Methods of Vitamin Assay

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on the vitamin concentration of these foods, the samples will presumably have some value as standards. The selection, preparation and storage of these samples and the method of their distribution are described in Chapter 9.

The methods described here are the result of the pooling and interchange of information on analytical technics, and thus represent the combined knowledge and experience of many persons. A difficult problem for the committees was the decision on the manner in which various alternative procedures, short cuts, explanations, and comments should be included. In each case one generally applicable method has been selected and alternative procedures, explanations, and comments have been included in smaller print.

In general, emphasis has been placed on those details of procedure on which the usefulness and precision of a method depend. Many of the procedures described here are not essentially different from official or accepted methods published elsewhere, but with the extensive notes incorporated with each method details are given which are frequently omitted from other descriptions. The background assumed is that of a laboratory technician with limited training in quantitative analysis. A complete list of apparatus needed for the assays is included beyond that which would ordinarily be available in a laboratory equipped with a bench, balance, and common chemical and bacteriological equipment.

The scope of the book is limited. Only such methods are included as have been successfully applied to a variety of foods or other materials by several committee members. In Chapter 8, selected references to other vitamin methods are listed.

Just as the methods outlined have resulted from the free discussion and exchange of ideas of the members of the Association, so also are further comments and criticism invited from readers for further improvement and standardization of procedure. A revision of this book of methods is planned whenever new knowledge of vitamin methodology justifies it, and in this connection the critical opinion of readers will be very helpful. Letters may be addressed to the General Advisory Committee of the Association.

Where special equipment or reagents are needed in the analytical procedures, mention of suitable products has been made. This has been done in full realization that other instruments or different brands of chemicals may give equally good results. It is not feasible for a

group of this nature to test all makes of instruments and all chemicals available. Accordingly, we are limited to expressions of opinion regarding those items with which we are familiar. In view of the fact that the characteristics of instruments and the qualities of the reagents profoundly affect the analytical values, it has been felt essential that the analyst be informed of at least one acceptable supply. It is hoped that future revisions will include an expanded and more representative listing, but until the added experience is gained we must offer our apologies to those manufacturers whose products are equally as acceptable as those listed but with which we are unfamiliar. In this respect we feel that our first responsibility is to the analyst, and that giving him a clue to sources of suitable materials may be helpful.

Since this book is intended more as a manual for the analyst than as a comprehensive treatise, references to the literature have been included only when it was felt they might be helpful to the reader should he desire further information. While an attempt has been made to give literature citations to all items of fundamental importance, many minor modifications which are in common use have been incorporated into the procedures without specific reference. If each of these steps were to be included, the bibliographic references would be so frequent as to lessen the emphasis upon more important items.

A number of scientists outside of the Association materially assisted the Methods Committees by critically reviewing certain chapters. A list of these reviewers is given on page ix, and the Association hereby expresses its sincere appreciation for their cooperation.

Acknowledgment is also given to the industrial concerns listed on page xi, whose financial support encouraged the Association in the publication of this volume.

THE ASSOCIATION OF VITAMIN CHEMISTS, INC.

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Contents

CHAPTER 1

Sampling for Vitamin Analyses

PRELIMINARY CONSIDERATIONS

Skilful sampling is important in vitamin analyses because of the great variability of the materials which are assayed. Not only do the units of the materials to be sampled differ from each other because of past history, i.e., differences in species, in variety, in conditions of culture, harvesting, manufacture or storage, etc., but frequently parts of a single unit differ widely from other portions. Thus, apples of different varieties from one orchard vary in vitamin content, and apples of the same variety differ if grown in several geographic localities or under differing climatic conditions. Apples on a single tree differ, depending upon the state of maturity or whether they are taken from the sunny side or have been shaded. Even portions from a single apple are not the same, a sample from near the surface having a different vitamin content than one from near the core. Failure to take such variations into consideration may result in data of limited value and may lead to false conclusions.

These variations usually cannot be avoided—they occur and must be accepted. It is therefore essential that the analyst recognize the existence of these variations, appraise them critically, and plan the sampling so as to minimize the errors they introduce. The importance of proper sampling is receiving increasing recognition, owing partly to frequent demonstrations of variations and partly to development of statistical methods which provide ways of drawing appropriate conclusions from analytical data.

The extent of the variations depends upon the product and upon the manner of its selection. Thus, if we are attempting to determine the average vitamin content for one general type of product—e.g., for all tomatoes, for all wheat, or for all pork—we are faced with a large array of variables. If we limit our investigation to a certain category, such as a definite species or variety, some of the variables vanish. Further limitations, such as to a particular geographic area or to a definite state of maturity, will further decrease the factors contributing to variation. If the

effects of processing are to be studied, some of the factors accounting for variability may be eliminated by selection of uniform raw material but others (temperature, oxygen, light, etc.) assume greater importance. In storage studies still other conditions must be given special consideration. A thorough realization of the variable nature of the materials, in relation to the purpose of the study and the accuracy desired, is indispensable for adequate sampling and thus for the meaningful interpretation of data.

In addition to variations occurring within the material itself and represented by the actual vitamin content of the portions taken for analysis, there are variations resulting from the analytical manipulations, the so-called errors of the method. Although the latter will not be emphasized in this chapter, both types of variation are present, and the combined effects influence the result. It is important to know the errors of the method, so that one may evaluate the contribution of other factors to the variations in analytical data.

THE GENERAL PROBLEM

The general problem in sampling is to secure a portion or portions which will satisfactorily represent the whole. Whole may refer to all of some specific part, such as to all of the peel of an orange as compared to the entire orange. The more varied the material, the more complex are the manipulations involved in taking a representative sample. Although sampling technics vary in individual cases, in general they may be classified according to the types of material to be assayed.

1. Homogeneous Materials such as single-phase liquids or well-mixed powders present the simplest case because each part is typical of all the others, and any portion of the original may be used as the analytical sample, that is, the sample actually used in the analysis. It should be realized, however, that many materials which appear to be homogeneous are not, especially if large volumes are involved. As a rule, even solutions or powders should be mixed thoroughly immediately prior to sampling.

Mixing. Small quantities of powders or solutions may be mixed by rotating and shaking in a closed container with a volume at least twice that of the product to be sampled. Mixing may also be accomplished by pouring the product from one container to another several times. Larger quantities of solutions contained in vats or large tanks can sometimes be mixed with a mechanical stirrer or large paddle. When this is impossible, geometric sampling as described below must be resorted to.

Samples of powders or ground materials may also be obtained by quartering. To carry out this procedure, place the product on a large sheet

of paper, oil cloth, canvas, etc., and mix by rolling; that is, pull one corner of the sheet over the top of the material as far as possible without causing spillage. Take the opposite corner and work the powder back to the center. Then take another corner and repeat the process until thorough mixing is attained. Flatten the pile of material somewhat and divide it into quarters, using a knife or spatula for small quantities and a piece of sheet metal or plyboard for large quantities. Discard two opposite quarters, then remix the remaining two by rolling on the mixing sheet as before. Repeat the quartering and mixing until the quantity of material is reduced to a sample of the desired size. Large quantities of powders may also be placed in a cone-shaped pile on a clean floor and mixed by moving the pile to another place, using a shovel or scoop. Place each scoopful on the top of the pile, so that the material rolls down the sides in all directions. Several such transfers of the material usually will result in good mixing. Sample dividers, which mechanically mix powdered or granular materials may be obtained from most apparatus supply houses.

2. Heterogeneous Materials require more complicated sampling technics; the difficulties increase in proportion to the size of the lot to be analyzed and to the variations existing within it. The only procedure which can be relied upon to give a truly representative sample is to grind the entire lot to such a degree of fineness that, after thorough mixing, the homogeneity of a solution is approached. Although such treatment is usually impractical, it must be recognized that any other procedure represents a compromise between practicality and accuracy. In each case the technic employed must be based upon consideration of (1) the accuracy acceptable or attainable, (2) the degree of homogeneity in the lot, (3) the amount of time, labor and money available, and (4) the purpose of the analysis.

With heterogeneous materials of great bulk or size, like a carload of grain or a shipment of feed, it is often necessary to prepare a succession of samples of progressively decreasing size, before amounts are reached which are convenient for transport to or storage in the laboratory. These samples are designated primary, secondary, tertiary, etc. From the last of these, the analytical sample is finally prepared. In order that each sample may be satisfactorily representative of the whole, the procedure known as *geometric sampling* usually is employed.

In geometric sampling the lot is considered as some sort of regular geometric solid (cube, cylinder, cone, etc.). This is divided, at least mentally, into a number of equal volumes and a sub-sample is withdrawn at random from each. All sub-samples should be of equal size. When combined and thoroughly mixed, these constitute the primary sample. When the geometric technic is employed, it is essential that the division of the lot

be such that portions are withdrawn from all parts, not merely from the surface or from one side. This type of sampling is the basis of most official procedures and must be employed whenever knowledge of the character of the lot is lacking.

If it is known that the lot is made up of several sub-lots of different origins (e.g., a carload of poultry feed supposedly of the same formula but produced on three different days) each sub-lot may be sampled, (geometrically, if necessary) and a sub-lot sample obtained by mixing. Then from each sub-lot sample a quantity of material is taken, this quantity being proportional to the contribution of that sub-lot to the whole lot. These proportional quantities are combined into a grand composite representative of the total. For example, if a 100-lb lot is made up of sub-lots weighing 25, 35 and 40 lbs, the primary sample might be prepared by taking 25-, 35- and 40-g portions, respectively, from the different sub-lots. This type of sampling is, of course, restricted to materials in which there can be satisfactory mixing of the various sub-samples. Devices for the taking of samples from large quantities of materials and instructions for the exact procedures to be followed have been described (*1–9*).

If an estimate of the variability of the material is desired, each sub-sample should be analyzed separately. From the values so obtained, it is then possible to obtain an average and, with the use of statistical formulas and tables, to determine the probable accuracy of that average.

Once several different lots of the material have been investigated and the variability determined, it is then possible to design a sampling and assay program which will give a maximum of information with a minimum of effort. In practice, when the number of sub-samples necessary to give the desired accuracy has been determined for a given type of material, the sub-samples of future lots can be pooled into a primary sample and analyzed as a single composite.

The number of sub-samples necessary depends upon the variation expected and the accuracy needed in the assay, and can be estimated only when both of these factors have been determined. The accuracy needed is conditioned by the purpose for which the assay is to be used and, for the following discussion, may be considered established—e.g., a value within 10 per cent of the true vitamin content. Estimation of the variability of the material being sampled and of the number of samples which must be taken to give the desired accuracy involves analyses of several randomly-selected portions and statistical treatment of the data. Although the theories underlying such treatments are too involved for inclusion here, the applications of a few basic principles will be given to illustrate the usefulness

of statistical methods. More thorough discussions of statistics may be obtained by reference to texts (*10–16*).

The usual approach in estimating the variability of any given material is to examine the range of values found for a number of randomly-selected portions. If the range is very large, a simple average of a few values may be grossly in error with respect to the true average for all the material. Nevertheless, such averages are frequently reported with erroneous impressions of accuracy. For example: an investigator studying the riboflavin content of milk took samples from each of ten randomly-selected cans of milk from one delivery truck, assayed the samples, and averaged the values, obtaining a mean of 1.78 γ per g. Frequently, naive analysts interpret such a value as meaning that each of the cans contained milk with a riboflavin content close to 1.78 γ per g. The values actually found ranged from 1.58 to 2.01, as listed below. The average value thus needs to be supplemented with additional information on the variability of the product.

The variability of a material may be stated in a number of different ways, but the *standard deviation* is one of the most useful expressions.

Although the theories involved in the calculation and interpretation of standard deviation are too complex to consider at this point, the actual calculation for a set of values is relatively simple. Tabulate the values found, determine the sum, divide by the number of samples, n, getting the average or mean, $\bar{x}$. Subtract $\bar{x}$ from each value in turn, getting a column of deviations from the mean. Square the deviations, add, and divide the sum by $(n - 1)$, which gives a value for variance, s^2. Determine the square root of the variance, thus getting s, the standard deviation. In this form deviation is expressed in terms of the original unit of measurement. The following table illustrates the calculations for the milk samples previously mentioned:

Sample No.	Riboflavin content, γ per g	Deviations from the mean	Deviations squared
1	1.79	0.01	0.0001
2	1.69	−0.09	0.0081
3	1.58	−0.20	0.0400
4	1.88	0.10	0.0100
5	1.78	0.00	0.0000
6	1.70	−0.08	0.0064
7	1.75	−0.03	0.0009
8	2.01	0.23	0.0529
9	1.71	−0.07	0.0049
10	1.91	0.13	0.0169
Totals	17.80	0.00	0.1402
Mean, $\bar{x}$	1.78		

Standard Deviation, s, $= \sqrt{\frac{0.1402}{10 - 1}} = \sqrt{0.0156} = 0.13$

There are other methods of calculating s, but all give approximately the same value. At least ten samples should be assayed if standard deviation is to be calculated. Twenty or more are much preferred. Since it is frequently more convenient to express variation in per cent, the coefficient of variation, C, which is $\frac{s}{\bar{x}} \times 100$, may be calculated. In the above example, $C = \frac{0.13}{1.78} \times 100 = 7.3\%$.

The standard deviation expresses the limit on either side of the mean, $\bar{x}$, within which two-thirds of the assay values may be expected to occur. Unless unusual distribution of values is encountered, s will be approximately one-third of the range from the lowest to the highest value if ten to twenty samples are assayed. With larger numbers of samples, s may be as little as one-sixth of the range. The standard deviation expresses the variation of a series of values, and thus gives information regarding the probability of the correctness of a single analysis.

If the standard deviation for the material being assayed can be estimated from previous experience, it can be inferred that there will be two chances out of three that the value from another single assay of this type of material will not differ from the true average by more than the amount of that standard deviation. Since, with a small number of samples, the range is usually three times as large as the standard deviation, the value found for a single portion may be expected not to differ from the mean by more than 1.5 s. Thus, if it has been found that the s for the riboflavin contents of ten milk samples taken at random from one truck is 0.13 γ per g, and if similar standard deviations are observed for several loads, then the value obtained by analysis of a single sample from another truck may be assumed to be within 0.20 γ per g (i.e., 1.5 s) of the mean which would have been found had ten samples been assayed. Also it may be assumed that there are two chances out of three that the value will be within 0.13 γ per g of the mean. Likewise, there is one chance in three that the value will lie outside the range $\bar{x}$-s, in this case 1.78 ± 0.13 (that is, < 1.65 or > 1.91).

From this it appears that the analysis of too small a number of samples can yield an erroneous average. As a rule, once an average has been computed, the chemist is prone to regard the value as correct. Actually, where much variability is encountered, the average of a few samples may deviate considerably from the true value. If only the first five milk samples had been assayed, a mean of 1.74 would have been obtained and considered correct. Similarly, had the last five been selected, the mean would have been 1.82. In general, a mean is merely a more reliable approximation of the true value than is any individual assay, and the larger the number of assays, the closer is the approach to the correct answer. Nevertheless, the

mean itself does not adequately express the results of a series of assays—it should be accompanied by the standard deviation and also by a record of the number of samples.

By further calculations and reference to statistical tables (*10,15*), it is possible to determine with a specified degree of confidence a range of values (fiducial limits) within which the true average value of the material may be expected to fall. That is, if we are willing to assume the risk that our answer will be wrong one time in twenty, we can compute limits on either side of the observed average within which we can report the true average. For the above illustration these limits (*16*) are 1.78 ± 0.09, or 1.69 to 1.87. If desired, it is possible to report limits which will embrace the true value 99 times out of a hundred rather than 19 times out of 20. For the above illustration, these would be 1.78 ± 0.13, or 1.65 to 1.91. Although in this case the fiducial limits differ from the mean by an amount equal to the standard deviation, this is not generally true.

For most purposes, the possibility that an assay value may be 0.20 γ per g in error from a value of 1.89 γ per g, or 11 per cent, will preclude acceptance of a single assay. But need there be ten assays? or twenty? or fifty? The only answer lies in statistical treatment of the data, and for anything more than an estimate of the number, attention is directed to references dealing with such problems (*10–16*). However, the following formula may be used to estimate the number of individual analyses, N, which are necessary for any corresponding desired accuracy, p:

$$N = (2.6)^2 \times C^2/p^2$$

where 2.6 is a constant arrived at by use of statistical formulas, C = coefficient of variation, and p = per cent deviation permissible from the mean value (*16*). However, it should be emphasized that coefficients of variation for several series of samples of one type of product must be obtained before there is any justification for selecting a coefficient to apply to a new series of samples. For example:

> It is desired to determine, within 10 per cent, the ascorbic acid content of a truck load of tomatoes. Previous assays of each of several truck loads indicate 20 ± 3 mg per 100 g as a typical average ascorbic acid content for a series of 20 tomatoes. The number of samples necessary to give the desired accuracy may then be estimated. In this case
>
> $$C = \frac{3}{20} \times 100 \text{ or } 15\%, \text{ and } p = 10\%.$$
>
> Substituting in the formula $N = (2.6)^2 \times (15)^2/10^2$, we have $\frac{6.76 \times 225}{100}$ or 15.2, the number of tomatoes that need be taken. If it is desired to hold p to a 5-per cent limit, N will be much greater, being 61.

As previously indicated, once a reasonably certain coefficient of variation has been determined for the type of material, individual portions need not be analyzed separately, but may be combined and mixed thoroughly, giving the primary sample. From this a *secondary sample* of more convenient size can be taken and analyzed.

Unless the primary sample is a liquid or a finely divided solid, it must be put through some type of comminuting operation to reduce the particle size sufficiently to permit ready mixing. For dry materials several types of grinders are available, such as the Wiley mills, burr mills, and hammer mills. A rolling pin is often sufficient. A mortar and pestle may be used for small samples. Wet materials may be minced with a knife, ground through a food chopper, or blended with a high-speed rotary cutter such as the Waring Blendor, or ground in a mortar with clean sharp sand.

During all of these operations the possibility of changes in moisture content must be considered and prevented or compensated for. Accidental losses of juices may change not only the vitamin content but also the vitamin concentration, since the vitamin concentration of the juice may differ from that of the remainder of the sample. In some cases special care must be taken to minimize the effects of enzymes and catalysts or to prevent undue exposure of the sample to oxygen (vitamin A, carotene, ascorbic acid) or to light (vitamin A, riboflavin, pyridoxine).

After comminution (and if possible, also before) the primary sample must be thoroughly mixed. The secondary sample should then be taken geometrically, thoroughly mixed, and stored under conditions most conducive to retention of the vitamins. The assays should be conducted as promptly as possible, but if delays are necessary, perishable samples usually may be preserved by dehydrating or freezing.

Even the laboratory sample (usually ½ to 2 pounds) is much larger than the amount needed in the actual assay. Accordingly, only a portion of the well mixed sample need be taken for analysis. In many cases either the entire sample or a portion of it is comminuted further in a Waring Blendor or other similar enclosed high-speed chopper. In such cases, it is common practice to add liquid so that a uniform slurry will result. If liquid is added, the material and liquid must both be added to the blendor in known quantities so that the composition of the slurry bears a known relation to that of the material sampled.

A word of caution should be added. In industrial work, the primary sample is frequently taken by employees not connected with the laboratory. Reliable samples must not be expected unless these workers have been care-

fully trained and indoctrinated, and have frequent, non-periodic inspection of their work. Without such precautions the job is likely to seem an unnecessarily complicated nuisance which can be easily cut short without detection.

APPLICATIONS TO VARIOUS TYPES OF PRODUCTS

A. Meat and Other Animal Tissues

1. Fresh, Frozen and Cured Meats. The vitamin content of animal tissues is known to vary with species, breed, feed, etc. Within a single animal, variations occur among organ systems, and even among individual muscles. For these reasons, no simple technic can be suggested for finding the average vitamin content of a whole animal or several animals. The methods and limitations of such a venture are generally indicated above. The sampling of lots composed of different cuts of meat or of similar cuts from different animals may be accomplished by taking a certain percentage of the whole cuts or a portion of each piece. These portions should be taken from different locations as different pieces are sampled, so that the same portion of each cut will not be used every time. The sub-samples may be assayed individually, or ground and combined into a composite sample.

Frequently, it is desired to study the change in vitamin content of a given cut of meat undergoing a specific operation (such as cooking). Sampling in this case may be handled by taking two adjacent cross-section slices not more than one inch thick from the approximate center of the muscles comprising the cut, one slice to be assayed before the processing operation and the other after. If the integrity of the cut is to be preserved during the process, one sample may be taken from a cut from one side of a carcass and the other from the corresponding cut on the opposite side of the same carcass, with due precaution to take both samples from the same part of each cut. Repeated checks have shown this procedure to be valid. The loin (*longissimus dorsi*) and the *biceps femoris* muscles are usually relatively large and quite uniform in vitamin content through the central section, and are therefore quite suitable for this type of study.

If the process involves change in gross composition of the meat, (addition of salt, loss of moisture, etc.), correction must be made for these changes. If no loss of protein is entailed, expression of vitamin potency per gram of nitrogen will serve this purpose. An alternative and more general basis is to compare total vitamin contents: vitamin concentration before processing times total weight before processing versus vitamin concentra-

tion after processing times total weight after processing. If juices or gravy result from the process, the vitamin content of this liquid must be measured and included in the calculation.

Because of the variability of meat, the primary sample will frequently be large (5–10 pounds or more). The first step in preparing the secondary sample will then be to put this entire quantity through a meat grinder or chopper and mix the ground meat thoroughly. If a high-speed blendor is available, 100 g of the ground meat (geometrically sampled) may be blended with 200 ml of water (or other extractant such as a buffer solution or 0.1 *N* HCl, etc.) and the portion for analysis withdrawn with a piece of glass tubing or inverted pipet while the blendor is running. Again, to improve the representativeness of the portion weighed for analysis, it is better to make several successive withdrawals rather than one for the whole portion. These may be transferred to the tared flask in which the analysis is to be started and the sample weight obtained by difference. If any particles adhere to the sides of the extraction flask, they should be washed down immediately after weighing to avoid later trouble in handling.

If no blendor is available, three successive grindings, followed by mixing in each case, should give a smooth paste suitable for withdrawing the secondary sample and the aliquots for assay.

While the considerations outlined above apply chiefly to fresh tissue, frozen meats may be handled in the same way after thawing at low temperature (40–50° F.) to avoid enzymic changes. Cured meats may also be handled in the same manner. It is reasonable to expect some equalization of vitamin content within each cut and, in brine-cured meats, within the lot by diffusion of the curing solution. Slight leaching of water-soluble factors into the curing solution may also occur.

2. Canned Meats. Many canned meats are solidly packed in the can with little opportunity for heat transfer through the can contents by convection. Therefore, the sterilization operation is usually a long one and the meat near the periphery of the can is subjected to the heat of the operation for a considerably longer period than that near the center. Consequently, the concentration of heat-labile vitamins varies in a regular fashion along the radius of the can and, for the same reasons, for some distance from the ends. The pattern of this variation is obviously governed by the dimensions of the can. Thus, the only accurate sampling procedure is to grind the entire contents of the can and to remove portions for assay. If necessary, the can may be chilled so as to solidify the juices and one-half of the contents used, taking care to get one-half of the juice as well as one-half of the meat.

The factors mentioned in connection with the variations in fresh meats are important for canned meats also, producing variation within the can, and, to an even greater extent, from one can to another. The variations may be increased by failure to get perfectly uniform heat distribution within the sterilizing retort thus causing variable destruction of heat-labile factors during the process. Since different packers use different times and temperatures for heat-sterilization of their canned meats, variation is to be expected from brand to brand. Variations occur from one product to another and from one can size to another. Losses varying with temperature and with the lability of certain vitamins have been observed during storage, so that variations in age must be considered in drawing the primary sample.

3. Dehydrated Meat. Dehydrated meat does not seem to present any sampling problems unique to its form. Sub-samples may be mixed and ground, and an aliquot extracted directly or preferably, after reconstitution with four volumes of water and blending.

4. Protein Concentrates for Animal Feeds. Meat meals, tankages, liver meals, etc., even though ground and mixed during manufacture, vary greatly from lot to lot. Quantities up to 100 pounds may be sampled after thorough mixing. If the lot consists of several bags of a product, the sample may be prepared by withdrawing a portion from each bag. With larger quantities, such as carload lots, a representative number of bags may be sampled. In general, the principles applying to mixed feeds may be considered valid for protein concentrates (*2–5*).

B. Pharmaceuticals

1. Liquids. Mix thoroughly by inverting several times. If any sediment is present, be certain that it is dispersed in the liquid. Viscous samples, such as malt extracts, may require prolonged mixing. Sampling of vitamin oils is discussed under vitamin A.

2. Powders. Powders, such as yeast, liver concentrates, synthetic vitamin preparations alone or in mixture with other ingredients, may be sampled as described under 1 above.

3. Tablets. Weigh at least twenty tablets, grind them to a fine powder and mix thoroughly. Weigh an aliquot sufficient for the analysis.

Tablet sampling in the control of manufacturing operations presents a more difficult problem. The number of tablets that can be taken from each lot for analysis is limited by practical consideration. The tablet granulation from which the tablets are manufactured may be sampled as under

powders and the analyses will give an indication of the degree of homogenity before tablet manufacture is undertaken. However, the fact that the granulation is homogeneous does not necessarily mean that all the tablets will be exactly uniform, since the difference in particle size of the granulation will influence the rate of flow into the tablet machines. Therefore, a practical method must be devised for the sampling of the tablets themselves. In following a production line to control the vitamin content a certain number of tablets may be taken at established intervals from the tablet machines during manufacture (the primary sample). Mix these tablets thoroughly and from this mixture, select at random and weigh a sample of not less than twenty tablets. Grind the sample to a fine powder in a mortar and weigh an aliquot sufficient for analysis.

The number of tablets taken and the time intervals at which they are taken should first be established for each product by plotting control charts (*10,11,15*) from the analysis of the individual samples. From the charts an indication of the number of tablets that should be taken and the intervals best suited to accurate control may be obtained.

4. **Capsules, Dry Filled.** Weigh at least twenty capsules, open them, and empty their contents. Clean the shells of adhering powder with absorbent cotton on the end of a toothpick or stiff wire, or by any other method of choice. Weigh the shells and subtract this weight from that of the filled capsules. This gives the weight of contents of the capsules used. Mix the contents thoroughly and take any convenient aliquot for analysis. For manufacturing control, a procedure similar to that described under tablets may be followed.

5. **Capsules, Soft Elastic.** For capsules containing only oil, weigh at least twenty capsules, cut them open with a razor blade or sharp knife, and empty their contents into a small beaker. Mix the oil and take an aliquot for analysis. Wash the shells several times with ether or acetone until free of oil. Allow shells to dry, weigh them, and subtract weight from that of the filled capsules to obtain the weight of the contents.

For capsules containing a mixture of water-soluble vitamins in oil, it is usually easiest to run analyses on whole capsules if any sort of aqueous extraction procedure is employed. Take twenty or more capsules, add the extractant, allow time for the capsules to dissolve and proceed with the usual extraction. If a nonaqueous reagent is used (e.g., alcoholic KOH), the gelatin capsule should be dissolved in a small amount of water before the extraction proper is started.

If desired, a procedure similar to that for oil-filled capsules may be

used. In this case, after washing the shells with organic solvent, rinse them with ice water to remove the water-soluble vitamins clinging to the shell. Follow with a rinsing with acetone, dry, and proceed as above. This procedure is valid only when the water-soluble vitamins have not separated from the oil suspension.

C. Cereals and Cereal Products, and Mixed Feeds

1. Whole Grains. Sampling whole grains presents no problems peculiar to vitamin analysis. Sampling technics developed for proximate analysis will therefore apply (*2,3*). The sample must be ground before weighing out a portion for extraction, or extraction will not be complete. Overheating during grinding must be avoided since it induces vitamin loss. Burr mills and hammer mills have been found satisfactory when properly used. Sieving the ground sample should be avoided to prevent fractionation of the grain.

2. Uncooked Cereals. Products such as farina, corn meal, corn grits, and other granular uncooked cereals may derive at least a portion of their vitamin content from enrichment. When the enriching material is in granular form, a primary sample of at least one pound should be ground and thoroughly mixed to improve the distribution of the enriching material before the secondary sample is taken. When the enriching material is in powder form in packaged cereals, special precautions should be taken to include enrichment powder adhering to the inside of the container when the cereal is removed for sampling. In products of this type, the primary sample should consist of at least four ounces, from which two to five grams are weighed for analysis after proper grinding and mixing.

3. Ready-to-Eat Cereals. At least part of the vitamin content of most ready-to-eat cereals is enrichment which may be added to the grain early in the process or sprayed on the product just before packaging or both. Thiamine is particularly apt to be sprayed because of its instability at the high temperatures encountered in cooking and toasting. The distribution of added vitamins, particularly those sprayed on, is frequently far from uniform. For this reason, a primary sample of at least eight ounces is advisable. If a composite primary sample is to represent a large lot, each portion going into the composite should comprise at least eight ounces.

4. Flour and Mixed Feeds. As with the whole grains, standard technics have been evolved for sampling for proximate analysis. These are equally satisfactory for vitamin analysis (*2,5*). After the primary sample has been

obtained by one of the official procedures or by geometric sampling, reduction in size to a secondary sample is usually sufficient, since the production of these materials makes them somewhat more homogeneous than most other natural products.

5. Baked Goods. Standard sampling technics which have been evolved for bread are generally satisfactory for baked goods (*2*). Vitamin analyses pose no special sampling problems except that the difference between crust and crumb in vitamin content may be more marked than it is with respect to other analytical factors. This means that before the primary sample is drawn, careful thought must be given to just what the analysis is to represent.

D. Fruits and Vegetables (*19–23*)

1. Fresh Materials

(a) *Small types*—berries, corn, peas, etc. In the sampling of raw material composed of small heterogeneous parts, the size of the primary sample will be controlled by the size of the lot of raw material which the sample is to represent. Mix this primary sample thoroughly without damaging the individual particles. Then, by halving or quartering, divide and sub-divide until a portion suitable for grinding or blending (usually 200–300 g) with the extractant remains. Blend the sub-divided sample with the desired extractant or stabilizing medium and withdraw the assay aliquot from the resulting slurry.

Occasionally it may be necessary to store the slurry for several days prior to actual assay. Storage is not recommended in ascorbic acid work but is acceptable when carotene and B-complex determinations are involved (*24*). In the case of carotene, prepare the slurry by blending the sub-divided sample with 1% alcoholic KOH, followed by storage at refrigerator temperatures. In the case of B-complex work, add 1 ml chloroform to the mixture of the sub-divided sample and extractant, usually 0.2 *N* H_2SO_4, during the blending. Place the blended sample or slurry into a storage container and cover the surface of the slurry with a thin layer of toluene to further inhibit microbiological growth. Samples of this type should be stored at refrigerator temperatures, also. By heating the container and sample in flowing steam for 10 minutes before withdrawing the assay sample, it is possible to minimize the introduction of toluene with the sample.

(b) *Large types*—apples, tomatoes, cabbage, carrots, etc. Raw materials composed of large heterogeneous units are sampled by selecting a large number of individual units to compensate for unit to unit variation. The

number of units to be selected will be controlled by variations within each product due to size and maturity. This will, of necessity, have to be predetermined for each product and vitamin. Since the volume of this primary sample is generally too large to grind and mix in available laboratory equipment it is necessary to withdraw portions from each unit of the primary sample. The most satisfactory means of sub-sampling each unit of the primary sample is to take sectors from opposite sides, thereby reducing the effect of variability within that unit to a minimum. Because of the likelihood of enzymatic action at the cut surfaces of these sectors, they should be immersed in the appropriate extractant or stabilizing medium as soon as cut. The size of these sectors will have to be such that all of the sectors may be mixed or blended in available laboratory equipment. From mixtures or slurries so obtained, the aliquot for analysis may be withdrawn.

(c) *Leaves and shoots*—spinach, asparagus, broccoli, etc. The problem of taking the primary sample for such products is similar to that described in the previous paragraph. In sub-dividing, it is essential to take proportionate parts of each unit and proportionate amounts of each type of tissue in each unit. This sampling may be accomplished by breaking or cutting the desired portions from each unit. Again one must inhibit enzymatic changes that may occur at cut surfaces by immersing the sample in a stabilizing medium.

(d) *Special Problems*—When bruised, finely cut, or hot samples are assayed, conditions are especially favorable for oxidative or enzymatic destruction of vitamins. In such cases, losses may be minimized by placing the primary sample directly into a stabilizing medium and blending or mixing the entire sample. The efficiency of the stabilizing medium may be increased by chilling. Where ice is used, allowance must be made for the change in volume due to thawing of the ice if the sample preparation is carried out on a volumetric basis.

As an alternative treatment for arresting enzymatic or oxidative actions, place the entire primary sample into a suitable receptacle containing a quantity of dry ice (solid CO_2). Rapid freezing of the product is effected by surrounding the sample and container with dry ice. If the sample is kept in a frozen state subsequent subdivision, under freezing conditions, can be carried out before the final mixing or blending with the desired extractant or stabilizing medium.

2. Canned Materials. In preparing a sample of a canned fruit or vegetable the problem is somewhat simplified. A sample representative of the food

under examination may be obtained by combining the contents of six consumer-size cans of small type, leafy or shoot materials, or 12 consumer-size cans of large type materials. If No. 10 or larger size cans are involved, combine 1 to 3 cans. Sampling may be facilitated by determining the proportion of solids to liquids. Empty the contents of each can onto a copper-free screen (tin, stainless steel, aluminum, etc.) suspended over a pan and allow a short drainage time (usually 30 to 60 seconds). Combine solids of all cans and weigh. Combine the liquid from all cans and weigh. Thoroughly mix each fraction taking particular care not to rupture the skin of the solid particles before weighing the final sample for blending. Take proportionate amounts of solids and liquid and mix or blend with the desired extractant or stabilizing mdiuem. Withdraw aliquots for analysis from the resultant slurry. More detailed instructions may be obtained from the literature (*19–23*).

E. Blood and Urine

1. Blood. Take the sample from a vein by means of a dry sterile syrin after sterilization of the skin with 70% alcohol. Transfer the blood i mediately to a small bottle containing enough potassium or ammoniu oxalate to give a final concentration of 0.1 to 0.2%. Rotate the tube gentl for several minutes to assure complete solution of the oxalate and store th sample in a refrigerator.

