations may be made turbidimetrically after 14 hours or titrimetrically after 72 hours.

THE two methods which have been used generally for the determination of riboflavin are the *Lactobacillus casei* assay, developed by Snell and Strong (*18*), and the fluorometric method, introduced by Hodson and Norris (*9*).

Variations of both methods have been proposed. The adsorbent used in the fluorometric assay has been a source of error, as have concentration, volume, and clarity of extracts (*10*). This has been partially corrected by the use of special filters (*10*) and by using controlled permanganate oxidation of the sample to be assayed in place of adsorption (*16*). For the microbiological method taka-diastase was found to hydrolyze interfering substances present in starch that stimulate lactic acid bacteria (*17*). Filtration of autoclaved samples at pH 4.5 has been suggested to remove proteins (*20*). Fat acids were observed to stimulate the growth of *L. casei* (*2*), and a procedure for their removal has been published (*19*). Several changes in the growth medium for the assay have been suggested (*4, 11, 12, 15, 19*). The fluorometric and microbiological assay methods have been compared, and it appears that the microbiological assay is favored slightly on the basis of somewhat better specificity and reproducibility (*1, 5, 7*).

However, for the assay method using *L. casei* as the organism, an amount of extract must be used that contains about 0.02 microgram of riboflavin per ml. Since many natural materials have a very low riboflavin content, relatively concentrated extracts must often be prepared, which may contain excessively colored pigments and other interfering substances, such as starches and fats.

This paper describes an assay for riboflavin based upon the response of *Leuconostoc mesenteroides* to concentrations of the vitamin in the order of one fiftieth of that required for an equivalent response of *L. casei*. The organism's growth may be measured turbidimetrically after 16 to 18 hours, or the acid produced may be titrated after 72 hours. The higher sensitivity of this microorganism permits preparation of samples for assay with greater dilution than heretofore; consequently decreased amounts of extraneous matter are present in the assay tubes

The *Leuconostoc mesenteroides* was kindly furnished by R. J. Williams of the University of Texas. Cultures may be obtained from the American Type Culture Collection, Georgetown University Medical School, Washington, D. C., where the organism is listed as No. 10,100. The correct naming of this organism is being investigated by R. P. Tittsler. Although it has been called *Leuc. mesenteroides* P-60, its fermentation characteristics resemble those of *Leuc. dextranicum* more closely (see *3*). P-60 cultures described by Dunn *et al.* (*6*) and by Pennington (*14*) do not require riboflavin.

METHOD

Preparation of Inoculum. Stab cultures containing 1% glucose, 1% yeast extract, and 1.5% agar are used to carry *Leuconostoc mesenteroides*. They are kept in the refrigerator, and the inoculum for assay is grown from them in 2.5 ml. of basal medium plus 0.05 microgram of riboflavin with sufficient water to make 5 ml. After incubation at 37° C. for 12 to 24 hours the organisms are centrifuged, washed once with sterile physiological saline solution by centrifugation, and resuspended in about 10 ml. of sterile saline.

Basal Medium. Table I shows the composition of the basal medium. Quantities of various constituents per assay tube are given, as well as quantities per liter and the final per cent concentration. Except for the special preparation of the alkali-treated peptone and the addition of asparagine, it is similar to that used in the Snell and Strong method for riboflavin (*18*). Each amount given represents the concentrations which the authors have found to produce the best growth response in the presence of riboflavin.

Table I. Basal Medium

	Mg. per Assay Tube	Grams per Liter	% in Final Medium (Diluted)
Boiled alkali-treated peptone + sodium acetate	60	12	0.6
Glucose	100	20	1.0
Asparagine	5	1.0	0.05
Yeast supplement (riboflavin-free)	5	1.0	0.05
Cystine	1	0.2	0.01
Salts A and B	0.05 ml.	10 ml. each	...
Water to make	5 ml.	1000 ml.	...
pH	6.8–7.0	....	...

For the preparation of the alkali-treated peptone, to 40 grams of Bacto peptone (Difco) in 250 ml. of water are added a solution of 20 grams of sodium hydroxide in 250 ml. of water. The resulting mixture is brought to a boil and allowed to stand at 37° C. for 24 to 48 hours, 28 ml. of glacial acetic acid and 7 grams of anhydrous sodium acetate are added, and the volume is made up to 667 ml., giving a concentration of 60 mg. of original peptone per ml. This solution is stored under toluene.

The expedient of bringing the peptone to a boil, followed by incubation of the alkaline solution, was found to destroy virtually all the riboflavin. Prolonged boiling is to be avoided, since heating for even 5 minutes was observed to impair the efficiency of the peptone, possibly by racemizing some of the essential amino acids. Asparagine, not normally present in alkali-treated peptone, was found to stimulate growth in the presence of riboflavin.

A more nearly synthetic medium using casein hydrolyzate, glucose, several amino acids, purines and pyrimidines, salts, and amino acids may also be used. Its formula is similar to that used by Gaines and Stahly (*8*) for their nutritional studies on a strain of *Leuconostoc mesenteroides*, identified as P-60.

Assay Procedure. Assays are carried out in 20 × 150 mm. Pyrex test tubes. Red glass test tubes are necessary if the acid production is to be measured after 72 hours' incubation. Samples to be assayed are introduced into tubes at four levels, 1 to 4 ml., containing approximately 0.001 to 0.002 microgram of riboflavin per ml. These samples are then diluted to 5 ml. with distilled water and 5 ml. of the basal medium shown in Table I are added to each. A series of tubes containing, respectively, 0.0, 0.0005, 0.001, 0.002, 0.004, 0.006, 0.008, and 0.01 microgram of riboflavin is used to establish a standard curve with each assay. The tubes are covered with a towel and autoclaved for 10 minutes (if acid production is to be measured after 72 hours instead of

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turbidimetric measurement, the tubes are plugged and autoclaved for 15 minutes). The tubes are cooled and inoculated with one drop of the previously grown culture (described above).

Turbidimetric readings may be made after 14 to 16 hours' growth at 37° C. If the number of tubes is large, they are usually cooled prior to reading, in order to slow growth. Measurements may be made in any reliable turbidimeter. A filter corresponding to the color of the basal medium is used to negate any effects due to slight changes of color in the various tubes. The authors have found that steeper curves result when filters transmitting shorter wave lengths are used (4200 to 4500 Å.) because of light scattering by the cells. However, the steeper curve is not particularly advantageous when there is a possibility of introducing errors because of color variation. In Figure 1 is shown a typical standard curve for riboflavin, when a 5400 Å. filter is used. The turbidimetric readings are given in terms of optical density which is equal to the log per cent of incident light absorbed and diffused ($2 - \log G$).

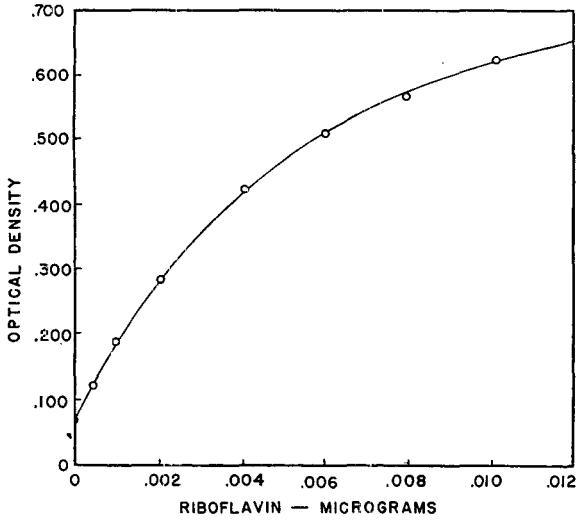

Figure 1. Growth Response of *Leuc. mesenteroides* to Riboflavin

Acid production may be measured after a 3-day period of incubation, using 0.05 *N* sodium hydroxide with bromothymol blue indicator. Figure 2 shows the type of standard curve which may be obtained by this means and the range of riboflavin concentrations employed. This organism (a heterofermentative species) is capable of producing about 8 ml. of 0.1 *N* acid under ideal conditions, and the curve shown here could be extended to include higher riboflavin levels. However, the slope of the curve becomes smaller, and best results are obtained within the recommended range.

Preparation of Samples for Assay. Extracts are prepared by adding 20 ml. of water and 3 ml. of 1 *N* sulfuric acid to 1 gram of the dry material to be assayed and autoclaving for 30 minutes. The pH is then adjusted to 4.5 to 5.0 and the extract diluted to contain approximately 0.001 to 0.002 microgram of riboflavin per ml. Materials low in riboflavin content may be diluted to contain approximately 0.0002 microgram per ml.

RESULTS

Table II contains the assay values and riboflavin recoveries from three different materials. Good agreement between the four assay levels has been obtained and recoveries of the added vitamin range from 90 to 100%.

Table III gives riboflavin values obtained by this method compared with those obtained by the method of Snell and Strong using *L. casei* (*18*). In general there is good agreement, although the last two samples appear to have a considerably lower riboflavin content by the present method. It has been shown (*2*, *19*) that fat-soluble substances present in many natural materials will stimulate the growth of *L. casei*. Although *Leuc. mesenteroides* is similarly stimulated, only relatively small amounts of these stimulants are present in the assay tubes, because of the greater dilution, and their effect upon the apparent riboflavin value is correspondingly less. Thus, for example, the authors have found that whereas ether extraction prior to assay lowered the apparent riboflavin content of flour from 0.33 to 0.20 microgram per gram with *L. casei*, the same treatment produced no lowering of the *Leuc. mesenteroides* assay value (0.21 to 0.22 microgram per gram).

The present assay method has given consistently satisfactory results for over 6 months, when used with a variety of plant and animal materials.