Ascorbic acid may be determined in the whole blood or plasma. T analysis of whole blood should be undertaken within 4 hours; plasma sho be analyzed immediately after separation from the cells by centrifugati The carotene and vitamin A of the plasma will remain unchanged several days, provided the sample is kept cold and protected from light.

2. Urine. The vitamins, like all other normal urinary constituents, ar excreted at a definite rate which generally is independent of urine volum but which is proportional to the concentration in the plasma. The deter mination, therefore, is of little value unless the period of time over whic the sample was collected is known. In other words, it is more important to know the rate of excretion per minute, hour or day, than the concentration per ml. In most nutritional investigations, two procedures have been used: (a) the fasting rate of excretion for a definite period, say, from 6 to 8 a.m., which is related to the vitamins in the tissues and plasma; (b) the 24-hour rate of excretion, which is related to the vitamin intake in the daily diet.

Many methods of collection have been suggested, depending upon the vitamin which is to be determined. Some vitamins, like pantothenic acid and folic acid, are sensitive to acids and must be preserved by refrigeration; others, like thiamine and riboflavin, can be collected and are stable many days in bottles containing enough HCl to give a final concentration of 0.1 *N*. The determination of ascorbic acid requires refrigeration of the sample and collection in a mixture of hydrochloric and metaphosphoric acids, preferably in bottles from which the air is displaced with carbon dioxide, and immediate analysis after the collection is completed.

The following method is generally applicable to all water-soluble vitamins (*18*): Collect the 24-hour sample in a one-gallon amber glass jug which is kept in the refrigerator at all times during the collection period. A smaller bottle may be used for shorter periods of collection. If ascorbic acid is to be determined, introduce a few pieces of dry ice into the bottle before collection, and add small portions of dry ice after each voiding. Measure and record the volume of urine. Ascorbic acid must be determined immediately. The other vitamins should be determined within 24 hours, or, if this is not possible, an aliquot portion should be transferred from the measuring cylinder into a small bottle and immediately placed into a deep-freeze unit.

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CHAPTER 2

Vitamin A

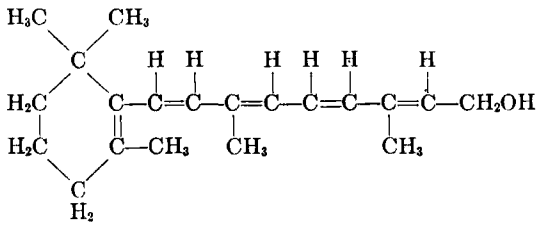
PRELIMINARY CONSIDERATIONS (*1–3*)

The existence of vitamin A was first recognized through the research of McCollum and Davis (*4–6*) and of Osborne and Mendel (*7,8*), on the basis of observations that a factor occurring in certain fats is essential for the growth of rats. This factor was named "fat-soluble A" to distinguish it from the "water-soluble B" factor.

It has been demonstrated that all vertebrates require vitamin A. A dietary deficiency may result in night blindness, poor growth, atrophy of epithelial tissues and tissues of the eye. Excessive amounts of vitamin A have been shown to be toxic (*9,10*).

Vitamin A occurs naturally in animals only and may be isolated from the unsaponifiable fraction of their fats. It is present in highest concentration in the liver, although other tissues may contain significant amounts. The most important natural source is fish liver oils, some of which may contain as much as 700,000 U.S.P. units of the vitamin per gram. The vitamin A activity of plant materials, which may be high, is due to their content of certain carotenoids which act as provitamins A.

The chemical constitution of vitamin A has been established mainly by the work of Karrer and associates (*11,12*). They suggested the following formula which was later confirmed (*13*):



Another form of vitamin A, designated "neo-vitamin A" appears to be reasonably well established. It is a stereoisomer of vitamin A and pos-

sesses chemical properties like the latter, thus giving the same response in physical and chemical determinations. Its biological activity is the same as that of vitamin A and it occurs to the extent of some 30 to 40% of the total vitamin A present in most fish oils.

The vitamin occurs in nature predominantly in an ester form rather than as the free alcohol (*14,15*). The esters are believed to be more stable than the free alcohol (*1*). Vitamin A [probably to the extent of 95% or more as esters (*16*)], and neo-vitamin A, are the only forms occurring in salt water fish and mammals, but there is evidence that tissues of fresh water fish contain, in addition, a substance provisionally designated as vitamin A_2 (*17–21*). This latter possesses considerable vitamin A activity as shown by biological assays on depleted rats and is stored in the livers of these animals as vitamin A_2 (*22*). Vitamin A_2 may be differentiated from vitamin A and neo-vitamin A by differences in the absorption spectra of their pure solutions and of their antimony trichloride reaction products (*17,20*).

The following properties of vitamin A have been established:

Crystalline form	Pale yellow needles
Empirical formula	$C_{20}H_{30}O$
Molecular weight	286
Boiling point	120–125° C at 5×10^{-3} mm (*23*)
Melting point	63–64° C (*24*)
Solubility	Soluble in most organic solvents and fats. Insoluble in water
Optical activity	Absent
Absorption maximum—vitamin A	325–328 mμ (*25*)
—vitamin A_2	345–352 mμ
Absorption maximum of $SbCl_3$ reaction product	
—vitamin A	620 mμ
—vitamin A_2	691 mμ
Fluorescence	Characteristic green in ultraviolet light (*26–28*)

Vitamin A is destroyed by ultraviolet light and is sensitive to oxidation in air. Naturally occurring vitamin A is stable to heat in an inert atmosphere and also in alkaline solutions, but crystalline preparations are not stable to heat (*29*).

An important characteristic of vitamin A is its ability to form with antimony trichloride a transient blue-colored reaction product having an absorption maximum at 620 mμ. Other inorganic chlorides and several other types of reagents have been used to produce a color for the measure-

ment of vitamin A (*30–36*), but antimony trichloride gives the closest correlation between depth of color and concentration of the vitamin and is the reagent most widely used at present. The reaction, however, is not specific for vitamin A, since some substances, especially carotenoids and sterols, may yield interfering colors or inhibit color development.

In 1934, The Permanent Commission on Biological Standardization of the Health Organization of the League of Nations defined the International Unit (I.U.) of vitamin A as the biological activity of 0.6 microgram (0.0006 mg) of standard β-carotene. In the United States the reference standard, supplied by the Vitamin Advisory Committee of the United States Pharmacopoeia, is a cod liver oil which was originally calibrated against β-carotene, so that the U.S.P. unit was equivalent to the I.U. However, since that time, there have been a number of indications that the two units are not identical. Apparently, there is a considerable difference in growth-promoting activity between the U.S.P. unit of vitamin A as supplied by U.S.P. Reference Oil No. 2 and the I.U. of β-carotene, the former being some 30% smaller than the I.U. according to Callison and Orent-Keiles (*37*). The introduction of U.S.P. Vitamin A Reference Oil No. 3 may diminish the differences.

By definition, the I.U. and the U.S.P. unit are both biological, but inasmuch as chemical and physical methods now predominate in the assay field, a means for interpreting these results in terms of the biological units is necessary. The values may be correlated by (*a*) the rat-growth biological assay of the sample in units per gram against the recognized standard and (*b*) the determination of the extinction coefficient for a 1% solution of the material measured through a 1-cm light path at a wave length of 328 mμ. Division of (*a*) by (*b*) gives a so-called "conversion factor" which is then used to convert physical assay results to biological units, thus:

(*a*) Biological assay	3,000 I.U. per g
(*b*) $E^{1\%}_{1\ cm}$ 328 mμ	1.5
Conversion factor	3,000/1.5 = 2,000

Actually, conversion factors determined in different laboratories differ markedly, presumably because of variations in the bioassays or variations from one type of oil to another. The average accepted for commercial use is 2,000 (*38*). Similar technics may be used to "standardize" the blue-color method of assay.

Two separate biological assays of crystalline vitamin A against crystalline β-carotene gave an average just under 3,000,000 I.U. per gram (*39*). Underhill and Coward (*40*) assayed two esters of vitamin A against β-carotene and arrived at figures, calculated in terms of vitamin A alcohol, of 3,181,000 and 3,424,000 I.U. per gram and their figure for the β-naphthoate was 2,225,000 I.U. per gram. As determined against U.S.P. Reference Vitamin A Oil, however, the biological potency of pure, crystalline vitamin A alcohol is 4,300,000 U.S.P. units per gram. Baxter and Robeson (*41*) obtained a potency of 3,400,000 U.S.P. units per gram for the vitamin A β-naphthoate when using U.S.P. Reference Vitamin A Oil as a standard. Recent work indicates approximately 3,500,000 units per gram to be the most acceptable figure now available for crystalline vitamin A.

This extremely confused situation may be clarified with the advent of crystalline vitamin A alcohol and crystalline vitamin A esters. These can be prepared in a high state of purity, they are stable under suitable storage conditions and their physical properties are easily measured for accurate checks of their chemical identity. It should then be possible to express vitamin A potencies as micrograms (γ) per gram of sample material (or milligrams per pound). At the present time, the Vitamin Advisory Board of the United States Pharmacopoeia is planning collaborative work relative to the adoption of a new vitamin A standard.

Methods Available

The curative rat-growth assay is inherently one of the most difficult of all bioassays, requiring skilful attention throughout its 6- to 8-week duration and special care in calculations and interpretations of data. Nevertheless, its use is essential in determining the biological availability of carotenoids and of vitamin A from different sources. Since it is tedious, time-consuming and expensive, and especially since the results are liable to considerable variation, the method is not adaptable to routine assays, but must be reserved for occasional check runs and for standardization of the chemical methods. It should be pointed out that, heretofore, the *accuracy* of the more precise chemical and physical methods has been limited by that of the bioassay, since the former were based upon the latter as a standard. With the availability of pure vitamin A, the accuracy of the chemical and physical methods will be improved, but bioassays will still be needed to determine biological availability. The basis of the present official method is presented in U.S. Pharmacopoeia XII.

Of the chemical methods, the most widely used is the Carr-Price reaction (*30*) adapted for photoelectric measurement of the blue color formed when vitamin A is treated with antimony trichloride. Though less precise than ultraviolet absorption methods, blue color assays are more specific and more sensitive and, for these reasons, have wider applicability. The reproducibility of the method is from 3 to 10% depending upon the product tested. The chief disadvantages of this procedure are: the rapid fading of the blue color resulting from the reaction, necessitating speed and experience for the determination of its maximum intensity; the corrosive nature of the antimony trichloride necessitating careful handling; its extreme sensitivity to even minute amounts of moisture and the production of atypical colors, or inhibition of color development due to the presence of various sterols, carotenoids and other interfering materials.

Spectrophotometric procedures of vitamin A assay, where applicable, have the advantages of being rapid, precise, and if saponification and extraction are unnecessary, of requiring no reagents other than the diluent. The reproducibility of this method may be considered to be 2% or better. On the other hand, there exists the danger that compounds other than vitamin A may contribute to the absorption measured at 325–328 mμ. While interfering substances may be diluted out or removed by saponification in some cases, sufficient concentrations may remain to give false vitamin A results. For some samples the specificity of the spectrophotometric procedure has been increased by the application of a blank obtained by destructive irradiation of the vitamin A in solution. This technic is based on the theory that only vitamin A is destroyed during the irradiation (*42*) but the validity of this assumption must be ascertained for each product tested. Chromatography is also being used either for purification of the vitamin A solution (*43–45*) or for the preparation of a vitamin A-free blank (*71*).

The agreement between values obtained spectrophotometrically and colorimetrically is usually within 3% for high-potency fish liver oils (50,000 U.S.P. units per gram and over) and concentrates exhibiting good spectrophotometric extinction ratios [see Ultraviolet Absorption Method, D-3-(b)-(2)].

However, for low-potency oils, the deviation between the two methods may range as high as 15%. Usually, when spectrophotometric and colorimetric results differ, the values obtained by biological assays approximate the latter rather than the former (*25,46*).

COLORIMETRIC METHOD (CARR-PRICE BLUE COLOR) (*47,48*)

A. Principle (*30*)

This method is based on the measurement of the unstable blue color formed by the interaction of vitamin A and antimony trichloride. The optical density of this blue solution at 620 mμ is a linear function of the concentration of vitamin A within certain limits.

B. Equipment

To prevent destruction of vitamin A by light during sampling and analysis, amber or nonactinic glassware is recommended throughout unless the analyses are carried out under subdued light (*49*). It is convenient to make a series of assays at one time, and the following requirements for equipment are based on six assays carried out simultaneously.

For Preparation of the Sample via Saponification

No. of pieces	*Item*
6	*Flasks*, 250 ml, with ground glass joints fitted to air- or water-cooled condensers, for saponification. In lieu of these, ordinary 250-ml Erlenmeyer flasks may be used in conjunction with cold finger condensers. It is also possible to dispense with condensers as a part of the saponification apparatus if adequate precautions are taken to maintain a constant volume of alcohol during the saponification procedure.
12	*Separatory Funnels, Squibb* (pear-shaped), 500-ml capacity. The lubricant used for the stopcocks should not be soluble in ether nor contain ether-soluble substances which absorb ultraviolet light. A suitable preparation is the following: "Suspend 9 g of soluble starch in 22 g of glycerol and heat to 140° C. Let stand one-half hour and decant. Cool and let stand overnight until it acquires the consistency of a heavy grease" (*50*). Nonaq stopcock grease (Fisher Scientific Co.) may also be used.
6	*Flasks*, 250 or 500 ml for solvent removal. The size to be used depends upon the volume of solvent to be removed.
—	*Glass-Stoppered Volumetric Flasks*, several sizes, for dilution purposes.
—	*Volumetric Transfer Pipets*, assorted sizes, especially 1, 2, 5 and 10 ml.
1	*Hot Plate*, or other suitable device, such as a steam bath, for heating during saponification and for use in solvent removal. Heat must be subject to control.
1	*Vacuum Pump or Filter Pump* (aspirator) with vapor trap for solvent removal. Optional.
1	*Cylinder of Nitrogen.*
6	*Glass Funnels*, 6 cm, or sintered glass plates.

For Assay

No. of pieces	*Item*
1	*Rapid Delivery Pipet*, for addition of the $SbCl_3$ reagent. A 9- or 10-ml pipet may be used at the discretion of the analyst, the only requisite being that its delivery time be very short (1 to 2 seconds). An all-glass automatic pipet is recommended (*51*).
1	*Photoelectric Colorimeter or Spectrophotometer.* This method presupposes the use of an instrument which exhibits a directly linear relationship between galvanometer readings and intensities of light in the region of 620 mμ, and within certain limits of concentration. Either filters or a simple monochromator may be used for the isolation. Filters have the advantage of simplicity and economy while monochromators afford a better isolation of the desired spectral region. Any instrument capable of making readings at 620 mμ and whose galvanometer registers maximum color formation within 3 to 6 seconds after the addition of the $SbCl_3$ reagent may be used. A constant light source is essential. This can be provided either by a voltage regulator or a storage battery with charger. It is convenient to have the colorimeter tube project above the top of the instrument, thus allowing the addition of the reagent while the tube is actually in place in the path of the light beam. Among other instruments, the Evelyn Photoelectric Colorimeter (The Rubicon Co., Ridge Ave. at 35th. St., Philadelphia 32, Pa.), the Coleman Model 11 Universal Spectrophotometer (Coleman Electric Co., Maywood, Ill.) and the Klett-Summerson Photoelectric Colorimeter (Klett Manufacturing Co., New York City, N.Y.) have found wide application.

C. Reagents

All reagents should meet A.C.S. specifications or be of Reagent Grade unless otherwise indicated.

1. Alcoholic Alkali. Dissolve 12 g of KOH pellets in 100 ml of ethanol (S.D. No. 30 is satisfactory). Prepare fresh daily. See also D-1-(b)-(1).

2. Diethyl Ether. Henceforth, the term ether refers specifically to diethyl ether. This must contain a minimum of peroxides. To test for peroxides, add 5 ml of a mixture of equal volumes of 50% KI solution and 1% alcoholic phenolphthalein to 20 ml of ether. Shake. A red color indicates the presence of peroxides. To remove the peroxides, wash the ether with aqueous $NaHSO_3$ solution.

3. 0.5 *N* Potassium or Sodium Hydroxide Solution. Dissolve 28 g of KOH or 20 g of NaOH in sufficient water to make 1 liter of solution.

4. Anhydrous Sodium Sulfate, Granular. This should be checked to determine that it retains no vitamin A as follows: shake several g of

Na_2SO_4 (anhydrous) with 50 to 100 ml of ether containing 100 U.S.P. units of vitamin A. Decant the ether as completely as possible and wash the Na_2SO_4 several times with 10- to 15-ml portions of ether. If the washed Na_2SO_4 does not turn blue when treated with 2 or 3 ml of a 25% solution of $SbCl_3$ in chloroform, it retains no vitamin A. Lots of Na_2SO_4 which give a blue color should be rejected.

5. Phenolphthalein. Dissolve 1 g of phenolphthalein in sufficient ethanol to make 100 ml.

6. Chloroform. This should be free of moisture and phosgene gas. The latter forms especially in redistilled chloroform on standing and results in destruction of vitamin A. The chloroform may be dried by allowing it to stand over anhydrous Na_2SO_4 or $CaCl_2$.

7. Antimony Trichloride Reagent. *Care! Corrosive Agent.* Weigh an unopened bottle (¼ pound) of $SbCl_3$. Open the bottle and empty the contents into a wide-mouth, glass-stoppered amber bottle containing approximately 100 ml of chloroform. By difference, obtain the weight of $SbCl_3$ and then add sufficient chloroform to supply 100 ml for each 25 g. Dissolve by warming or shaking for several hours and filter or decant into a clean, dry, amber bottle with ground glass stopper. This solution may be stored at room temperature but should be kept in the dark when not in use. The reagent is apparently stable for long periods of time, but it is convenient to make up sufficient amounts to last for one month. Rinse all glassware coming in contact with this reagent with chloroform, a mixture of ethanol and ether or dilute or concentrated HCl before washing, since SbOCl which forms is insoluble in water.

D. Procedure

1. Saponification

(a) Weigh a representative assay sample directly into a saponification flask.

(1) Other fat-soluble substances occurring with vitamin A interfere with its determination, making some purification necessary, especially in the case of low-potency materials. This is accomplished by saponification and extraction, the vitamin A remaining in the unsaponifiable fraction (*25*).

(2) Materials of high potency (over 10,000 units per g) frequently may be assayed directly, i.e., without saponification, especially if an increment calculation is used. Such a sample may be weighed directly into a volumetric flask and diluted with chloroform, as indicated in step 3-(d). In cases of doubt as to whether this direct technic is applicable, the values should be checked by the regular method.

(3) *Preparation of representative assay sample.* Experience has indicated that widely varying technics are employed in obtaining the representative samples used for vitamin A assay purposes. Wherever possible, mix the entire sample thoroughly and remove representative portions for analysis. Oils should be warmed to room temperature before mixing since sterols and vitamin A may settle out at refrigerator temperatures. The sample weight to be taken varies with the type of product under study, i.e.:

For fish oils, weigh a sample of approximately 1 g to the nearest mg for the saponification procedure or at least 100 mg to the nearest 0.1 mg for reading by direct dilution. The sample may be weighed directly onto a small portion of a clean, fractured cover glass, into a small glass cup, or on a wire helix. These must be small enough to permit insertion directly into the saponification or other flask along with the adhering sample portion. It is possible to weigh by difference either from the original container or into the receiving vessel in some instances (*52*).

For pills and tablets, obtain the average weight per unit by weighing 10 to 20. Grind these to a fine powder in a mortar, mix well and weigh a 1-g sample for assay. If the assay is needed on a per tablet basis, dissolve (or extract) a representative number, dilute to a known volume and take an aliquot.

For gelatin capsules, take 5 whole capsules for test. Dissolve the gelatin with a small amount of hot water prior to the addition of alcoholic alkali for saponification. As an alternative procedure, weigh 10 capsules, open them and extrude the contents for analysis. Wash the gelatin capsules free of adhering material with petroleum ether and weigh the dry shells. The difference between the weight of the capsules and the weight of the shells represents the weight of the contents. See also above note for pills and tablets.

For food products, accurately weigh a sample estimated to contain at least 200 U.S.P. units of vitamin A. For products such as butter, cheese and fortified margarine this usually will be 5 to 10 g. As an alternative procedure for butter and margarine, obtain a clear oil by liquefying the sample in a 45 to 50° C incubator or water bath and filtering through Whatman No. 1 filter paper. Determine the vitamin A content of the oil and calculate the potency of the original sample after a determination of its fat content. In the case of milk, use 50 to 100 ml. Extract the fat and carry out the vitamin A assay on the saponified fat (*53,54*). Products containing starch or sugar should be dissolved in a minimum amount of hot water prior to saponification.

For vitamin A-fortified feed concentrates, weigh a finely ground sample containing not less than 200 U.S.P. units of vitamin A into an extraction thimble and extract for 2 hours with ether in an extraction apparatus, evaporate the ether and saponify the residue. Should this be a premix of sufficiently high potency, it may be saponified directly.

(b) Add a volume of alcoholic alkali which will supply an amount of KOH approximately equal to one-half the weight of the assay sample. Use at least 15 ml of alcoholic alkali.

(1) If desired, the alcohol and alkali may be added separately, the latter either as solid KOH pellets or as a 50% aqueous solution (50 g of KOH dissolved in sufficient water to make 100 ml). This solution keeps indefinitely.

(c) Heat under gentle reflux for at least 15 minutes, or until saponification is complete.

(1) The time required for complete saponification varies with the nature of the product. In general, those materials containing long-chain fatty acids will require a longer saponification time. For most samples, 15 minutes is adequate, but there is some evidence that saponification for longer periods of time exerts no detrimental effect on the content of vitamin A. Test for completeness of saponification by adding a small amount of water and shaking. If cloudiness appears, the reaction is incomplete and the sample should be refluxed for an additional period of time. However, if unusually high amounts of unsaponifiable material are present, cloudiness may occur even though saponification is complete. Occasionally, the soaps produced during saponification will solidify, but solution results upon the addition of water.

2. Extraction

(a) Wash the reflux condenser with about 10 ml of water.

(b) Cool the saponified mixture to room temperature, add 50 to 100 ml of water and transfer to a separatory funnel.

(1) If the volume of alcoholic alkali used in 1-(b) is minimal, the amount of water added may be reduced accordingly, although larger amounts of water apparently cause no difficulty in later steps.

(c) Rinse the saponification flask with a volume of ether equal to at least twice the volume of alcoholic alkali used in saponification, but not less than 50 ml. Add to the separatory funnel in (b) above.

(1) If the materials remaining in the saponification flask are of a sticky nature (candy, etc.) the saponification flask should be rinsed with water prior to the first ether extraction and these rinsings included in the first separatory funnel.

(d) Shake the separatory funnel cautiously, opening the stopcock at intervals to release pressure. Allow the phases to separate completely.

(1) Emulsions may occur upon too vigorous shaking at any stage in this extraction and the subsequent extraction and washing procedures or if the proportions of ethanol, water and ether are incorrect. Should an emulsion form, a few milliliters of alcohol may break it. If this proves ineffective, the addition of water may be successful.

(e) Draw off the lower (aqueous) phase directly into a second separatory funnel and let the ether extract remain in the first.

(1) It is also possible to carry out the ether extractions with the use of only one separatory funnel per sample. In this case, drain the aqueous phase into the flask used for saponifying the sample and then place the ether solution in a 500-ml flask.

Return the aqueous portion to the original separatory funnel and re-extract, separating the layers as before and adding the ether extract to the first portion in the 500-ml flask. Repeat this procedure one to three times more, then discard the aqueous phase and place the combined ether extracts in the separatory funnel for the subsequent washing procedure.

(f) Again rinse the saponification flask with 35 to 50 ml of ether and add the rinsings to the second separatory funnel.

(g) Repeat (d) above.

(h) Draw off the lower (aqueous) phase into a flask and add the ether extract to that in the first separatory funnel, in (e).

(i) Return the contents of the flask from (h) above to the second separatory funnel.

(j) Re-extract this material 3 more times with 35- to 50-ml portions of ether, adding all ether extracts to the first separatory funnel. Discard the last aqueous layer removed.

(1) Experience with some products has indicated that fewer than 5 ether extractions are necessary. If many assays are to be made, it may be time saving for the analyst to determine the minimum number of ether extractions necessary for removal of all the vitamin A.

(k) Pour 50 to 100 ml of water through the combined ether extracts in the first separatory funnel, without shaking. Draw off and discard the lower (aqueous) phase.

(l) Add 50 ml of 0.5 *N* NaOH or KOH solution to the ether extracts and shake the mixture gently (*55*).

(1) The alkaline wash is used to ensure the removal of acid soaps which are ether-soluble (*56*). This step is omitted by some operators. It has been suggested that the use of tap water accomplishes the same purpose.

(m) After complete separation of the layers, draw off and discard the aqueous phase.

(n) Continue the washing by shaking gently with 50-ml portions of water until the washings are free of alkali as determined by testing with phenolphthalein. This may require as many as 5 to 8 portions.

(o) After removal of the final water wash, allow the ether extract to stand for 10 minutes and carefully draw off any separated water.

3. Solvent Removal

(a) Filter the ether extract into a 250- or 500-ml flask through several g of anhydrous Na_2SO_4 distributed evenly on a filter paper in a glass funnel or on a sintered glass plate.

(b) Rinse the separatory funnel and Na_2SO_4 with two 25-ml portions of ether, adding the rinsings to the flask in (a).

(c) Place a glass bead in the flask and evaporate the ether to dryness on a water bath in a hood, removing the flask from the source of heat during evaporation of the last few ml of solution. The ether removal may also be accomplished by heating the solution on a steam bath with the concurrent introduction of a stream of nitrogen until all of the ether is removed.

(1) The evaporation may be carried out with the use of suction only. If nitrogen gas is used with heat for the solvent removal, or along with vacuum, the introduction of the gas may be delayed until a layer of ether about ⅛ inch deep remains in the flask. The remaining ether can then be evaporated with the flask removed from any source of heat, thus ensuring the maintenance of the residue in an inert atmosphere until the final solvent addition is made. Carbon dioxide gas may be used but often times its use results in turbid $SbCl_3$ reaction products.

(d) *Immediately*, take up the residue in chloroform to a concentration between 7 and 15 U.S.P. units vitamin A per milliliter.

(1) The final solvent addition must be made *immediately* after removal of the ether is complete to prevent possible oxidation of vitamin A. *This is a very sensitive point.*

(2) The concentration indicated (7 to 15 U.S.P. units per ml) here applies to both the Evelyn Photoelectric Colorimeter and the Coleman Model 11 Universal Spectrophotometer. If other instruments are used, suitable adjustments must be made in this dilution so that readings may be taken in the region of greatest accuracy, i.e., within a range of approximately 30 to 70% transmission (*39*).

(3) For routine assays where only small amounts of unsaponifiable material remain, the dilution may be accomplished by adding a measured amount of chloroform directly to the evaporation flasks rather than by transferring to volumetric flasks and diluting to mark. Thus, for a dilution to 10 ml, the addition of 10 ml of chloroform from a pipet will be sufficiently accurate for control purposes, although the chloroform plus the unsaponifiable matter may total 10.1 or 10.2 ml.

(4) If the sample, especially dairy and animal products, contains biologically active carotenoid pigments in addition to vitamin A, take up the residue in petroleum ether. Use an aliquot of this solution for determination of the biologically active pigments as described under D 2, 3 and 4 of the Chromatographic Carotene Method. However, if it is known that the major portion of the pigment present is β-carotene, chromatography may be dispensed with. Take up the residue directly in chloroform and measure spectrophotometrically the carotene in that solvent. Evaporate another aliquot of the petroleum ether extract to dryness and take up the residue as in 3-(d) and continue as in 4-(a).

4. Reading of the Unknown by Increment (*51*)

The increment technic corrects for the presence of substances in the unknown which may modify the intensity of the blue color but can *only* be used with those instruments giving a linear response over the assay range.

(a) Introduce 2 ml of chloroform into a colorimeter tube, place in the instrument and add 9 ml of $SbCl_3$ reagent from the rapid delivery pipet.

(b) With this solution in the path of the light beam at 620 mμ, set the galvanometer at full deflection, 10 or 100 depending upon the instrument.

(c) To a second colorimeter tube add 1 ml of the chloroform solution of the unknown and 1 ml of chloroform.

(d) Place this tube in the instrument in the path of the light beam and add 9 ml of $SbCl_3$ reagent from the rapid delivery pipet.

(1) The quantity of $SbCl_3$ reagent used is frequently increased to 10 ml. Any amount of reagent may be used *provided* the same amount is always used.

(2) Occasionally, a turbid solution occurs upon the addition of the $SbCl_3$ reagent, especially when the humidity is high. The addition of a drop of acetic anhydride prior to the addition of the reagent will usually prevent cloudiness although it may decrease the observed optical density by as much as 2%.

(e) Read the galvanometer at the pause point, obtaining the per cent transmission.

(1) The pause point refers to the point of minimum light transmission after the agitation of the mixture has ceased and should be reached in 3 to 6 seconds after addition of the reagent. The time required is influenced by the type of sample tested, concentration of the vitamin A, the intensity of the incident light beam and also by the degree of damping of the galvanometer used (*56,57*).

(2) If desired, each reading may be recorded as per cent transmission and referred to a standard curve plotted on semilogarithmic paper with per cent transmission on the log scale.

(3) If fading of the blue color is so rapid that a definite pause point cannot be read, reference should be made to literature on the subject of light effects (*57*).

(4) It must always be remembered that the blue color formed as a result of this reaction is unstable. It usually appears to be more stable with materials of high potency and/or high degree of purity. The presence of certain impurities may render the determination invalid, inasmuch as the colors formed as a result of the reaction may not be blue, but rather violet, reddish or even yellow or brown in color. Observations of such off-colors should be carefully noted.

(f) To a third colorimeter tube add 1 ml of unknown plus 1 ml of chloroform which contains an amount of vitamin A approximately equal to that contained in the unknown.

(1) There is no standard official preparation for this work. Either crystalline vitamin A (alcohol or ester form) or a highly purified oil (containing at least 200,000 U.S.P. units of vitamin A per g) which has been standardized with crystalline vitamin A must be used. Distillation Products Inc., 755 Ridge Road West, Rochester 13, N.Y., supplies a highly purified vitamin A concentrate suitable for calibration purposes. To prepare the standard solution weigh accurately at least 100.0 mg of the calibration oil. Place this in a volumetric flask and dilute to the mark with

chloroform. Calculate the potency per ml and dilute a portion sufficiently to give a final working standard of the desired potency—usually 8 to 10 units per ml. If kept cool and in the dark, such solutions deteriorate slowly, not over 5% per week. For critical work, however, a standard should be prepared on the day of its use.

(g) Using this solution repeat (d) and (e).

(h) Convert all transmission readings to optical density ($2 - \log G_{620}$) where G_{620} = galvanometer reading at 620 mμ.

(1) For convenience, the term "optical density" is used throughout this procedure, although it must be realized that true optical density can be measured only with sources of monochromatic light. The "L value," introduced by Dann and Evelyn (*48*) is an analogous expression.

(i) If the final chloroform solution of the unknown is colored, make a blank correction by subtracting the optical density of a solution of 1 ml of the unknown plus 10 ml of chloroform from the optical density observed in (e).

(1) Carotene, or provitamin A, also gives a blue color with $SbCl_3$ although it is of lesser intensity than that formed with an equal amount of vitamin A. In extracts where carotene occurs, correction of the blue color for that contributed by carotene should be made. In order to accomplish this correction, treat 2-ml aliquots of a series of concentrations of carotene ranging from 0 to 50 γ per ml of chloroform with $SbCl_3$ and plot the optical density against the concentration [see 4-(e)-(2)]. Calculate the amount of carotene present in the 1 ml of chloroform extract taken for the $SbCl_3$ reaction of the unknown in 4-(c) from the carotene assay of the sample as described in 3-(d)-(4). Employing the blue color curve for carotene, determine the optical density due to the concentration of carotene present in the 1 ml of unknown. Subtract this optical density from the optical density found in 4-(e), thus getting the optical density as corrected for carotene. If other colored materials are present as measured according to the directions for this step, corrections must be made for these also, as indicated. Carotene, *per se*, is almost transparent at 620 mμ and hence there is not a double correction for it.

5. Calculation

(a) Calculate the vitamin A content of the chloroform solution of the unknown from the following formula:

$$\text{U.S.P. units vit. A per ml unknown} = \frac{\text{corr. opt. dens. [(e) - (i)]}}{\text{opt. dens. (g) - opt. dens. (e)}} \times \text{concn. of stand. (U.S.P. units per ml)}$$

(b) Calculate the vitamin A content per g of sample from:

$$\text{U.S.P. units vitamin A per ml unknown} \times \frac{\text{final volume}}{\text{sample weight}}$$

(1) The following sample illustrates the calculations. 2 g of fresh liver were treated according to the above directions, the volume of the chloroform solution, 3-(d), being 25 ml. The per cent transmissions were determined in an Evelyn Photoelectric Colorimeter.

Step	Per cent transmission	Optical density
4-(e) 1 ml unknown + 1 ml chloroform + 9 ml reagent	65.0	2.000 − 1.813 = 0.187
4-(g) 1 ml unknown + 1 ml standard + 9 ml reagent (1 ml standard = 10.3 units)	48.5	2.000 − 1.686 = 0.314
4-(i) 1 ml unknown + 1 ml chloroform + 9 ml chloroform	99.0	2.000 − 1.996 = 0.004
4-(i) optical density of blue color due to carotene as determined from curve		0.041

Corrected unknown = 0.187 − 0.004 − 0.041 = 0.142

$$\frac{0.142}{0.314 - 0.187} \times 10.3 = \frac{0.142}{0.127} \times 10.3 = 11.5 \text{ units per ml chloroform solution}$$

$$11.5 \times 25/2 = 144 \text{ units per g liver}$$

For samples for which it has been established that interfering substances do not exist, it is possible to calculate the vitamin A concentration from a calibration curve. If this procedure is applicable, it is only necessary to determine the optical density of the blue color formed when 1 ml of unknown is treated with 9 ml of $SbCl_3$ reagent. The vitamin A concentration corresponding to this optical density may then be read from a calibration curve.

(c) Preparation of calibration curve

(1) Weigh accurately a sample of the calibration oil, 4-(f), and dilute sufficiently with chloroform to give a solution containing about 25 U.S.P. units per milliliter.

(2) From this latter, make a series of dilutions, in duplicate, with chloroform, to concentrations of 20, 15, 10 and 5 U.S.P. units per milliliter.

(3) Determine the optical density of the blue color formed when 1-ml aliquots of these solutions are treated with $SbCl_3$ reagent.

(4) Plot these optical densities against the corresponding known unitages of vitamin A. If desired, the actual galvanometer readings of per cent transmission may be

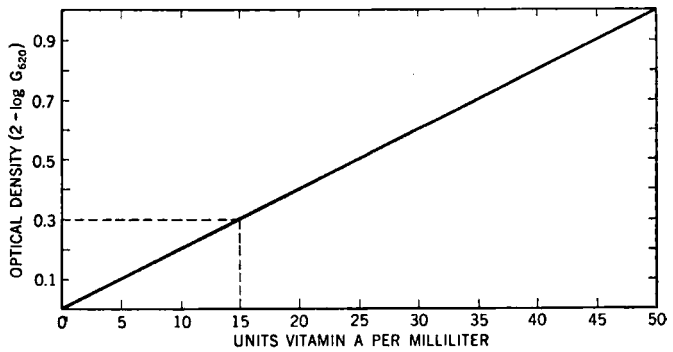


FIG. I. A Standard Curve for Vitamin A Determination.

plotted against known vitamin A unitages if semilogarithmic paper is used (plot per cent transmission on the log scale). Calibration curves should be checked at frequent intervals in order to minimize errors due to changes in reagents, instrument or manipulation.

An example of a standard curve for vitamin A determination is shown as Figure I on the preceding page.

Sample Calculation

Evelyn Photoelectric Colorimeter used.
Sample weight = 10 g.
Saponification, extraction and solvent removal made according to above directions, and the unsaponifiable residue diluted to 15 ml with chloroform.
Per cent transmission (1 ml unknown plus 9 ml $SbCl_3$ reagent) = 50.
Optical density (2 − log 50) = 0.301, equivalent to 15 units vitamin A per ml as determined from curve.
Therefore, 15 × 15/10 = 22.5 U.S.P. units per g.

ULTRAVIOLET ABSORPTION METHOD (*47,61*)

A. Principle

The method is based on the measurement of light absorption of the vitamin in solution. This light absorption is proportional to the concentration of the vitamin, is most consistent at that wave length (324–330 mμ) which is maximally absorbed by the vitamin and applies strictly only for monochromatic light (*62,63*).

B. Equipment

To prevent destruction of vitamin A by light during sampling and analysis, amber or nonactinic glassware is recommended throughout unless the analyses are carried out under subdued light (*49*). It is convenient to make a series of assays at one time, and the following requirements for equipment are based on six assays carried out simultaneously.

No. of pieces	*Item*
—	*Volumetric Flasks*, assorted sizes 25-, 50- and 100-ml capacity, the number of each depending upon the degree of dilution necessary.
—	*Volumetric Transfer Pipets*, assorted sizes including 1, 2, 5, 10 ml, etc., depending upon the degree of dilution necessary.
1	*Spectrophotometer or Spectrograph* (*61*). This instrument must be capable of measuring transmission at various wave lengths in the ultra violet region. The essential parts of such an instrument are a light source, a dispersing system for separation of the wave lengths of incident light into a spectrum, an optical train

for the purpose of focusing a light beam of chosen wave length on a sample contained in a quartz cell and also on a reference cell containing the solvent alone, and a device for comparison of the light intensities transmitted by the sample and the solvent. The resolving power of these instruments depends upon three conditions:

(a) The character of the dispersing system. The larger the prism or the finer the grating, the higher will be the dispersion of light by these elements.

(b) The absence of stray light. Stray light reflected from the surfaces of the optical parts must be screened off from the exit slit.

(c) The width of the exit slit. This is the wave length interval which the slit subtends on the spectrum.

The intensities of transmitted light in the ultraviolet region may be measured photoelectrically or with a photographic plate. Several types of photoelectric devices are available. The barrier layer cell with a thin coating of metal exposed to the source of light has the advantage of an electrical current output sufficiently large to measure with a galvanometer; however, it has a range of light sensitivity extending only slightly beyond the visible range of the spectrum. The phototube or photoelectric cell has a very small output, but this can be amplified. Some tubes have sensitivity ranges from 200 to 1,000 mμ. Instruments suitable for determining extinction coefficients of vitamin A, because of their high resolving power, freedom from stray light interference and range of sensitivity, include the following:

(a) *Spectrophotometers*, (1) Beckman Spectrophotometer, National Technical Laboratories, South Pasadena, Cal., (2) Hardy Recording Spectrophotometer, General Electric Corp., Schenectady, N.Y.

(b) *Spectrographs*, (1) Hilger-Spekker Quartz Photometer, Adam Hilger Ltd., 75A Camden Rd., London, N.W.1., (2) Gaertner Medium Quartz Grating Spectrograph, Gaertner Scientific Corp., 1201 Wrightwood Ave., Chicago, Ill.

Only medium-sized instruments are mentioned, but larger instruments with a greater focal length may be used. The advantage of a greater degree of linear dispersion by a larger optical system is not a requisite for accurate vitamin A determinations since the band width at the absorption maximum of this vitamin is so broad, i.e., about 2 mμ.

C. Reagent

1. **Propanol-2** (isopropanol) of spectrophotometric quality, as solvent.

D. Procedure

Theoretically, this method is applicable only to those materials which exhibit in the spectral region of 300 to 350 mμ a light-absorption curve typical of vitamin A, and in which the absorption at 328 mμ is due, almost entirely, to vitamin A. In general, the technic is applicable to *clear* fish oils containing over 10,000 U.S.P. units of vitamin A per g, to vitamin A concentrates, and to many pharmaceuticals. Some

fish oils of low potency may be assayed satisfactorily by ultraviolet absorption measurement provided they are saponified as indicated under the colorimetric method and the measurement made on the unsaponifiable residue.

Fortified products, such as margarine and animal feed concentrates may also be assayed by this procedure if unfortified samples of the ingredients are available for determination of a blank value (*51,64*). Ordinarily, however, food and feed products contain so much material absorbing light at 328 mμ that the method cannot be used. Various methods for the elimination of or compensation for these impurities have been suggested (*42–45*).

1. Preparation of Sample

(a) Weigh a representative sample and transfer to a 50- or 100-ml volumetric flask.

(1) *Preparation of representative assay sample.*

Experience has indicated that widely varying technics are employed in obtaining the representative samples used for vitamin A assay purposes. Wherever possible, mix the entire sample thoroughly and remove representative portions for analysis. Oils should be warmed to room temperature before mixing since sterols and vitamin A may settle out at refrigerator temperatures. The sample weight to be taken varies with the type of product under study, i.e.:

For fish oils, weigh a sample of approximately 1 g to the nearest mg for the saponification procedure or at least 100 mg to the nearest 0.1 mg for reading by direct dilution. The sample may be weighed directly onto a small portion of a clean, fractured cover glass, into a small glass cup or on a wire helix. These must be small enough to permit insertion directly into the saponification or other flask along with the adhering sample portion. It is possible to weigh by difference either from the original container or into the receiving vessel in some instances (*52*).

For pills and tablets, obtain the average weight per unit by weighing 10 to 20. Grind these to a fine powder in a mortar, mix well and weigh a 1-g sample for assay. If the assay is needed on a per tablet basis, dissolve (or extract) a representative number, dilute to a known volume, and take an aliquot.

For gelatin capsules, take 5 whole capsules for test. Dissolve the gelatin with a small amount of hot water prior to the addition of alcoholic alkali for saponification. As an alternative procedure, weigh 10 capsules, open them and extrude the contents for analysis. Wash the gelatin capsules free of adhering material with petroleum ether and weigh the dry shells. The difference between the weight of the capsules and the weight of the shells represents the weight of the contents. See also above note for pills and tablets.

(b) Make to volume with propanol-2.

(1) Different solvents affect not only the position of the absorption maximum but also the intensity of the absorption at a given wave length. The displacement is not large but is worth noting because of the present confusion between "maximum" and the usually quoted term "328 mμ" (*62,65,66*). The solvent most commonly used for commercial assays of vitamin A is propanol-2. Others used include cyclohexane, absolute ethanol, and petroleum ether. All solvents must be completely

dry and carefully purified so as to free them from substances destroying vitamin A (peroxides) and substances absorbing light in the 325–328 mμ region (aldehydes, benzene, etc.), (*25,42*). Distillation is usually sufficient, but treatment with norite or percolation through silica gel may be necessary occasionally.

(c) Dilute an aliquot from (b) with propanol-2 to a concentration between 5 and 15 units per ml.

(1) The most satisfactory optical density varies with the instrument used. With a Beckman Spectrophotometer, it is 0.3 to 0.6, but with a Hilger or Gaertner Quartz Spectrograph, densities of 0.9 to 1.3 are preferred (*67,68*). These optical densities of 0.3 to 0.6 and 0.9 to 1.3 correspond approximately to 6 to 12 and 18 to 24 units per ml respectively.

2. Measurement

(a) Fill one of the matched cuvets with propanol-2 and fill the other with the diluted solution from 1-(c).

(1) The cuvets must be scrupulously clean, making sure that no lint or grease is left adhering to the polished surfaces through which the light beam is to pass. It is advisable to use a pipet for filling cuvets, so as not to wet the outsides.

(2) If a fortified sample is being assayed with the unfortified product as the control, one of the cuvets may be filled with a propanol-2 dilution of the unfortified oil rather than pure propanol-2. The concentration of unfortified sample in this "blank" dilution must be exactly the same as the concentration of the fortified sample being assayed. It may then be assumed that the optical density measured will be due only to vitamin A, all interfering substances being cancelled out by the use of equal amounts in the blank and test solutions.

(b) Place the cuvet in the instrument and set the propanol-2 to zero optical density at 328 mμ.

(c) Take optical density reading of the test solution at 328 mμ.

(1) At the absorption peak of the vitamin A curve, the width of the band of light is about 2 mμ as measured on the Beckman instrument. The absorption of light at the wave length of maximum absorption obeys Beer's law, which, in the case of vitamin A, is expressed mathematically as follows:

$$d = \frac{\log I_o}{\log I} = k\,c\,l \qquad (1)$$

The terms of equation (1) are defined as follows:

- d = optical density or 2 − log per cent transmission
- I_o = intensity of incident light
- I = intensity of transmitted light
- k = a constant, characteristic for each wave length
- c = concentration of solute (vitamin A oil) in g per 100 ml
- l = length of the light path through the solution

Since k is a constant, it is possible to obtain a coefficient of light absorption ("extinction coefficient") in terms of per cent concentration or in terms of molar concentration, where the wave length of the light path through the solution is constant.

Thus, $E^{1\%}_{1\ cm}$ for a sample is the extinction coefficient obtained with light traversing a 1-cm distance through a 1% solution, or

$$E^{1\%}_{1\ cm} = k \times 1 \times 1 \qquad (2)$$

By substituting in equation (1) the value of k in equation (2), the following relation is obtained:

$$E^{1\%}_{1\ cm} = \frac{d}{c\ l} \qquad (3)$$

Equations (1) and (2) apply only when monochromatic light is used or at least where the resolving power of the instrument is as good as or better than the band width of light at the absorption maximum of the vitamin. True $E^{1\%}_{1\ cm}$ values can only be obtained with instruments (see B) which meet the above specification as to resolving power. However, measurements of vitamin A absorption may be made on instruments (e.g., Coleman Model 11 Universal Spectrophotometer, Coleman Electric Co., Maywood, Ill.) which have a lower resolving power, by referring to a calibration curve prepared from a series of standard vitamin A solutions and measured on the same instrument.

(d) Repeat all the operations of steps (a) (b) and (c) at 300 mμ and at 350 mμ (310 mμ optional).

3. Calculation

(a) Calculate $E^{1\%}_{1\ cm}$ 328 mμ $= \frac{d}{c\ l}$, where $E^{1\%}_{1\ cm}$ 328 mμ is the extinction value at 328 mμ, d is the optical density of the solution read at 328 mμ, c is the concentration of the sample in g per 100 ml and l is the length of the light path through the solution. Then, the potency in U.S.P. units per g =

$$E^{1\%}_{1\ cm}\ 328\ m\mu \times 2{,}000$$

(1) For the calculation of vitamin A concentration in the sample for which a true $E^{1\%}_{1\ cm}$ value has been obtained, it is necessary to utilize the following relation:

$$\text{U.S.P. units per g} = E^{1\%}_{1\ cm}\ 328\ m\mu \times \text{conversion factor}$$

Theoretically, the conversion factor may be obtained by determining the biological potency and the $E^{1\%}_{1\ cm}$ 328 mμ value of vitamin A. This yields a conversion factor of 2,460 for crystalline vitamin A alcohol and a slightly lower figure for crystalline vitamin A esters (*41*). However, the conversion factor for materials of relatively low potency may be quite different. For U.S.P. Reference Vitamin A Oil No. 2 (unsaponifiable fraction), the conversion factors reported average 2,000 in propanol-2 and slightly over 2,100 in cyclohexane (*63*). The lower figure for the U.S.P. Reference Vitamin A Oil as compared to crystalline vitamin A may be due to absorption of light by materials other than vitamin A. This results in higher E values and correspondingly lower conversion factors. The conversion factor for use with commercial vitamin A-bearing oils is generally accepted as 2,000 (*37,38,60,66*).

(b) Calculate the ratios of the optical densities at 300 mμ (310 mμ) and 350 mμ to that at 328 mμ. If these ratios exceed 0.73 (0.91) and 0.65,

respectively, the validity of the assay as a true measure of vitamin A is open to question (*46*).

(1) *Sample Calculation*

Sample weight = 0.1250 g

Diluted to 50 ml with propanol-2. 1 ml = 0.0025 g of sample

Further dilution of 2 ml of the above to 25 ml

1 ml = 0.0002 g of sample, or

100 ml = 0.02 g of sample

Optical density observed at 328 mμ = 0.550

Optical density observed at 300 mμ = 0.355

Optical density observed at 350 mμ = 0.315

Cuvet length (distance of light path) = 1 cm

$$E^{1\%}_{1\text{ cm}}\ 328\ m\mu = \frac{0.550}{0.02 \times 1} = 27.5$$

$$\text{Potency} = 27.5 \times 2{,}000 = 55{,}000 \text{ U.S.P. units per g}$$

$$\text{Extinction ratio } \frac{300\ m\mu}{328\ m\mu} = \frac{0.355}{0.550} = 0.65$$

$$\text{Extinction ratio } \frac{350\ m\mu}{328\ m\mu} = \frac{0.315}{0.550} = 0.57$$

Since these extinction ratios fall within the limits prescribed, the assay is considered valid.

(2) Absorption measurements are made at 300 mμ (310 mμ optional) and 350 mμ as a check on the presence of light-absorbing impurities in the sample or on the presence of oxidation products of vitamin A (*51*). The ratio of the optical density at 300 mμ to that at 328 mμ should not exceed 0.73, and the ratio of the optical density at 350 mμ to that at 328 mμ should not exceed 0.65. The allowable limits for these ratios are those promulgated for fish liver oils by the War Food Administration (*69*). The ratio of the optical density at 310 mμ to that at 328 mμ should not exceed 0.91 (*46*). If the ratios are higher than indicated, there is sufficient extraneous absorption to suggest that the absorption measured at 328 mμ is not a true measure of vitamin A. It must be borne in mind, however, that extraneous absorption may exist even though the ratios are favorable. The more nearly the ratios approach those for pure vitamin A, the greater is the probability that the observed optical density at 328 mμ represents only vitamin A.

The extinction ratios, written in a more convenient form as E 300/328, E 310/328 and E 350/328, given below for vitamin A alcohol and vitamin A acetate are average values on crystalline preparations and are indicated as being tentative (*70*).

	E 300/328	E 310/328	E 350/328
Average, 5 samples alcohol	0.53	0.77	0.53
Average, 4 samples acetate	0.51	0.77	0.55

In an attempt to render possible an accurate determination of vitamin A in those cases in which the ratios exceed the prescribed values, a technic of irradiation has been proposed (*42*). In this method, the vitamin A is destroyed by exposure to ultraviolet light and the difference between the absorption measured before and after irradiation is assumed to represent the true vitamin A content of the material.

Saponification may be sufficient purification for some samples, but in other cases chromatography may be necessary (*43–45,71*).

APPLICATION OF METHODS

Those products which have been assayed by one or more of the methods described or by reasonably similar modifications are listed below. The omission of any product from the list or failure to check it under a method heading does not necessarily imply that the method is not applicable. Rather, it merely indicates that the committee and reviewers have not applied these methods to the product.

Type of material	Colorimetric method	Ultraviolet absorption method	Type of material	Colorimetric method	Ultraviolet absorption method
Vitamin A concentrates	+	+	Cream	+	
Pharmaceuticals	+	+	Ice cream	+	
Fish oils	+	+	Cheese	+	
Butter	+		Salmon	+	
Margarine	+		Tuna	+	
Liver	+		Fortified feeds	+	
Milk	+				

Certain materials, e.g., eggs and egg products (which contain large amounts of pigments reacting with $SbCl_3$) require a further purification than is accomplished by saponification in order to give a blue color reaction which is representative of the vitamin A content alone (*43–45*). With further modification of the procedures outlined above, it is also possible to make corrections for the presence of carotenoids in the solutions or materials being tested (*58–60*).

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CHAPTER 3

Carotene

PRELIMINARY CONSIDERATIONS (*1–14*)

From the standpoint of human and animal nutrition the carotenoid pigments are of importance because of the conversion of some of them into vitamin A. These pigments, which are found largely in yellow plants and in green plants along with chlorophyll, are polyenes belonging to four principal groups:

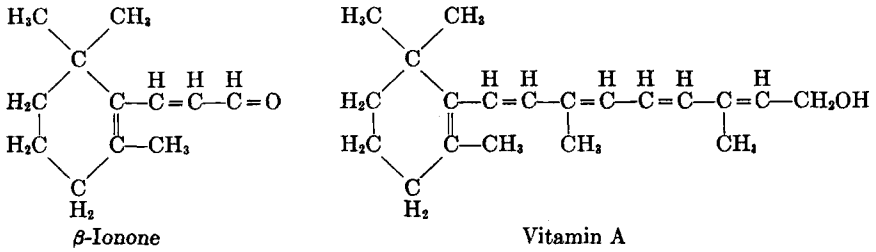
1. The carotenes, carotenoid hydrocarbons, $C_{40}H_{56}$, which include α-, β-, and γ-carotenes and lycopene.

2. The xanthophylls, oxy and hydroxy derivatives of the carotenes, which include among others, cryptoxanthin, $C_{40}H_{55}OH$, and lutein, $C_{40}H_{54}(OH)_2$.

3. The xanthophyll esters, esters of the xanthophylls with fatty acids.

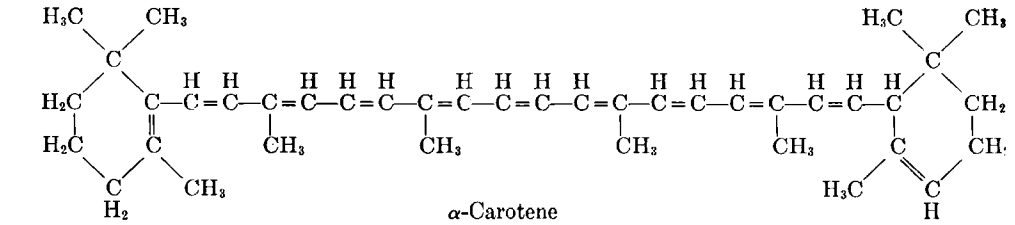
4. The carotenoid acids, carboxyl derivatives of the carotenes.

The structure of the carotenoid pigments is characterized by an aliphatic chain with attached methyl groups and a system of conjugated double bonds which is responsible for the deep red to yellow color of these compounds. The terminal groups of the aliphatic chain vary for the different carotenoids, and determine the vitamin A activity as well as the adsorbability and differential solubility properties which form the basis for their separation from one another. The provitamin carotenoids, those which exhibit vitamin A activity, contain a β-ionone ring at either one or both ends of the polyene chain, as shown in Figures I and II.

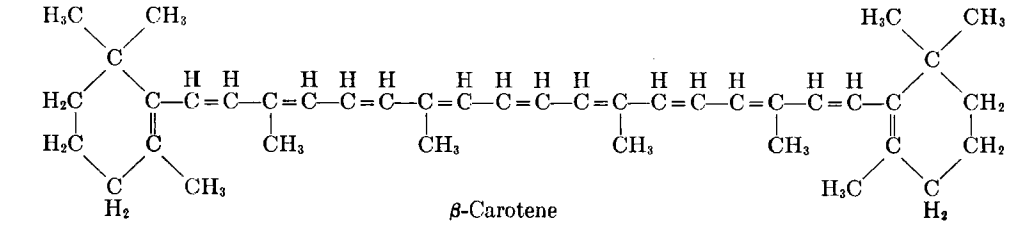


β-Ionone Vitamin A

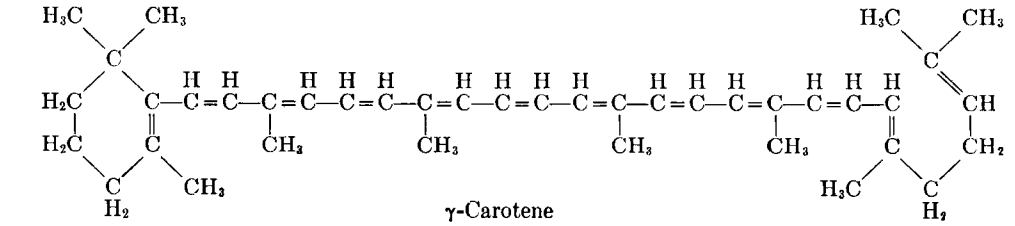
FIG. I. The cyclohexenyl ring appearing in both compounds is common to all substances showing vitamin A activity.



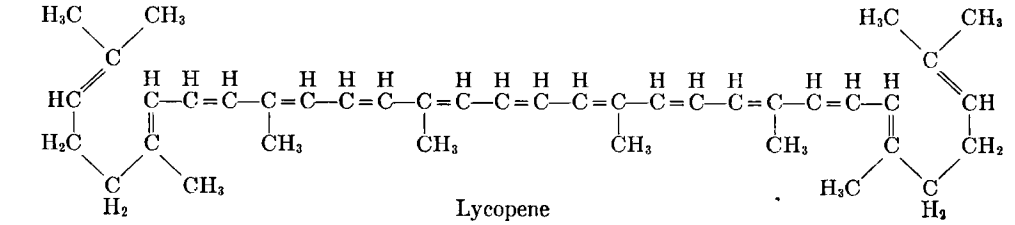
α-Carotene



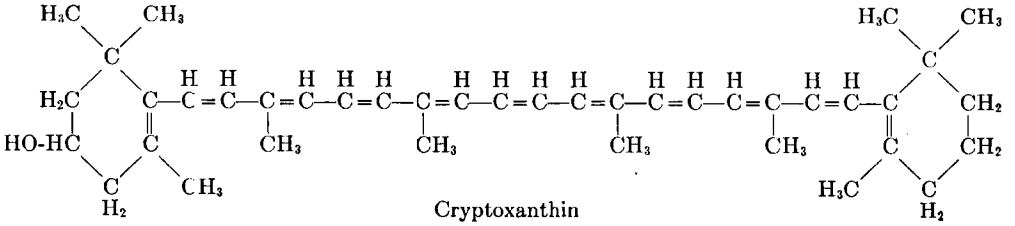
β-Carotene



γ-Carotene



Lycopene



Cryptoxanthin

FIG. II.

Of the several vitamin A precursors found in nature, the better known ones are α-, β-, γ-, and neo-β-carotenes and cryptoxanthin. β-Carotene contains two β-ionone rings and is capable of splitting into two molecules of vitamin A, whereas the others possess only one β-ionone ring and therefore have less activity. The vitamin A activity of most vegetables and fruits is due to their β-carotene content, the α-, γ-, and neo-β-isomers and cryptoxanthin appearing in much smaller quantities. Yellow corn, however, contains more cryptoxanthin than β-carotene. The precise vitamin A equivalent of each provitamin is not definitely known, since utilization by the animal organism is dependent upon the vegetable sources (*7*), the species of animal in question and the nutritional status of the animal at the time of feeding (*5,8*). An arbitrary provitamin value assigned by United States Pharmacopoeia for β-carotene is 0.60 γ of β-carotene = 1 U.S.P. unit of vitamin A.

In general, the provitamins A have the following physical and chemical properties:

1. They are fat-soluble.
2. They are readily soluble in chloroform, benzene, carbon disulfide, petroleum ether, but difficultly soluble in alcohol.
3. They are sensitive to oxidation, autoxidation, and light.
4. They are stable to heat in an oxygen-free atmosphere, except for some stereoisomeric changes (*9,10*).
5. They have characteristic absorption spectra, closely related to one another, the positions of the maxima differing with the solvents used.

Wave Lengths of Absorption Maxima of Some Provitamins A in Various Solvents (*2*) (expressed in mμ)

Compound	Petroleum ether	Hexane (*11*)	Carbon disulfide	Benzene	Alcohol	Chloroform
α-Carotene, $C_{40}H_{56}$	447	422	477	—	—	—
M.p. 187° $[\alpha]_{644} = +323°$ (*1*)	478	446	509	—	—	—
	—	474	—	—	—	—
β-Carotene, $C_{40}H_{56}$	451	450	485	—	452	466
M.p. 184°	484	478	521	—	480	497
γ-Carotene, $C_{40}H_{56}$	462	—	496	477	—	477
M.p. 178°	495	—	533	510	—	510
Cryptoxanthin, $C_{40}H_{55}OH$	452	452	483	—	452	463
M.p. 169°	485	478	519	—	486	497
Neo-β-Carotene, $C_{40}H_{56}$ (*10*)	—	443	—	—	—	—
	—	467	—	—	—	—

Typical absorption spectra of some provitamins in hexane (*10,11*) are shown in Fig. III.

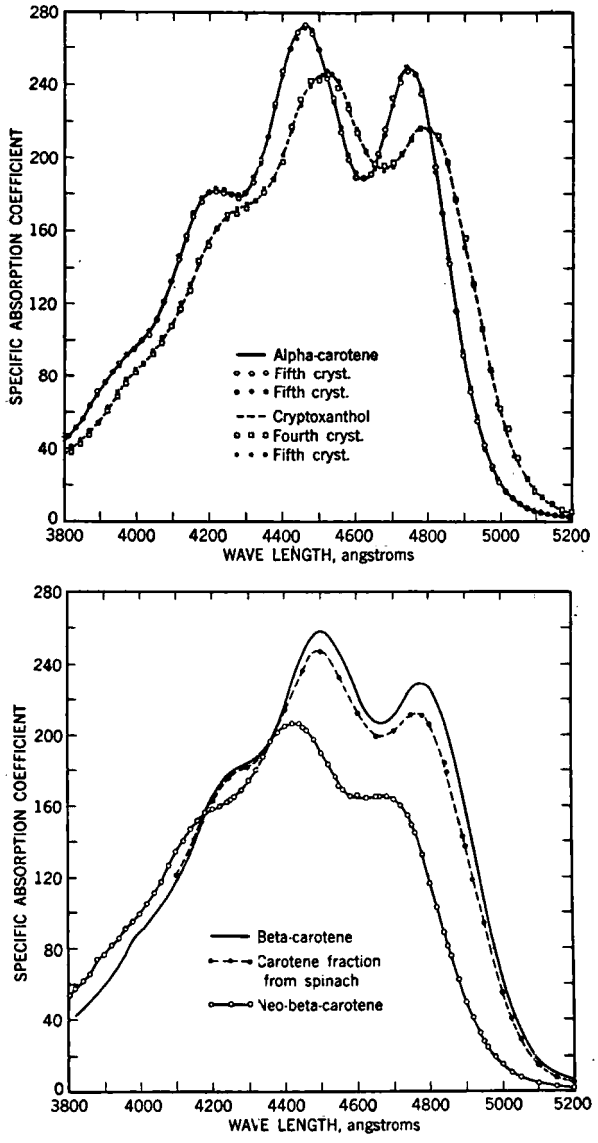


FIG. III. Typical Absorption Spectra of Some Provitamins in Hexane.

Methods Available

The provitamin A content of foods may be determined by bioassay, by the solvent partition method, and by the chromatographic technic. The latter two are the most widely used for carotenoid analyses, as they are not so time-consuming as the bioassay.

Of these methods, the biological assay is the only one which indicates the actual vitamin A activity of foods or feeds. Results obtained in this way may be expressed in International Units (I.U.) or U.S.P. units of vitamin A. The assays are carried out on vitamin A-depleted rats by feeding several levels of the sample in question and comparing the weight gains with those obtained by feeding known quantities of β-carotene. That quantity of food which results in similar weight gains to that produced by 0.6 γ of the standard β-carotene contains 1 International Unit of vitamin A. Differences in utilization of carotene from various food sources are thus taken into account, although the types and relative amounts of the various provitamins present are not indicated. This method, though desirable, is tedious, time-consuming, expensive and subject to considerable error and variations as are inherent in all bioassays.

Nonbiological assays for carotene, based on physical properties of these pigments, on the other hand, are rapid and provide for separate measurement of the kinds and amounts of the various carotenoids. However, it should be recognized that carotene values obtained by physical assays represent merely quantities of carotene with vitamin A activity without indicating the actual biological potency in terms of vitamin A, as utilization by the organism is not taken into account (*32*). At present there are no satisfactory conversion factors which may be applied to the nonbiological values obtained on various types of materials. The literature reveals that the biological vitamin A activity of green vegetables is more closely related to the carotene content determined physically than is the biological vitamin A activity of yellow and orange vegetables. Graves (*7*) in a review article pointed out that the actual biological efficiency of yellow and orange vegetables is only 21 to 25% of the amount indicated by physical assay, while it is 70 to 100% for green vegetables.

In general, the physical methods employ spectrophotometric or colorimetric measurement of the several carotenoid pigments which have been separated from extracts of the sample either by solvent partition or chromatographic adsorption technics.

Purification of the crude carotene extract by solvent partition is usually accomplished either by employing aqueous diacetone alcohol solution (100 : 6 of water) and petroleum ether (*26*), or 85–92% methanol and petroleum ether as the immiscible phases (*23*, *27*). Either set of solvents is applicable to the purification of material in which the pigment composition is relatively simple, i.e., fresh, canned, frozen green vegetables, carrots and other materials in which lycopene and cryptoxanthin do not occur. These latter pigments are not completely partitioned from the carotene and xanthophyll fractions respectively, and thus the solvent partition technic is not satisfactory for materials which contain them. On the whole, the diacetone alcohol partition is more effective than the methanol, giving a more quantitative, clear-cut separation of the xanthophylls, chlorophyll and other nonbiologically active pigments from the provitamin A pigments. Any solvent partition method, when employed, should be checked against the chromatographic procedure to insure its applicability for the material in question.

Chromatography is becoming the most widely used method of separation of the various carotenoids. The differences in structure between the various pigments allow for the development of distinct adsorption zones when petroleum ether solutions are poured through a column of proper adsorbent. These zones may be separated mechanically or eluted individually. A number of different adsorbents have been used (*16*, *19*, *20*). With the 1 : 3 adsorbent mixture of MgO and Super-Cel, and 2 to 5% acetone in petroleum ether as eluant, (*19*) the separation of the biologically active pigments from the other carotenoids is rapid, clear-cut, and gives great accuracy and duplicability. In addition, it also permits the study of the pigment system occurring in the product assayed. By decreasing the amount of Super-Cel in the adsorbent mixture, individual pigments become more distinct and isomers which appear in small amounts may even be discerned. There are many variations of adsorbent and eluant possible to achieve separation of pigments for more detailed work.

Regardless of the type of purification employed, a certain degree of choice remains for the extraction procedure. In general, heat should be avoided in carotene determinations. There are instances in which hot saponification is necessary, such as in the analysis of materials with high fat content, i.e., dairy products and mixed feeds. Hot extraction in conjunction with the alkali (*27*) results in disintegration of the tissue, as well as in saponification of the fats, thus facilitating complete removal of the

pigment. There are other materials which have not been effectively extracted at room temperature, but which do not need to be saponified. In the extraction of materials of this type (alfalfa), the use of the acetone-petroleum ether extraction in the Soxhlet has been found effective (*19*). This method has the additional advantage that the pigment extract does not need to be filtered before proceeding to subsequent operations.

The methods involving cold extraction have some points of difference which allow a choice to be made based on the type of materials to be analyzed and upon the final degree of purification required. Diacetone alcohol extraction may be used, but it is not recommended for routine analyses where chromatography is to be used for purification because of the difficulty encountered in removing final traces of this solvent from the petroleum ether extract. Other extracting solvents, acetone, alcohol, petroleum ether or a "foaming mixture" (*28*) of alcohol and petroleum ether have been used mostly in conjunction with a Waring Blendor. Extraction with acetone on a fritted glass funnel offers the additional advantage of small volumes and avoids the relatively cumbersome transfers encountered in the use of the Waring Blendor (*29*).

CHROMATOGRAPHIC METHOD

A. Principle

The method is based upon the separation of the biologically active carotenoid pigments from the total carotenoid pigments in an extract by an adsorbent with varying affinities for the different pigments. Under the proper conditions the various materials may be separated into relatively distinct zones or bands. The individual pigments exhibit characteristic absorption maxima at which their concentration may be determined by photometric measurement.

B. Equipment

The following list includes equipment sufficient for 8 simultaneous determinations, a convenient number for one operator to handle. Exposure to intense light should be avoided during carotene assays, since the pigments may be destroyed under such conditions. Where this is impossible, protection should be provided through the use of amber glassware or its equivalent (*12*).