Table II. Riboflavin Analyses and Recoveries

Material	Sample	Riboflavin Found		Recovery
	Mg.	γ	γ/g.	%
Green pea soup, dehydrated	2	0.0008	0.40	..
	4	0.0016	0.40	..
	6	0.0024	0.40	..
	8	0.0034	0.43	..
	1 + 0.001 γ riboflavin	0.0013	..	90
	2 + 0.002 γ riboflavin	0.0028	..	100
Sweet potatoes	0.5	0.0012	2.4	..
	1.0	0.0020	2.0	..
	1.5	0.0035	2.3	..
	2.0	0.0044	2.2	..
	0.5 + 0.001 γ riboflavin	0.0021	..	90
	1.0 + 0.002 γ riboflavin	0.0039	..	95
Turkey feed	0.25	0.0008	3.2	..
	0.50	0.00185	3.7	..
	0.75	0.00225	3.0	..
	1.0	0.0034	3.4	..
	0.50 + 0.002 γ riboflavin	0.0037	..	93
	0.75 + 0.003 γ riboflavin	0.0051	..	95

Table III. Comparative Assay Values Using *Leuc. mesenteroides* and *L. casei*

Material	Riboflavin, γ per Gram	
	Leuconostoc mesenteroides	*L. casei*
Yeast	45	45
Navy beans	2.6	2.5
Green pea soup, dehydrated	1.00	1.01
Pea puree	0.90	1.05
Sweet potato	0.95	0.94
Tomato juice	0.32	0.35
Carrots	1.68	1.83
Carrot puree	0.16	0.22
White potatoes	0.36	0.52
White flour, unenriched	0.21	0.33

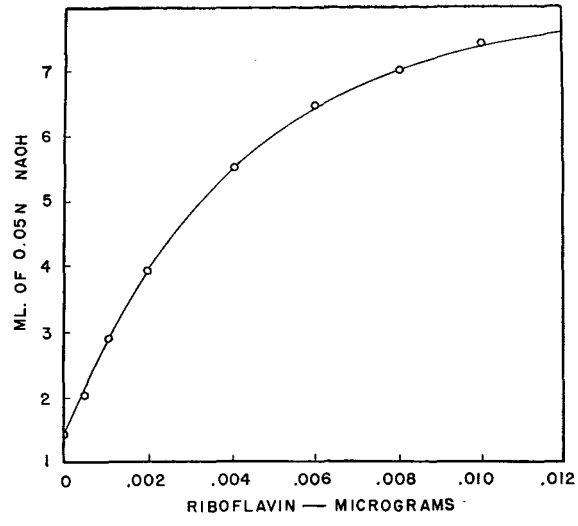

Figure 2. Acid Production by *Leuc. mesenteroides*

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Volumetric Determination of Small Amounts of Soluble Sulfates

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A new technique is described for the volumetric determination of sulfur, in which standard barium chloride is used with the indicators dipotassium rhodizonate or tetrahydroxyquinone. The technique permits continuous following of the colors developed, so that the true end point is easily identified. This makes the titration readily reproducible and results in an appreciable gain in accuracy. The barium chloride solution is standardized, and samples are titrated by the same procedure, eliminating a titration correction factor.

THE volumetric method for determination of sulfur as sulfate with barium chloride, in which the internal indicator tetrahydroxyquinone disodium salt (THQ) or the dipotassium or disodium rhodizonate is used, has been described by a number of investigators (*1–12*), each of whom has attempted to present a method whereby a sharp or reproducible end point may be obtained. Although satisfactory in the hands of skilled technicians, these methods have not been generally accepted because of difficulty in identifying the true end point. In the titration of sulfate ions with barium chloride, the indicator changes the color of the solution from yellow to orange red. As the end point is approached, but before it is reached, there is a localized formation of the red barium salt of the indicator, which is dispersed through the solution by stirring. This produces a pseudo end point, which slowly disappears as the barium associated with the indicator reacts with remaining sulfate ions. Because of the low concentration of sulfate ions, the reaction becomes very slow as the end point is approached. Unlike most reactions, the true end point is not indicated by a sudden change in color but instead is that point where no fading of the barium-indicator color (orange red) occurs.

The success of the titration depends upon the ability of the analyst to distinguish that point in the titration at which fading no longer occurs. Throughout the titration, there is a slight but gradual change in the stable color from yellow to orange red. As the equivalence point is passed, there is a shift in the color from orange to red, the same as that which occurs with the premature or pseudo end points but distinguishable from them because it is nonfading. If the end point is passed and the titration is continued, the shift in color toward the red is more rapid than that before the end point is reached, and will continue until sufficient barium has been added to combine with the indicator present.

The end point should be easily recognizable if it can be compared with a standard which has the same orange-red hue as the solution which has been titrated just past the equivalence point. However, the fading of the color must be followed continuously to avoid selection of a pseudo end point.

An apparatus and titration technique have been developed which not only identify the end-point color but permit the continuous comparison of the color of the solution with a standard color filter.

The apparatus (Figure 1), consists of a rectangular titration vessel (25 × 45 × 50 mm. high optical absorption cell), a standard 25 × 45 mm. glass color filter, two 5-ml. burets graduated to 0.01 ml., and a titration stand. The titration vessel and the light filter are mounted side by side on an opal glass plate. Illumination is from below, preferably by fluorescent light. All the opal glass is masked except that covered by the titration vessel and the color filter. Best results are obtained when no overhead artificial illumination is used.

PROCEDURE

Sulfate solutions high in carbonates are acidified with nitric acid and boiled. All solutions, either acidic or basic, are neutralized to pH 6.5 to 7.5, the neutral solution is transferred to the titration vessel, and the volume is adjusted to approximately 15 ml. About 0.08 gram of the commercial tetrahydroxyquinone indicator is added and dissolved, and the solution is diluted with an equal volume of 95% ethanol. The solution is titrated with standard barium chloride until the permanent color matches the color filter. At the end point, stirring must be continued long enough (1 to 2 minutes) to make sure that no fading of the orange-red color will occur. A rubber-tipped stirrer is used to prevent scratching the bottom of the absorption cell. The end point is reached when one additional drop of barium chloride causes the solution to appear a deeper red than the color filter. The 0.02 *N* barium chloride is standardized against a solution containing 1.8140 grams of reagent grade potassium

sulfate per liter of solution. Standardization of the barium chloride in this manner instead of gravimetrically as barium sulfate eliminates a correction factor such as used by Sundberg and Royer (*11*).

In the titration of a sample for soluble sulfate, unless at least 3 ml. of 0.02 *N* barium chloride are consumed, the assumed normality will be too high, as shown in Figure 2. In the standardization of the barium chloride, unless 3 ml. or more are used, the apparent normality will show rapid changes with correspondingly slight changes in volume of barium chloride. Only when the amount of 0.02 *N* barium chloride required by the sulfate is 3 ml. or more does the normality remain nearly constant. To avoid possible errors through use of an incorrect titer, all titrations, whether standardization or analysis of a sample, should use 3 ml. or more of the 0.02 *N* barium chloride.

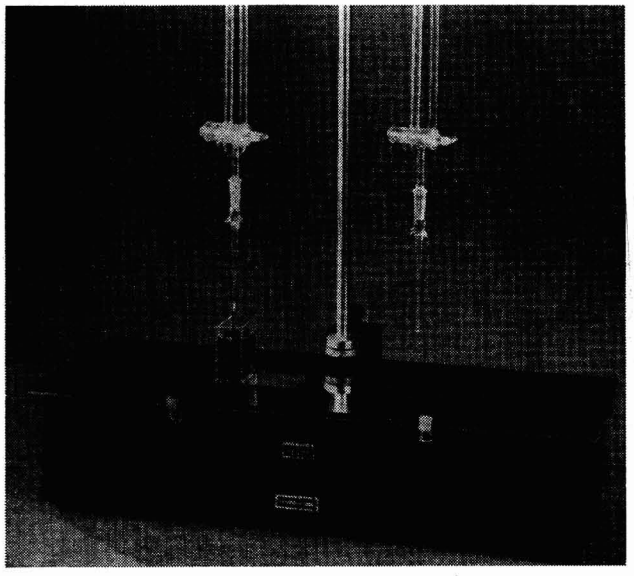

Figure 1. Sulfur Titration Apparatus

In the analysis of microsamples, the amount of sulfur is often so small that less than 3 ml. of the 0.02 *N* standard barium chloride solution will be required. Therefore, when the end point is reached, additional sulfate as standard potassium sulfate is added to bring the volume of barium chloride required to more than 3 ml., and the titration is continued to the second point. Correction is made for the amount of sulfate added. The amount of standard potassium sulfate added can be measured with greater accuracy than the true titer of less than 3 ml. of barium chloride can be determined from a normality curve, and recourse to the curve is eliminated.

Others (*2*, *5*, *11*) have noticed this apparent change in titer when small concentrations of sulfur as sulfate are titrated and (*2*, *5*) have attempted to overcome it by using a correction factor. However, the curve shows that when the total volumes of the standard barium chloride solution are small the change in the apparent normality is rapid with correspondingly small change in volume of the barium solution used. Therefore, titrations in which volumes of standard barium chloride less than 3 ml. are used are subject to error.

The standard color filters of polished glass with 37% spectral transmission at 550 ± 2 mμ need not all be identical, if the same filter is used for both the standardization and subsequent titrations of sulfate samples. To compensate for turbidity caused by formation of insoluble white barium sulfate during the titration of samples containing large amounts of sulfur, the polished color filter can be covered by a microscope slide having an appropriately ground surface. It is desirable to have several such slides, each ground to produce a different amount of diffused light. Thus an exact match or compensation of the turbidity of the solution can be made through the selection of the appropriate slide. The slides are easily prepared by grinding with a water suspension of fine Carborundum.

With the apparatus described and the recommended technique, the values obtained on a series of titrations of equal aliquots of a sulfate solution varied by not more than ±0.02 ml. from the mean and the average deviation was 0.01 ml., or 0.003 mg. of sulfur. Table I gives the sulfur values obtained by several analysts for 3 ml. of solution containing 0.333 mg. of sulfur per ml. Two of the four analysts were without experience in this titration. The data show that the reproducibility of results is practically independent of the skill of the analysts. Table I also shows typical analyses obtained on pure organic compounds, the sulfur of which had been converted to sulfate by catalytic combustion.

Table I. Reproducibility of Titration and Determination of Sulfur in Pure Organic Compounds

A. Agreement Obtained by Analysts

Analyst	Sulfur Taken Mg. per ml.	Sulfur Found Mg. per ml.
MJW	0.333	0.333
		0.332
		0.333
CLO		0.334
		0.333
		0.332
FJC		0.334
		0.333
		0.334
RWB		0.334
		0.334
		0.334

B. Analyses of Pure Organic Compounds

Name	Theory %	Found %	Difference %
S-Benzyl thiuronium chloride (benzyl isothiourea hydrochloride), Beazley combustion apparatus	15.82	15.77, 15.76, Av. 15.77	0.05
Grote combustion apparatus	15.82	15.79, 15.85, Av. 15.82	0.00
p-Dichlorophenyl sulfone, Beazley combustion apparatus	11.16	11.07, 11.05, Av. 11.06	0.10

This paper was suggested by numerous microchemists visiting this laboratory, who had been unsuccessful in the use of the indicators tetrahydroxyquinone or dipotassium rhodizonate for the volumetric determination of soluble sulfates but were able to perform the titration successfully after one demonstration of the procedure described here. They attributed the success of this procedure to the fact that the true end point was easily observed, since the fading of the pseudo end point color could be continuously and accurately followed.

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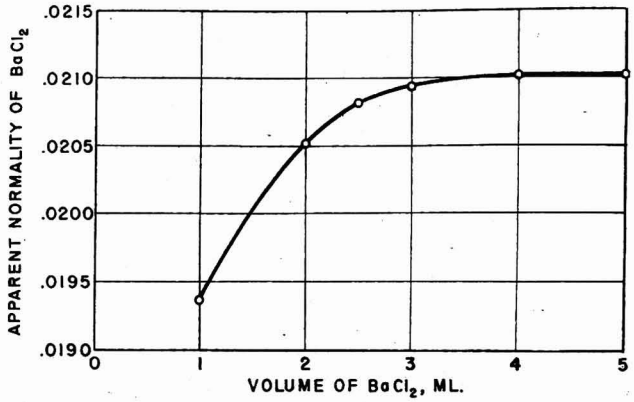

Figure 2. Normality Curve for Barium Chloride, Standardized against 0.0200 *N* Potassium Sulfate

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RECEIVED May 15, 1947. Presented before the Division of Analytical and Micro Chemistry at the 111th Meeting of the AMERICAN CHEMICAL SOCIETY, Atlantic City, N. J.

Microvolumetric Method for Determination of Sulfur in Organic Compounds

JOSEPH F. ALICINO
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A rapid microvolumetric method for determining sulfur in organic compounds has been developed which gives a sharp end point reproducible to within 0.05 ml. and requires no blank correction within the usual ranges.

WITH the discovery in recent years of an increasing number of biologically important substances which contain sulfur, there arises a need for a rapid method for the determination of this element. Many volumetric methods (*2, 5, 7*), both direct and indirect, have been proposed to accomplish this end but of these the most promising appear to be procedures using tetrahydroxyquinone or rhodizonates (*4, 8, 9*). While some difficulty has been experienced by some (*1, 3, 6*) in obtaining satisfactory end points with this indicator, the simplicity of the method seemed to warrant further investigation. It was felt that greater simplification, especially with the view of eliminating the necessity for using elaborate or special equipment, might render the method more popular, particularly with academic institutions.

A simple determination of sulfur was proposed by Brewster and Rieman (*2*), in which the sulfuric acid formed by combustion is titrated after the volatile acids have been removed by evaporation over the steam bath. This method can be made to yield reliable results only when certain conditions, sometimes difficult to control, are adhered to. As mentioned by Brewster and Rieman and confirmed in this laboratory, variable factors such as the alkalinity of the spiral, length of time of evaporation, rate of flow of air, etc., influence the blank. In this laboratory, the blank corrections were somewhat larger than expected. This was attributed to the difficulty of completely removing ammonia from the air system. Consequently, it seemed feasible to use a sulfate titration to avoid the errors mentioned above. No advantage was gained by using the method of Ingram at this point, since it is essentially alkalimetric. Reliable and consistent results were obtained after some investigation, using the proposed procedure.

PROCEDURE

The usual Pregl method was followed with certain modifications. Platinum wire gauze (14 cm. long) was substituted for one of the contacts because it ensured a longer and more complete contact of the oxidation products with the catalyst in substances which have a tendency to volatilize rapidly. Oxygen was delivered from a gasometer without drying and passed through a bubble counter containing water alone, since water-saturated oxygen appears to minimize sulfur trioxide mist (*5*). After the combustion of the sample was completed, the rinsed spiral contents were evaporated as directed by Brewster and Rieman, except that with a rapid current of air over the steam bath only 30 minutes were found necessary. Standard 0.01 *N* sodium hydroxide was added from a buret directly into the evaporating dish until a pink coloration was produced with phenolphthalein. After transfer to a 50-ml. Erlenmeyer flask, the rinsing was accomplished by adding an equal amount of 95% ethyl alcohol. Approximately half of the equivalent amount of 0.01 *N* barium chloride solution could be quickly run in with shaking. About 0.2- to 0.4-mg. of dipotassium rhodizonate was added (preferably from the tip of a microspatula) and a fairly rapid, drop-by-drop addition of the barium chloride solution was continued until about 90% (as measured by the alkali consumption) was reached. At this point, the color was still yellow and as more barium chloride was added a cherry-red coloration began to appear and was dispelled by agitation. The time interval between drops was increased to allow more thorough shaking, so that the fleeting red coloration could be entirely dispelled before addition of the next drop. The end point is reached when the red coloration persists after vigorous swirling for 30 seconds.

Table I. Determination of Sulfur

Compound	Per Cent Sulfur Found	Theory
Methionine	21.39 21.31	21.49
Thiourea	42.33 42.12	42.12
Thiocarbanilide	14.01[a]	14.05
Cystine	26.61[b]	26.69
Sulfanal	27.92 27.94	28.09
Benzylpenicillin	8.89 9.04	8.99
Cpd. CHNOS	18.95 18.52	18.52
Cpd. CHBrNS	11.48 11.33	11.44
Cpd. CHINOS	8.39 8.52	8.55
Streptamine sulfate	11.66[c] 11.71	11.60
Protein	0.98 0.97	0.98[d]
Sulfur	99.82 99.77	100.00

[a] Average of 12 analyses: max. 14.18, min. 13.88.
[b] Average of 10 analyses: max. 26.78, min. 26.47.
[c] Sample titrated directly without combustion.
[d] Average of gravimetric analyses 0.98.

Under the above conditions, the end point can be made reproducible to within 0.05 ml. without much practice and does not require a blank correction within the ranges of the usual determination. The adoption of the evaporation procedure greatly increases the sharpness of the end point and improves the method.

Elimination of extraneous anions results in an ideal solution of sodium sulfate.

The use of hydrogen peroxide solution, which is both a convenient absorber and satisfactory oxidant, is possible.

The barium chloride addition is better controlled, especially with compounds of unknown sulfur content, by virtue of the predetermined alkali value.

The sharpness of the end point is such that no color comparison is necessary.

The method was used for two years on several hundred compounds. Table I gives typical results on subtances of known purity.

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RECEIVED June 9, 1947. Presented before the Division of Analytical and Micro Chemistry at the 111th Meeting of the AMERICAN CHEMICAL SOCIETY, Atlantic City, N. J.

NOTES ON ANALYTICAL PROCEDURES

Mercury Slug Flowmeter

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INCIDENTAL to other researches for the Manhattan Project, the author developed with J. F. Bacon and other associates a meter to measure small gas flows at various pressures. The requirements of an operating pressure drop of less than 3 mm. of mercury and independence from the physical properties of the gas made capillary flowmeters, rotameters, and circuit-terminating flowmeters undesirable. Various designs of absolute displacement meters were investigated.

Marsh's (*2*) original design based on the displacement of a bubble of amyl phthalate between scratches on a straight capillary tube appeared unsatisfactory for several reasons. The range quoted was 0.25 to 1 cc. per minute, much below requirements. The accuracy of a straight tube meter varies as the distance between scratches, requiring a tube several feet long for reasonable accuracy.

Appleby and Avery (*1*) substituted mercury for amyl phthalate, permitting the use of larger tubing and consequently higher flow rates. The range (up to 25 cc. per minute) was still low and the delicacy of design incompatible with the need for portability and ruggedness.

In operation the mercury slug continuously traverses the spiral path as indicated. The spiral is made by winding 6-mm. Pyrex tubing in a plane, and allowing it to set on a slightly convex carbon block. This fixes the spiral outlet 1 to 2 mm. above the inlet. Sufficient mercury must be used in the meter, so that a new slug will close the spiral entrance before the original slug completely leaves the spiral.

The length of the slug and therefore the volume swept out per cycle were found to vary somewhat with flow rate. This necessitates calibration over the range of flow rates to be encountered. Below rates of about 25 cc. per minute, the volume swept out increases anomalously. It is suggested that the spiral be made of smaller tubing if it is desired to measure rates below 25 cc. per minute. With the simple precautions of cleanliness and careful leveling, rates of over 250 cc. per minute were attained before the slug broke into several sections. It is not feasible to use tubing much larger than 6 mm., as the mercury will not then form a slug. The cycles may be counted by means of a relay activated by the passage of the slug past the contacts shown in the illustration.

This type of meter operates over a broad pressure range (2 to 76 cm. of mercury) and on a low pressure drop (2 to 3 mm. of mercury). Its operation is independent of the physical properties of the gas being measured. It gives an integrated or average flow rate rather than an instantaneous rate.

The advantages of this specific design are compactness, ruggedness, and higher flow rate attainable. More than a dozen of these meters have been used satisfactorily at SAM Laboratories. They have held their calibrations to within from 1 to 10% over several months of regular use. Most variations were probably due to contamination of the mercury.