No. of pieces	*Item*
8	*Beakers*, 50 ml
8	*Fritted Glass Funnels*, coarse porosity, 60-mm diameter. This quantity can be decreased if the extractions are done in succession rather than simultaneously.
16	*Filter Flasks*, 500 ml. Two are necessary for each extraction, but the total number may be decreased if extractions are done in succession.
1	*Volatile Liquid Wash Bottle*. All glass, 250 ml
16	*Separatory Funnels*, pear-shaped, 500 ml, with racks or stands. Two funnels are needed for each determination, but the total number may be decreased if the phasic separations are done in succession rather than simultaneously.
8	*Funnels*, short-stem, 6-cm diameter
8	*Receiving Vessels*, 250-ml Erlenmeyer flasks, beakers, etc.
8	*Erlenmeyer Flasks*, 500 ml
16–24	*Glass Beads*. Two to three for each determination.
1	*Steam Bath*, 6 spaces
8	*Adsorption Tubes* (*13,14*). These may be home-made from ordinary laboratory test tubes by constricting the end and attaching 1-mm diameter glass tubing or they may be ordered from supply houses. One source is Scientific Glass Apparatus Co., Bloomfield, N.J. A convenient size for laboratory use is 150 × 19 mm (inside diameter). Directions given for chromatographing in this paper apply to these tubes. One tube may be used for all 8 determinations by blowing out the adsorbent each time and refilling the same tube. Scientific Glass Apparatus Co. has an assortment of larger-size tubes which are satisfactory for more concentrated extracts or when there are large amounts of xanthophyll pigments, i.e., eggs. Long tubes (200 × 19 mm) give better separation of pigments under these conditions because the extra length of the column allows for more washing without contamination. It is also well to use the larger tubes when the extract contains impurities such as sterols and solvents which are difficult to wash out of the extract. Here again, the extra surface is more likely to permit distinct separations.
1	*Plunger* (*13,14*), for the preparation of the adsorption column. This may be a wood, glass or metal disc firmly affixed to a handle, or a cork attached to a glass or wooden rod. The diameter of the disc or the wide end of the cork should be 1 to 2 mm smaller than that of the tube so that it can be inserted easily and moved up and down in the tube. If a stopper is used, the surface which is to come in contact with the adsorbent should be smooth and free from breaks. Starting at the tapered end, a hole is bored half-way through the stopper, and the desired length of fire-polished glass rod is inserted snugly.
1	*Vacuum Desiccator*, or top-tubulated bell jar. The use of a vacuum desiccator or bell jar under reduced pressure allows the collection of the eluate directly into a volumetric flask instead of a filter flask. Optional.
8	*Volumetric Flasks*, or glass-stoppered mixing graduates, 25–250 ml, depending upon the concentration of the extract.

No. of pieces	*Item*
1	*Instrument.* A spectrophotometer or a photoelectric colorimeter equipped with filters transmitting bands as near as possible to 450 mμ. The Beckman and Coleman Spectrophotometers, as well as the Evelyn and Klett photoelectric colorimeters have been used successfully.

C. Reagents

1. Acetone. U.S.P. grade.

2. Petroleum Ether. Boiling range 65–70°C. Skellysolve B or Viking petroleum ether of this boiling range is satisfactory. The petroleum ether should be free of water and other solvents.

3. Stopcock Lubricant. It should be insoluble in fat solvents. Nonaq, a Fisher product, is satisfactory for this purpose. Glycerine may also be used.

4. Anhydrous Sodium Sulfate, Granular. Na_2SO_4.

5. Filter Paper. Whatman No. 1 or any other fast filter paper which does not retain carotene. Glass wool or nonabsorbent cotton may be used in place of filter paper.

6. Adsorbent. Mix intimately one part by weight of MgO (Micron Brand, No. 2641, Westvaco Corp., Newark, Cal.) with three parts of Hyflo Super-Cel (Johns Manville) either by shaking in a sufficiently large bottle or by repeated mixing on paper. It is convenient to make up a large batch of adsorbent at one time. This adsorbent is very satisfactory for most carotene analyses. The adsorbent requires no activation, it is relatively rapid, and it gives good separation of carotene from xanthophylls, lycopene and chlorophyll. Each new batch should be tested for recovery of known amounts of carotene. Good preparations will give 95 to 100% recoveries.

7. Eluant. 3% acetone in petroleum ether. Make 3 ml of acetone to 100 ml with petroleum ether. The quantity of acetone may be varied from 2 to 5%, the amount being determined by the rate of elution desired. The rate of elution should be one which allows the carotene pigment to be washed through completely before any material from the more strongly adsorbed zones is eluted.

8. Carotene Standard Solution. Dissolve 50 mg of a 90% β-, 10% α-carotene mixture in petroleum ether and dilute to 500 ml with that solvent. From this make dilutions as needed for preparing calibration curve. The standard solution should be prepared at the time of calibration, since it deteriorates after 2–3 days' storage. The 90% β-, 10% α-

carotene mixture may be obtained from General Biochemicals Inc., Chagrin Falls, Ohio. Pure β-carotene ampules are also available but are much more expensive. For most practical purposes, the mixture is satisfactory.

D. Procedure

1. Extraction

(a) Weigh up to 25 g of sample containing 10 to 500 γ of carotene into a 50-ml beaker.

(1) Homogenize all moist samples such as fresh, frozen, and canned fruits and vegetables in a Waring Blendor to a puree consistency with a known weight of added water, if necessary. In many cases, it is desirable to blanch fresh or frozen foods to inactivate enzymes prior to blending. Pulverize all dry samples to a 40-mesh powder or finer, and rehydrate a weighed sample by covering with a small quantity of hot water for 5 to 10 minutes. This insures complete extraction.

Grind sun-dried fruits such as apricots, prunes, peaches, etc., in a food chopper, and rehydrate a representative sample with a known weight of water. Then blend the rehydrated sample and excess water to a puree consistency.

Saponify materials in which xanthophyll esters occur, as in flowers and some fruits (some varieties of peaches, apricots, etc.) by blending with 150 ml of 12% alcoholic KOH for 5 minutes at room temperature in a blendor. The saponification splits the esters and thus facilitates separation from the biologically active pigments (*15,16*). Transfer quantitatively the contents of the Waring Blendor using petroleum ether, and proceed with the extractions as described in D-1-(i) through (o). See D-1-(h)-(1).

Saponify animal and dairy products and other materials containing fat by refluxing with alcoholic KOH. For liquid whole milk, extraction of the fat-soluble material followed by saponification as described by Olson, Hegsted and Peterson (*17*) is recommended. Ethyl ether, rather than petroleum ether, is used for the extraction as vitamin A which occurs along with the carotene in dairy products is more easily extracted by the former solvent (*18*). Follow steps D-1 and D-2 of the colorimetric procedure for vitamin A, take up the residue in petroleum ether and determine the concentration of carotene as described under D-3.

(2) A simplified procedure for alfalfa and other materials which can be finely powdered is to extract a 1- to 4-g sample with 150 ml acetone–petroleum ether mixture (30 + 70 parts by volume) (*19*) for 1 hour in a Soxhlet apparatus. The extract is then evaporated to a small volume as in step D-1-(o) in the extraction flask of the Soxhlet, and the residue is then ready for chromatography. These hot-extraction methods are special cases. Cold extraction is recommended for those types of materials for which it can be used since it has been established that the heating of carotenoids in solution results in isomeric changes (*9,10,31*), which are detectable spectroscopically and which may, therefore, influence quantitative results if not taken into consideration.

(3) As an alternative procedure, the sample may be transferred to a Waring Blendor cup and extracted by blending 5 minutes with approximately 200 ml acetone. To prevent loss of sample and to avoid contact of fumes with sparks from the motor, care should be taken to prevent leakage of sample through faulty washers or leaky bearings.

The Waring Blendor extraction has also been used with a "foaming mixture" of alcohol and petroleum ether (*28*). Up to 15 g of moist sample is blended with 75 ml of petroleum ether and enough 95% alcohol to make a "foaming mixture." Usually 150 ml of alcohol is sufficient with samples of 10 g or less. When larger samples are used, the alcohol volume must be increased. A convenient way to estimate the amount of alcohol required to produce a "foaming mixture" is to determine the quantity of alcohol necessary to make a miscible mixture with 75 ml of petroleum ether and the amount of water estimated to be in the sample. The maximum volume of alcohol should not exceed 250 ml, as larger volumes are impractical for use with the equipment described. *Caution!* This mixture is highly inflammable and may be ignited by sparks from the motor.

(b) Transfer to a fritted glass funnel, rinsing with 5- to 10-ml portions of water.

(1) The volume of water used for rinsing the beaker should be kept as small as possible. A large volume will cause difficulty in obtaining a speedy filtration of the acetone extract through the fritted glass funnel unless the water is removed as in step (c). If the beaker cannot be rinsed sufficiently with 5 to 10 ml of water, proceed to step (c) and then continue to rinse the beaker with the acetone used for step (d).

(c) Draw off the water under reduced pressure until only a thick paste remains. Discontinue suction.

(1) For fruits or vegetables canned or cooked in syrup, the sample should be washed 2 or 3 times with 10-ml portions of hot water before the acetone extraction, in order to remove the sugar which interferes with the filtration (*15*).

(d) Add 15 to 20 ml of acetone and stir for at least 30 seconds.

(e) Draw off most of the acetone with suction, but do not take to dryness or oxidation of the pigments may occur.

(f) Continue the extraction by stirring with successive 20-ml portions of acetone and removing the solvent each time until the extract and residue are colorless.

(1) In materials such as tomatoes and carrots, hard, tiny particles containing pigment occasionally appear in the funnel. Break up these by adding a small quantity of water and allowing the residue to reconstitute again. Then the application of acetone will result in further and complete extraction. Corn seed coats also have a tendency to retain color and this pigment can be removed by persistent tamping with the flattened end of a glass rod, rehydration and repeated extraction. Although it is not necessary to remove all the pigment from the coats (because it is not carotene), it is a good policy always to get the residue colorless. Slight shades of

tan and pink will remain occasionally in materials containing lycopene and chlorophyll. The sample may be considered completely extracted at this point.

(2) To test for completeness of washing, transfer the funnel to another suction flask which contains a test tube to receive the filtrate. Slight shades of color or absence of color can then be seen with more certainty.

(g) Quantitatively transfer the contents of the filter flask to a separatory funnel.

(1) If the blendor is used for the extraction, transfer the entire contents of the blendor with the aid of a glass paddle to a 1000-ml beaker and then to the separatory funnel or directly to a 500-ml separatory funnel through a wide-diameter funnel.

(h) Rinse the flask with two 10-ml portions of water and three 15-ml portions of petroleum ether.

(1) The blendor when used should be rinsed with the extracting solvent, petroleum ether, and water. In the case of the "foaming mixture," rinse successively with approximately 50 ml petroleum ether, 25 ml alcohol and 150 ml water. These amounts of the 3 solvents will help prevent emulsions.

(i) Shake the contents of the separator gently for at least 30 seconds and allow the layers to separate.

(1) If there is more than a slight yellow color in the acetone–water layer, water or acetone added gradually down the sides of the funnel will assist in the transfer of the carotenoid pigments to the petroleum ether layer (epiphase). It has been suggested that the use of water containing 5% Na_2SO_4 aids in the transfer of pigments to the petroleum ether and helps eliminate emulsions (*30*).

(2) It is important to avoid vigorous shaking to prevent formation of stable emulsions. If an emulsion does occur, the addition of 5 to 10 ml of 95% ethanol will usually break the emulsion. With the "foaming mixture," do not shake but allow the layers to separate by standing.

(3) Stable emulsions are frequently formed when petroleum ether–alcohol mixtures are shaken vigorously in a separatory funnel. These emulsions may be avoided and good mixing still accomplished by drawing the water–alcohol phase, including any suspended material, into an Erlenmeyer flask containing 30 to 35 ml of petroleum ether and swirling vigorously for at least 30 seconds. Transfer the mixture to a separatory funnel and allow two minutes for separation of layers. Draw off the lower layer, leaving any suspended material with the petroleum ether extract.

(j) Draw the water–acetone layer into a second separatory funnel.

(1) Occasionally, the solid material settles too firmly against the stopcock to allow removal of the lower layer. This material can be readily dislodged by pushing through the opened stopcock from the top with a long, thin stiff wire.

(k) Re-extract this water–acetone layer with 50-ml portions of petroleum ether, two to four times, repeating steps (i) and (j). Use a receiving flask to collect the water–acetone layer each time until the petroleum ether extract is transferred to the first separatory funnel.

(1) Three to five extractions are usually enough. Any slight color appearing in the petroleum ether extract after the fifth extraction may be disregarded.

(2) For successive extractions of the alcohol–water phase follow the procedure described under D-1-(i)-(3).

(l) Pour 100 ml of water through the combined petroleum ether extracts, allow layers to separate and discard the aqueous phase. Wash one to two times more.

(1) Two washings are usually enough to reduce the polar solvent content of the extract so that the chromatographic procedure described will accomplish the desired separations. If difficulty is encountered in obtaining good chromatographic separation, additional washings may be helpful. The extract now may be used for purification by partition between immiscible solvents as in the solvent partition method.

(m) Filter the petroleum ether extract through approximately 10 g of Na_2SO_4 contained in a funnel into an Erlenmeyer flask.

(1) For concentrated sources of carotene such as leafy green vegetables and carrots the petroleum ether extract can be filtered through Na_2SO_4 into a 250-ml volumetric flask, diluted to volume and a 20- to 25-ml aliquot chromatographed directly. Volumes larger than 25 ml should not be chromatographed directly because less clear-cut separation will be obtained. See 2-(i)-(1). Solutions which are too dilute should be concentrated by evaporating to a smaller volume.

(n) Rinse the separatory funnel and Na_2SO_4 with small portions of petroleum ether until both are free from pigment.

(o) Add several glass beads to the flask and evaporate the extract to 10- to 20-ml volume on the steam bath.

(1) Care should be taken not to evaporate the extract to dryness as this would result in the oxidation of the carotene. The use of reduced pressure and/or nitrogen or carbon dioxide will aid in minimizing the exposure to heat and oxygen. See 1-(a)-(1). If a hot plate is used, do not allow the flask to become superheated. Constant stirring and low heat will help reduce isomerization.

2. Purification (*13, 14, 19*)

Preparation of Column

(a) Attach the adsorption tube to a filter flask and pack a plug of non-absorbent cotton or glass wool into the constriction of the adsorption tube.

(b) Apply vacuum and add enough adsorbent to make the column 2–2½ cm in length.

(1) The adsorbent and eluant recommended are satisfactory for routine carotene analyses. To obtain slower separations or more distinct bands in materials containing a large proportion of carotenol pigments and sterols, a 1:1 ratio of MgO to Super-Cel should be employed. See note under adsorption tubes. The Super-Cel acts as a filter aid by increasing the rate of filtration through the columns.

(2) *Dicalcium phosphate* recommended by Moore (*20*) can also be used satisfactorily for some products. However, it is more sensitive to moisture and other solvents so that its adsorptive properties are modified and difficulties may be encountered in the separation of the biologically active pigments from the other pigments. When using this adsorbent, the carotene is washed through rather than being adsorbed and then eluted as with the MgO. Upon activation it does not react consistently each time and its ability to adsorb pigments changes upon standing. It is recommended that the recovery of carotene be checked frequently when this adsorbent is used.

Aluminum oxide and calcium hydroxide (*13,14,16*) are generally used for research purposes rather than routine assays. With these adsorbents it is possible to separate isomers which cannot be distinguished on other adsorbents. Many other adsorbents are available and their use depends on the separation of pigments desired (*13,14,33*).

(3) The vacuum pump should remain on throughout the preparation of the adsorption column so that even packing is obtained.

(c) Press down the adsorbent firmly once or twice with a plunger.

(d) Loosen the surface of the adsorbent around the edges with a thin-edged spatula.

(1) Loosening the edges of the adsorbent before additional amounts are added helps to produce a smooth, continuous column, relatively free of lines or zones at the boundaries between the portions of adsorbent, and thereby insures more even adsorption of the pigments on the column. Do not loosen the last portion of adsorbent.

(e) Add more adsorbent, repeating steps (c) and (d) after each addition, until the column is approximately 10 cm in length.

(f) Place 1 cm of Na_2SO_4 over the top of the column.

(1) The Na_2SO_4 prevents the disturbance of the top layers of adsorbent during the chromatographic process and also picks up any traces of moisture which may have remained in the extract. Some workers omit this operation.

(g) Wet the column by washing with 25 to 50 ml of petroleum ether. While the last 5 ml of the petroleum ether is still above the Na_2SO_4, turn off the vacuum and transfer the adsorption column to a clean filter flask or to a top-tubulated bell jar containing a volumetric flask.

Adsorption and Elution

(h) Pour the extract to be chromatographed onto the column and apply suction.

(i) Rinse the flask with 5- to 10-ml portions of eluant, pouring each rinsing onto the column before the previous one has been entirely absorbed into the Na_2SO_4.

(1) The first washings should be kept small so that the concentrated extract may be adsorbed in a narrow band in the upper portion of the column, allowing the re-

mainder of the column to be used for the development of the chromatogram or for the separation and elution of the desired pigments. If the purification is to be accomplished by mechanical separation of the adsorbed zones (development of the bands, extrusion of the column and cutting of the zones), the nonpolar solvent (in this case, petroleum ether) alone should be used for the washing and development of the column. If the pigments are to be separated by direct elution from the column, a polar solvent, such as acetone, is introduced with the nonpolar solvent to speed up the elution.

(j) Wash the column continuously with eluant, adding the successive portions of eluant when the preceding one is just barely visible above the Na_2SO_4.

(1) If the column is allowed to become dry, air will be pulled through and destruction of carotene by oxidation will occur.

(2) The rate of elution and the distinctness of the bands may also be varied [see D-2-(b)-(1)] by changing the percentage of acetone in the petroleum ether from 0 to 5%. For extracts containing a high proportion of biologically inactive pigments (xanthophyll and lycopene) accompanied by other impurities as sterols, polar solvents, etc., a slower separation may be desired and the adsorbent and eluant should be changed accordingly.

(3) With some materials (dried silage, dehydrated squash and certain fermented vegetable leaf products) many bands are observed and often it is difficult to obtain a clear-cut separation. The following technics, singly or in combination, may be used. *1.* If the bands are not distinct after preliminary washing with the acetone–petroleum ether eluant, or if it is obvious that the carotene band is associated with noncarotene pigments, wash the column with pure petroleum ether. This sometimes will give better resolution of the bands. *2.* The eluate may be concentrated and readsorbed on a fresh column. *3.* Preliminary saponification of the sample with 10% alcoholic KOH often aids in securing a better chromatogram with the above mentioned materials (*30*).

(k) Continue the washings until the desired pigments have moved off the column and the filtrate is colorless.

(1) The desired pigments refer to those having biological activity and include α-, β-, and γ-carotenes, cryptoxanthin and the neo isomers of these pigments. Because β-carotene constitutes the greatest portion of the biologically active pigments present in foods, the other biologically active pigments which are not easily separated from it by the method of chromatography described, are eluted into the same filtrate and calculated as β-carotene. Yellow corn, however, contains a greater proportion of cryptoxanthin than of β-carotene. In this food, separation of the cryptoxanthin from β-carotene is desirable especially in view of the fact that the theoretical biologic potency of cryptoxanthin is 50% of that of β-carotene (*21*). Calculation of the cryptoxanthin as β-carotene in this case would yield an erroneously high value. These two pigments should be collected separately and the quantity of each reported individually. Similarly, the α-carotene band of carrots should be collected separately from the β-carotene and reported individually (*24*).

(2) Typical adsorption columns are shown in Figure IV (1 : 3 MgO Super-Cel).

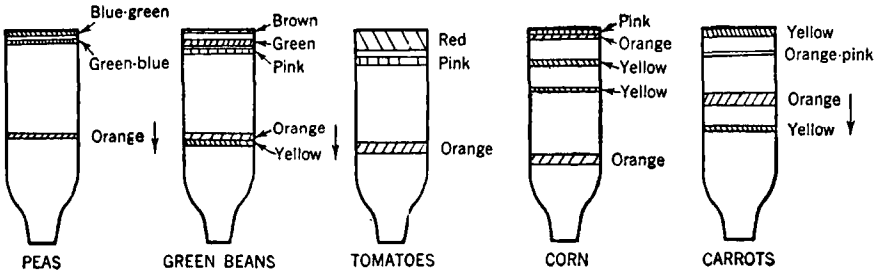


FIG. IV. Typical Adsorption Columns.

It is important that the chromatographic behavior of pigment systems of newly encountered materials should be studied so that the purification technic may be modified as necessary.

(3) See D-1-(f)-(2).

(l) Transfer the contents of the filter flask to a volumetric flask and dilute to volume with eluant. The concentration of carotene should be 1 to 4 γ per ml.

(1) If the eluate is too dilute to be read in the necessary range on the instrument used, it should be evaporated to a convenient volume, using nitrogen or reduced pressure.

3. Determination of Concentration

(a) Using a suitable instrument, determine the intensity of color at 450 mμ in the final solution of carotene.

(1) Since β-carotene isomerizes readily during heating to give neo-β-carotene and since nec-β-carotenes have also been found to occur naturally in some foods, a more accurate value of the total carotene content can be obtained by reading the solutions at 436 mμ, the wave length at which both β-carotene and the neo-β-isomers have equal absorption (*10*). However, for most routine analyses, the results obtained on reading at 450 mμ are within 10% of those obtained by reading at 436 mμ and calculating the concentration from a calibration curve made at 436 mμ. Differentiation of the 436 from the 450 wave length is impossible on filter instruments or nonmonochromatic light source instruments.

(b) Determine the concentration of carotene in γ per ml in the solution by reference to a calibration curve, prepared by plotting the per cent transmission (or optical density) against concentration for a series of carotene solutions.

(1) Pure β-carotene or a mixture of 90% β- and 10% α-carotene dissolved in petroleum ether may be used for preparation of the calibration curve at the wave length at which the sample readings are made. Figure V illustrates a typical calibration curve.

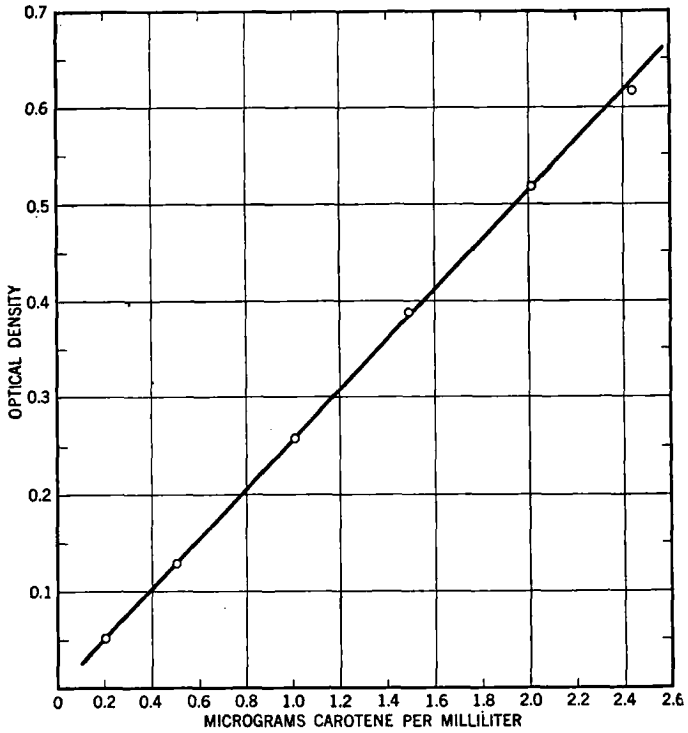


FIG. V. A Typical Calibration Curve.

4. Calculation

(a) Using the value for concentration obtained in D-3-(b), calculate the carotene in the original sample by use of the following:

$$\gamma \text{ of carotene per g} = \text{concn. [3-(b)]} \times \frac{\text{final vol., [2-(l)]}}{\text{wt. sample, [1-(a)]}} \times \text{dilution, [1-(m)]}$$

(1) The following example illustrates the method of calculation. 1 g finely powdered dehydrated mixed greens was extracted following rehydration and the final anhydrous petroleum ether extract was made to 250-ml volume. 50 ml of the petroleum ether extract was evaporated to a small volume and chromatographed. The final volume of eluant was 200 ml and consisted of α- and β-carotenes as shown in Figure VI.

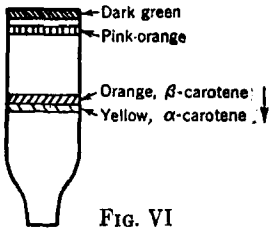


FIG. VI

Optical density of eluant at 450 mμ = 0.412
Optical density of 0.412 = 1.68 γ of carotene per ml of solution from calibration curve

Therefore, $1.68 \times \frac{200}{1} \times \frac{250}{50} = \gamma$ carotene pigment per g sample

(2) The final value designated as carotene content necessarily includes all pigments in the final solution. These will include not only β-carotene, but the α-carotene in many vegetables, and the neo isomers of the above. However, for all practical purposes the value may be considered as β-carotene.

SOLVENT PARTITION METHOD

A. Principle

The method is based upon the differential solubilities of fat-soluble pigments in immiscible solvents. By proper choice of solvents, it is possible to separate the biologically active carotene from the other fat-soluble pigments. The individual pigments exhibit characteristic absorption maxima at which their concentration is determined by photometric measurement.

B. Equipment

The following list includes equipment sufficient for 12 simultaneous determinations, a convenient number for one operator to handle. Since carotene pigments undergo destruction upon exposure to intense light, the solutions should not be exposed to bright light. Where this is impossible, protection should be provided through the use of amber glassware or its equivalent (*12*).

No. of pieces	*Item*
2	*Waring Blendor Assembly.* Two sizes of blendor cups are available. For volumes of 150 ml or less, the smaller cup is preferable. Care should be taken to tighten the blade unit before each using. The washers around the blades should be renewed as soon as there are signs of leakage. Two complete units are recommended so they can be alternated, and in this way allow for the cooling of the motor and cup.
12	*Büchner, Slit-Sieve or Fritted Glass Funnels.* 60-mm diameter. This number may be reduced if the filtrations are done in succession rather than simultaneously.
24	*Separatory Funnels,* 500-ml capacity, pear-shaped, with ring supports and stands. This number may be reduced by running extractions in succession.
1	*Wash Bottle.* Fine tip, all glass.
12	*Receiving Vessels,* 250-ml Erlenmeyer flasks or beakers.
12	*Funnels,* 6-cm diameter, short stemmed, with rack.
12	*Volumetric Flasks* or glass-stoppered mixing graduates, 100- to 250-ml capacity, depending on the concentration of carotene to be determined.

C. Reagents

1. Diacetone Alcohol. Acetone-free grade. If the diacetone alcohol becomes yellow, distil under reduced pressure before use.

2. Aqueous Diacetone. Mix 100 volumes of diacetone with 7 volumes of water.

3. Petroleum Ether. Boiling range 65–70°C. Skellysolve B or Viking petroleum ether of this boiling range is satisfactory. The petroleum ether should be free of water and other solvents.

4. Methanolic Potassium Hydroxide. Dissolve 20 g reagent KOH in 100 ml synthetic absolute methanol.

5. Anhydrous Sodium Sulfate, Granular. Na_2SO_4.

6. Filter Paper. Whatman No. 1 or any other fast filter paper which does not retain carotene. Glass wool or nonabsorbent cotton may be used in place of filter paper.

7. Stopcock Lubricant. This should be insoluble in fat solvents. Nonaq, a Fisher product, is satisfactory for this purpose. Glycerine may also be used.

8. Carotene Standard Solution. Dissolve 50 mg of a 90% β-, 10% α-carotene mixture in petroleum ether and dilute to 500 ml with that solvent. From this, make dilutions as needed for preparing calibration curve. The standard solution should be prepared at the time of calibration, since it deteriorates appreciably after 2–3 days of storage. The 90% β-, 10% α-carotene mixture may be obtained from General Biochemicals Inc., Chagrin Falls, Ohio. Pure β-carotene ampules are also available, but are much more expensive. For most practical purposes the mixture is satisfactory.

D. Procedure (*10,22,26*)

1. Extraction

(a) Place 10 g of sample containing 10–500 γ of carotene into the cup of a Waring Blendor and add 125 ml of cold (20° C.) diacetone alcohol.

(1) Extraction and purification by the diacetone alcohol method is rapid and is suitable for the analysis of those materials in which the pigment mixture is relatively simple, i.e., in which lycopene and cryptoxanthin do not occur. In general, this method is applicable to fresh, canned, and frozen green vegetables and carrots. It is not satisfactory for tomatoes and other lycopene-bearing materials unless chromatography is employed in the final purification. Nor is the diacetone extraction applicable to yellow corn or other materials containing cryptoxanthin because of the difficulty of separating cryptoxanthin from xanthophylls by solvent partitioning.

Other methods of partitioning have been used, notably 85–92% methanol with petroleum ether (*23,27*). In view of the relatively poor separations obtained by the latter method, its use is not encouraged. If it is to be employed, chromatography should be used as a control.

(2) The amount of diacetone used for extraction must be varied depending upon the amount of moisture in the sample. The final concentration of water in the diacetone pigment extract should be 6 to 8%. The proportions given will work for materials containing more than 80% moisture, thus being satisfactory for most vegetables. If the moisture content is less than 80%, the amount of diacetone must be reduced.

(b) Blend for 5 minutes.

(c) Filter through paper on a slit-sieve filter, Büchner funnel, or through a fritted glass funnel, using reduced pressure. Wash the blendor cup twice with 5- to 10-ml portions of aqueous diacetone.

(1) It is desirable to use a minimum amount of aqueous diacetone for washing in order to facilitate subsequent extraction with petroleum ether. A wash bottle with a very fine delivery tip is convenient for this step.

(d) Wash the residue, if necessary, with additional 5- to 10-ml portions of aqueous diacetone until washings are colorless.

(1) To test for completeness of washing, transfer the funnel to another suction flask which contains a test tube to receive the filtrate. Slight shades of color or absence of color can then be seen with more certainty.

(e) Quantitatively transfer the diacetone extract to a 500-ml separatory funnel.

(f) Add 50 ml of petroleum ether, shake for one minute and allow phases to separate.

(1) The petroleum ether extracts the carotene (plus some impurities) from the diacetone solution.

(2) Six to 8% of water in the diacetone extract is important at this stage for the transfer of the pigment to the petroleum ether layer. The moisture in fresh plant materials is usually sufficient under the above conditions, but water should be added if necessary. The presence of excess water tends to cause emulsions and also to interfere with distribution of pigments.

(g) Draw off lower layer into a second separatory funnel.

(h) Re-extract this layer three times, using 25-ml portions of petroleum ether, drawing off the lower layer into a beaker each time and combining the petroleum ether extracts in the first separatory funnel.

2. Purification

(a) Add 25 ml of aqueous diacetone alcohol to the combined petroleum ether extracts, and shake at least 30 seconds.

(1) The diacetone extraction is not recommended where chromatography is to be employed, because of the difficulties encountered in removing final traces of this solvent which interferes with the adsorption of the pigments. However, if for some reason chromatography is desired, wash the extract at least 6 to 8 times with 100-ml portions of water. Smell the extract to be certain that the diacetone has been removed. Then proceed to steps 1-(m), 1-(n), and 1-(o) under the chromatographic method.

(2) These diacetone washings further purify the carotene solution by the extraction of oxygen-containing pigments and chlorophyll.

(b) Allow the layers to separate and discard the lower layer.

(c) Repeat the extraction 2 additional times.

(d) Add 25 ml of methanolic KOH, shake for one minute, allow phases to separate and discard the lower layer.

(1) The methanolic KOH removes any traces of chlorophyll which may have remained after the diacetone washings.

(e) Add 100 ml of water to the petroleum ether extract in the separatory funnel. Shake for 1 minute, allow the phases to separate and discard the aqueous layer.

(f) Repeat the washing procedure twice.

(1) The washing removes all solvents other than petroleum ether insofar as possible.

(g) Filter through Na_2SO_4 contained in a funnel into a volumetric flask.

(h) Wash Na_2SO_4 with petroleum ether and dilute to a volume such that the concentration of carotene is 1 to 4 γ per ml.

3. Determination of Concentration

(a) Proceed to D-3 under Chromatographic Procedure.

APPLICATION OF METHODS

Those products which have been assayed by one or more of the methods described or by reasonably similar modifications are listed below. The omission of any product from the list or failure to check it under a method heading does not necessarily imply that the method is not applicable. Rather, it merely indicates that the committee and reviewers have not applied these methods to the product.

A (+) under a heading for a method indicates that the method is applicable as described. Reference is made to a note in Section D or to the literature cited if special precautions are necessary or if simplifications in the procedure are permissible. Failure to list a note or reference to a

simplified procedure does not necessarily imply that short-cuts cannot be taken. It may mean only that they have not been tried for the product.

Type of material	Chromatographic method	Solvent partition method
Milk, cream, ice cream and cheese	D-1-(a)-(1)	
Milk, fresh, whole or skimmed	+ (*17*)	
Milk, evaporated, condensed or dried	+ (*17*)	
Cream, sweet or sour	+	
Cheese	+	
Cheddar type, cottage, cream or processed	+	
Eggs	D-1-(a)-(1)	
Whole, fresh, frozen or dehydrated	+ D-2-(b)-(1) (*25*)	
Meat, poultry and fish	D-1-(a)-(1)	
Fish, cooked, canned or fresh	+	
Liver	+	
Vegetables	D-1-(a)-(1)	
Fresh, frozen or canned	+	+ D-1-(a)-(1)
Dehydrated	+	
Fruit	D-1-(a)-(1)	
Fresh, frozen or canned	+ D-1-(c)-(1)	
Dried	+	
Grain products		
Corn, yellow	+	
Animal feeds	D-1-(a)-(1)	
Mixed feeds	+	
Liver meals	+	
Miscellaneous		
Chocolate and cocoa	+ D-1-(a)-(1)	

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CHAPTER 4

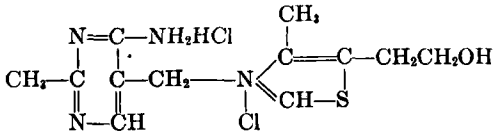
Thiamine

PRELIMINARY CONSIDERATIONS (*1–6*)

Studies finally leading to the isolation and characterization of thiamine* as a vitamin began about 1890, when it was first recognized that beriberi is caused by lack of a food essential, later shown to be thiamine. Characteristic signs of thiamine deficiency are loss of appetite and weight. In advanced stages there are increased concentrations of pyruvate and lactate in the blood, and polyneuritis may occur, involving degeneration of peripheral nerves. In humans this condition, known as beriberi, is seldom encountered except in the Orient. However, subclinical deficiencies are frequently seen.