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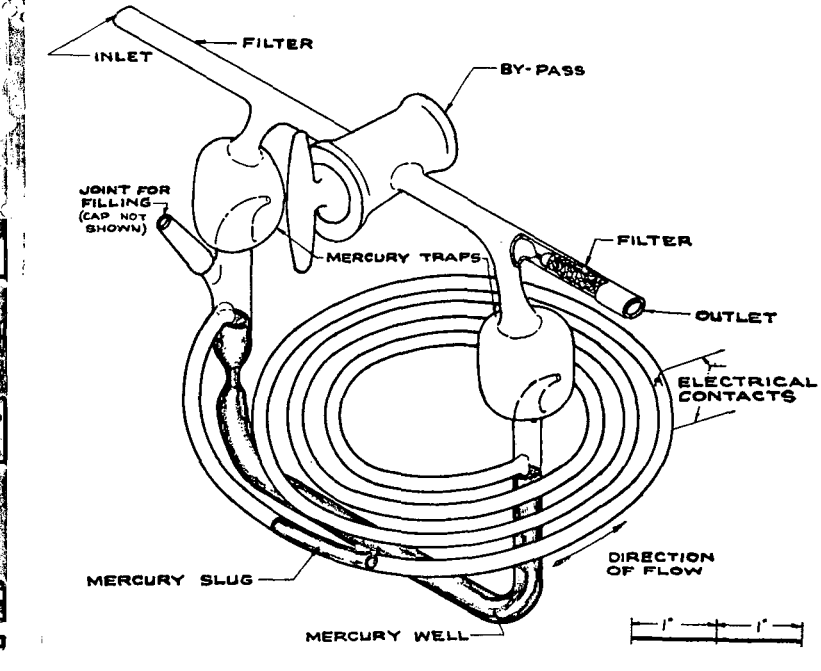
RECEIVED June 11, 1947. Based on work performed under contract No. W-7405-eng-26 for the Manhattan Project. The information will appear in Division VIII of the Manhattan Project Technical Series as part of the contribution of the SAM Laboratories, Carbide and Carbon Chemicals Corp., New York, N. Y.



Removal of Manganese in Determinations of the Zinc, Calcium, and Magnesium of Manganese Ores and Products

ROBERT L. EVANS, *White Bear Lake, Minn.*

STANDARD treatises give procedures for the precipitation of manganese from concentrated nitric acid solutions by potassium chlorate as a "separation" method in its determination (*1*, *2*). The author has found this precipitation an excellent manganese "removal" method in determining zinc, calcium, and magnesium contents of high-manganese ores and products. This method does not require the double precipitation necessary in basic ammoniacal precipitations using bromine, ammonium persulfate, hydrogen peroxide, or a combination of ammonium persulfate and hydrogen peroxide, which has proved to be the best ammoniacal precipitation for these samples.

The standard procedure is modified as follows:

After the addition of potassium chlorate the whole mixture of solution and suspended precipitate is very cautiously evaporated (slow overnight evaporation below the boiling point precludes bumping) to dryness on a low-temperature hot plate or steam bath. It is then moistened with a few milliliters of nitric acid and digested in 50 to 100 ml. of cold water for filtration, with or without suction, and the residue and precipitation beaker are washed with an equal amount of water.

The combined filtrate has a low enough concentration of nitrate ion for direct determination of zinc, calcium, and magnesium, which obviates the need of another evaporation to dryness. With good filtration this method removes up to 1 gram of manganese and leaves a clear solution with the manganese equivalent of not more than 1 or 2 drops of 0.1 *N* potassium permanganate, as shown by bismuthate oxidation. Reprecipitation of the manganese from the combined residues precipitated in the routine determination of calcium and magnesium in six samples gave a filtrate that contained less than 1 mg. of either calcium or magnesium.

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RECEIVED June 30, 1947.

Turbidimetric Method for Determination of Potassium Sulfate in Propellent Powders

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THE incorporation of potassium sulfate in colloided nitrocellulose propellants required a method for the control determination of this ingredient. At first, the new additive was determined gravimetrically by precipitation as barium sulfate. By modifying the method of Rudy (*3*) for the determination of sulfuric anhydride in cements, a satisfactory and rapid turbidimetric procedure has been developed.

A Lumetron photoelectric colorimeter, Model 400-A, equipped with a Sola constant-voltage transformer, was used for the determination of optical density (in terms of the optical density of the blank as zero) of barium sulfate suspensions in matched test tubes. Calibrating solutions were prepared by dilution to 100 ml. of portions of a standard potassium sulfate solution containing 1 gram of dried, reagent grade potassium sulfate per liter. The solutions, controlled at a temperature of 25° ± 2° C., were poured into 250-ml. beakers, 0.5 ml. of 6 *N* hydrochloric acid was added to each, and a suspension of barium sulfate was produced by adding barium chloride crystals to the mechanically stirred solution, as specified by Rudy (*3*). Part of the suspension was poured into a clean, dry test tube, and the optical density was determined 90 seconds after addition of the barium chloride. Before each reading, the colorimeter was set at zero optical density against distilled water as the reference standard.

The straight-line calibration curve obtained was used by the control laboratory in the analysis of powders. The powder, freed by extraction from ether soluble components, was taken up in a solvent (ether-alcohol or acetone) and the nitrocellulose precipitated in the usual manner (*2*, *5*) by the addition of water. After the solvent had been evaporated, the wet nitrocellulose was washed into a sintered-glass crucible on a suction bell jar, using hot water to leach out the potassium sulfate. About five 10-ml. portions were drawn through under vacuum. A 100-ml. volumetric flask was used as receiver for the filtrate, and after cooling and adjusting to volume, the potassium sulfate was estimated turbidimetrically.

Variations in response between different Lumetrons made it impractical to select a light filter or test tube size which would be universally applicable. In general, it was found that a straight line was obtained over the 0.2 to 0.5 optical density region for all filters. The scale can be read with reasonable precision in that region. The average deviation of calibration points from the straight line was under ±2% for all calibration curves.

Control laboratory, nontechnical personnel were rapidly trained in the analytical operations, after which they obtained data comparing the turbidimetric method with gravimetric determinations on powder lots. Thirty-five trial determinations showed that the turbidimetric procedure gave results that agreed with those obtained gravimetrically, within the precision of the calibration curve.

Where a more rapid separation of the potassium sulfate is desired, the powder may be taken up in combinations of organic solvents—e.g., acetone–acetic acid (*4*)—the insoluble potassium sulfate filtered off, and potassium sulfate determined turbidimetrically.

Certain water-soluble compounds, such as nitroguanidine (*1*), interfere with the turbidimetric determination by crystallizing out as the solution cools.

ACKNOWLEDGMENT

The author wishes to thank W. C. Cagle, who supervised this work, for his aid and suggestions in the course of this investigation.

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RECEIVED May 31, 1947.

BOOK REVIEWS

Organic Analytical Reagents. *Frank J. Welcher.* Vol. I, xv + 442 pp., $8 single volume price; $7 series price. Vol. II, xi + 530 pp., $8 single volume price; $7 series price. D. Van Nostrand Co., Inc., 250 Fourth Ave., New York, N. Y., 1947.

These two volumes are the first of a five-volume treatise on the use of organic reagents in analysis. The literature on this subject is covered completely through the year 1945 and for the greater part of 1946. As evidenced by the first two volumes, the completed work will represent an invaluable addition to the library of all research analysts and constitute a prerequisite to the library of industrial analytical control laboratories in all fields. Welcher has earned the distinction of having endowed analytical chemistry with a series of reference books which enhance the facilities for studies in the field of analysis to a superlative degree. Chemical reference libraries cannot be considered complete without this series of volumes.

Volume I consists of five chapters dealing with the electronic theory of valence, types of organic analytical reagents, and the effect of structure on solubility. The analytical reagents are taken up as type compounds in the next eleven chapters, beginning with hydrocarbons and their substitution products, alcohols, phenols, and compounds of a miscellaneous group of phenolic types. There then follow chapters on amino phenols, phenol sulfonic acids, 8-hydroxyquinoline and its derivatives, as well as azo derivatives of 8-hydroxyquinoline. Volume I is completed by the applications of ethers, aldehydes, and ketones. There is an index by names and synonyms of organic reagents and an index according to the uses of the organic reagents described.

Volume II consists of ten chapters. The first five deal with organic acids, halogen substituted acids, hydroxy acids, amino acids, and miscellaneous acids. There then follow five chapters on acyl halides, acid anhydrides, esters, and the bulk of the volume on the amines, concluding with Chapter X on quaternary ammonium compounds. A name and use index to Volume II is similar to that of Volume I.

The objectives attempted by the author as stated in the preface have been adequately met. The shortcomings of the text are recognized by the author and the fact that the material has not been critically examined in any particular for authenticity or substantiation of the claims made by the original publications cited is not a just criticism of this work. The magnitude of the task of its compiling precludes the possibility of such treatment. The work is complete as regards references to the literature, even though abstracts were frequently the only available source of information. A most valuable feature of this work is the inclusion of Beilstein references to the preparation of materials and in a great many cases the procedure of preparations.

To the analyst who is progressive, the research analyst who is resourceful, and the industrial control laboratory in general these volumes represent a most profitable investment.

G. Frederick Smith

Calculations of Analytical Chemistry. *L. F. Hamilton* and *S. G. Simpson.* 4th ed. International Chemical Series, L. P. Hammett, consulting editor. 387 pp. McGraw-Hill Book Co., 330 West 42nd St., New York 18, N. Y., 1947. Price, $3.50.

The title of the fourth edition has been changed from "Calculations of Quantitative Analytical Chemistry" to "Calculations of Analytical Chemistry." This change does not imply that all types of analytical calculations have been included but rather that the subject matter has been expanded to include qualitative analysis. For the most part, the problems are those discussed in the basic courses of qualitative and quantitative analysis.

Most teachers will applaud the addition of sections on the calibration of weights, the use of logarithms, and the use of conductance measurements for titrations. The brevity and content of the section on amperometry lessen its usefulness. Most students in the basic course do not have a comprehensive understanding of this topic. Some of the new sections represent a rearrangement of old material; in these cases the presentation has been improved. It would have been fortunate had the authors been more careful in proofreading the type solutions (see p. 85, Example II; derivation p. 194, substitution of H^+ instead of OH^- in equation 9). Further, there would be less confusion if the definition for equivalent weight read: "that amount of reagent which contains or reacts with one gram atom of replaceable (or acid) hydrogen or with one gram molecule of hydroxyl." An error in a single problem is serious enough; an error in the type solution for a section of problems is even more troublesome. Factor weight problems are included in sections 55 and 77. The student might be served better if the authors expanded the material in one section and included the commonly used principles of nomographic analysis.