Thiamine occurs in natural foods and other biological materials, either in the free or in a combined form—as a protein complex, or as a phosphorus–protein complex, or as the pyrophosphoric acid ester, cocarboxylase. The relative amounts of any of these forms vary considerably in different sources. Yeast, cereal germs, pork and nuts are especially rich in thiamine.

Thiamine hydrochloride, sometimes referred to as vitamin B_1, thiamine chloride, thiamine, aneurin, and antineuritic vitamin, or the anti-beriberi vitamin, has the structural formula:



Thiamine chloride hydrochloride

In its pure form, thiamine is a white crystalline powder with a yeasty odor and a salty, nutlike taste. It may be crystallized from alcoholic aqueous solutions as colorless monoclinic needle crystals of the hemihydrate. The crystals are hygroscopic, absorbing a maximum of one mol of water. Other physical properties are on the following page.

*For the purpose of brevity, the term thiamine will refer to thiamine chloride hydrochloride.

Empirical formula	$C_{12}H_{18}ON_4SCl_2$
Molecular weight	337.26
Melting point	246–250°C (decomposition)
pH of 1% solution in water	3.13
pH of 0.1% solution in water	3.58
Optical rotation	Absent
Solubility:	
Water	100 g per 100 ml
Ethanol, 95%	1 g per 100 ml
Ethanol, 100%	0.3 g per 100 ml
Glycerol	5 g per 100 ml
Ethyl ether	Insoluble
Acetone	Insoluble
Benzene	Insoluble
Hexane	Insoluble
Chloroform	Insoluble

Ultraviolet absorption curves are shown in Figure I.

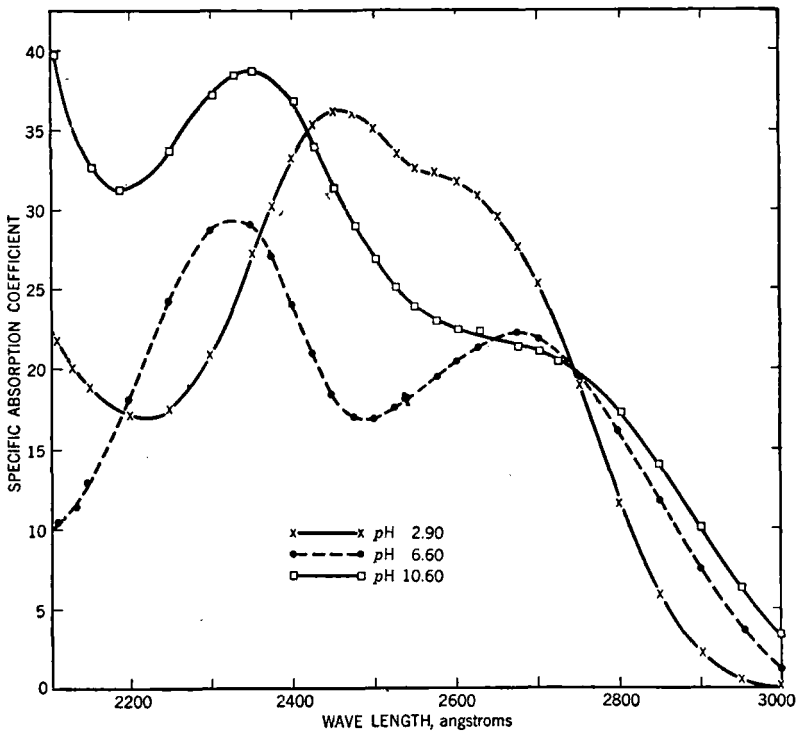


FIG. I. Ultraviolet Absorption Curves for Thiamine in Aqueous Solution (*5*).

In neutral or alkaline solutions, thiamine is rapidly destroyed, presumably because of decomposition of the thiazole portion. However, in acid solution (at pH 3.5), the vitamin will withstand sterilization at temperatures of 120°C for one-half hour without loss of activity. Sulfite will cause a cleavage of the molecule in solutions of pH 4–6.

In dry form, the vitamin is very stable and is not sensitive to atmospheric oxidation. In solution, however, thiamine is quite sensitive to reduction and oxidation. A dihydro-compound, devoid of vitamin activity, is formed by mild reduction. Mild oxidation under physiological conditions yields a disulfide of biological activity equal to that of the vitamin. *In vitro* oxidation of the vitamin yields thiochrome, which is biologically inactive. Even on prolonged standing in alcoholic solutions very small amounts of thiochrome will be formed. This ease of oxidation has been the basis of the thiochrome method for determining thiamine.

Prior to the isolation and identification of thiamine, vitamin B_1 activity was designated in terms of the International Unit, arbitrarily established as 10 milligrams of an adsorbate prepared from an extract of rice polishings. When pure thiamine became available it was shown that 3 micrograms were approximately equal to 10 milligrams of the adsorbate. Accordingly, the International Unit (I.U.) was defined as the vitamin activity of 3 micrograms of the pure chemical; thus one gram contains 333,000 I.U.

Methods Available

The many methods which have been proposed for the analysis of thiamine can be classified into animal, microbiological, chemical, or physical. Each class of methods has certain advantages and disadvantages which limit its usefulness.

Animal assays were developed first since the vitamin nature of thiamine was first detected by observations of the failure of man or animals to thrive on thiamine-deficient diets. While animal assays have many disadvantages, they are of importance in determining the thiamine available to an animal. Three principal types of animal assays have been proposed: (1) growth measurements, (2) curative tests, and (3) physiological response (bradycardia). Of these, the first two, using rats, pigeons, or chicks, have the widest application. The growth test with rats requires a 2- to 4-week depletion period on a thiamine-free diet followed by a 4-week growth period on the test and standard diets. Thus 6 to 8 weeks are required for completion of an assay. The rat curative

procedure is slightly shorter, requiring only a 5- to 8-day recovery period after the depletion. This time-consuming feature is one of the principal disadvantages of the biological methods. This, coupled with the need for large groups of animals and carefully prepared diets, makes them also very expensive. Like all biological data, the results vary considerably, depending upon the particular group of animals or upon the technics employed. Relatively recent work has demonstrated that bacteria in the intestinal tract can synthesize thiamine and make it available to the animal. This introduces another complicating factor into the evaluation of data obtained by biological methods. Nevertheless, since the object of most thiamine assays is to determine the amount available for the metabolism of man or animals, the biological assays are used as a measure of physiologically available thiamine.

The microbiological methods include fermentation procedures and methods involving the growth of or acid production by bacteria, yeasts, molds, or fungi. They consume much less time, are less expensive, and yield more reproducible results than the animal assays. From 4 hours to 3 days are required to complete an assay. Their main disadvantage is the tendency for substances other than thiamine, especially breakdown products of thiamine, to respond in the same way as the vitamin. An assay thus may give high values unless correction factors are applied. With improvement of the methods by including blanks representing response after destruction of thiamine, accurate values can be obtained.

Chemical analyses can be carried out rapidly and economically and are more applicable to routine determinations than most of the other methods. An experienced analyst can complete 12 to 20 assays in 8 hours. Two principal types have been used: a determination of thiochrome; or measurement of the color produced by coupling an amine such as *p*-aminoacetophenone with thiamine (*38,39*). The thiochrome method is more widely applicable to food and feed products than the colorimetric methods, which require several micrograms of thiamine per ml of extract for reliable assays. With some types of samples, care must be taken to insure complete extraction of the vitamin and separation from as many impurities as possible, since extraneous fluorescent or colored substances will affect the results unless proper blanks are applied.

Spectrophotometric methods can be applied only to relatively pure solutions of thiamine and hence are of little importance in the analysis of foods, feedstuffs, and many pharmaceuticals.

In the hands of capable analysts, all the methods yield similar data for most products (*17, 40–42*). Some substances give atypical results

with one or more of the methods, and must be assayed by a more suitable procedure. In general, the chemical procedures give slightly lower values than the biological or microbiological, probably due to the measurement of small amounts of nonthiamine materials in the latter procedures, or the impossibility of complete extraction or recovery of thiamine in the former procedures.

THIOCHROME METHOD

A. Principle

Thiochrome procedures depend upon the oxidation of thiamine to thiochrome, which fluoresces in ultraviolet light. Under standard conditions and in the absence of other fluorescing substances, the fluorescence is proportional to the thiochrome present, and hence to the thiamine originally in solution. If necessary, the thiamine may be freed from interfering substances by treatment with adsorbents which retain thiamine but not the impurities. Subsequent elution usually provides an extract sufficiently pure for analysis.

B. Equipment

The following list of equipment is sufficient for 10 simultaneous (or 5 duplicate) determinations, a convenient number for one operator to handle.

No. of pieces	*Item*
12	*Volumetric Flasks*, 100 ml
10	*Funnels*, 2- or 3-inch, with qualitative papers to fit
10	*Receiving Vessels*, 100-ml Erlenmeyer flasks, beakers or bottles, etc.
11	*Base Exchange Tubes* with a 25- to 30-ml reservoir constricted gradually into a column 6 to 8 mm inside diameter and 10 to 15 cm long. If 50–80 mesh Decalso is used, the 10-cm adsorption columns are satisfactory, but if coarser Decalso is used, 15-cm columns should be used.
2	*Rapid Delivering Pipets*, 3 ml, either volumetric transfer or Mohr
1	*Volumetric Transfer Pipet*, 4 ml
11	*Volumetric Transfer Pipets*, 5 ml. This number can be reduced if a pipet is rinsed with each assay solution rather than using a clean one for each sample.
2	*Pipets*, 10 ml, volumetric transfer or Mohr
2	*Volumetric Transfer Pipets, One Rapid Delivering*, 15 ml. An automatic 15-ml rapid delivering pipet may be substituted for the rapid delivering volumetric pipet if desired.

(*Continued on page 74*)

No. of pieces	*Item*
10	*Volumetric Transfer Pipets*, 20 ml. This number can be reduced if a pipet is rinsed with each assay solution rather than using a clean one for each sample.
1	*Volumetric Transfer Pipet*, 25 ml
11	*Volumetric Flasks*, 25 ml. If desired, graduates or nonprotein nitrogen tubes may be substituted.
2–10	*Glass-Stoppered Maizel-Gerson Reaction Vessels*, (Wilkens-Anderson Co., 111 N. Canal St., Chicago, Ill.). The number of reaction vessels needed depends upon the technic of the operator, the minimum being two.
1	*Rack* to support the number of Maizel-Gerson vessels being used at one time.
1	*Siphon Bottle* (optional). See Figure II.
2 or more	*Matched Cuvets.* For some instruments, the cuvets may be prepared from test tubes of identical diameter which give the same galvanometer reading when tested with quinine sulfate solution. The number of cuvets needed depends upon the technic of the operator and should equal the number of reaction vessels used.
1	*Photofluorometer*, with filters and voltage regulator. Among suitable instruments are the Coleman Model 12, the Coleman Model 11 with ultraviolet lamp and the Pfaltz and Bauer fluorophotometer, Model B. The different types vary considerably in sensitivity, a point to be considered in selecting equipment.

FIG. II.
Siphon Bottle.

C. Reagents

All chemicals should meet A.C.S. specifications or be of Reagent Grade. Insofar as possible, contact of reagents and solutions with cork or rubber should be avoided as these materials may contribute fluorescing substances which will interfere with the assay.

1. **Anhydrous Sodium Sulfate, Granular.** See D-4-(b)-(2).

2. **15% Sodium Hydroxide Solution.** Dissolve 15 g NaOH in water and dilute to 100 ml.

3. **1.0% Potassium Ferricyanide Solution.** Dissolve 1.0 g of $K_3Fe(CN)_6$ in water and dilute to 100 ml. This reagent is stable indefinitely if kept cool and in the dark, preferably in a brown bottle.

4. **Alkaline Potassium Ferricyanide Solution.** Dilute 3 ml of 1.0% potassium ferricyanide to 100 ml with cool 15% NaOH solution. Prepare fresh daily and keep out of sunlight.

5. **0.1 *N* Hydrochloric Acid Solution.** Dilute 8.5 ml of concd. HCl to 1 liter with water.

6. **0.1 *N* Sulfuric Acid Solution.** Dilute 2.8 ml of concd. H_2SO_4 to 1 liter with water.

7. **2.5 *M* Sodium Acetate Solution.** Dissolve 205 g of anhydrous $NaC_2H_3O_2$ or 345 g of $NaC_2H_3O_2 \cdot 3H_2O$ in water and dilute to 1 liter.

8. **Isobutyl Alcohol.** This product should be of such purity that its fluorescence does not exceed 10% of the fluorescence of the quinine standard. The purification of isobutyl alcohol can best be effected by redistillation using all-glass apparatus. Collect for immediate use that portion which distils between 105 and 108° C. The distillate collected prior to this temperature range still retains an appreciable amount of water. This fraction may be dried by the addition of NaOH pellets in excess, with shaking. On standing, the NaOH solution formed will separate from the alcohol, which can be decanted or siphoned off and redistilled.

9. **Enzyme Solution.** Prepare fresh daily, using Taka-diastase (Parke Davis and Co., Detroit, Mich.), Clarase (Takamine Laboratories, Clifton, N. J.), Polidase (Schwarz Laboratories Inc., New York City, N. Y.), Mylase (Wallerstein Laboratories, New York City, N. Y.), or other suitable phosphatase. Suspend, with thorough shaking, 6 g of the enzyme in 2.5 *M* sodium acetate solution and dilute to 100 ml with additional sodium acetate solution. If this enzyme preparation is not practically thiamine-free, a new lot of enzyme should be secured or a correction applied to the calculations.

10. **25% Potassium Chloride Solution.** Dissolve 250 g of KCl in water and dilute to 1 liter. This reagent is stable indefinitely.

11. **Acid 25% Potassium Chloride Solution.** Dilute 8.5 ml of concd. HCl to 1 liter with 25% KCl solution. This reagent is stable indefinitely. If desired, a solution containing 25% KCl by weight may be used.

12. **Activated Decalso.** A mixture of equal parts of 30–50 mesh and 60–80 mesh may be used. However, if aliquots containing more than 7 γ of thiamine are to be assayed, the 60–80 mesh product is required to provide a greater adsorbing capacity at the expense of speedy filtration. The entire Decalso activation procedure can be carried out in a Büchner funnel. To 100 g of Decalso add 200–250 ml of hot 3% acetic acid and allow the acid to remain in contact with the Decalso for 10–15 minutes. Apply mild vacuum to drain off the solution. Repeat the acid wash. Repeat the washing once using approximately 250 ml of hot 25% KCl solution. Then wash once more with hot acetic acid solution. After draining

off the wash solution, flush out the remaining KCl by washing the Decalso with 500- to 600-ml portions of hot water, applying mild vacuum continually. The final wash solution should be free of chlorides as indicated by failure to give a precipitate with 1% $AgNO_3$ solution. Dry at room temperature or in an oven at any temperature below 100° C and store in a stoppered, wide-mouth bottle. To have the material in its maximum state of expansion, moisten a small portion of the dry Decalso and store it in a stoppered, wide-mouth bottle for daily use. To retain the hot acid and KCl solutions in contact with the Decalso in the Büchner funnel during the 10- to 15-minute holding period, place a pinch clamp on the rubber tubing connected to the side arm of the filter flask. A slight pressure will be built up within the flask and practically stop the passage of the solution through the funnel. Other methods of activation have been described (*17*).

13. **Stock Thiamine Solution.** Dry thiamine hydrochloride (preferably U.S.P. Reference Standard, from E. Fullerton Cook, United States Pharmacopoeia, 43rd. St. and Woodland Ave., Philadelphia, Pa.) over P_2O_5 in a desiccator for at least 24 hours. Dissolve 100 mg in 25% ethanol (Specially Denatured Alcohol No. 1 or 30 is satisfactory), and dilute to 1 liter with the same reagent. This solution is stable for several months if kept at 5° C or below. The standard thiamine may also be dissolved in 0.01 *N* HCl, if desired.

14. **Standard Thiamine Solution.** Dilute 5.0 ml of stock thiamine solution (warmed to room temperature) to 100 ml with water. Transfer 4.0 ml of this dilution to a flask containing 75 ml of 0.1 *N* H_2SO_4 and 5 ml of sodium acetate solution and adjust to 100 ml with water. The final concentration of thiamine is 0.2 γ per ml. Make fresh daily.

15. **Stock Quinine Sulfate Solution.** Dissolve 100 mg of U.S.P. quinine sulfate in 0.1 *N* H_2SO_4 and dilute to 1 liter with the same solvent. This solution is stable indefinitely if stored in a dark brown bottle. *Caution!* If the solution becomes turbid, discard and prepare anew.

16. **Working Quinine Sulfate Solution.** Dilute 3 ml of stock quinine sulfate solution to 1 liter with 0.1 *N* H_2SO_4 to give a final concentration of 0.3 mg per liter. Fluorescent glass standards are available for use with certain instruments. These may be obtained from Bausch and Lomb Optical Co., Rochester, N. Y. or from Pfaltz and Bauer Inc., New York City, N. Y.

17. 95% Ethanol. Specially denatured alcohols Nos. 1 and 30 are satisfactory substitutes.

18. 3–5% Acetic Acid Solution. Dilute 30 ml acetic acid to 1 liter with water.

D. Procedure

The following procedure is a composite of several published methods (*8–22*) or unpublished modifications, and represents a workable basis from which variations suitable to specific products may be evolved.

1. Extraction

(a) Accurately weigh or pipet into a 100-ml volumetric flask an amount of suitably prepared sample estimated to contain 10 to 30 γ of thiamine. Add 75 ml of 0.1 *N* HCl and heat for 30 minutes in a boiling water bath with occasional shaking.

(1) The function of extraction is two-fold: (a) The use of an acid extraction effects a practically complete solution of the various forms of thiamine that may be present in the product and (b), the resulting pH of the acid extract is such that the thiamine is very stable, even when heated in a steam bath for 1 hour or longer. Either HCl or H_2SO_4 can be used. In case acid concentrations lower than 0.1 *N* are used, smaller quantities of sodium acetate must be added in the enzyme mixture of the next step. The final pH should be 4.5 to 5.0.

(2) Smaller quantities of thiamine may be determined, but with reduction in the precision and accuracy of the method. In case solutions or suspensions of 15 ml or more are used, it may be more convenient to use 50 ml of 0.15 *N* acid, thus giving approximately 0.1 *N* acid concentration including the water of the sample.

(3) With some powdered materials, such as flours, it may be advisable to pipet part of the acid into the flask before adding the sample in order to prevent the latter from lumping or adhering to the flask.

(b) Cool the extract to 50° C or lower and add 5 ml of freshly prepared enzyme suspension (C-9). Incubate at 45–50° C for 2 hours or longer.

(1) This step aids in the conversion of bound thiamine to its free form. The phosphatase will hydrolyze any phosphate esters of thiamine that may be present, liberating the thiamine (*10,18*). This is important because the oxidation products of the esters are not soluble in isobutyl alcohol, while thiochrome is (*10,13*). Also, the enzymes, because of diastatic activity, are effective in preparing suitable extracts of starchy materials.

(2) Sodium acetate of 4 *M* strength may be substituted for the 2.5 *M* reagent. The former has a lesser tendency to support mold growth. The volume added need not be altered, provided the pH of the digest does not exceed 5.0.

(3) Two hours suffice for most samples, but occasionally longer digestions give higher values. When working with unfamiliar material, it is well to extend the period to three hours or more. At this stage it is frequently convenient to allow samples to stand overnight at 37° to 50° C under benzene (*12,18*).

(c) Cool to room temperature and dilute with water to 100 ml. Mix thoroughly and filter, discarding the first few milliliters of filtrate.

(1) If all of the filtrate is needed, the first few milliliters must be retained. If turbid, this portion should be refiltered through the same filter. Some samples cannot be completely clarified in this manner, and may be taken to the next step even though turbid.

(2) If much solid material remains after the enzyme digestion, it is advisable to centrifuge the sample and wash the precipitate several times and then to adjust the volume of solution and washings to 100 ml. Filtration is then unnecessary. For most samples, the quantity of solids is not great enough to influence the assay significantly.

(3) If much fat is present, the volume is adjusted to 100 ml, exclusive of fat which contains insignificant quantities of thiamine.

(4) If the assay cannot be completed in one day, this makes a convenient stopping point.

2. Purification

(a) Fill each adsorption tube with water; then allow Decalso (C-12) to fall in place by gravity until a layer 4–8 mm deep is present in the reservoir above the column. Allow tube to drain until no water is visible above Decalso.

(1) Preparation of the adsorption tube in the manner described gives a free-flowing column. The Decalso, in the reservoir of the tube, prevents clogging with turbid extracts (*19*). If desired, a small wad of glass wool or a disc of fine nichrome screen (punched out with a paper punch) may be used to prevent particles of Decalso from plugging the capillary.

(2) Some laboratories prefer to use dry Decalso, placing 4–6 g of prepared Decalso on a plug of glass wool in the adsorption tube, packing lightly, and then moistening with 10 ml of 3% acetic acid. It is advisable to apply mild vacuum when moistening the Decalso to avoid air pockets in the column. Such air pockets will reduce the efficiency of the adsorbing agent and may result in losses of thiamine during the elution step.

(b) Pipet 25 ml of the filtrate from D-1-(c) into the reservoir of the prepared adsorption tube, and allow it to pass slowly through the Decalso column. Discard the filtrate. Wash the reservoir and column with three successive portions (about 10 ml each) of hot water, discarding the washings.

(1) A properly prepared Decalso will adsorb thiamine from solution, thereby separating it from many substances which might interfere with subsequent steps. Treatment of the column with acid KCl solutions replaces the adsorbed thiamine with potassium ions. The thiamine then appearing in the KCl solution usually is sufficiently pure to permit accurate analyses.

(2) Although 1 g of Decalso will adsorb more than 40 γ of pure thiamine quantitatively, 10 γ is considered to be the practical upper limit since other substances, such as amino acids, greatly reduce the capacity of the column. 10 γ of thiamine will be 98% adsorbed from 15 ml of an 8% solution of casein hydrolysate, at pH 4.5 but 20 γ will overload the columns, approximately 25% passing into the effluent.

(3) The 25-ml aliquot taken for assay should contain approximately 5 γ of thiamine. If the potency of the solution is such that a 25-ml aliquot is estimated to contain less than 3 or more than 8 γ, adjustments should be made in the volume taken for purification. In some cases, urine for example, the volume of starting material and the concentration of impurities may influence the adsorption on the column. The use of smaller amounts of thiamine may be advantageous.

(4) The aliquot for the adsorption step may be heated after measuring or used at room temperature, the former being preferred for increased speed of drainage if large volumes are used. The reservoir and Decalso may be washed with either hot or cold water, the former being preferred because of faster drainage (*19*).

(5) With certain types of samples, the purification procedure may be omitted providing the solutions are clear and the blanks [D-3-(c)] are low (not more than 1½ times the blank for the standard thiamine solution). Preliminary determinations should be run on a few samples to determine whether purification is necessary. If this step is omitted, the thiamine standard solution is used directly without adsorption and elution. Thiamine can be determined directly on extracts of such materials as meat and meat products, milk and fortified flour (*10,12,16,21,22,24*). Omission of the purification procedure eliminates the possibility of any loss of thiamine due to incomplete adsorption and elution and decreases errors of manipulation (*14,24*). Inclusion of the purification procedure in the thiamine assay eliminates some of the errors caused by interfering substances in the extracts. The analyst should try both methods before deciding which is better for his type of product.

(c) After washing, place 10 ml of acid KCl (C-11) in the reservoir, collecting the effluent solution in a 25-ml volumetric flask. Add a second 10-ml portion when all of the first has entered the Decalso, collecting the eluate in the same 25-ml flask. When this second portion has drained into the receiver, dilute the contents to the mark with acid KCl solution and mix well.

(1) If the tip of the base exchange tube fits tightly into the mouth of the volumetric flask during elution, an air pocket may form in the flask causing overflow and loss of the eluate. This may be avoided by adjusting the tube so that the tip barely touches the inside of the top of the flask.

(2) If hot solutions are used, boiling the acid KCl should be avoided. Boiling may cause clogging of the Decalso column as the KCl crystallizes from the supersaturated solution, with a resultant loss of the sample.

(3) For routine purposes, after the wash water has drained from the columns under the influence of gravity, 25 ml of acid KCl may be used as a single charge to elute

the thiamine. The volume need not be adjusted since variations in the amount of solution draining from the column are relatively insignificant.

(4) Some investigators use NaCl in activating the Decalso and for eluting solutions. A reagent grade chemical, free of carbonates, should be used.

(d) Repeat steps (a) (b) and (c) with a new column and using 25 ml of standard thiamine solution (C-14) containing 5.0 γ of thiamine, in place of the sample aliquot.

(1) This step provides a standard for use in calibrating the instrument. One such standard is sufficient for a series of assays once skill is acquired in handling the solutions. Some investigators prefer to use an "internal" standard in which the response to a known amount of thiamine added to an aliquot of the unknown is determined and used as a basis for calculations (*11*). It is claimed that such a scheme corrects for atypical responses. On the other hand, ordinarily a small amount of thiamine is added so that an error in reading either the sample or the sample plus standard will result in a relatively large error in the analysis.

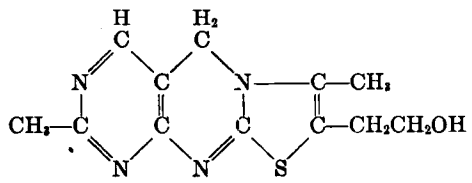
(2) Some Decalso preparations do not act as efficiently as others (*14, 17, 24*). Accordingly, this standard should be checked against one prepared directly from the stock thiamine solution in order to determine the recovery after the adsorption and elution steps. The latter standard may be prepared by diluting the stock thiamine solution as directed in C-13 except that acid KCl solution must be used in place of water in making the final dilution. Recoveries of over 92–96% of the thiamine indicate a satisfactory preparation. If recoveries are lower than 92%, the Decalso should be reactivated or discarded in favor of a different lot.

3. Conversion to Thiochrome

(a) Pipet 5 ml of the acid KCl eluate from D-2-(c) into each of two reaction vessels, numbered 1 and 2.

(b) To vessel No. 1 add 3 ml of alkaline ferricyanide solution (C-4), mix gently, then add 15 ml of isobutyl alcohol. Shake vigorously for 90 seconds.

(1) Thiamine is oxidized by potassium ferricyanide in the presence of strong alkali, yielding thiochrome (*4,8–10*):



This material is soluble in isobutyl alcohol. The yield is not quantitative but is very constant under standardized conditions (*13*). When irradiated with ultraviolet light, thiochrome, which has absorption maxima at 358 and 375 mμ, fluoresces, emitting a blue light (*4*).

(2) The step in which thiochrome is produced and extracted is one of the most varied in the entire procedure. Some investigators claim that speed is of utmost importance (*17,19*). Others maintain that the solutions may stand for several minutes after the addition of alkaline ferricyanide solution and before shaking with isobutyl alcohol without altering the assay value (*25,26*). One investigator has found that more concordant results are obtained if the KCl eluate is added to the potassium ferricyanide–sodium hydroxide reagent rather than in the usually employed reverse manner (*43*). However, this technic cannot be used with certain samples (*44*). Most operators arrange their work so that several reaction vessels can be shaken simultaneously. Mechanical shakers may be used if desired. Whatever time schedule is adopted, it should be constant for both standards and unknowns.

(3) The following data concerning this problem have been obtained (*26*). In each case, 5-ml aliquots of KCl standard solutions have been treated with 3 ml of alkaline potassium ferricyanide solution and then shaken with isobutyl alcohol after the indicated intervals.

Time	Galvanometer deflections Sample		Blank
Shaken immediately	86.0	84.5	12
30 seconds before shaking	86.0	84.5	—
60 seconds before shaking	85.5	86.0	—
180 seconds before shaking	86.0	85.5	—
300 seconds before shaking	83.0	83.5	—

(4) Extraction of thiochrome with isobutyl alcohol may also be effected by bubbling a stream of air or nitrogen through the reaction vessels instead of shaking them (*31*).

(5) It has been shown that hematin accelerates the destruction of thiochrome in alkaline solutions. The precipitation of proteins removes the hematin from most assay solutions in which it might be expected to occur (*27*). The assay of blood requires special precautions (*19*). Excesses of iron in solution are to be avoided, as iron will combine with the ferricyanide and inhibit the oxidation.

(6) Separate 3-ml pipets should be used for the alkaline ferricyanide solution, and for the NaOH solution used in the next step. These, as well as the 15-ml pipet used for the isobutyl alcohol, should be rapid delivering. All reagents must be brought to room temperature (*20*).

(7) A word of caution is necessary regarding the use of any lubricant in the stopcock of an automatic buret for isobutyl alcohol, should such a piece of equipment be used. Vaseline, Lubriseal, or any similar lubricant will be dissolved by the solvent and contribute considerable fluorescence to the final reading. If it is necessary to use a lubricant, orthophosphoric acid, glycerol or Silicone, in moderate quantities, can be employed successfully (*30*).

(8) Some investigators use stronger alkali and more ferricyanide than given in the directions (*13,14,19,43*). Others, however, have been unable to show differences in assay values when the alkali concentration is increased (*9,26*). Excellent results have been claimed by using 3 ml of a mixture of 9 parts of 10 *N* NaOH and one part of potassium ferricyanide solution to 5 ml of the KCl eluate (*19*). This gives more

consistent results with urine than the less concentrated alkali. Although the volume is considerably increased, identical results are obtained by oxidation of 10 ml of eluate with 5 ml of this alkaline ferricyanide solution, provided the standard is prepared in similar volumes. English workers advocate the addition of ferricyanide solution dropwise to the alkaline extract until a faint yellow color persists, thereby avoiding any great excess of ferricyanide which might destroy the thiochrome (*15*). Regardless of the procedure used, the yellow color should persist for at least 15 seconds.

(9) In view of the conflicting claims, it is advisable for each assayer to standardize his technic and give each sample and the standard exactly the same treatment.

(c) To vessel No. 2 add 3 ml of 15% NaOH solution (C-2) followed by 15 ml of isobutyl alcohol. Shake vigorously for 90 seconds.

(1) Theoretically, this provides a blank identical with the assay solution [D-3-(b)] except for lack of thiochrome; but, actually, nonthiamine fluorescent substances present in some samples, notably urine, are affected differently by alkaline ferricyanide solution and NaOH solution. In such cases, blanks prepared with NaOH are not valid. In work with urine it has been shown (*31*) that much, if not all, of the substances interfering with true blank readings can be eliminated by adding 0.50 to 0.55 ml of a mixture of equal parts of concd. HCl and 85% H_3PO_4 to the reaction vessels containing the assay solution and the blank solution before shaking with isobutyl alcohol. This brings the pH within the range 8 to 9.5, where a white precipitate appears. Under these conditions, thiochrome may be completely extracted from aqueous solution with isobutyl alcohol, but nonthiamine fluorescent substances are largely eliminated.

(d) Repeat steps (a) (b) and (c) using 5 ml of standard thiamine solution eluate, [D-2-(d)].

4. Separation of Thiochrome Solution

(a) Centrifuge the reaction vessels 1 to 3 minutes.

(1) If the phases separate readily and the alcohol layers are clear, the vessels need not be centrifuged.

(b) Siphon out the aqueous layers (lower) and add 2–3 g of anhydrous Na_2SO_4. Shake 30 seconds.

(1) A siphon bottle may be used conveniently for removal of the aqueous layer if many assays are made.

(2) The Na_2SO_4 need not be weighed. A quarter-teaspoon of the anhydrous material usually provides enough of this chemical to carry out the drying procedures. If the adsorption on Decalso is omitted, more Na_2SO_4 may be needed (*12,26*). The quantity is not so important as that equal amounts of Na_2SO_4 be added to all vessels. Varying the amount of this chemical may produce variation in the blank and unknown reading since the Na_2SO_4 may contain small amounts of fluorescing materials. Granular Na_2SO_4 is preferred to the powdered type because of greater ease in

handling. The Na_2SO_4 may be checked by shaking 1–2 g with 15 ml of isobutyl alcohol and comparing the fluorescence of this extract with that of the alcohol alone. If the difference is more than 2–3 deflections, the lot should be rejected.

(3) Certain samples, blood for example, yield an emulsion when the isobutyl alcohol extract is shaken with the Na_2SO_4 (*19*). This can be largely prevented and a crystal-clear extract obtained immediately, if the Na_2SO_4 is added in four small amounts instead of all at once. The extract should not be shaken, although it is permissible to rotate the mixture a few seconds after each addition. When added in this manner, the first sulfate entraps the aqueous solution remaining at the bottom of the vessel. This is covered by successive layers of fresh sulfate. If not clear, the extract is rotated very gently, a small amount of the sulfate is added, and the mixture is again centrifuged. The total sulfate added should not exceed one-quarter teaspoon.

(4) Some workers prefer not to use Na_2SO_4, but add 1 ml of ethanol to a definite aliquot of the isobutyl alcohol extract (*43*).

(c) Centrifuge or allow to stand until sparkling clear.

(1) Some analysts allow the isobutyl alcohol and Na_2SO_4 to stand until sparkling clear. This method, however, decreases the amount of isobutyl alcohol recovered from the drying agent. Centrifuging the suspension results in a more compact layer of crystals and minimizes the possibility of decanting Na_2SO_4 particles into the cuvet.

5. Measurement of Thiochrome

(a) Decant at least 10 ml of the clear, colorless isobutyl alcohol solutions from D-4-(c) into separate matched cuvets.

(1) Colored isobutyl alcohol solutions may absorb the exciting ultraviolet light or the fluorescent light, resulting in erroneous readings. They likewise indicate the possible presence of fluorescing substances other than thiochrome. Recovery experiments or internal standards [2-(d)-(1)] must be conducted before reliance can be placed upon analysis of such solutions.