For one who has used this book a feature of particular interest is the inclusion of 36 new sections and 266 new problems. The new edition adheres to the original plan of offering type problems at the beginning of each section; answers are given for some problems in each section. Since most textbooks in qualitative and quantitative analysis include problems at the end of each chapter, one may ask, what purpose does a separate book on calculations serve? This reviewer believes that a book on calculations serves a real purpose. Hamilton and Simpson provide type solutions for each new set of problems. These type solutions are seldom provided in textbooks of quantitative analysis. It is the reviewer's experience that a book on calculations which includes type solution of problems saves a great amount of valuable lecture time and trains the student well to solve a variety of problems. Finally, the authors have provided a book of good quality on chemical calculations. It is a useful tool for teachers in qualitative and quantitative analysis and a good reference source for elementary chemical calculations in these fields.

V. W. Meloche

Proceedings of the Society for Experimental Stress Analysis. Volume IV, No. 1. Edited by *C. Lipson* and *W. M. Murray.* xviii + 129 pages. Addison Wesley Press, Inc., Kendall Square, Cambridge 42, Mass., 1946. Price, $6.00.

This volume, like the preceding volumes of this series, consists of a group of research papers on experimental stress analysis. The scope of the papers is best conveyed by listing the titles.

A Graphical Method of Rosette Analysis
A Nomographic Solution to the Strain Rosette Equations
A Nomographic Rosette Computer
Photoelastic Analysis of a Spar Bulkhead in a Semi-Monocoque Airplane Fuselage
Stress Study of a Fabricated Steam Chest
New Portable Stress Analysis Equipment
Measurement of Residual Stresses in Torsion Bar Springs
Residual Stress Indications in Brittle Lacquer
Mechanical Strain Gage Technique of Separating Strains Due to Normal Forces and Bending Moments
Impact on Prismatic Bars
Pressure of Plastic Concrete in Forms
The Stress Gage

The methods of experimental stress analysis are essential to the engineer seeking to design the most efficient structure for a given purpose. Through the application of these methods, he can avoid the use of large safety factors which are forced on him by lack of accurate knowledge of stresses in various parts. If actual use reveals unexpected weaknesses in supposedly well-designed equipment, the methods of experimental stress analysis enable the engineer to discover their cause and devise methods for correcting them. These proceedings are required reading for design engineers who wish to keep up on the latest developments in their field.

Ralph H. Munch

Cereal Laboratory Methods with Reference Tables. American Association of Cereal Chemists, University Farm, St. Paul 1, Minn., 1947. Fifth edition. xiv + 341 pages. Price, $4.50.

The fifth edition of "Cereal Laboratory Methods," published by the American Association of Cereal Chemists, has been revised and expanded by the association committee, F. C. Hildebrand, chairman. The amount of expansion is indicated by the increase from 264 to 341 pages and the addition of 3 new chapters.

The new chapters are on experimental malting, vitamin assay methods, and sanitation methods. The first contains directions for steeping, germination, and kilning. The second includes methods for determining vitamin A, carotene, thiamine, riboflavin, nicotinic acid, pantothenic acid, vitamin B_6 complex, and choline; in the previous edition only the thiamine method was described in detail.

The third new chapter on sanitation methods has nine sections dealing with detection and/or determination of foreign matter in flour, rodent contamination, bacteria and molds, and residues from plant fumigations.

At numerous points elsewhere in the book the methods have been modernized, but essentially the same topics are covered again in the other fourteen chapters.

The most important change in the appendix is the elimination of tables and charts for conversion of weights and analytical values to 13.5 and 15.0% moisture levels; these are replaced by a single table for converting all analytical values (except absorption) to 14.0% moisture, which has become a standard practice in cereal laboratories. Another new table gives correction of absorption values to 14.0% moisture basis.

Revision has enhanced the usefulness of the book. It is indispensable in laboratories concerned with cereal foods and feeds. Paper, printing, and binding are first class.

R. C. SHERWOOD

CORRESPONDENCE

Explosion of Cobaltinitrite

SIR:

Paiva Netto and Catani (*5*) explain the explosion of cobaltinitrite as reported by Broughton and associates (*1*) as due to the formation of fulminic acid, and state that another explosive compound formed is ethylnitrolic acid. Available literature sources show that both these compounds will be formed, but make no mention of the reason for the explosion or how to avoid the danger.

Wieland (*8*) has stated the following chain as leading to the formation of fulminic acid:

$$CH_3CHO \xrightarrow{HNO_2} HC(=N.OH)CHO \xrightarrow{HNO_3} HC(=N.OH)COOH \xrightarrow{HNO_3} O_2N.C(=N.OH)COOH \longrightarrow O_2N.CH(=N.OH) \xrightarrow{(+CO_2)} C(=N.OH) \quad (+HNO_2)$$

The chain leading to the simplest of the nitrolic acids starts from nitromethane, CH_3NO_2 or $CH_2(NO_2)_2$, which in its aci form, $CH_2(OH)N{=}O$, reacts with HNO_2 to form $HC(=NOH)NO_2$, methylnitrolic acid, water (*7*).

The salts of the nitrolic acids are explosive themselves and change easily into fulminates.

The formation of the nitroparaffin that starts this chain is easily explained by the old method of nitromethane preparation

$$CH_2ClCOOH \xrightarrow{HNO_2} CH_2(NO_2)COOH \longrightarrow CH_3NO_2 (+ CO_2)$$

Unpublished work in a Viennese laboratory (*6*) has shown that under certain conditions this chain can be started from acetic acid (nonsubstituted), though the yield is small. Nef (*4*) has demonstrated that the mercuri salt of nitromethane can be changed in one step into mercuri fulminate.

$$(CH_2{=}NO.O)_2Hg = (C{=}NO)_2Hg + 2H_2)$$

We have therefore at least two chains leading to the fulminate. Both chains require the presence at the same time of nitrate and nitrite, and this extremely dangerous mixture of nitrate, nitrite, and organic material will lead to the formation of explosive compounds if the worker does not take the utmost care. Gattermann (*2*) describing the preparation of ethyl nitrate warns that ethyl alcohol will not be oxidized by pure nitric acid but only esterified. In the presence of traces of nitrous acid oxidation ensues with the formation of nitric oxide. Nitric oxide is oxidized to nitrogen peroxide by the nitric acid and the oxidation advances autocatalytically to an explosion.

The reaction between nitric and nitrous acids is accelerated in the presence of traces of heavy metals and Hofmann (*3*) states: "Stable salts of cobalt (III) are all of a complex nature and are formed from the corresponding cobalt (II) salts by oxidation by air, some of them by dissociation of water or reduction of solvent." Broughton and associates (*1*) say: "Presumably, the only constituents in the filtrate were sodium acetate, acetic acid, sodium nitrite, sodium nitrate, and a trace of cobalt." This combination of nitric acid, nitrous acid, and organic solvent shows us a practically ideal setup for an explosion, whereas no explosion occurs in the absence of nitric acid plus cobalt.

No mention was made in connection with the Texas City disaster of traces of nitrite in the ammonium nitrate in the presence of organics (wax coating, paper bags, etc.).

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Cartesian Diver Type of Manostat—Correction

In the article entitled "Theory and Operation of a Cartesian Diver Type Manostat" [IND. ENG. CHEM., ANAL. ED., **18**, 633 (1946)] the statement should be added under "Equilibrium of Buoyant Forces" (immediately following Equation 3) that the area of the inner tube has been neglected. If this area is not neglected, Equation 8 may be written as:

$$\frac{\Delta P}{P_0} = \frac{a}{A + \frac{A_1}{A_2}A_0}\left(1 + \frac{A_1}{A_2}\right) + \frac{a}{A + \frac{A_1}{A_2}A_0} \times \frac{A_1}{V_0} P_0$$

where A_0 = cross-sectional area of inner vent tube and V_0 = volume of gas inside float.

ROGER GILMONT

Electron Microscope Society of America

L. T. HALLETT, Associate Editor

THE annual meeting of the Electron Microscope Society of America was held at the Franklin Institute in Philadelphia on December 11, 12, and 13. Several visitors from Great Britain, Australia, and Canada participated in the program, thus giving an international touch to the sessions.

A. S. McFarlane from the National Institute of Medical Research, London, read a paper prepared by V. E. Cosslett, Cavendish Laboratory, Cambridge, outlining the activities of the Electron Microscope Group of the British Institute of Physics. The practice of electron microscopy in England goes back to the construction of the first instrument in 1935–36 by L. C. Martin, Imperial College, London, in conjunction with Metropolitan-Vickers, Ltd., soon after the initial work of von Borries and Ruska. This instrument was in a state for commercial development when war broke out in 1939, but was not produced until 1944–45 as the EM-2 model. Lately an improved model has been built. During this early period, seven RCA Type B instruments came to Britain under Lend-Lease and were allotted to various research laboratories. Around these instruments developed the nucleus of a society of electron microscopy in Great Britain, and from initial informal meetings of the scientists using these instruments emerged a group, now a part of the Institute of Physics, but maintaining close relations with the Royal Microscopical Society. The enrolled membership is now 85. The group organizes two conferences each year for the presentation of original papers and for the discussion of special topics.

The group has been engaged in the compilation of a bibliography of electron microscopy in the form of a card index of titles and authors with short abstracts wherever possible. A special effort has been made to collect all the work published in Germany during the war. This section alone now comprises over 400 entries, and the whole index numbers over 1200.

Another project has been the establishment of a central collection of offprints of papers on electron microscopy and immediately related subjects which will be housed at the central office of the Institute of Physics at London. The group is also interested in setting up summer school courses for the training of comparative beginners in the fundamentals of the electron microscope and in the details of specimen technique. It is hoped to arrange such a session at Cambridge in August 1948.

There are at present in use in Great Britain ten RCA models, six Siemens, twenty Metropolitan-Vickers, and three built and designed by scientists themselves.

Like the British Society, the Electron Microscope Society of America is compiling a bibliography made up of 5 × 8 Keysort cards which will be furnished alphabetized as to the author but not punched. The bibliography contains 1000 cards, including all the references in previously published bibliographies. Orders for the set should be addressed to Perry C. Smith, Manager, Scivntific Instruments Engineering, Building 10-4, RCA Victor Dieision, Camden, N. J.

Abstracts of some of the papers of general interest presented at Philadelphia follow:

Polymorphism of Organic Pigments. F. A. HAMM, General Aniline and Film Corp., Easton, Pa.

Organic pigments are often recrystallized from sulfuric acid to effect purification and to control the particle size of the new crop of crystals. Copper phthalocyanine (Heliogen Blue) and Indanthrene Blue RS have been observed to yield crystals of varying shapes, ranging from a light greenish blue to a dark reddish blue (purple). These shades have in the past been attributed only to differences in particle size because the chemical activity and elementary analyses strongly indicated that the compounds were chemically identical. This investigation shows that both these pigments are polymorphic, and each can exist in at least two different physical structures. The shade differences are, therefore, very probably due to the differences in the light absorption exhibited by the structurally different polymorphic forms. The polymorphic transformations can, for both pigments, be readily observed to take place in the electron microscope. The new phase, stable during and after exposure to the illuminating electron beam, can be seen to grow from the vapor state at the expense of the original solid material. Electron diffraction patterns prove that the newly formed phases are structurally different from both the original samples. However, both "red" and "blue" shades of each blue pigment transform to give the same new structure. Both these commercially important pigments are therefore at least dimorphic.

Range of Migration of Silica Molecules in the Formation of Silica Replicas. C. J. CALBICK, Bell Telephone Laboratories, Inc., Murray Hill, N. J.

Silica condensing upon a surface at an oblique angle of incidence produces shadows of obstacles such as particles present on the surface, similar to those produced by the metal shadow-casting technique of Williams and Wyckoff. In contrast to the behavior of heavy metals, silica diffuses from the regions of direct incidence into the shadows, there forming a thin film relative to that in regions directly exposed to condensing silica. Densitometer traces of electron micrographs show: (1) uniform thickness far from any shadow, (2) slowly decreasing thickness as a shadow edge is approached, (3) a sharp decrease in thickness across the shadow edge, (4) slowly decreasing thickness within the shadow as distance from the edge increases. This behavior can be explained by assuming that high-temperature silica molecules condensing upon a surface at room temperature have a probability for inelastic collision less than unity, and the reflected silica is trapped in a surface layer to form a "two-dimensional gas" which diffuses over the surface. Whenever the molecules composing this gas collide with the surface they have a definite probability of sticking; consequently, the film within the shadow grows thinner as one recedes from the shadow edge. The densitometer measurements yield values for the probability for inelastic collision (0.54), the range of migration—i.e., the distance within which half of the silica diffusing from a shadow edge condenses on the surface (0.53 μ)—and the average distance a molecule travels between collisions with the surface (0.42 μ). The bearing of these results upon the mechanism of formation of silica replicas was discussed.

Electron Microscopy of Keratin Fibers. J. L. FARRANT, Division of Industrial Chemistry, Melbourne, Australia, E. H. MERCER, Division of Physics, Sydney, Australia, AND A. L. G. REES, Division of Industrial Chemistry, Melbourne, Australia.

Electron microscopical studies of the keratin fibers, the most important of the natural fibrous proteins, have contributed much to their general histology. Our earlier work demonstrated the physical and chemical differentiation of various constituent parts of the fiber and this has been substantiated recently by the direct chemical investigations of Lindley. Application of techniques such as metal shadow casting indicates that the intracellular matrix of the cortical cells is an amorphous protein having molecules ~110 Å. in diameter and that the fibrous part of the cortex is composed of protofibrils about 110 Å. wide and ~0.3 μ in length twisted together in a ropelike manner to form fibrils of diameter ~0.2 to 0.4 μ extending along the cortical cells. These protofibrils are further shown to be linear aggregates of approximately isometric particles ~110 Å. in diameter. This evidence, which has been exhaustively tested to eliminate the possibility of its being of artifact origin, is in contradiction to Astbury's concep-

tion of the molecular chains extended parallel to the fiber axis in such proteins and indicates a closer association with the globular proteins. The implication of the electron microscopical evidence, together with that obtained from x-ray and chemical studies on these proteins was discussed.

Replica Studies of Dyed Nylon. F. A. HAMM, General Aniline and Film Corp., Easton, Pa.

The crystal size of pigments and dyes when applied to textile fibers is almost invariably below the limit of light microscopical resolution. The dyestuff chemist has, however, had an insatiable desire to learn something about the distribution of his dyestuff on these fibers. This paper describes a technique for preparing replicas of nylon dyeings containing crystals ranging from about 100 to 5000 Å. long. These replicas are prepared by subliming the silica directly onto the nylon fibers, followed by a mild acid extraction of the nylon. Two types of replicas are possible: those consisting of silica only, and those consisting of silica with the dye crystals themselves in the same relative position as they were on the nylon fiber. The second type is obviously more informative because of the greater contrast (resolution) and because fewer artifacts are possible. This technique has been successfully used on nylon dyed with such commercially important vat dyes as Indanthrene Golden Yellow IGK, Indanthrene Brown IRRA, and Indanthrene Brilliant Pink IRA. The large crystal size range, their shape, and their orientation on the nylon surface are of interest. Striations on the surface of the nylon fibers are clearly visible. Similar investigations on other fibers are being carried out.

Further Studies on Use of an Objective Aperture. J. HILLIER, RCA Laboratories, Princeton, N. J.

The effect on an electron microscope image of the use of an objective aperture, of smaller than optimum size, has been studied. Physical apertures providing angular apertures in the range 10^{-2} to 5×10^{-4} radian were used in the experimental double objective lens system described earlier. Angular apertures near the optimum value of 5×10^{-3} radian were found to increase the gross contrast but not the detailed contrast, particularly in extremely thin specimens. Angular apertures an order of magnitude smaller, however, were found to increase the detailed contrast in all kinds of specimens. With all apertures the loss of resolving power was greater than would be expected from the Abbe formula. Some of the difficulties encountered in this regard and the attempts to overcome them are described.

The Hydrogen-Diffusion Leak Locator. HERBERT NELSON, RCA Victor Division, Harrison, N. J.

A new instrument has been developed for locating leaks in vacuum devices and vacuum equipment. It employs a sealed-off, highly evacuated ionization gage having a section of its envelope made of thin palladium sheet which, when heated, is highly permeable to hydrogen. In the instrument, the gage is connected to an all-metal manifold with the palladium section isolating the vacuum of the gage from that of the manifold. When a leaky vacuum device connected to the manifold is probed with hydrogen, leaks are indicated by an increase in the ion current of the gage. Since gases or vapors other than hydrogen do not penetrate the palladium, high vacuum is not required in the manifold nor in the device under test. A vacuum of the order of 1 to 10 microns is sufficient. The new instrument is similar to the mass-spectrometer leak locator in that it owes its high sensitivity to its ability to sort out, from other gases and vapors, the tracer gas (H_2) which enters through the leak. Thus, the palladium window in the hydrogen-diffusion leak locator serves the same purpose as does the crossed electric and magnetic fields of the mass-spectrometer leak locator.

Structure of Cellulose Fibers as Revealed by the Electron Microscope. CHARLES W. HOCK, Hercules Experiment Station, Hercules Powder Co., Wilmington, Del.

The structure of cellulose fibers was studied advantageously by means of metal-shadowed specimens, surface replicas, and stained preparations. These techniques of preparing specimens for electron microscopical examination clearly revealed the fundamentally fibrillate structure of natural cellulose, as well as other details not readily discernible in specimens prepared by ordinary techniques.

Interferometric Method for the Calibration of Electron Microscope Magnification. J. L. FARRANT AND A. J. HODGE, Division of Industrial Chemistry, Council for Scientific and Industrial Research, Melbourne, Australia.

Interferometric methods have been used to measure uniform cylindrical silica and glass fibers as small as 5 microns in diameter with better than 1% accuracy. Such fibers yield sharp images when examined in the electron microscope and constitute a convenient and rugged specimen for accurate direct calibration of the instrument for magnifications up to 10,000 diameters. Higher magnification ranges are then readily determined by means of an internal calibration obtained by taking micrographs of glass spheres about 0.5 micron in diameter. Such spheres are also convenient specimens for determining the image distortions which must be known to correct the widths of the fiber images. This method appears to be capable of greater accuracy than any employed hitherto.

Visual Focusing of an Electron Microscope. J. HILLIER AND S. G. ELLIS, RCA Laboratories, Princeton, N. J.

Two new electron microscope accessories facilitating more accurate visual focusing were described.

Optical Magnifier. This is essentially a low-power light microscope mounted in place of the right window around the final viewing chamber and focused on a very small portion of the final image. Just as in light microscopy a variety of combinations of objective and eyepiece lens may be used. The phosphorescent screen and 45° reflecting mirror are swung out of the electron beam during the exposure of the photographic plate. If this auxiliary optical microscope provides a magnification of 50 ×, the final image is observed at magnifications up to 1,000,000 ×.

Focusing Reflector. This electrical control increases the angular aperture of the illuminating beam so that more precise focusing is possible. It is analogous to light optics, where focusing the image at a larger "stop opening" is more accurate than at smaller "stop openings."

The angular aperture of the illuminating cone is increased by placing charged parallel metal plates above the specimen in the specimen chamber. The illuminating electron rays pass between the plates. One plate is at ground potential, whereas the other is charged with a potential of 200 volts, 60 cycle, with a square wave form. The electrical charge attracts and repels the electron beam, so that the beam is reflected off the metal plates, causing an increase in the angular aperture of the cone that illuminates the sample.

The use of the focusing reflector is comparable with that of a range finder—that is, the objective lens current is varied until both images are in juxtaposition.

Methods and Techniques for Particle Size Determinations with Electron Microscopes. JOHN H. L. WATSON, Research Institute, Henry Ford Hospital, Detroit 2, Mich.

Emphasis was placed on the use of the electron microscope to obtain semiquantitative as well as qualitative data. Included in the discussion was a treatment of the problems involved in particle size work, such as the need of good resolution, low distortion, careful focusing, and defining the term "particle size" as applied to each problem. A statistical treatment of the particle size count was advised. The results could be shown in an effective manner by plotting stated size *vs.* per cent less than stated size on log probability paper.