(2) The amount of the isobutyl alcohol extract of thiochrome taken for fluorescence measurement is dependent upon the instrument used (*28*). In all cases, the minimum volume taken should be sufficient to bring the liquid level in the cuvet well above the top edge of the incident light beam. In most instances this minimum volume is approximately 10 ml. The manufacturers' instructions should be followed. These volumes need not be accurately measured, except to insure the minimum volume. The cuvets should be washed between runs and dried with acetone or alcohol and ether. Allow all traces of these reagents to evaporate.

(b) Determine the fluorescence of the isobutyl alcohol solutions from D-4-(c) in terms of galvanometer deflections, operating the photofluorometer according to the manufacturer's directions for the instrument used. Check the photofluorometer between readings with the working quinine

solution (C-16) or with a glass standard. If the instrument does not give the initial setting with the working quinine solution, readjust, and then repeat the readings on the isobutyl alcohol solutions. All solutions, including the quinine reference, should be at room temperature.

(1) Since ultraviolet light destroys thiochrome, excessive exposure should be avoided. Less than 15 seconds is suggested.

(2) Remove the working quinine sulfate solution from the instrument between readings to prevent increases in temperature which cause variations in the fluorescence (*29*).

(3) Despite the fact that thiochrome is stable in isobutyl alcohol, it is desirable to make the readings promptly. Ordinarily, delays up to 20 minutes will not alter the results, providing the solutions are not exposed to bright daylight.

6. Calculation

(a) Thiamine content of the sample in γ per g =

$$\frac{U - UB}{S - SB} \times \frac{1}{5} \times \frac{25}{V} \times \frac{100}{\text{wt. of sample}}$$

Where U = deflections of unknown, UB = deflections of the unknown blank, and S and SB are the deflections for the standard and its blank, and V = volume of solution used for the adsorption on Decalso. The factor 1/5 converts the reading to γ per ml instead of γ per 5-ml aliquot. Since the final volume of eluate is 25 ml, the factor 25/V corrects for volume changes during adsorption and elution. If the suggested 25 ml is adsorbed [D-2-(b)] this factor becomes unity.

(1) The following examples illustrate the method of calculation:

In comparing the method using adsorption and elution for purification of the assay solution to the modification in which these steps are omitted, the following experiment was conducted:

2 g of pork muscle were extracted [D-1-(a)(b)(c)], yielding 100 ml of assay solution. 5 ml of this solution were assayed directly [D-2-(b)-(5)].

A 15-ml aliquot of this solution was also purified by adsorption on and elution from Decalso, the final volume of the eluate being 25 ml [D-2-(c)]. 5-ml quantities of the eluate were assayed (D-3,4,5).

Two standard thiamine solutions were used [C-14 and D-2-(d)]. Readings were made with a Coleman photofluorometer, Model 12.

Adsorbed and eluted	Galvanometer deflections
Standard, 1 γ in 5 ml KCl	62.4
For the standard blank	7.0
5 ml KCl eluate	52.0
5 ml KCl eluate blank	8.0

Calculations:

$$\frac{52 - 8}{62.4 - 7} \times \frac{1}{5} \times \frac{25}{15} \times \frac{100}{2} = 13.2\ \gamma \text{ per g}$$

Directly on assay solution	Galvanometer deflections
Standard (C-14), 1 γ in 5 ml buffer solution	62.0
For the standard blank	8.0
5 ml assay solution	80.0
5 ml assay solution blank	11.0

Calculations:

$$\frac{80.0 - 11.0}{62.0 - 8.0} \times \frac{1}{5} \times \frac{100}{2} = 12.80\ \gamma \text{ per g}$$

Duplicate samples simultaneously carried through the assay procedure should give values agreeing within ±2.5% of the mean.

Assays of a homogeneous material made on different days should agree within ±5% of the mean. Usually agreement within ±2.5% of the mean can be easily obtained by skilled operators.

FERMENTATION METHOD

A. Principle

With certain yeasts, rate of fermentation in a specially prepared medium is proportional to amount of free and bound thiamine present (*32*). By measuring, in terms of carbon dioxide gas produced, the stimulation brought about by a known amount of sample and comparing this to the stimulation induced by a known amount of thiamine, it is possible to estimate rather accurately the thiamine content of the sample. Certain cleavage products of thiamine also stimulate the rate of fermentation although these substances are not biologically active (*33*). Treatment with sulfite (*34*) destroys intact thiamine and renders it nonstimulatory to yeast. The activity of the cleavage products is not affected by sulfite treatment; consequently a sulfite-treated blank is run to correct for their activity.

B. Equipment

This procedure is based upon the use of a specially designed fermentometer, of which models are available for simultaneous measurement of six or twelve fermentation reactions. The following list of equipment suffices for measuring twelve reactions. This permits assay of 4 samples, with standards in duplicate.

No. of pieces	*Items*
1	*Fermentometer*, with 12 gasometers, a shaking device to hold 12 reaction bottles, and a thermoregulator to maintain the temperature of the water bath at 30° ± 0.2° C. There are two sizes in use, the older style large fermentometer and a newer small type. When using the smaller instrument the quantities of samples and reagents need to be only half those used in the larger fermentometer. (The small Aminco Fermentometer may be obtained from the American Instrument Co., Silver Springs, Md.)
10	*Bates Sugar Flasks*, 100 ml, or other wide-mouth, short-neck volumetric flasks
1	*Spot Plate*, as large as obtainable
10	*Graduated Cylinders*, 50 ml
1	*Graduated Cylinder*, 100 ml
3	*Volumetric Transfer Pipets*, 20 ml, two of which are rapid delivering
1	*Volumetric Transfer Pipet*, 10 ml
1	*Volumetric Transfer Pipet*, 4 ml
1	*Erlenmeyer Flask*, 250 ml
7	*Erlenmeyer Flasks*, 500 ml
1	*Volumetric Flask*, 100 ml
2	*Volumetric Flasks*, 500 ml
1	*Volumetric Flask*, 1000 ml

C. Reagents

All chemicals should meet A.C.S. specifications or be of Reagent Grade.

1. Solution A. Dissolve 180 g of $NH_4H_2PO_4$, 72 g of $(NH_4)_2HPO_4$, 0.2 g of nicotinic acid and 0.004 g of pyridoxine hydrochloride in water. Add 200 ml of acid-hydrolyzed casein solution (C-3) and dilute to 1000 ml. Divide the solution between three cotton-stoppered 500-ml Erlenmeyer flasks and heat for 30 minutes at 100° C on three successive days. Store in the refrigerator.

2. Solution B. Dissolve 200 g anhydrous dextrose, 7.0 g $MgSO_4 \cdot 7HO_2$, 2.2 g KH_2PO_4, 1.7 g KCl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeCl_3 \cdot 6H_2O$ and 0.01 g $MnSO_4 \cdot 4H_2O$ in water and dilute to 1000 ml. Divide the solution between three cotton-stoppered 500-ml Erlenmeyer flasks and heat for 30 minutes at 100° C on three successive days. Store in the refrigerator.

3. Acid-Hydrolyzed Casein Solution. Place 100 g of vitamin-free casein in a 2-liter round bottom flask and add 300 ml of water and 300 ml of concd. HCl (this gives approximately constant-boiling HCl). Reflux

8 hours on a sand bath. In a hot water bath, evaporate the resulting mixture to a thick syrup under reduced pressure. Take up the syrup with 100 ml of hot water and again reduce to a thick syrup. Again take up the syrup with water (about 400 ml) and adjust the pH to 3 with NaOH solution (1 *N* or stronger). Dilute to 950 ml, add 20 g of activated charcoal, stir for 1 hour and filter. If the hydrolysate is darker than straw colored, repeat the charcoal treatment. Adjust the solution to pH 6.8 with NaOH solution and dilute to 1 liter with water. Refrigerate under toluene. Experienced users of this method report that many materials may be assayed successfully without acid-hydrolyzed casein in the basal medium. However, the operator should check this point for each new substance under examination, if the reagent is omitted routinely.

4. Indicator, Acid-Starch-Iodide. Place one drop each of the following solutions in an indentation of the spot plate:

5% KI	Dissolve 5 g of KI in water and dilute to 100 ml.
0.2% soluble starch	Mix 0.2 g soluble starch with enough cold water to give a thin paste. Add enough boiling water to bring the volume to 100 ml and boil with stirring for about 1 minute.
50% H_2SO_4	Add slowly, 100 ml concd. H_2SO_4 to 166 ml of water.

5. 0.15 *N* Sulfuric Acid Solution. Dilute 5 ml of concd. H_2SO_4 to 1200 ml with water.

6. Bromothymol Blue Indicator, 0.04% Solution. Grind 0.1 g dry bromothymol blue with 16 ml of 0.01 *N* NaOH solution and dilute to 250 ml. (The solution may be purchased).

7. Chlorophenol Red Indicator, 0.04% Solution. Grind 0.1 g dry chlorophenol red with 23.6 ml of 0.01 *N* NaOH and dilute to 250 ml. (The solution may be purchased).

8. 1 *N* Sodium Hydroxide Solution. Dissolve 40 g of NaOH in water and dilute to 1 liter.

9. 0.1 *N* Sodium Hydroxide Solution. Dissolve 4 g of NaOH in water and dilute to 1 liter or dilute solution (C-8), 1 to 10.

10. 3% Hydrogen Peroxide Solution. U.S.P. H_2O_2 or 30% H_2O_2 (Superoxol) diluted 1 to 10.

11. 0.6% Sodium Bisulfite Solution. Dissolve 0.6 g of $NaHSO_3$ in water and dilute to 100 ml. Prepare fresh daily.

12. Yeast Suspension. Place 25 g of fresh, high-quality, bakers' yeast in a mortar with a little water and grind to a smooth suspension. Transfer

this suspension to a 500-ml volumetric flask and make to volume with water. Make a fresh yeast suspension for each run. *Caution!* Do not use special high-vitamin yeasts.

13. Thiamine Stock Solution. Dry thiamine hydrochloride, preferably U.S.P. Reference Standard from E. Fullerton Cook, United States Pharmacopoeia, 43rd. St. and Woodland Ave., Philadelphia, Pa., over P_2O_5 in a desiccator for at least 24 hours. Dissolve 25 mg in 25% ethanol adjusted to pH 3.5 with HCl and dilute to a volume of 1 liter with this reagent. This solution is stable for several months if capped and stored in the dark at 5° C.

14. Thiamine Working Solution. Warm a small portion of the stock solution (C-13) to room temperature. Dilute 4 ml to 500 ml with water adjusted to approximately pH 3.5 with HCl. The concentration of thiamine in this solution is 0.2 γ per ml. Prepare this solution fresh daily.

15. Gasometer Fluid. Dissolve 350 g of $CaCl_2 \cdot 2H_2O$ in water and dilute to 3500 ml. Color with a small amount of $CuCl_2 \cdot 2H_2O$ and fill gasometers. Replace only when necessary.

16. Ethanol. Dilute 250 ml of 95% ethanol to 1 liter with water.

D. Procedure

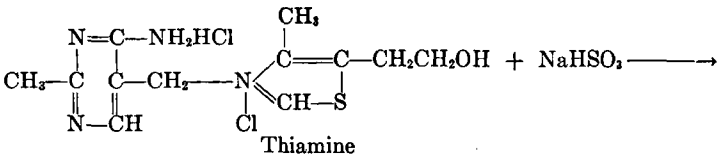
1. Preparation of Sample and Blank Extracts

(a) Place an amount of sample estimated to contain from 6 to 8 γ of thiamine into each of two 100-ml Bates sugar flasks. To prepare the sample for *total fermentation activity* add 50 ml of 0.15 *N* H_2SO_4 to flask No. 1 and mix thoroughly. To prepare the blank for *residual fermentation activity* add 50 ml of fresh 0.6% $NaHSO_3$ solution to flask No. 2 and adjust to pH 5.2, using chlorophenol red on a spot plate as an external indicator. Heat both flasks 30 minutes in a boiling water bath or steam sterilizer with frequent agitation.

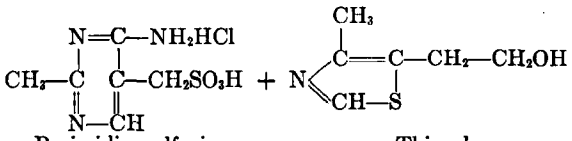
(1) It is assumed that the product being assayed has been sampled in the manner prescribed in Chapter 1 for that type of material. The ratio of acid weight to dry sample weight should be at least 10 to 1. The use of 0.15 *N* H_2SO_4 provides an acid medium for extraction which effects a practically complete solution of the various forms of thiamine that may be present in the product, and results in a pH at which the thiamine is stable, even when heated in a boiling water bath for 30 minutes. When powdery materials are used, it is advantageous to add a few milliliters of the extracting solution before the sample. This tends to prevent lumping.

(2) The same amount of sample is used for determination of total fermentation activity and for determination of residual fermentation activity so that the effect of stimulating substances other than thiamine may be equal in both cases (*35*).

(3) The following equation shows the effect of the sulfite:



Thiamine



Pyrimidinesulfuric acid portion

Thiazole portion

(4) The adjustment of the solution to pH 5.2 in flask No. 2 is important, because at this pH maximum cleavage of the thiamine molecule is obtained under the specified conditions of time and temperature (*36*).

(5) The completeness of the destruction of fermentation stimulating activity of thiamine may be checked for each type of substance assayed by adding a known small amount of thiamine to a sample before sulfite treatment and comparing with a sample to which no thiamine has been added. If desired, a correction factor may be introduced, but this is usually unnecessary.

(b) Cool both solutions to room temperature. Remove the excess sulfite from No. 2 by adding 3% H_2O_2, using the acid–starch–iodide indicator on a spot plate to detect the end point. The indicator has a faint pink color which disappears in the presence of excess sulfite, but which turns violet-blue when an excess of peroxide is present. Add peroxide until the violet-blue color just becomes apparent. All of the sulfite must be destroyed. Now dilute each solution to 100 ml after adjusting to pH 6.5 with 1 *N* and 0.1 *N* NaOH, stirring continuously and using bromothymol blue indicator on the spot plate.

(1) In adjusting the pH of the solution, bromocresol green may be conveniently used on the spot plate as a warning indicator to hasten the speed of the operation. After the solution is alkaline to this indicator (blue) the adjustment is then carefully completed with bromothymol blue. For workers unaccustomed to using these indicators, Clark's "Color Chart of Indicators" (available from Williams & Wilkins Co., Baltimore, Md.), will be useful.

(2) It is important that all of the sulfite be carefully removed from the solution, and that a definite violet-blue color appears on the spot plate after the solution has been well mixed.

(3) Some workers prefer to use an Erlenmeyer flask of convenient size in preparing the sample for residual fermentation activity, and then to transfer the solution to a volumetric flask when diluting to 100 ml (*36*).

2. Preparation of Reaction Bottles for Fermentometer

(a) Using a graduated cylinder measure 21 ml of Solution A and 63 ml of Solution B into a 250-ml Erlenmeyer flask and mix thoroughly. With a rapid delivery transfer pipet, place 20 ml of this mixture into each of 4 reaction bottles. After thorough shaking, measure with a 50-ml graduated cylinder, a 40-ml aliquot of the sample solution from flask No. 1 [D-1-(b)] and pour into reaction bottle No. 1. In like manner place the same volume of the sample solution from flask No. 2 [D-1-(b)] into reaction bottle No. 2 and add 10 ml of thiamine standard solution (2 γ). To bottle No. 3 add 10 ml of thiamine standard (2 γ), and to bottle No. 4, add 20 ml of thiamine standard solution (4 γ). Add enough water to each bottle to make a total volume of 80 ml using the water to rinse the graduated cylinders employed in measuring aliquots into bottles No. 1 and No. 2.

(1) When the small Aminco Fermentometer is used, one-half of the quantities used in 1-(a) and 1-(b) are taken and placed in the smaller 125-ml reaction bottles.

(2) With all gasometers of this Fermentometer in use, two of the six gasometers on each side should be used for 1 γ- and 2 γ-standard reaction bottles, leaving eight gasometers available for unknown samples (*37*).

(3) Theoretically, the basal medium composed of solutions A and B should provide a medium completely adequate for yeast fermentation except for lack of thiamine. Actually, the presence of some nonthiamine constituents of certain samples increases the rate of fermentation and also increases the effectiveness of thiamine. A true blank must account for both of these effects. The addition of 2 γ of thiamine to an assay solution in which the thiamine has been destroyed by sulfite permits an accurate measurement of the true blank by subtraction of the gas volume produced in the presence of 2 γ of thiamine from that produced in the presence of the blank solution +2 γ of thiamine. If materials augmenting the thiamine stimulation are present, they will affect the thiamine added to the blank in the same degree as they will affect the thiamine present in the assay solution. Actually the blank value need not be calculated, as the difference between the blank and thiamine of the unknown is all that is needed in the calculation.

(b) After making certain that the fermentometer bath is at the proper temperature and that the instrument is otherwise ready for use, thoroughly mix the prepared yeast suspension and add 20 ml to each bottle, using a rapid delivering transfer pipet. Complete this operation with the greatest possible speed.

(1) The yeast suspension may be poured from the volumetric flask to a suitable size beaker with thorough mixing just before use. The suspension should be quickly stirred with the rapid delivering transfer pipet before each addition of yeast suspension.

(c) Immediately place the bottles on the shaker, attach them to the

gasometers, making certain that the rubber stoppers are firmly seated, and start the shaker. After 3 minutes stop the shaker and equalize the pressure in each gasometer by lowering the adjustment bulb until the surface of the liquid in the bulb is level with that in the gasometer. Make the initial readings on the graduated gasometer scales as rapidly as possible. Start the shaker and allow it to run for 3 hours. Stop the shaker, again equalize the pressures, and make the final readings. These readings should be made in the same order as the initial readings. The volume of gas produced in each bottle is the difference between the final and initial readings.

(1) Just before attaching them to the reaction bottles, the stoppers should be wetted by dipping in water to obtain a deeper seat. The stoppers should be of fresh flexible rubber to insure a complete seal. It has been found advisable to seal all other connections on the fermentometer with paraffin to guard against possible escape of gas.

(2) Care must be taken that the fermentometer is so situated in the laboratory that all parts of the instrument are subject to identical conditions of temperature.

(3) Replicate bottles containing 2 and 4 γ of thiamine and simultaneously run should show a variation no greater than 5 ml of gas. The difference between the gas produced in reaction bottle No. 3 and bottle No. 4, representing 2 γ of thiamine, should not be less than 60 ml and preferably closer to 70 ml.

(4) The gas produced in reaction bottle No. 1 must not be less than that produced in bottle No. 3, nor more than that produced in bottle No. 4. If this occurs an adjustment must be made either in the aliquots of sample solution used or in the weight of the original samples taken.

3. Calculation

The following formula indicates the method of calculation, letting

W = weight of sample taken for analysis.
T = volume of gas produced in bottle No. 1, containing the test solution.
B = volume of gas produced in bottle No. 2, containing the solution for residual fermentation and 2 γ of thiamine.
S_2 = volume of gas produced in bottle No. 3, containing 2 γ of thiamine.
S_4 = volume of gas produced in bottle No. 4, containing 4 γ of thiamine.
$\frac{100}{40}$ = factor to correct for the use of a 40-ml aliquot from a total volume of 100 ml.

$$\text{Then, } \gamma \text{ per g of sample} = 2 + 2 \times \frac{T - B}{S_4 - S_2} \times \frac{100}{40} \times \frac{1}{W}$$

(1) In the calculations, the value T represents the total gas produced owing to the thiamine of the aliquot and to the blank effects. By subtracting from this the value for the blank plus 2 γ of thiamine, a gas volume is obtained which corresponds to the amount by which the unknown aliquot exceeds 2 γ, i.e., (T − B). The thiamine value corresponding to (T − B) can be obtained by dividing (T − B) by the volume

of gas produced by 1 γ of thiamine, i.e., $\frac{(S_4 - S_2)}{2}$. Then, since this is the amount by which the thiamine content of the aliquot exceeds 2 γ, $2 + \frac{(T - B)}{(S_4 - S_2)/2}$ or $2 + 2 \times \frac{(T - B)}{(S_4 - S_2)}$ is the thiamine content of the aliquot. This must be multiplied by 100/40 to obtain the total thiamine content of the 100 ml of extract. Dividing by the sample weight gives the potency.

(2) When the response to added thiamine as measured by CO_2 production has been found to be linear between 2 γ and 4 γ, a typical calculation for a 2-g sample is as follows:

T, representing solution tested for total fermentation activity = ml of gas produced in bottle No. 1 = 361.

B, representing solution tested for residual fermentation activity plus 2 γ of thiamine = ml of gas produced in bottle No. 2 = 334.

S_2, representing 2 γ of thiamine = ml of gas produced in bottle No. 3 = 312.

S_4, representing 4 γ of thiamine = ml of gas produced in bottle No. 4 = 380.

Thiamine content per g of sample

$$= 2 + 2\frac{(T - B)}{(S_4 - S_2)} \times \frac{100}{40} \times \frac{1}{2}$$

$$= 2 + 2 \times \frac{(361 - 334)}{(380 - 312)} \times \frac{100}{40} \times \frac{1}{2} = 2 + 2 \times \frac{27}{68} \times \frac{100}{80}$$

$$= 3.49$$

(3) Duplicate samples assayed simultaneously on the fermentometer should give values within ±2.5% of the mean. Homogeneous samples assayed on different days should give values agreeing within ±5% of the mean. An experienced operator should obtain agreement within ±3% of the mean.

APPLICATION OF METHODS

Those products which have been assayed by one or more of the methods described or by reasonably similar modifications are listed below. The omission of any product from the list or failure to check it under a method heading does not necessarily imply that the method is not applicable. Rather, it merely indicates that the committee and reviewers have not applied these methods to the product.

A + under a heading for a method indicates that the method is applicable as described. Reference is made to a note in Section D or to the literature cited if special precautions are necessary or if simplifications in the procedure are permissible. Failure to list a note or reference to a simplified procedure does not necessarily imply that short-cuts cannot be taken. It may mean only that they have not been tried for the product.

Type of material	Thiochrome method	Fermentation method
Milk, cream, ice cream, and cheese		
Milk, fresh, whole, or skimmed	+ 2-(b)-(5)	+
Milk, evaporated, condensed, or dried	+ 2-(b)-(5)	+
Milk, chocolate	+	
Buttermilk	+	
Ice cream	+	
Cheese, cheddar type, cottage, cream, or processed	+ 2-(b)-(5)	
Eggs		
Whole, fresh, frozen, or dehydrated	+	
Meat, poultry, and fish		
Muscle cuts, fresh or frozen, beef, pork, veal, lamb, or poultry	+ 2-(b)-(5)	
Muscle cuts, cooked, canned, cured, and dehydrated	+ 2-(b)-(5)	+
Sausage, bologna, frankfurters, Braunschweiger, and salamis	+ 2-(b)-(5)	
Heart	+ 2-(b)-(5)	+
Liver		+
Kidney	+ 2-(b)-(5)	+
Tongue	+	
Vegetables		
Fresh, frozen, or canned		
Asparagus	+	
Beans, green, lima, and snap	+	+
Beet greens		
Beets	Fails	+
Broccoli	+	
Cabbage	+	
Carrots	+	
Cauliflower	+	
Corn, sweet, white, and yellow	+	+
Lettuce	+	
Peas, green	+	+
Potatoes	+	
Spinach	+	
Tomatoes	+	+
Dehydrated		
Cabbage	+	
Carrots	+	
Potatoes	+	
Sweet potatoes	+	
Beets	Fails	
Fruit		
Fresh, frozen, or canned		
Apples	+	
Apricots	+	
Berries		
Blackberries	Fails	
Blueberries	Fails	+
Strawberries	Fails	
Grapefruit		+
Lemons		+
Oranges	+	+
Peaches	+	
Pears	+	

Type of material	Thiochrome method	Fermentation method
Fruit—Continued		
Pineapple	+	
Dried Apples	+	
Grain products		
Corn, white	+	
Corn, yellow	+	
Wheat	+ 2-(b)-(5)	
Barley	+	
Oats	+	
Flour		
White patent	+ 2-(b)-(5)	+
White patent, enriched	+ 2-(b)-(5)	+
Wheat	+	+
Whole wheat	+ 2-(b)-(5)	+
Bread and baked products		+
Rye		+
Barley		−
White	+ 2-(b)-(5)	+
White, enriched	+ 2-(b)-(5)	+
Whole wheat	+	+
Toasted bread		+
Breakfast cereals		
Corn cereals	+	
Wheat cereals	+	
Oat cereals	+	
Rice cereals	+	
Hominy	+	+
Macaroni and spaghetti		+
Oats		+
Buckwheat		+
Millet		+

Type of material	Thiochrome method	Fermentation method
Animal feeds		
Mixed feeds	+	
Meat scraps and meat meals	+	
Liver meals	+	
Whey, dried and condensed	+	
Distillers' dried grains	+	+
Distillers' dried solubles	+	
Molasses and molasses residues	+	+
Wheat middlings and bran	+	+
Pharmaceuticals		
Vitamin tablets or capsules	+ 2-(b)-(5) 3-(b)-(5)	+
Malt preparations		+
Yeast preparations	+	
Liver preparations	+	
Preparations containing large quantities of iron	+ 3-(b)-(5)	
Biological materials		
Blood	+ 3-(b)-(5)	
Urine	+ 3-(c)-(1)	
Ascitic fluid	+ 3-(b)-(5)	
Miscellaneous		
Bouillon cubes	+	
Chocolate and cocoa	+	
Nuts	+ 2-(b)-(5)	
Wheat germ	+	
Yeast	+ 2-(b)-(5)	+
Vitamin candies		+

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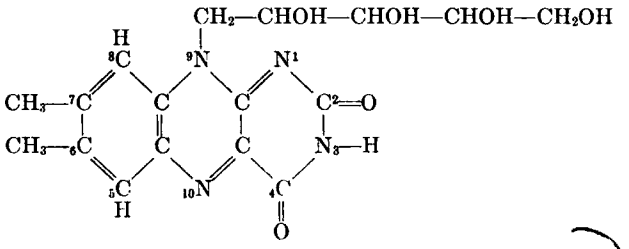
CHAPTER 5

Riboflavin

PRELIMINARY CONSIDERATIONS (*1–6*)

Riboflavin (synonymous with lactoflavin, vitamin G and vitamin B_2) is a yellow-green, fluorescent, water-soluble pigment widely distributed in plant and animal cells. It has the following structural formula:



Riboflavin or 6,7–dimethyl–9–[*d*,1′–ribityl]–isoalloxazine

It crystallizes from absolute alcohol as yellow-orange, needle-shaped, crystal clusters. Other properties are:

Empirical formula	$C_{17}H_{20}N_4O_6$
Molecular weight	376
Melting point	274° to 282° C decomposition
Solubility:	
Water (25° C)	0.012 g per 100 ml
Ethanol (100%)(27.5° C)	0.0045 g per 100 ml
Ethyl ether	Insoluble
Acetone	Insoluble
Benzene	Insoluble
Hexane	Insoluble
Chloroform	Insoluble
Optical rotation $[\alpha]_D^{20}$	−114° (in 0.1 *N* NaOH)
Redox potential	−0.21 V (pH 7.0)

Ultraviolet absorption curves are shown in Figure I.(3)

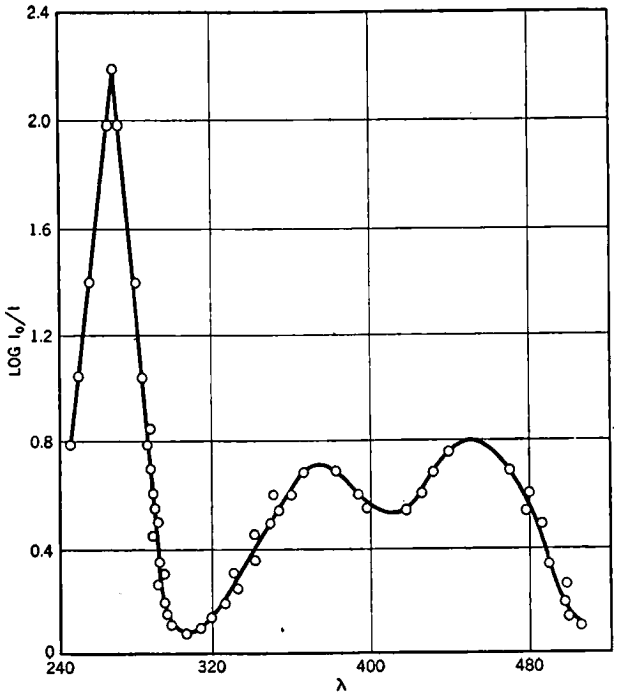


FIG. I. Absorption Characteristics of Riboflavin.

Maximum molecular extinction coefficient.

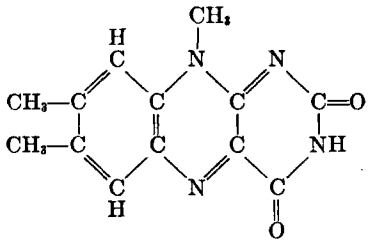
$$K = \frac{2.30}{cd} \times \log \frac{I_o}{I}$$

c = 0.0025% d = 1.0 cm

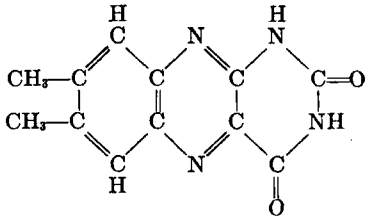
K at 267 mμ = 7.6×10^4

Pure riboflavin is stable in strong mineral acids, and toward most oxidizing agents (H_2O_2, Br_2H_2O, concd. HNO_3), but it is oxidized by chromic acid. It is also destroyed by $KMnO_4$ in 0.1 *N* acetic acid in 10 minutes at room temperature; but at pH 4.5 there is less than 10% destruction by $KMnO_4$ in 10 minutes (*7*). Although stable in the presence of H_2O_2, it is decomposed by H_2O_2 in the presence of ferrous ion (*8*). It is unstable in alkaline solution. Riboflavin is reversibly reduced to a leuco-base, a dehydro compound, by active hydrogen, sodium hydrosulfite, stannous and titanous chlorides, and alkaline sulfides. It is very

sensitive to both visible and ultraviolet light (*9,10*). Irradiation of alkaline solutions yields lumiflavin (6,7,9-trimethylisoalloxazine) and in acid solutions lumichrome (6,7-dimethylalloxazine) is formed, which is characterized by a blue fluorescence.



Lumiflavin



Lumichrome

One of the distinguishing properties of riboflavin is its yellow-green fluorescence in neutral solutions which reaches a maximum at pH 6.7 to 6.8.

Modification of the riboflavin molecule by shifting the methyl group from position 7 to 8 destroys the vitamin and co-enzyme activity. At least one of the methyl groups in position 6 or 7 is required for vitamin activity in a flavin molecule. So far as the side chain is concerned, only *d*-ribityl or to a lesser extent *l*-arabityl groups attached to the nitrogen atom in position 9 have thus far been demonstrated to be associated with vitamin activity of flavins. In addition to riboflavin, the following synthetic flavins possess limited vitamin activity:

6,7-dimethyl-9-[*l*,1′-arabityl]-isoalloxazine
7-monomethyl-9-[*d*,1′-ribityl]-isoalloxazine
6-monomethyl-9-[*d*,1′ribityl]-isoalloxazine
6-ethyl-7-methyl-9-[*d*,1′-ribityl]-isoalloxazine

None of these synthetic flavins is equal to riboflavin in vitamin potency and none of these has been found to occur naturally.

In living cells riboflavin generally occurs combined either with phosphoric acid or with phosphoric acid and adenylic acid, both of which may be combined with specific proteins to form oxidative enzymes. In some products, like milk, riboflavin is reported to be in part in a free, dialyzable form.

In most analytical procedures for riboflavin, it is necessary to treat natural products with acid or enzymes to get maximal values. This

insures the liberation of riboflavin from its protein combination and makes it more readily extractable.

Methods Available

Three general methods are satisfactory for the assay of riboflavin: fluorometric, microbiological and animal. The fluorometric method is the most rapid and inexpensive, but the least widely applicable. It requires a fluorometer equipped with suitable voltage stabilizer. The results can be duplicated to within 5 to 10 per cent.

Animal assays (*11–15*) (rat and chicken) were developed prior to the fluorometric and microbiological methods. The rat assay (*11*) was the original standard for other methods. Animal assays are most time-consuming (requiring several weeks), expensive and the least accurate. Air-conditioned animal quarters are generally required during most seasons of the year. The chief advantage of the animal assays is that they are based on biological response which is important from the nutrition standpoint.

A comparison of the rat-growth, microbiological and fluorometric methods for the determination of riboflavin in pharmaceutical products has been made (*16,17*). The three methods gave similar and reproducible results on samples of high potency. Greater differences were observed in low-potency samples.

Another comparative study (*18*) was made of the rat, chick, microbiological and fluorometric methods for the estimation of riboflavin in dried milk products. Good agreement was obtained between the fluorometric and microbiological assays when fat-soluble factors were removed before applying the latter procedure. The rat and chick assays gave more variable and inconclusive results, usually yielding much higher values than the fluorometric and microbiological methods.

FLUOROMETRIC METHOD

A. Principle

Riboflavin fluoresces in light of wave length 440 to 500 mμ. The intensity of fluorescence is proportional to the concentration of riboflavin. The riboflavin is measured in terms of the difference between its fluorescence before and after chemical destruction. For some products, it is necessary to remove interfering substances by adsorption and elution or by oxidation or both.

B. Equipment

The listed equipment is sufficient for 10 simultaneous determinations which one person can complete in a day.

No. of pieces	*Item*
12	*Volumetric Flasks*, 100 ml
10	*Funnels*, 2- or 3-inch, and qualitative filter papers to fit
10	*Receiving Vessels*, 100-ml Erlenmeyer flasks, beakers, or bottles, etc.
11	*Adsorption Columns* (Wilkens-Anderson, 111 N. Canal St., Chicago, Ill.). These consist of glass tubes, 10 mm inside diameter and 95 mm length, with a bell-shaped top, 40 mm inside diameter and 50 mm length, and a capillary constriction at the bottom, 2 mm inside diameter and 15 mm length. The overall length is 160 mm. When the tubes are charged, the rate of flow should be about 1 ml per minute.
2	*Rapid Delivering Pipets*, 3 ml, either volumetric transfer or Mohr
2	*Volumetric Transfer Pipets*, 1 ml
2	*Pipets*, 5 ml
2	*Pipets*, 10 ml, volumetric transfer or Mohr
2	*Volumetric Transfer Pipets*, 15 ml, one rapid delivering
2	*Volumetric Transfer Pipets*, 25 ml
11	*Volumetric Flasks*, 50 ml
2	*Volumetric Flasks*, 1000 ml
2 or more	*Matched Cuvets.* The cuvets can be prepared from test tubes of identical diameter which show the same number of deflections when tested with sodium fluorescein solution. The number of cuvets needed will depend upon the technic of the operator and should equal the number of adsorption columns.
1	*Fluorophotometer.* Any one of the following is suitable: *Coleman Model 12*, equipped with filters B-2 and PC-2 and voltage stabilizer. This instrument is the one most commonly used in the Chicago area. Pfaltz and Bauer, either Model B with voltage stabilizer or Model C; *Lumetron* (Photovolt Corporation); *Klett; Coleman Model 11* with voltage stabilizer. All of these require appropriate filters, cuvets, and, in some cases, adapters or carriers.

C. Reagents

All chemicals should meet A.C.S. specifications or be of Reagent Grade. Insofar as possible, contact of reagents and solutions with cork or rubber should be avoided as these materials may contribute fluorescing substances which will interfere with the assay.

1. 0.1 *N* **Sulfuric Acid.** Dilute 2.8 ml concd. H_2SO_4 to 1 liter with water.

2. 2.5 *N* Sodium Acetate. Dissolve 205 g CH_3COONa or 340 g $CH_3COONa \cdot 3H_2O$ in sufficient water to make 1 liter.

3. Enzyme Solution. Mix 0.3 g solid enzyme preparation with 5 ml of 2.5 *N* sodium acetate. Prepare fresh daily. Any of the following enzyme preparations may be used: Polidase, Clarase, Takadiastase, Mylase.

4. 4% Potassium Permanganate. Dissolve 4 g $KMnO_4$ in sufficient water to make 100 ml. Prepare fresh daily.

5. 3% Hydrogen Peroxide. This is prepared on each day of use by a 1:10 dilution of 30% H_2O_2 (Merck's Superoxol).

6. 60–80 Mesh Florisil or Supersorb (fuller's earth preparations).

7. 2% Acetic Acid. Dilute 2 ml glacial acetic acid to 100 ml with water.

8. 20% Pyridine in 2% Acetic Acid. Dilute 200 ml pyridine and 20 ml glacial acetic acid to 1 liter with water.

9. Stock Riboflavin Solution A (25 γ riboflavin per ml in 0.02 *N* of acetic acid). Accurately weigh 50 mg of U.S.P. reference standard riboflavin which has been dried in a vacuum desiccator over concd. H_2SO_4 for 24 hours and transfer quantitatively to a 2-liter volumetric flask. Add about 1500 ml water, 2.4 ml glacial acetic acid, and warm to aid solution. After cooling to room temperature, make to volume with water. Preserve this stock solution, protected from light and under toluene, in a refrigerator.

When the more concentrated stock solution of 100 γ per ml heretofore recommended by most authors (*30*) is cooled in a refrigerator, riboflavin crystallizes from solution (*31*). If a microbalance is available it may be more desirable to dissolve 12.5 mg riboflavin in 500 ml 0.02 *N* acetic acid.

10. Stock Riboflavin Solution B (10 γ riboflavin per ml). Dilute 40 ml of stock riboflavin solution A to 100 ml with water. Preserve this solution, protected from light and under toluene, in a refrigerator.

11. Riboflavin Working Standard (1.0 γ riboflavin per ml). Dilute 5 ml of stock solution B to 50 ml with 20% pyridine in 2% acetic acid. Prepare fresh daily.

12. Stock Solution of Sodium Fluorescein. Dissolve 50 mg sodium fluorescein in sufficient water to make 1 liter.

13. Dilute Solution of Sodium Fluorescein. Dilute 1 ml of stock sodium fluorescein solution to 1 liter with water.

14. Sodium Hydrosulfite. (Dithionite) $Na_2S_2O_4$.

D. Procedure

The following procedure represents a composite of several published methods (*19–29*) or unpublished modifications. For special products, some of the steps in the procedure may be omitted or changed without altering the accuracy of the method. The analyst who uses modifications of the regular procedure must establish the reliability of deviations from accepted procedures.

In our experience, the fluorometric procedure described here has a wider application than any abbreviated modification thereof. For the sake of brevity with certain kinds of samples, some of the steps; namely, enzymatic incubation; $KMnO_4$–H_2O_2 oxidation; and/or adsorption-elution purification may be omitted without sacrificing accuracy. In general, the higher the potency of the sample, the shorter the method which can be employed. However, there are some high-potency samples containing interfering pigments and materials for which one or all of the purification steps need be included. On the other hand, there are some low-potency samples (such as flour) with very little interfering substances, and all the purification steps, as well as enzymatic treatment may be omitted. Consequently, pigments and other interfering substances present in the sample must be considered as well as potency in deciding what steps are necessary or optional. All short-cuts should be checked against the long method.

The fluorometric method is not applicable to samples which contain a high concentration of iron or to those which have been heated so that interfering colors, like caramel, may be present.

Since riboflavin is light-sensitive and is most readily destroyed by light in the blue and violet regions, it is necessary to perform all operations in the absence of strong light. The use of red or amber glassware is advantageous. The higher the pH value of the solution, the greater will be the destruction of riboflavin in the presence of light (*9,10*).

1. Preparation of Sample (See Chapter 1)

2. Extraction

(a) Weigh a sample containing about 15–40 γ of riboflavin and transfer by means of a funnel to a 100-ml volumetric flask. After adding 75 ml of 0.1 *N* H_2SO_4 to the flask, place in a boiling water bath and agitate every 5 minutes for 1 hour, or autoclave for 15 minutes at 15 pounds pressure.

(1) In the pharmaceuticals containing fuller's earth or similar adsorbents as a base, riboflavin cannot be extracted by the usual procedure. Here the riboflavin may still be extracted by pyridine–acetic acid, but then the adsorption-elution must be omitted. Proceed as follows: Weigh a small sample, about 0.5 g, into a 50-ml centrifuge tube. Extract with four 25-ml portions of pyridine–acetic acid solution. After the addition of each portion, stir the sample well, centrifuge, and decant the supernatant liquid into a 100-ml volumetric flask. Make to volume with the pyridine–acetic acid solution, filter, and dilute with pyridine–acetic acid solution as necessary. Follow with $KMnO_4$–H_2O_2 (see D-4) treatment and measure the fluorescence (see D-6).

(2) Some pharmaceuticals are so free from impurities and contain the riboflavin in such available form, that fluorometry may be carried out directly on a solution of the sample. Thus one omits hydrolysis, oxidation, and adsorption-elution purification. The fluorometry differs slightly from that described in the regular procedure. Proceed as follows: Dissolve the sample in 0.1 *N* HCl, using heat if necessary, and dilute it to contain about 0.4 γ riboflavin per ml. Transfer 5 ml of the diluted extract to a cuvet, and add 5 ml McIlvaine's buffer pH 6.0 (34 g $Na_2HPO_4 \cdot 7H_2O$ and 8 g citric acid, $C_6H_8O_7 \cdot H_2O$, to 1 liter with water) and read the fluorescence of the sample. See D-6-(a). Add a few crystals of sodium hydrosulfite to reduce the riboflavin and read the fluorescence of the blank. Prepare a standard riboflavin solution containing 0.4 γ riboflavin per ml in water (4 ml of stock riboflavin solution B diluted to 100 ml with water). Transfer 5 ml to a cuvet, add 5 ml of McIlvaine's buffer pH 6.0, mix, and read the fluorescence of the standard (*32*). Calculate the riboflavin content using the following formula:

$$\frac{\text{Sample} - \text{blank}}{\text{Standard} - \text{blank}} \times 0.4 \times \text{dilution factor} = \gamma \text{ riboflavin in sample}$$

(3) Another recommended procedure for extracting riboflavin (*33*) is to reflux the material with a 3 to 1 mixture of acetone and *N* HCl.

3. Hydrolysis

(a) Cool flask below 50° C, and add 5 ml of freshly-prepared enzyme solution. The pH at this point should be approximately 4.5. Incubate the mixture at 45–50° C for 1 hour or at 37° C overnight.

(1) Each batch of enzyme preparation used should be checked for its potency by comparing it with an enzyme of known effectiveness. The potency of the enzyme preparation can be tested by analyzing a sample of known riboflavin content requiring enzymatic treatment (e.g., dried yeast) or by adding varying amounts of the enzyme to the sample until a definite excess is reached, and then determining the level at which maximal riboflavin values are obtained on the sample. If satisfactory, the enzyme should then be assayed for its riboflavin concentration and a correction made by subtracting the riboflavin introduced with the enzyme, wherever enzyme incubation is used. For assaying the enzyme, omit acid extraction in a hot water bath. Simply add 75 ml 0.1 *N* H_2SO_4 plus 5 ml 2.5 *N* sodium acetate to a weighed sample of enzyme and incubate. Then proceed to D-4.

(2) Digestion with enzyme accomplishes two things. It liberates bound riboflavin and also aids in the extraction by causing breakdown to a finely divided state. All samples should be incubated with enzyme if they are to be put through the adsorption-elution step, since some forms of combined riboflavin are not adsorbed on Florisil.

(3) Enzyme incubation may be omitted for high-potency samples and others, such as bread, flour, yeast, milk powders, whey, liver powders, etc., which do not give too high a blank (not over 15 scale deflections) and do not require the enzyme digestion to facilitate extraction. For such samples, follow the acid extraction by cooling, add 5 ml 2.5 *N* sodium acetate and allow mixture to stand for several minutes to complete precipitation of protein material. Continue with steps 4-(a) and

4-(b). Dilute the filtrate from 4-(b) directly to a concentration of 12 to 16 γ riboflavin per 100 ml and measure the fluorescence as described in the detailed procedure, except that all measurements are made in water.

(4) Incubate fresh animal tissues for at least 15 hours at 45° to 50° C in order to insure complete extraction. Make extract to volume with water, allowing the fat to rise above the mark and filter. An alternative method of removing the fat is to add a few ml of chloroform after bringing the fat layer above the mark. Allow the fat dissolved in chloroform to settle to the bottom, decant and filter. The filtrates are now ready for fluorescence readings as described in D-6-(b) for the eluates. Some processed meat samples, however, require additional purification for assay depending upon the quantity of interfering materials present.

4. Oxidation

(a) Bring the mixture to room temperature and add 3 ml of 4% $KMnO_4$ solution.

(1) The permanganate–peroxide treatment may be omitted for certain high-potency samples and for fresh animal tissues.

(2) Removal of the interfering fluorescent substances and pigments which occur in extracts may also be accomplished by treatment with $SnCl_2$ and $Na_2S_2O_4$. These reagents reduce the interfering fluorescent substances and the riboflavin to the non-fluorescent form. Shaking in the presence of air reoxidizes the riboflavin and the fluorescence may then be measured. Purification of extracts by this method alone or by the permanganate method alone is not complete for certain materials, such as low-potency foods and feeds. In such cases, the $KMnO_4$–H_2O_2 treatment may be followed by the $SnCl_2$–$Na_2S_2O_4$ treatment (*26*).

(b) After 1 minute, add 3 ml of 3% H_2O_2, shake well until foaming ceases. If foaming is excessive, introduce one drop of caprylic alcohol. Add water to volume, filter and discard the first few ml.

(1) For low-potency samples and samples having high blanks, a modification using $KMnO_4$–H_2O_2 oxidation following filtration rather than preceding filtration may be introduced. Since the purpose of the $KMnO_4$ treatment is to oxidize pigments which may be present, it is more effective on a small volume than on a large one. An aliquot of filtered extract (15–25 ml) is treated with 1 ml $KMnO_4$ (more may be needed depending on the size of the aliquot) and enough 3% H_2O_2 is added dropwise to just decolorize the $KMnO_4$. The treated aliquot need not be filtered again prior to fluorescence measurement if excess peroxide is avoided. Some samples form slight precipitates with $KMnO_4$ and must be filtered or centrifuged until they remain clear.

(2) In certain instances, as for example where very highly-pigmented extracts are obtained, the application of the oxidation step both before and after the adsorption-elution step will aid in reducing the pigment considerably. Oxidation of an aliquot following adsorption-elution is more effective than before adsorption-elution because of the smaller concentration of pigment present. In other cases the oxidation step is employed following adsorption-elution to prevent the decreased rate of flow

through the column which is often caused by the oxygen bubbles generated in the oxidation step. When this is done, however, care must be taken to prevent oxygen bubbles from collecting on the cuvet walls during fluorescence readings.

(3) The use of acetone in diluting a treated aliquot of the unknown to volume, following the oxidation step, has been used successfully with cereals, high-potency samples and animal tissue. The acetone results in precipitation of starches, protein and other impurities: thus filtration is necessary. Precautions to prevent evaporation of the solvent should be taken. Sulfur does not precipitate in the acetone as it does in the pyridine–acetic acid when excess $Na_2S_2O_4$ is used.

5. Adsorption-Elution Purification

(a) Prepare an adsorption column by first placing a wisp of glass wool in the bottom and adding Florisil to a height of about 8 to 10 cm. Wash the Florisil with about 25 ml of 2% acetic acid solution, followed with about 15 ml of water.

(1) The adsorption-elution step may be omitted, generally, for animal tissues, high-potency samples, most pharmaceuticals and samples having low blanks.

(2) To check the efficiency of the Florisil, put 10 γ riboflavin through the adsorption-elution step and compare the fluorescence of the eluate made to 50 ml in pyridine–acetic acid solution with the fluorescence of a solution containing 10 γ riboflavin in 50 ml of pyridine–acetic acid solution. If there is less than 90% recovery of the riboflavin, the Florisil should be discarded.

(3) It is advisable to apply mild vacuum when moistening the Florisil to avoid air pockets in the column. Such air pockets will reduce the efficiency of the adsorbing agent and may result in losses of riboflavin during the elution step. An alternative method for packing the adsorption tube is to fill it with water, allowing the moistened Florisil to fall in place by gravity until a layer 4–8 mm deep is present in the reservoir. Allow the tube to drain until no water is visible above the Florisil. The Florisil in the reservoir of the tube prevents clogging with turbid extracts.

(b) Place 25–50 ml of filtrate containing 8 to 12 γ of riboflavin in the reservoir of the adsorption column. After the sample has passed through the Florisil, wash with two 15-ml portions of water.

(1) More than 20 γ riboflavin are not adsorbed quantitatively on a Florisil column. If it is found that a sample containing more than 20 γ was used, the assay must be repeated using a smaller aliquot, or a lower concentration of the extract.

(c) Elute the riboflavin from the column with 25 ml of the 20% pyridine in 2% acetic acid solution and collect in a 50-ml volumetric flask. Add additional portions of the pyridine–acetic acid solution until 35–50 ml of eluate are collected in the flask. Make the eluate to volume with the pyridine–acetic acid solution.

(1) If the presence of oxygen bubbles in $KMnO_4$–H_2O_2-treated aliquots slows up the column, suction may be used.

6. Fluorometry

The following instructions apply to the Coleman Model 12 photofluorometer.

(a) Using the dilute sodium fluorescein (50 γ per liter), adjust the instrument to give a deflection of 50 on the galvanometer scale. (Some other point may be selected if the readings of the unknown appear to be out of range of the scale.) This adjustment is checked immediately before and after the reading of each sample.

(1) Before attempting to measure the fluorescence of solutions containing riboflavin, it should be ascertained that the instrument used gives a linear response to different concentrations of standard fluorescent substances (*34*).

(b) To cuvet A add 10 ml of eluate. Into cuvet B pipet 1 ml of the freshly prepared riboflavin working standard (1.0 γ per ml), followed by 10 ml of the same eluate. Agitate cautiously to insure homogeneity. Measure the fluorescence of A and B. Add a small amount of $Na_2S_2O_4$ (approximately 2–10 mg) to A, and after mixing well, read the remaining fluorescence, C.

(1) Excessive quantities of $Na_2S_2O_4$ should be avoided since high salt concentrations may change the fluorescent properties of the blanks. Since the blank may change on standing, it should be read within one minute after the addition of the $Na_2S_2O_4$.

(2) Instead of solid $Na_2S_2O_4$, 0.5 ml of a 5% solution prepared by dissolving 5 g of $Na_2S_2O_4$ in 100 ml of an ice-cold $NaHCO_3$ solution (2 g of $NaHCO_3$ per 100 ml) may be used. The addition of the solution instead of the solid prevents an excess of $Na_2S_2O_4$. With care, however, an excess can be avoided even with the solid. The use of the solution has two disadvantages—it is stable only for 2 to 4 hours even in an ice bath and corrections for the change in volume are necessary.

(3) For the blank, some investigators recommend the use of a drop of saturated NaOH, instead of $Na_2S_2O_4$ to give a final pH of 11.0 (*28*).

(4) During fluorescence measurements care should be taken to avoid excess exposure to the ultraviolet light since it causes rapid destruction of riboflavin.

(5) It should be noted that the addition of 1 ml of working standard to 10 ml of eluate gives a volume of 11 ml, and that the B reading must, therefore, be multiplied by 11/10 so that it will be on the same volume basis as readings of A and C. The standard increment of 1 γ amounts to 0.1 γ per ml of eluate and hence

$$\frac{A - C}{\frac{11}{10}B - A} \times 0.1 = \text{concentration of riboflavin in each milliliter of eluate}$$

7. Calculations

(a) Calculate the riboflavin content of sample from the following formula:

$$\frac{A - C}{\frac{11}{10}B - A} \times \text{riboflavin increment} \times \frac{\text{dilution factor}}{\text{wt. of sample}} = \gamma \text{ per g}$$

(1) The following example illustrates the method of calculations of the long procedure which employs incubation adsorption-elution purification, and fluorescence measurement by the increment method. Two g of feed were extracted, yielding 100 ml of assay solution. A 25-ml aliquot of this solution was adsorbed on and eluted from Florisil, the final volume of eluate being 50 ml.

	Galvanometer deflection
A. 10 ml eluate	65.0
B. 10 ml eluate + 1 ml standard (1.0 γ riboflavin)	86.0
C. Blank of eluate	2.2

$$\frac{65.0 - 2.2}{\frac{11}{10} \times 86.0 - 65.0} \times 0.1 \times \frac{50 \times \frac{100}{25}}{2} = 21.2 \text{ γ per g}$$

(2) All three readings may be made on one aliquot by first taking reading A, adding 1 ml of standard, mixing and taking reading B, followed by addition of $Na_2S_2O_4$ and taking reading C. In this case, the calculation is identical except that reading C must be multiplied by 11/10 to correct for changes in volume.

(3) Calculation by the increment technic compensates for the variations which may occur in the fluorescence of riboflavin in the presence of interfering substances. Theoretically, these interfering substances will affect both standard and sample fluorescence similarly, thus correcting for the effect of salt content, extraneous color, solvent concentration, etc. With certain extracts it is possible to determine the riboflavin content by direct comparison of the fluorescence of an aliquot with that obtained for a standard riboflavin solution carried through the identical procedure. The advantage of this method over that of the increment technic lies in the fact that only one value need be determined for the standard for a whole series of extracts, thus eliminating the numerous readings made in the increment technic and also minimizing variations in the reading of the standard. In such cases the calculation becomes:

$$\frac{\text{Sample} - \text{sample blank}}{\text{Standard} - \text{standard blank}} \times \text{γ in standard} \times \frac{\text{dilution factor}}{\text{weight of sample}} = \text{γ per g}$$

This method is applicable only to those extracts having low blanks and little, if any, interfering fluorescent substances. Whenever it is employed for a new product the results should be checked by the increment technic.

(4) These calculations do not take into account the possibility of loss of riboflavin during the procedure. See D-5-(a)-(2). If such corrections are desired, treat 4 ml of stock riboflavin solution B exactly as the sample, including the fluorometry as described in D-6. Calculate the per cent recovery and to correct values for loss of riboflavin during the procedure, divide each by this percentage.

MICROBIOLOGICAL METHOD

A. Principle

On certain media, adequate in all respects except for riboflavin, the extent of the growth of certain microorganisms is a direct function of the concentration of riboflavin. The growth is measured either by acidimetric titration or by turbidimetric observation.

B. Equipment

The quantities listed are for 10 simultaneous determinations which one person can set up in a day provided the reagents have been prepared previously.

No. of pieces	*Item*
1	*Autoclave*
1	*Constant Temperature Incubator or water bath* (± 0.5°)
1 gross	*Test Tubes*, size 16 to 18 mm diameter × 150 mm length. Individual metal or glass caps or rectangular covers for test tubes. For routine work, several gross should be available.
3–4	*Metal Racks*, to hold 40 or more tubes each. More are convenient, especially for routine work.
1	*Sterilizing Can* for pipets or syringes
10	*Funnels*, 2–3 inch diameter, and qualitative filter paper
1	*Glass-Stoppered Graduate Cylinder*, 500 ml
1	*Glass-Stoppered Graduate Cylinder*, 1000 ml
1	*Spot Plate*
1	*Volumetric Flask*, 500 ml
12	*Volumetric Flasks*, 250 ml
2	*Bottles*, 3 or 5 gallon
2	*Pipets*, volumetric transfer, 50 ml
2	*Pipets*, volumetric transfer, 15 ml
5	*Pipets*, volumetric transfer, 10 ml
20	*Pipets*, volumetric transfer, 5 ml
12	*Pipets*, volumetric transfer, 1 ml
	for use in diluting samples and standards when necessary
1	*Buret*, self-filling, 25 ml

C. Reagents

1. 0.1 *N* Sodium Hydroxide (approx.). Dissolve 4 g NaOH in water and dilute to 1 liter.

2. *N* **Sodium Hydroxide** (approx.). Dissolve 40 g NaOH in water and dilute to 1 liter.

3. 0.1 *N* **Hydrochloric Acid** (approx.). Dilute 8.5 ml concd. HCl to 1 liter with water.

4. **Stock 0.1% Bromothymol Blue.** To 1 g bromothymol blue in a small beaker add 15 to 25 ml alcohol and stir until dissolved. Add 20 ml 0.1 *N* NaOH gradually with stirring, then about 900 ml water, and adjust the pH with 0.1 *N* NaOH to 6.8, a blue-green color. Finally dilute to 1 liter.

5. **Stock Riboflavin Solution A** (25 γ riboflavin per ml in 0.02 *N* acetic acid). Weigh accurately 50 mg U.S.P. reference standard riboflavin which has been dried in a vacuum desiccator or oven over concd. H_2SO_4 for 24 hours, and transfer quantitatively to a 2-liter volumetric flask. Add about 1500 ml water, 2.4 ml glacial acetic acid, and warm to aid solution. After cooling to room temperature, make to volume with water. Preserve under toluene, protected from light, in a refrigerator.

When the more concentrated stock solution of 100 γ per ml heretofore recommended by most authors (*30*) is cooled in a refrigerator, riboflavin crystallizes from solution (*31*).

6. **Stock Riboflavin Solution B** (10 γ riboflavin per ml in 0.002 *N* acetic acid). Dilute 40 ml of stock riboflavin solution A to 100 ml with water. Preserve under toluene, protected from light, in a refrigerator.

7. **Riboflavin Working Standard** (0.1 γ per ml). Dilute 1 ml of stock riboflavin B to 100 ml with water. Prepare immediately before use.

8. **Alkali-Treated Peptone Solution.** Dissolve 40 g of peptone (Difco Bacto or Wilson's is suitable) in 250 ml of water, and 20 g of NaOH in 250 ml of water. Mix the two solutions. Allow to stand for 18–24 hours, then neutralize with glacial acetic acid (approximately 25 ml). Add 14 g of anhydrous sodium acetate (or 23.2 g $NaC_2H_3O_2 \cdot 3H_2O$) and sufficient water to make to 800 ml. Preserve under toluene in a refrigerator.

9. **0.1% Cystine Solution.** Dissolve 1 g of *l*-cystine in 20 ml of water and 5–10 ml of concd. HCl. Add sufficient water to make a volume of 1 liter. Keep under toluene at room temperature.

10. **Yeast Supplement Solution.** Dissolve 100 g of yeast extract (Difco is suitable) in 500 ml of water, and 150 g of basic lead acetate in 500 ml of water. Mix the two solutions, and adjust the pH to 10 with concd. NH_4OH and filter or centrifuge. Adjust the filtrate to pH 6.5

with glacial acetic acid, precipitate the excess lead with H_2S, and filter. Aerate to remove traces of H_2S and add water to make 1000 ml. Preserve under toluene in a refrigerator.

11. **Salt Solution A.** Dissolve 25 g of KH_2PO_4 and 25 g of K_2HPO_4 in water to make 250 ml. Keep under toluene.

12. **Salt Solution B.** Dissolve 10 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.5 g of $FeSO_4 \cdot 7H_2O$ and 0.5 g $MnSO_4 \cdot 4H_2O$ in water to make 250 ml. Keep under toluene. Shake before using.

Instead of the two salt solutions indicated, Salt A and Salt B, the following single salt solution may be used: 10 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.5 g $MnSO_4 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, and 23 ml 85% H_3PO_4, dissolved in water and diluted to 250 ml. 2.5 ml of this solution is equivalent to 5.0 ml of the mixture of equal volumes of Salt A and Salt B. The acidity of the H_3PO_4 prevents the precipitation of iron phosphate which would occur if Salt A and Salt B were mixed, and this acidity does no harm since the pH of the medium is adjusted with NaOH later.

13. **Stock Culture of *Lactobacillus casei*.** Dissolve 3 g of agar (Difco Bacto or other equally good brand) in about 150 ml of hot water. After the agar is in solution, add 3 g of yeast extract and 1 g of dextrose. Add sufficient hot water to make 200 ml and add approximately 10-ml portions of the hot solution to test tubes. Plug the tubes with non-absorbent cotton, sterilize in an autoclave at 15 lbs. pressure for 20 minutes, and allow to cool. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus casei* and incubate for 16–24 hours at 37° C. Store both the unused tubes and the stab cultures in a refrigerator. Prepare a fresh stab of the stock culture every week from a stab made the preceding week. Do not use for inoculum if the culture is more than two weeks old.

Pure cultures of *Lactobacillus casei* may be obtained from the American Type Culture Collections, Georgetown University Medical School, Washington, D. C. as number 7469.

14. **Basal Medium Stock Solution** (for 50 tubes)

Alkali-treated peptone solution	50 ml
0.1% cystine solution	50 ml
Yeast supplement solution	5 ml
Salt solution A	2.5 ml
Salt solution B	2.5 ml
Dextrose, anhydrous	10 g

Dissolve the dextrose in the mixture of the solutions, adjust the pH to 6.8 with *N* NaOH, and add sufficient water to make 250 ml.

An entirely different basal medium for the estimation of riboflavin has been proposed by Roberts and Snell (*43*). Although a total of 18 constituents need to be weighed or measured out, the authors point out how these may be combined into three stock solutions and thus the preparation of the medium greatly simplified. An advantage of this medium is that it may be used for folic acid as well as for riboflavin, and there are indications that it may be suitable for niacin, pantothenic acid, and biotin as well.

15. Culture Medium. To 250 ml of basal medium stock solution, add 5 ml stock riboflavin solution B and water to 500 ml. Mix well. Add approximately 10 ml to test tubes, plug with nonabsorbent cotton, autoclave at 15 lbs. pressure for 15 minutes, and store in a refrigerator. This medium contains 1γ riboflavin in 10 ml.

16. Sterile Saline. Dissolve 9.0 g NaCl, reagent grade, in sufficient water to make 1000 ml of solution. Add approximately 10 ml of this solution to test tubes, plug with nonabsorbent cotton and autoclave at 15 lbs. pressure for 15 minutes.

17. Inoculum. With a sterile wire transfer the *Lactobacillus casei* from an agar stab to a tube of culture medium, incubate 18–24 hours at 37° C, then centrifuge aseptically. During the centrifuging the cotton plug may be held in place by folding a portion of the cotton over the outside of the tube and fastening with a rubber band. A pin through the plug may also be used. Decant the liquid, add approximately 10 ml sterile 0.9% NaCl solution, and mix thoroughly before using.

D. Procedure

The following procedure represents a composite of several published methods (*30,36–41*) and unpublished modifications, all of which are adaptations of the original microbiological method of Snell and Strong (*35*).

The following time relations must be anticipated. The inoculum must be started the day before it is used; if the growth is measured by titration, the time of inoculation must be chosen so that the titration can be carried out three days later. If growth is measured by the turbidimetric method the extent of growth is determined 18–24 hours after the time of inoculation. The samples may be prepared well in advance, even a week or more before the analysis. The standard and unknown tubes may also be set up one or two days in advance, since after autoclaving, the contents of the tubes are stable providing precautions are taken to eliminate light, and temperature of storage is low (about 4° C).

1. Extraction and Hydrolysis

(a) Into a 120-ml Erlenmeyer flask weigh a homogeneous sample containing 10 or more γ riboflavin, add 50 ml 0.1 *N* HCl and autoclave at 15 lbs. for 15 minutes.

(1) When the concentrations of the various members of the B-complex (thiamine, riboflavin, niacin, pantothenic acid, biotin, inositol, and folic acid) are wanted on the same sample or samples, the following general enzymatic digestion procedure has been used for the simultaneous release of them from plant and animal tissue.

Dilute approximately 1 g of finely minced tissue with 8 ml of 0.2 *N* sodium acetate buffer having a pH of 4.5 to 4.7. Add 1 ml of a freshly prepared enzyme suspension containing 20 mg of papain and 20 mg of takadiastase per ml. To prepare the enzyme suspension, mix 20 mg of papain with 1 drop of glycerine, add 20 mg of takadiastase and make to 1 ml with water. In practice, when several samples are assayed simultaneously, a suitable multiple of these quantities is used. After mixing the enzyme with the sample, add a few drops of benzene or toluene, cover loosely and incubate for 24 hours at 37–45° C (*43*).

Since papain and takadiastase contain varying amounts of riboflavin (1 to 8 γ per g) it is important, especially with low-potency samples, that the vitamin content of each lot of enzyme be determined and proper correction made in the calculation.

Heat the samples in flowing steam or autoclave for 10 minutes. Add a level teaspoonful (about 1 g) of Filter Cel or equivalent filter aid (must not absorb riboflavin), shake and filter through a conical paper. Collect the filtrate in a volumetric flask of suitable size. Wash the residue with small amounts of water and collect the washings with the filtrate. Finally, dilute the extracts so that the riboflavin concentration is between 0.05 and 0.5 γ per ml. If the samples are to be stored, transfer to brown bottles, add a few drops of toluene and refrigerate.

(b) Cool to room temperature, adjust the pH to 4.5, transfer to a 100-ml volumetric flask, make to volume and filter.

(1) For most samples filtration at pH 4.5 is effective in removing growth stimulants and inhibitors, such as starch, fatty acids and phospholipids. Some workers find that the stimulating effect of starch may be avoided, and lower and more uniform values can be obtained with cereal products if, after autoclaving in 0.1 *N* HCl, the samples are digested at 50° C for one-half hour with 5 ml of 6% takadiastase in 2.5 *N* sodium acetate prior to pH adjustment and filtration. Also, some find that fat has a stimulating effect. In the case of high-fat materials such as cheese, liver meal and fish meal, the difficulty is overcome by ether extraction (*37*). After autoclaving with 0.1 *N* HCl, adjust to pH 4.5, make to volume and filter. Extract a 50-ml aliquot of the filtrate three times with 30-ml portions of ether. Then adjust to pH 6.8 and dilute to 100 ml.

(2) When urine is assayed without dilution as is sometimes necessary when the riboflavin concentration is extremely low, care should be taken that the urea concentration does not exceed 20 mg per tube. If more than this amount is present the growth of the organism is inhibited. If it is impossible to keep the urea concentration below this level, a formula to correct the result has been devised (*44*).

(c) Measure 50 ml (or an aliquot containing about 10 γ riboflavin) of the filtrate into a 100-ml volumetric flask, adjust the pH to 6.8, dilute to 100 ml, and filter again if necessary to obtain a clear solution.

2. Set-up of Standard and Unknown Tubes

(a) From the number of samples to be assayed estimate the number of culture tubes required, allowing 14 for the standards and 10 for each sample.

Place the required number of tubes into suitable racks and identify by labeling to withstand autoclaving, or better, by noting their positional arrangement in numbered racks.

(b) Into the first fourteen tubes, measure accurately 0.0-, 0.5-, 1.0-, 1.5-, 2.0-, 2.5- and 3.0-ml quantities, each in duplicate, of the riboflavin working standard (0.1 γ per ml).

(c) Into succeeding blocks of 10 tubes, measure five levels of the sample extract, each in duplicate, ranging from 0.05 to 0.25 γ riboflavin. Volumes of unknown greater than 5 ml cannot be used, and less than 0.1 ml is not usually conveniently and accurately measurable.

(1) Some workers do not run duplicates at each level of the sample tubes since each of the five levels already constitutes an independent observation of the same thing. They believe that the same result is obtained for considerably less effort. The standard tubes, however, are always run in duplicate.

(d) To each tube, both standard and sample, add 5 ml basal medium, and finally enough water to make the total volume 10 ml. Cover the tubes with metal covers or glass caps or insert cotton plugs, and autoclave at 15 pounds for 15 minutes.

(1) The original method of stoppering tubes with cotton plugs is very laborious and time-consuming. Some workers use individual glass or aluminum caps, 25 mm deep and 22–25 mm in diameter, just big enough to cover each tube separately. More convenient still is a rectangular metal cover made of stainless steel or aluminum, with 25-mm deep edges, and of sufficient area to cover two rows of tubes. Two covers per rack (of four rows) are preferred to one larger cover, since with the latter it would be necessary to reach over one or two rows of uncovered tubes during the inoculation, and thus increase the chance of contamination. Also, the smaller covers are more easily handled. With these covers, it is possible to inoculate a rack of forty tubes in two minutes.

3. Inoculation and Incubation

(a) When cool, add aseptically to each tube one drop of inoculum, C-17, from a sterile pipet or syringe. Move rapidly from one tube to the next, lifting each cap or cotton plug just high enough to insert the tip of the pipet or needle.