An Investigation of Degraded Wool. GLORIA SEEMAN AND MAX SWERDLOW, National Bureau of Standards, Washington, D. C.

A thermoplastic replica technique was used to make possible the surface study of wool fibers during various periods of degradation. A 1% solution of a thermoplastic such as polystyrene in benzene was cast upon a clean glass microscope slide. When dry, the fibers to be studied were placed in contact with the film and were held against it by means of a second slide, forming a sandwich. Pressure was applied by inserting the sandwich in a C-clamp and tightening by hand. The clamped slides were placed in an oven at a temperature slightly above the softening temperature of the plastic. After sufficient time was allowed for this softening, the slides were removed and allowed to cool. Pressure was released, and the one slide was removed from the plastic-coated slide. The fibers could readily be removed mechanically from the plastic film and were used for further studies. The plastic film on the slide was shadow-cast with chromium or gold and was then stripped from the slide for mounting on specimen screens. The replicas showed clearly some of the fine structure of the wool fibers.

New Bacteriological Techniques in Electron Microscopy. J. HILLIER, RCA Laboratories, Princeton, N. J., GEORGES KNAYSI, Department of Bacteriology, Cornell University, Ithaca, N. Y., AND R. F. BAKER, School of Medicine, University of California, Los Angeles, Calif.

A new technique was described which involved a minimum of handling of bacteria and prevented a change of conditions. Agar was placed in the bottom of a culture dish which was filled with water. A thin film of collodion was cast on the water from an amyl acetate solution. The water was removed from the dish until the collodion film rested on the agar. The bacteria were then grown directly on this film of collodion, receiving nutrients from the agar by diffusion through the collodion film. When growth had reached the desired stage, as determined by use of the light microscope, the agar containing the collodion film was placed agar-side down in water, and the thin film of collodion separated from it. Sections of the collodion upon which the bacteria had grown were then picked up in specimen screens in the usual manner.

Recent Developments in the Shadow-Casting Process. ROBLEY C. WILLIAMS AND ROBERT C. BACKUS, Department of Physics, University of Michigan, Ann Arbor, Mich.

A transfer-replica process was described which makes possible the examination of extremely small organic objects without the presence of surface structure of a substrate to interfere with interpretation. Gold was found to granulate and show structure under the beam from the biased electron gun. Uranium sulfide was found to be ideal in that it did not granulate. A film of platinum or of a mixture of platinum and palladium was also found satisfactory.

The specimen is mounted upon a glass microscope slide which has undergone a thorough cleaning treatment. The shadowing-metal is then evaporated directly onto the specimen and slide. Platinum or a mixture containing 20% palladium was found very satisfactory. Enough foil, calculated to give a film 3 to 5 Å. thick, is weighed and placed in the tungsten heating coil in the sublimation apparatus. After the film has been prepared, the slide containing the specimen and metal film is dipped into, and immediately withdrawn from, a 1% solution of collodion in amyl acetate. Specimen screens which have been mounted in Scotch tape with paper covering the centers are placed in contact with the wet collodion. When the collodion has dried, the tape is pulled away from the film, and with it come the screen and the metal film supported by the collodion. The use of glass gives the metal film a remarkably smooth surface. Metals which readily oxidize, such as chromium and uranium, adhere too strongly to the glass and cannot be used. The sulfide of uranium may be used and appears to be the best.

Modified Silica Replica Technique for Study of Biological Membranes and Rotary Shadow Casting of Specimen. F. HEINMETS, with technical assistance of J. T. QUYNN, Chemical Corps, Camp Detrick, Frederick, Md.

Biological membranes were studied by means of the silica replica technique. Shadow casting from more than one angle or from a rotary shadow casting showed clearly the outlines of the objects examined and made possible more accurate interpretation. The rotary shadow-casting technique depended upon a means of continually rotating the specimen during evaporation of the metal.

A Positive-Replica Technique for Electron Microscopy. C. M. SCHWARTZ, A. E. AUSTIN, AND P. WEBER, Battelle Memorial Institute, Columbus, Ohio.

A rapid means of making positive replicas was applied to a study of metal surfaces. A negative replica was prepared by casting an aqueous solution of polyvinyl alcohol on the metal surface. A solution of Formvar in ethylene dichloride was cast in the PVA negative replica and the PVA was removed from the Formvar replica by floating the PVA-Formvar combination, with the PVA side down, in water until the PVA dissolved. Micrographs were shown to illustrate that structural details do not appear to have been altered during the process. The process has not been applied to the study of fine structure below 100 Å. The technique appears promising for studying the surface contour of bearings operating under fluid film lubrication.

Dark-Field Electron Microscopy. CECIL E. HALL, Massachusetts Institute of Technology, Cambridge, Mass.

This technique is somewhat analogous to that employed in dark-field light microscopy. Greater contrast and greater resolution are possible on certain particular samples, especially where the crystal planes cause diffraction of the obliquely incident electrons.

The dark-field illumination is effected by placing an ordinary specimen screen about 1.5 mm. above the specimen. This is easily done by adapting a holder to the end of the regular specimen holder. The upper specimen screen is oriented so that the specimen is illuminated only by obliquely incident electrons, scattered from the upper screen.

The position of the particles may be such that they are not imaged by this dark-field technique, so that a smaller number of crystals may be indicated than with bright-field illumination. Probably the chief advantage of this technique is the ability to study the size and location of crystalline components exhibiting parallel crystalline planes which cause a coherent scattering of the oblique electron rays. Crystallites in evaporated films on the order of 50 Å. have been resolved. Better resolution in smaller objects has been estimated, although bright-field illumination may provide better resolution in larger objects.

SYMPOSIUM ON THE TEACHING OF ELECTRON MICROSCOPY

S. C. Ellis, RCA Laboratories, Princeton, N. J., stated his opinion that there should be no specialized training to prepare a man solely for electron microscopy, but that a general education in science is necessary with good training in physics. Persons taking a course in electron microscopy should have practical experience in operating the microscope and should be able to use it as an aid in research. The courses he outlined as offered at the University of Toronto are on the graduate level and include the following:

Electron Optics, 25 hours. Integrated with general physics. Lenses, aberrations, tolerances, description of the microscope
Electron Microscopy and Diffraction, 25 hours
a. Microscopy
1. Specimen Preparation
2. Actual Use of the Microscope
3. Interpretation of Results

b. Diffraction
1. Laue Pattern
2. Particle Size Determinations
3. Uses of Electron Diffraction, Limitations, etc.

Research. The use of the electron microscope in a special research problem. Ellis emphasized the fact that the best training for research is actual research, and that the electron microscope is not used for research work but to observe the results of research.

Cecil Hall of the Massachusetts Institute of Technology pointed out that at M.I.T. is offered a graduate course in electron microscopy, but that proficiency in some other field such as chemistry, physics, or biology is required. The course includes lectures on electron optics, the electron microscope, and the interpenetration of electrons with matter. There is also a laboratory course planned to give the student practical experience in specimen preparation and the use of the electron microscope.

Robley C. Williams, University of Michigan, Ann Arbor, Mich., described briefly a course in electron microscopy at the graduate level offered at the University of Michigan.

In general it was felt that more universities should add courses on electron microscopy to their curricula. The National Bureau of Standards offers a graduate course, and a course in electron optics is offered at the University of Illinois.

It was felt that eventually electron microscopists might be considered as a separate professional group; however, they are at present simply chemists, physicists, or biologists who are using the instrument as a reasearch tool. At M.I.T. and Michigan electron microscopy is taught as a subordinate to some more general field such as chemistry or physics. Students who have had these courses are never recommended to industry as electron microscopists. Electron microscopists apparently must learn the hard way—through practical experience.

Electron microscopy has been, through public appeal, considered by many to be the great and glorious panacea. Any course in electron microscopy should emphasize the often overlooked limitations in the technique.

W. G. Kinsinger, Hercules Powder Co., Wilmington, Del., stated that training in light microscopy gives experience in techniques and principles directly applicable to electron microscopy, and examples of many successful electron microscopists having this type of background can be cited.

THE ANALYST'S CALENDAR....

Symposium on Modern Methods of Analytical Chemistry

Louisiana State University, Baton Rouge, La., is sponsoring the first of a series of annual Symposia on Modern Methods of Analytical Chemistry during the week of February 2, 1948. The program is designed to bring together leaders in the field to promote interchange of views and discussion of recent and current developments. Reservations should be made through Philip W. West, Louisiana State University, Baton Rouge, La.

The Role of the Analytical Chemist. WALTER J. MURPHY

Organization and Techniques for the Solution of Industrial Analytical Problems. L. T. HALLETT

Instrumentation. R. H. MÜLLER

Absorptometry and Colorimetry. M. G. MELLON

Spectroscopy. J. R. CHURCHILL

Chromatography. A. L. LEROSEN

Mass Spectroscopy. H. W. WASHBURN, C. E. STARR, AND B. W. THOMAS

X-Ray Methods of Analysis. H. A. LIEBHAFSKY

Polarography. HERBERT LAITINEN

Developments in Noninstrumental Methods of Analysis. PHILIP W. WEST

Trends in Analytical Chemistry. PHILIP J. ELVING

Third Analytical Symposium at Pittsburgh

The Third Analytical Symposium, sponsored by the Analytical Division of the Pittsburgh Section, will be held February 12 and 13 at the Hotel William Penn in Pittsburgh. Papers on instrumental analysis, curricula in analytical chemistry, and analysis of industrial wastes will be featured.

Modern Analysis, a New Science. RALPH H. MÜLLER, New York University

Instrumenting Chemical Analysis. NELSON GILDERSLEEVE, General Electric Co.

Electronics in Instrumentation. R. L. GARMAN, General Precision Laboratory

Instrumental Analysis in an Industrial Laboratory. H. A. FREDIANI, Merck & Co., Inc.

Photoelectric Spectrochemical Analysis. J. R. CHURCHILL, Aluminum Company of America

X-Ray Diffraction, a Fundamental Analytical Tool. H. P. KLUG, Mellon Institute

Applications of the X-Ray Spectrometer. L. E. ALEXANDER, Mellon Institute

Analytical Applications of Radioactivity. F. C. HENRIQUES, Tracerlab, Inc.

Current Spectrographic Method of Nonmetallic Sample Analysis. C. E. HARVEY, Applied Research Laboratories

Beckman Flame Photometers. P. T. GILBERT, R. C. HAWES, AND A. O. BECKMAN, National Technical Laboratories

Photometric Determination of Arsenic in Copper and Copper-Base Alloys. O. P. CASE, American Brass Co.

Colorimetric Determination of Tungsten and Columbium in Steel. FRANK MALOOLY, Rustless Iron and Steel Division, American Rolling Mill Co.

Quantitative Spectrographic Analysis of Cemented Carbide Compositions. C. W. HANNA AND JOHN C. REDMOND, Kennametal, Inc.

8-Hydroxyquinoline for the Colorimetric Determination of Aluminum in Steel. S. E. WIBERLEY AND L. G. BASSETT, Rensselaer Polytechnic Institute

Determination of Manganese, Nickel, and Chromium in Type 304, 316, 347, and Similar Steels with the Beckman Spectrophotometer. J. B. CULBERTSON AND R. M. FOWLER, Union Carbide and Carbon Research Laboratories

Determination of Copper in Iron and Steel by the Diethyldithiocarbamate Method. R. E. LIEBENDORFER, Ellwood Works, National Tube Co.

Proposed Method of Expressing Extinction Coefficients of Photometric Test Methods. GRANT WERNIMONT, Eastman Kodak Co.

Accurate Analysis of Carbon Monoxide and Carbon Dioxide Mixtures. C. H. TOENSING, Carnegie-Illinois Steel Corp., and D. S. MCKINNEY, Carnegie Institute of Technology

Molar Volume Corrections in Gas Analysis. C. H. TOENSING, Carnegie-Illinois Steel Corp., AND D. S. MCKINNEY, Carnegie Institute of Technology

Complexation in Chemical Analysis. D. F. BOLTZ, Wayne University

Analyzing Fluoride-Containing Materials. R. J. ROWLEY, Aluminum Company of America

Progress in Unification of Procedures for Determination of Iodine in Organic Compounds and Mixtures. D. L. DEARDORFF AND GEORGE D. BEAL, Mellon Institute, AND K. L. WATERS, Zemmer Co.

Problems of Teaching Analytical Chemistry. Papers by M. G. MELLON, Purdue University, HURD W. SAFFORD AND C. J. ENGELDER, University of Pittsburgh, AND LYNN L. MERRITT, Indiana University, Bloomington, Ind.

General Papers on Stream Pollution Abatement. R. D. HOAK, Mellon Institute, GLADYS SWOPE, Allegheny Sanitary Authority, C. C. RUCHHOFT, Cincinnati Experiment Station, S. A. BRALEY, K. M. MADISON, AND G. A. BRADY, Mellon Institute, AND M. L. RIEHL, Ohio Department of Health

Microchemical Symposium

The third annual symposium sponsored by the Metropolitan Microchemical Society of New York will be held February 27 and 28 at the American Museum of Natural History, New York, N. Y. The meetings will begin at 8 P.M. on February 27, followed by sessions at 10 A.M. and 2 P.M. on February 28.

Friday, February 27

Recent Developments in Microscopy. K. H. HEINICKE, Bausch & Lomb Optical Co.

Application of Infrared Spectroscopy. KONRAD DOBRINER, Sloan Kettering Institute for Cancer Research

Saturday, February 28

Paper Chromatography. R. J. BLOCK, New York Medical College

Statistical Methods in Analytical Chemistry. JOHN MANDEL, National Bureau of Standards

Spectrophotometry. E. I. STEARNS, Calco Chemical Division, American Cyanamid Co.

Microbiological Assay Methods. LOUIS SIEGEL, Food Research Laboratories, Inc.

Symposium on Analytical Chemistry. Campus, Louisiana State University, Baton Rouge, La., Feb. 2 to 5.
Symposium on Analytical Chemistry. Pittsburgh Section, A.C.S., Feb. 12 and 13.
Third Annual Symposium. Metropolitan Microchemical Society of New York, Feb. 27 and 28.
Symposium on Modern Instrumental Methods of Analysis. Minnesota Section, A.C.S., and Institute of Technology, University of Minnesota, Minneapolis, March 22 to 24.

AIDS FOR THE ANALYST . . . . .

An All-Glass Laboratory Still. G. E. Mallory and R. F. Love, Alcohol Tax Unit, Bureau of Internal Revenue, San Francisco, Calif.

THE ordinary laboratory still consisting of boiling flask, connecting tube, and condenser, joined by rubber stoppers, has the disadvantage that the rubber may be affected by the vapors produced in the distillation process. Such action may cause contamination of the distillate and it hardens and shrinks the rubber so that tight connections are not always maintained and vapor may be lost through leakage.

To obviate these difficulties, all-glass stills have been devised in which the two rubber stoppers have been replaced by glass joints; while they are better than the older type, it is rather difficult to adjust the apparatus so that the two glass joints make tight connections, and unequal pressure causes one of the joints to become loose with resultant loss of vapor.

The still described here has only one glass joint, the usual connecting tube is eliminated, and one tube extends from the flask through the condenser jacket to the receiver. The separate jacket is attached to the tube by rubber or threaded connections.

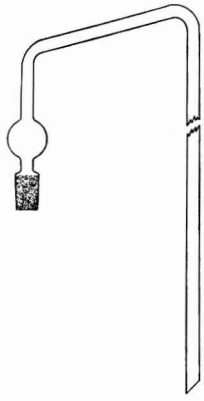


Figure 1

As used in this laboratory, the tube is 13 mm. in outside diameter, the long arm is 70 cm. long, and the short arm is 14 cm. long. The length of tube between arms is 21 cm. The bulb is 45 mm. in outside diameter. A $ joint 29/42 is used. Dimensions can be varied to suit any need. The supply catalogs do not list the tubes but those described (Figure 1) were furnished by Ace Glass, Incorporated, Vineland, N. J.

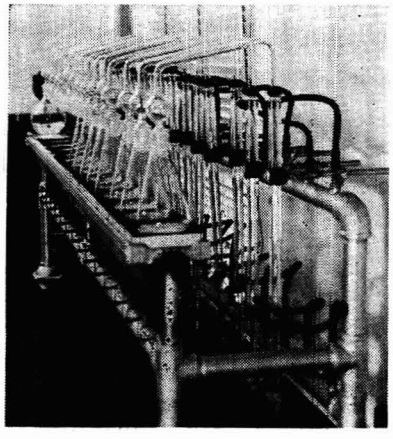


Figure 2

The condenser is held by a single clamp, the jaws of which are covered with rubber, or better with woven asbestos sleeves, and which are closed just tightly enough to prevent the tube from slipping. This arrangement permits sufficient flexibility to prevent breakage and when the flask is connected or disconnected, the condenser is moved up or down in the clamp, thus avoiding strain on the tube.

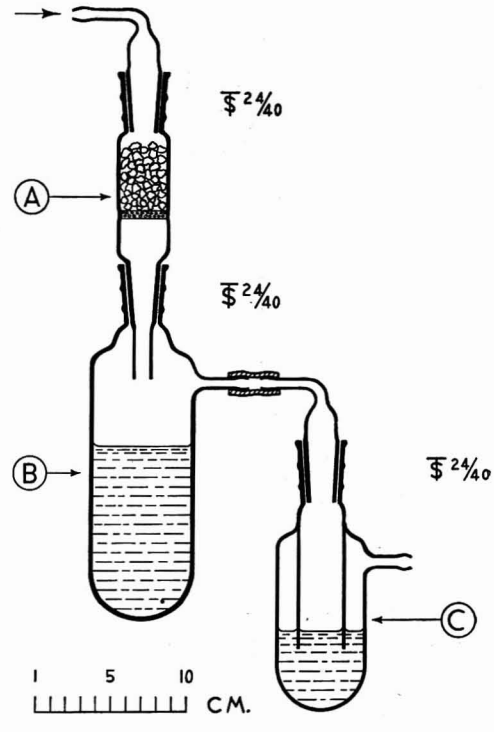
Figure 2 shows a bank of 15 stills used in this laboratory. As the framework was already installed, the tubes were designed to fit its dimensions. They have been in use for three years with very little breakage. Even when thick liquids which bumped considerably have been distilled, there have been no breakage and no disconnection of the glass joint. The short tube between the bulb and the joint is large enough to keep liquid from collecting in the bulb and in an ordinary distillation the boiling proceeds very smoothly.

The upward incline of the transverse part of the tube from the still head to the descending portion causes it to act as an efficient trap and reflux section.

The tube can be cleaned by boiling water in the flask and immersing the end of the condenser tube in a beaker of water or cleaning fluid. When the boiling is stopped, the liquid is drawn by suction through the tube, thereby cleaning it.

Absorption Apparatus for Hydrogen Chloride. Max H. Hubacher, Research Laboratory, Ex-Lax, Inc., Brooklyn, N. Y.

TRAPS using running water are customarily employed for the absorption of hydrogen chloride or other water-soluble gases [Johnson, J. R., *Org. Syntheses*, Coll. Vol. 1 (2nd ed.), 97 (1941); Allen, C. F. H., *Ibid.*, **2**, 4 (1943)]. However, where it is desired to observe the beginning or the end of the development of hydrogen chloride, another type of absorption apparatus has shown great utility and convenience, without the disadvantage of running water. The trap was designed to absorb up to 4 moles of hydrogen chloride developed in synthetic preparative work such as Friedel-Crafts reactions, halogenations, and similar processes.



Vessel *B*, when charged with 350 ml. of water, will absorb 4 moles of hydrogen chloride. The absorption of the gas in *B* can be observed by the *Schlieren* formation. Drying tube *A* has a sintered-glass disk of coarse porosity and holds granular anhydrous calcium chloride or Drierite. This prevents water vapors from diffusing back into the reaction vessel. Any gas not absorbed in *B* is forced to pass through the water or sodium hydroxide solution contained in absorption vessel *C*. The inner tube of *C* has a relatively large diameter to prevent sucking back of the liquid into *B*.