(1) The bacteriological technic of flaming tubes in inoculation has been omitted from the recommended procedure, since this has been found to be unnecessary and time-consuming. In areas where the danger of contamination is high, however, it may be advisable to introduce the flaming technic.

(2) It is convenient to use a 5- or 10-ml pipet with 5-mm tip bent at a 60° angle, so that the pipet may be held almost horizontally, while the drop is delivered vertically. One may also use a sterile 5–10 ml hypodermic syringe with 20-gage needle.

(b) Incubate at 37° C for approximately 72 hours in the dark.

(1) All tubes must be maintained at exactly the same temperature. The time of incubation can be extended by as much as 18 hours, and shortened by as much as 12 hours, without appreciably affecting the results of the assay. After incubation the tubes may be kept in a refrigerator overnight and titrated the following day.

4. Titration

(a) Transfer the contents of each tube to a 125-ml Erlenmeyer flask, and rinse the tube once with about 10 ml water, adding the rinsings to the flask. Add about 0.2 ml of 0.1% bromothymol blue (most conveniently from a dropper), and titrate with 0.1 *N* NaOH to a green color, about pH 6.8. It is important to keep the first flask as a reference end point.

(1) Instead of 0.1% bromothymol blue, of which about 0.2 ml is used in each tube for the titration, some workers prefer to use a larger volume (about 40 ml) of a more dilute indicator solution (0.001% bromothymol blue), and to use this very dilute indicator solution for rinsing the test tubes. In this latter procedure, after the incubation, fill all tubes at once with the 0.001% indicator solution, and pour the contents of each tube into a 125-ml Erlenmeyer flask. Then fill each test tube once again with the dilute indicator, pour into the same flask, and titrate with 0.1 *N* NaOH. In this way the addition of indicator and the rinsing of the tubes is accomplished at once, and the total volume and amount of indicator are kept satisfactorily constant.

(2) The NaOH used for titration need not be exactly 0.1 *N*. However, the whole series of tubes must be titrated with the same lot of NaOH.

(3) Instead of titration, turbidity may be used as a measure of microbial growth. For greatest convenience, matched colorimeter tubes are used for setting up the assay. Great care must be exercised to add exactly the same amount of inoculum to each tube. The time of incubation is 18 to 24 hours. The turbidity is measured in a turbidimeter or a photoelectric colorimeter with a filter in the region of 640 mμ. Shake well to suspend the organisms uniformly and if air bubbles are present in the solution allow to stand for approximately 30 minutes. After this time the contents must again be suspended uniformly.

From the turbidimetric values of the standard riboflavin tubes, a standard curve is plotted—density against γ of riboflavin. The density of the inoculated blank need not be subtracted from the readings. The turbidimetric method has the advantage of being more rapid but where colored or turbid solutions are encountered before the addition of the organisms, the titration procedure is indicated.

(4) A glass electrode assembly has been described for titrating (*45*). The pH meter may be set for constant indication of pH or the key pressed intermittently. When approximately pH 6.5 is reached, add alkali more slowly to pH 7. The assembly is simple and rapid and eliminates the color end point.

(5) The *high blank* and *drift* are two types of errors which occasionally appear in microbiological work, which the investigator should understand. When the titration value of the standard tube containing no riboflavin (usually about 0.5 ml) exceeds 2.0 ml, the blank is too high. The cause is excessive riboflavin in the basal

medium stock solution. The remedy is to examine carefully each constituent of the basal medium, and to replace those constituents which contain the excessive riboflavin. For the elimination of drift, see note 5-(c)-(1).

5. Calculation

(a) From the titration values of the standard riboflavin tubes, prepare a standard curve plotting ml 0.1 *N* NaOH against γ of riboflavin.

Values for a typical standard are shown in the accompanying table.

Tube No.	Standard, ml	γ per tube	0.1 *N* NaOH, ml
1	0.0	0.0	0.5
2	0.0	0.0	0.5
3	0.5	0.05	2.3
4	0.5	0.05	2.3
5	1.0	0.10	4.0
6	1.0	0.10	4.0
7	1.5	0.15	5.9
8	1.5	0.15	5.9
9	2.0	0.20	8.2
10	2.0	0.20	8.1
11	2.5	0.25	9.0
12	2.5	0.25	9.0
13	3.0	0.30	10.0
14	3.0	0.30	10.0

(b) Using this curve, determine the riboflavin content corresponding to the titration value of each other tube or to the average value of duplicate tubes. Divide the riboflavin content per tube by the volume of the sample aliquot added to it [2-(c)] getting the concentration of riboflavin in terms of γ per ml sample extract. Riboflavin values of less than 0.05 or more than 0.30 γ per tube cannot be used since they are beyond the useful range of the standard curve.

(1) Except for the most precise work, if the titrations for duplicate tubes check within 0.2 ml the values may be averaged before calculating the content per milliliter. Titrations above 10 ml may be averaged if they do not differ by more than 0.4 ml.

(c) Determine the average concentration of riboflavin per ml of sample extract, using only those values which do not differ from the average by more than 10%.

(1) The term *upward drift* is applied to data in which the calculated values for γ riboflavin per ml of test solution consistently increase as the size of the sample is increased. *Downward drift* relates to a corresponding progressive decrease.

The cause of upward drift is the presence in the sample of some growth factor which is inadequately supplied in the basal medium. The remedy is to enrich the basal medium. For this purpose, the use of 14 g of anhydrous sodium acetate in the preparation of the alkali-treated peptone and 10 g of glucose per 250 ml of basal medium has been recommended. The above procedure specifies such a medium.

Some workers find that this is all that is necessary. Others prefer to further enrich their medium with 6 ml of 1% asparagine, 25 γ of pantothenic acid per 250 ml of medium and by doubling the amount of cystine. Still others use 1 mg of niacin per 250 ml of medium as the only enrichment. Some workers use as much as 15 g of glucose in the preparation of the medium, but this is not desirable since caramelization during autoclaving makes subsequent titration difficult. As little as 5 g of glucose may be used.

Downward drift may be due to some toxic or inhibitory factor in the sample. In these cases the remedy is to remove these factors, for examples see references (*26,37, 44,46,47*). Downward drift may also be due to some factor which stimulates at low but not at high concentration of sample. For example see (*48*). The nature of some of these growth promoters and inhibitors and methods for removing them are discussed in 1-(b)-(1) under Extraction and Hydrolysis. This whole problem of stimulation and inhibition, and enrichment of media, has been ably discussed by Strong (*48*).

(d) Calculate the riboflavin content of the sample from the formula:

$$\gamma \text{ per g} = \frac{\text{avg. } \gamma \text{ per ml of extract} \times \text{volume}}{\text{weight of sample}} \times \text{dilution factor}$$

(1) For samples of potencies such that the procedure can be followed exactly, the volume is 100 ml, 1-(b), and the dilution factor is 100/50 or 2, 1-(c). Effectively, this is equivalent to setting the volume equal to 200 ml. This will simplify calculations somewhat.

A sample of 4 g of meat scraps was treated with 80 mg of takadiastase, 80 mg of papain, 30 ml of 0.1 *N* sodium acetate (pH 4.6), incubated 24 hours at 37° C, autoclaved 30 minutes at 15 lbs. pressure, cooled to room temperature and filtered. The filtrate was adjusted to pH 6.8 with alkali and made up to 200 ml with water.

Tube No.	Sample, ml	0.1 *N* NaOH, ml	γ per tube	γ per ml
1	0.5	1.5	0.030	
2	0.5	1.8	0.036	0.072
3	1.0	3.1		
4	1.0	3.0		
		Avg. = 3.05	0.076	0.076
5	1.5	4.3		
6	1.5	4.3		
		Avg. = 4.3	0.110	0.074
7	2.0	5.4		
8	2.0	5.6		
		Avg. = 5.5	0.147	0.073
9	2.5	7.5		
10	2.5	7.6		
		Avg. = 7.55	1.85	0.074
				Avg. = 0.074

$$\frac{0.074 \times 100}{4} \times \frac{100}{50} = 3.7 \text{ } \gamma \text{ per g sample, uncorrected}$$

The riboflavin content of the quantity of enzymes used was 0.8 γ, corresponding to a correction of 0.2 γ per g of sample. The corrected value, therefore, is

$$3.7 - 0.2 = 3.5\ \gamma \text{ per g}$$

Duplicate samples assayed simultaneously should give values within ±5% of the mean. An experienced analyst should be able to obtain values within ±2.5% of the mean. If the same sample is assayed at different times, however, values checking no better than ± 10% of the mean may be obtained.

APPLICATION OF METHODS

Those products which have been assayed by one or more of the methods described or by reasonably similar modifications are listed below. The omission of any product from the list or failure to check it under a method heading does not necessarily imply that the method is not applicable. Rather, it merely indicates that the committee and reviewers have not applied these methods to the product. A + under a heading for a method indicates that the method is applicable as described.

Type of material	Fluorometric	Microbiological
Milk, cream, ice cream and cheese		
Milk, fresh, whole, or skimmed	+	+
Milk, evaporated, condensed or dried	+	+
Milk, chocolate	+	
Buttermilk	+	+
Cream, sweet or sour		+
Ice cream	+	+
Cheese, cheddar type, cottage, cream or processed	+	+
Eggs		
Whole, fresh, frozen or dehydrated	+	+
Meat, poultry and fish		
Muscle cuts, fresh or frozen, beef, pork, veal, lamb or poultry	+	+
Muscle cuts, cooked, canned, cured and dehydrated	+	+
Fish, cooked or canned	+	+
Sausage, bologna, frankfurters, Braunschweiger and salami	+	+
Heart	+	+
Liver	+	+
Kidney	+	+
Tongue	+	+
Vegetables		
Fresh, frozen or canned		
Asparagus	+	+
Beans, green, lima and snap		+
Brussels sprouts		+
Cabbage		+
Corn, sweet, white and yellow		+

Type of material	Fluorometric	Microbiological
Vegetables-Continued		
Lettuce, head		+
Lettuce, all other		+
Peas, green		+
Potatoes		+
Rutabagas		+
Squash, summer		+
Squash, winter		+
Sweet potatoes		+
Fruit		
Fresh, frozen or canned		
Apples		+
Bananas		+
Blueberries		+
Cantaloupe		+
Grapefruit		+
Grapes		+
Limes		+
Oranges		+
Peaches		+
Pears		+
Pineapple		+
Plums		+
Tangerines, other mandarin type oranges		+
Dried		
Raisins		+
Grain products		
Corn, white		+
Corn, yellow		+
Wheat		+
Barley		+
Rice	+	+
Oats		+
Flour, buckwheat		+
Flour, rye		+
Flour, white patent	+	+
Flour, white patent, enriched	+	+
Flour, whole wheat		+
Bread, rye		+
Bread, white	+	+
Bread, white, enriched	+	+
Bread, whole wheat	+	+
Corn cereals	+	+
Wheat cereals		+
Oat cereals		+
Rice cereals		+
Hominy		+
Macaroni and spaghetti		+
Animal feeds		
Mixed feeds	+	+
Meat scraps and meat meal	+	+
Liver meals		+
Alfalfa	+	+
Whey, dried and condensed	+	+
Distillers' dried grains	+	+
Distillers' dried solubles	+	+
Molasses residues	+	+
Wheat middlings and bran	+	+
Miscellaneous		
Bouillon cubes	+	+
Chocolate and cocoa		+
Nuts	+	+
Wheat germ	+	+
Yeast	+	+
Pharmaceuticals		
Vitamin tablets or capsules	+	+
Malt preparations	+	+
Yeast preparations	+	+
Liver preparations	+	+
Preparations containing large quantities of iron		+
Preparations containing absorbents	+	+

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CHAPTER 6

Niacin

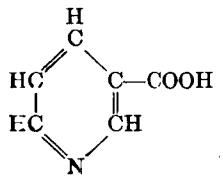
PRELIMINARY CONSIDERATIONS

Niacin or nicotinic acid is one of the important vitamins of the B-complex for human and animal nutrition. Although its chemical and physical properties have been known for many years, it is only recently that its biological significance has been brought to light. Niacin constitutes part of two known coenzymes, coenzyme I and coenzyme II, which are essential for the operation of a large number of enzyme systems (*1*). From this and other evidence it is believed that all living tissues need niacin for normal metabolism. Humans, monkeys (*2*), dogs (*3*) and pigs (*4*) require dietary sources of niacin. The lack of an adequate supply of the vitamin leads to pellagra in humans and to similar deficiency diseases in animals. This fact has greatly stimulated the development of rapid and accurate methods for the estimation of niacin in biological materials and foods. Many species, among them the rat, cow and chicken do not appear to need dietary niacin except under unusual dietary conditions. Ordinarily, these species apparently obtain sufficient niacin either through tissue synthesis or microbial synthesis in the digestive tract.

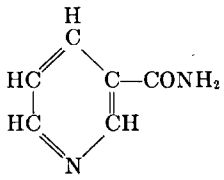
The amide of niacin is biologically active. This is the form predominantly found in animal tissues. The niacinates and several N-substituted niacinamides have been shown to be active (*5,6*). It appears that any compound which is readily converted to niacin in the body will exhibit niacin activity.

Niacin and niacinamide are widely distributed in foods. Meat, fish, yeast, enriched and whole grain cereals are good dietary sources. In natural products niacin is usually bound to other chemical compounds and must be freed by hydrolysis with strong acid or alkali (*7*) or by enzymatic treatment (*8*) before analytical methods can be applied.

The chemical name of niacin, 3-pyridinecarboxylic acid, indicates the chemical structure for the acid and its amide as shown on the following page:



Niacin
Nicotinic acid



Niacinamide
Nicotinamide

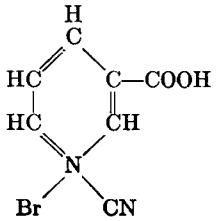
Some of the more important chemical and physical properties of these compounds are shown in the following table:

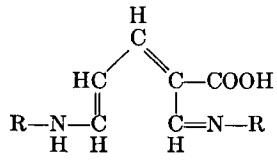
Properties	Niacin	Niacinamide
Appearance	Colorless needles	Colorless needles
Taste	Tart	Bitter
Empirical formula	$C_6H_5O_2N$	$C_6H_6ON_2$
Molecular weight	123.11	122.12
Melting point	235.5–236.5° C	128–131° C
Boiling point	Sublimes	150–160° C at 5×10^{-4} mm
Hygroscopicity	Nonhygroscopic	Slightly hygroscopic
Absorption maximum	385 mμ	212 mμ
pH of 1% solution	3.0	6.0
Solubility:		
Water at 25° C	1.67 g per 100 ml	100 g per 100 ml
Ethyl alcohol	0.73 g per 100 ml	66.6 g per 100 ml
Ethyl ether	Insoluble	Very slightly soluble
Glycerine at 25° C		10 g per 100 ml

Niacin and niacinamide are both stable in the dry form and in aqueous solutions and are unaffected by light or pH. Niacin is relatively stable to strong oxidizing agents such as fuming nitric acid, chromic acid and permanganate. In aqueous solutions, niacin can be titrated with alkali to form alkaline niacinates. Chemically, it undergoes the typical reactions of pyridine as modified by the carboxyl group at the 3 position.

Niacin reacts with cyanogen bromide to give a pyridinium compound (I), which undergoes rearrangement yielding derivatives that will couple with aromatic amines, giving colored compounds (II). The principal chemical methods for the assay of niacin involve these reactions (*9–13*).

(I)





(II)

Methods Available

Recognition of the fact that many bacteria require niacin for growth has resulted in the development of microbiological methods for the assay of niacin (*14,15*). Usually, these methods are quite sensitive and measure the niacin-active compounds more specifically than the chemical methods. On the other hand, the microbiological method may be subject to error by growth-stimulating and growth-depressing materials, although the presence of these materials is indicated in a given assay by "drift" and can be eliminated with little difficulty.

It has been observed generally that the microbiological method is applicable to a wider variety of materials without modification than are the chemical methods. The microbiological method is particularly well adapted to the simultaneous assay of many samples and for this reason it is very often used for continuous product control routines. It is not so well suited for an occasional assay because the test organism requires frequent attention, and the many stock solutions used in the assay will not keep indefinitely.

Chemical methods require fewer reagents and are much simpler insofar as initial preparation is concerned. However, they are considerably less sensitive than the microbiological procedures which makes it more difficult to apply the chemical methods to the analysis of materials containing small amounts of niacin. The chemical methods are also less specific than the microbiological procedures, being influenced by biologically inactive materials occurring naturally or produced during extraction. With such materials it is sometimes necessary to use involved extraction procedures, purifications and blank (*16*) determinations. On the other hand, when applicable, the chemical methods are relatively rapid and provide values for comparison with those obtained microbiologically.

Some laboratories using both methods have reported good agreement although in some instances the chemical method gives higher results than the microbiological (*17*). Other investigators have found wide discrepancies between the two methods, especially for highly pigmented extracts. When assays by both chemical and microbiological procedures agree, the validity of the value found is enhanced. In cases of disagreement the operator must rely upon his own judgment as to which value is most nearly correct.

A detailed description of the microbiological method with *Lactobacillus arabinosus* is given in the following section, but at the present no chemical

method is presented. Several very useful chemical methods for niacin have been published (*16,18–20*) and the inclusion of such a method can be expected in subsequent editions of this manual.

MICROBIOLOGICAL METHOD

A. Principle

The microbiological method is based upon the observation that *Lactobacillus arabinosus* 17–5 requires niacin for growth. Using a niacin-free but otherwise complete basal medium, growth responses of the organism are compared quantitatively in standard and unknown solutions. Either the acid or the turbidity produced by the organism is measured to determine the extent of growth and thereby the amount of niacin in the test solution.

B. Equipment

Since this method is adapted more to large-scale routine assay than to the analysis of a single sample, equipment is listed for analyzing, simultaneously, 12 different samples or 6 samples in duplicate. This list includes only the equipment used in the actual assay procedure. In preparing the reagents, additional bottles, beakers, pipets and other pieces of ordinary laboratory equipment are needed.

No. of pieces	*Item*
1	*Buret*, 50 ml
24	*Erlenmeyer Flasks*, 250 ml
12	*Erlenmeyer Flasks*, 50 ml
12	*Filter Funnels*, 3-inch
1	*Filter Rack and Stand*, 12 holes
1	*Pipet*, graduated, 10 ml
1	*Pipet*, graduated, 5 ml
1	*Pipet*, volumetric transfer, 50 ml
1	*Pipet*, volumetric transfer, 25 ml
1	*Pipet*, volumetric transfer, 10 ml
1	*Pipet*, volumetric transfer, 5 ml
1	*Automatic Pipet*, 5 ml, or a pipetting machine (Fisher Volustat or Brewer pipetting machine, for example). Optional for dispensing basal medium.
1	*Volumetric Flask*, 1000 ml
1	*Volumetric Flask*, 500 ml
1	*Volumetric Flask*, 250 ml
1	*Volumetric Flask*, 100 ml

No. of pieces	*Item*
1	*Incubator or Water Bath*, which will maintain constant and uniform temperature (±0.5° C) in the range 30° to 37° C. Uniform temperature is essential for uniform growth. It is particularly difficult to maintain a uniform temperature with closely packed tubes. A forced-draft incubator or circulatory water bath will give more satisfactory results than an ordinary incubator.
1	*Autoclave*, large enough to admit culture tubes in their racks and capable of accurate adjustment to a constant pressure of 15 lbs.
144	*Uniform, Lipless Pyrex Tubes*, 15 × 150 mm to 25 × 200 mm are satisfactory. Smaller tubes may be used, but there is more difficulty in getting good mixing and more danger of losing sample during autoclaving.
2–3	*Culture Tube Racks* to hold a total of approximately 120 tubes. The racks should hold the tubes in a vertical position and should be open enough to permit free circulation of air. Preferably, they should be made of material resistant to rust.
120	*Cotton Culture Tube Plugs or Metal Caps.* Cotton plugs can be reused provided they do not become contaminated with niacin. This sometimes occurs. Aluminum or glass caps can be used with considerable saving of time. Metal covers for an entire rack have also been suggested.
1	*Inoculating Needle*, platinum or nichrome steel, to be used for the transfer of stock culture.
1	*Hypodermic Syringe*, 5 to 10 ml, fitted with a freely moving plunger and a three-quarter inch 22-gage needle, to be used for inoculating the tubes.
1	*Refrigerator*, of sufficient capacity to hold reagents, basal medium and stock cultures.
1	*Centrifuge*, with adapters to hold 3/4-inch test tubes. (Optional, See D-2)

C. Reagents

The principal reagent is a basal medium prepared by mixing a number of stock solutions and chemicals. The stock solutions may be preserved at room temperature in the dark with toluene and chloroform, although most workers prefer storage in the refrigerator. It has been reported that 0.1% chloroform and 0.5% toluene are adequate to prevent microbial growth (*22*). The stock solutions are prepared as follows:

1. Acid-Hydrolyzed Casein. Stir 100 g of "vitamin-free" casein with 250 ml of 95% ethyl alcohol for 15 minutes in an 800-ml beaker and filter with suction. Repeat using another 250-ml portion of alcohol. If 95% ethyl alcohol is unavailable, commercial brands of denatured alcohol can be used with satisfactory results. It has been found that some commercial brands of "vitamin-free" casein contain enough niacin to give appreciable growth in the blanks provided this precaution is not taken.

Transfer the alcohol-washed casein into a round-bottom flask of at least 1-liter capacity, preferably one having two necks ground to standard taper. Mix well with 500 ml of constant-boiling HCl. Fit the flask with a glass stopper and a water-cooled condenser and reflux over a low flame or hot plate for 8-12 hours. A mixture of one volume of concd. HCl (37%) with one volume of water gives a 20.1% HCl solution which is satisfactory for the hydrolysis.

Since casein tends to froth during the initial stages of hydrolysis, heat carefully and gradually. Mix the contents of the flask occasionally by shaking and have a wet towel ready to cool the flask if the reaction becomes too vigorous.

After refluxing, fit the flask with a condenser and receiving flask suitable for vacuum distillation and remove as much HCl as possible by concentrating the hydrolysate to a thick paste under reduced pressure. Air introduced through a bleeder tube placed well into the bottom of the flask will minimize bumping during the final stages of the concentration. The temperature at which the distillation is carried out should not exceed that of a boiling water bath. Temperatures of 70° to 80° C, have been recommended. To get rapid and complete distillation at this low temperature it is necessary to reduce the pressure considerably. This may not be possible with a water aspirator unless high water pressure is available. A steam aspirator or a vacuum pump can be used. Care must be taken to trap HCl fumes effectively, especially with a vacuum pump.

It is the usual custom to redissolve the paste in approximately 200 ml of water and repeat the concentration to remove additional amounts of HCl; however, a satisfactory hydrolysate can be attained with a single concentration to a rather thick paste. In any case, the acid concentration should be low enough so that subsequent neutralization will not yield salt enough to retard bacterial growth on the basal mediums.

Dissolve the hydrolysate paste in about 700 ml of water and adjust the pH to 3.5 with 40% NaOH. Decolorize by stirring with 20 g of activated charcoal (e.g. Norite A or Darco G-60) at room temperature. Stir until a small test filtrate is light straw colored. The decolorization may be complete in 5 minutes or may require more than an hour dependent upon the charcoal used. This step removes any niacin which may have remained in the alcohol-washed casein. Some workers omit the alcohol wash and still

when harvested and were not considered representative of the variety. These lots furnished samples for overall estimates of deterioration, histological study, chemical analysis, and palatability testing.

For histological study, sections were prepared from raw and steamed unstored and stored samples from the second year's crop. Microscopic sections and photomicrographs were made to provide material for histological study. Chemical analyses on raw samples were made during both years to determine pectic substances, total sugars, reducing sugars, pH, total acidity, and moisture. Palatability of the apples was determined both years on three replications each for unstored and stored samples when raw, baked, and made into sauce.

Measurement of deterioration of apples in storage

Upon removal from refrigerated storage, each lot of apples to be used for testing was checked and counts were made to ascertain the amount of rot, softening, and shriveling that had taken place during storage. During the first year any deterioration of the apples was noted and recorded as an estimated fraction of the whole. Throughout the second year, apples showing each type of deterioration were counted and percentages of loss were calculated on that basis. After rotted apples were discarded, representative samples were selected for use in palatability judging, for histological study, and for chemical analysis.

During the second year weight loss was determined on special lots consisting of 28 apples representative of each variety, each stage of maturity, and each storage temperature. Individual apples were numbered, permanently marked, weighed when first received, and weighed again at each storage interval. Weights for apples that developed rot during storage were deducted. Weight loss was computed for the remainder on the basis of percentage loss at each period compared with that of the unstored sample.

Histological techniques

For the histological phases of this study, maturity, storage tem perature, and storage period of each variety of the second crop was represented. Every sample was studied in both raw and steamed condition, and after being stained by two procedures. Information was desired on the histology and histochemistry of apples as a group, on varietal differences among apples, and on the effects of storage and cooking on their microscopic appearance.

Apples for microscopic study were washed, dried, cored, and cut crosswise. One slice about one-fourth of an inch thick was cut from each cored apple. Two segments of skin, one from the stem end and one from the blossom end, were taken from the same apple. Skin segments were treated with 0.75 percent hydrochloric acid (*26*) and preserved in 70 percent ethyl alcohol. Later they were stained with Sudan IV, peeled or scraped, preserved in 50 percent glycerin, and examined for russet.

The peripheral 5 mm. portion of the slice was cut into cubes of appropriate size for fixing. Some cubes from each apple were immersed immediately in a solution containing 50 ml. of formalin, 50 ml. of glacial acetic acid, and 900 ml. of 70 percent ethyl alcohol. This solution will be referred to hereafter as FAA. Other cubes,

Ratings for appearance of sauce were most influenced by color and by consistency. Average scores for acidity of cooked apples were closely related to the pH values of the raw juice.

EXPERIMENTAL PROCEDURES

Selection and sampling of apples

Varieties of apples used for this study were Delicious, Golden Delicious, Jonathan, Rome Beauty, Stayman, and Winesap. Delicious and Rome Beauty varieties were obtained in 1948 from orchards of the Crops Research Division at Beltsville, Md. These apples were picked when they had crisp flesh and were considered suitably mature for storage. The four remaining varieties were obtained from a local commercial orchard. The Golden Delicious and Stayman varieties were crisp and firm; cracks through the skin were prevalent in the Stayman. All apples of the Jonathan variety were bruised and some showed cuts and wormholes. Winesap apples were received late in the season; some were bruised and they appeared to be fully ripe.

Two pickings each of Golden Delicious and Stayman, immature and mature, and one picking of mature Jonathan apples were obtained from a local commercial orchard in 1949. Delicious and Rome Beauty varieties were again obtained from the orchards of the Crops Research Division. They were picked when most of the apples were at the hard-ripe stage. The under-ripe apples, as judged by color, were separated from those more fully red and mature. These selected under-ripe apples were classed as immature, together with those of other varieties that had been picked at an immature stage.

All apples were sorted and stored in bushel baskets or wooden boxes as soon as possible after they were received. Controlled storage at 32° F. with 85 to 90 percent relative humidity was furnished by the Marketing Research Division; 40° storage (uncontrolled humidity) was available in the Human Nutrition Research Division. Dates of picking and dates when apples were put into storage are given below:

Variety and maturity of apples	*Date picked*	*Date stored*
Delicious:		
Mature, 1948	Sept. 24	Sept. 24
Mature, 1949	Sept. 28	Sept. 28
Immature, 1949	Sept. 28	Sept. 28
Golden Delicious:		
Mature, 1948	Sept. 21	Sept. 28
Mature, 1949	Oct. 7 [1]	Oct. 12
Immature, 1949	Sept. 16 [1]	Sept. 22
Jonathan:		
Mature, 1948	(2)	Oct. 19
Mature, 1949	Sept. 26 [1]	Sept. 29
Rome Beauty:		
Mature, 1948	Oct. 14	Oct. 14
Mature, 1949	Oct. 17	Oct. 18
Immature, 1949	Oct. 17	Oct. 20
Stayman:		
Mature, 1948	Oct. 1 [1]	Oct. 6
Mature, 1949	Oct. 4 [1]	Oct. 7
Immature, 1949	Sept. 27 [1]	Sept. 30
Winesap:		
Mature, 1948	(2)	Nov. 30

[1] Approximate. [2] Unknown.

Apples of each variety and stage of maturity were divided into seven lots of about 1 bushel each. One lot was analyzed within a few days after harvest, and the remaining lots were analyzed after storage of 3, 5, and 7 months at 32° F. with 85 to 90 percent relative humidity (representative of commercial storage) or at 40° with no humidity control (representative of household storage). Winesap apples were studied only in 1948, since those available in 1949 were of poor quality

obtain low blanks. Filter through a large fluted filter or by suction as preferred.

Adjust the pH of the filtrate to 6.8, dilute to 1 liter and store under toluene and over chloroform in the refrigerator. Occasionally, a precipitate will form in this solution on standing. This is mainly tyrosine. It is a good practice to shake up the solution and use the suspended material as well as fluid portion. The insoluble material will dissolve when the entire medium is prepared. If desired, the norite-treated hydrolysate may be diluted to one liter without pH adjustment. Commercial casein hydrolysates are available but high blanks are sometimes obtained with these preparations. Norite-treated peptone has also been used with success in some laboratories (*24*).

2. **Cystine–Tryptophane Solution.** Suspend 4.0 g of *l*(−)cystine and 1.0 g of *l*(−)tryptophane (or 2.0 g of *dl*-tryptophane) in 700–800 ml of water, heat to 70–80° C and add 20% HCl dropwise with stirring until the solids are dissolved. Approximately 12 ml of 20% HCl are required. Cool to room temperature and make to 1 liter with water.

3. **Adenine–Guanine–Uracil Solution.** Heat 0.1 g each of adenine sulfate, guanine hydrochloride, and uracil in a 250-ml Erlenmeyer flask containing about 75 ml of water and 2 ml of concd. HCl. Cool when all solids have gone into solution. If a precipitate forms, add a few drops of concd. HCl and heat. Repeat until no precipitate forms on cooling and then transfer to a 100-ml volumetric flask and make to volume with water.

4. ***d*-Calcium Pantothenate–*p*-Aminobenzoic Acid–Pyridoxine Hydrochloride Solution.** Weigh 10.0 mg each of *d*-calcium pantothenate, *p*-aminobenzoic acid and pyridoxine hydrochloride. Transfer to a 1-liter volumetric flask and dilute to volume with water. Store the solution in the dark to protect pyridoxine from light. An alternative procedure for making up this solution in 25% neutral alcohol is described in the U.S.P. method (*21*).

5. **Riboflavin–Thiamine Hydrochloride–Biotin Solution.** Dissolve 1 mg of crystalline biotin (free acid) in 100 ml of 0.02 *N* acetic acid. Add 4.0 ml (equivalent to 40 γ) of this solution to a 1-liter volumetric flask. Ampules containing 25 γ may be purchased. Add 20 mg of riboflavin and 10 mg of thiamine hydrochloride to the flask and make to volume with 0.02 *N* acetic acid. Store in the dark to prevent destruction of riboflavin by light.

6. **Salt Solution A.** Dissolve 25 g of K_2HPO_4 and 25 g of KH_2PO_4 and dilute to 500 ml with water. Store under toluene.

7. Salt Solution B. Dissolve the following amounts of salts and dilute to 500 ml with water:

Magnesium sulfate	($MgSO_4 \cdot 7H_2O$)	10.0 g
Sodium chloride	(NaCl)	0.5 g
Ferrous sulfate	($FeSO_4 \cdot 7H_2O$)	0.5 g
Manganese sulfate	($MnSO_4 \cdot 4H_2O$)	0.5 g

Add 5 drops of concd. HCl and store under toluene. Sodium chloride may be omitted when HCl-hydrolyzed casein is used.

8. Standard Niacin Solution. Weigh 50 mg of U.S.P. Reference Standard anhydrous crystalline niacin (dried over P_2O_5 or concd. H_2SO_4 in a vacuum desiccator for 24 hours), and dilute to 500 ml with ethyl alcohol. Store this stock solution in the refrigerator. Prepare a working standard for each assay by diluting 1.0 ml of the above solution to 1 liter in a volumetric flask with water.

9. Basal Medium Stock Solution. In order to make up this basal medium stock solution for 100 assay tubes, mix the following ingredients in a 500-ml Erlenmeyer flask marked at 450 ml:

Quantity	Reagent	Reagent number
50 ml	Casein Hydrolysate	1
50 ml	Cystine–Tryptophane	2
10 ml	Adenine–Guanine–Uracil	3
10 ml	*d*-Ca Pantothenate–*p*-Aminobenzoic Acid–Pyridoxine	4
10 ml	Riboflavin–Thiamine Hydrochloride–Biotin	5
10 ml	Salt Solution A	6
10 ml	Salt Solution B	7
10 g	Anhydrous Glucose	
10 g	Anhydrous Sodium Acetate (or 16.6 g $NaC_2H_3O_2 \cdot 3H_2O$)	
250 ml	Water	

Mix the ingredients thoroughly, dilute to 450 ml and adjust the pH to 6.8 with 40% NaOH using a pH meter or bromothymol blue as an external indicator. Approximately 1 ml of alkali is necessary for the adjustment. Transfer to a 500-ml volumetric flask or a graduated cylinder and make to volume with water. In the assay procedure one volume of basal medium stock solution is diluted to two volumes. The concentrations of the ingredients are shown on the following page.

10. Agar Medium for Stock Culture. Dissolve 5.0 g of Difco Bacto Yeast Extract in 200 ml of water, add 1 g anhydrous glucose, 1 g of anhydrous sodium acetate (or 1.7 g $NaC_2H_3O_2 \cdot 3H_2O$) and 3 g of agar and heat the mixture on a steam bath with occasional stirring until the ingredients are dis-

Ingredient	Basal medium stock solution	Basal medium (final dilution)
Glucose	2.0%	1.0%
Sodium acetate	2.0%	1.0%
Casein hydrolysate	1.0%	0.5%
Cystine	0.04%	0.02%
l-Tryptophane	0.01%	0.005%
Adenine sulfate	20 γ per ml	10 γ per ml
Guanine hydrochloride	20 γ per ml	10 γ per ml
Uracil	20 γ per ml	10 γ per ml
Riboflavin	0.4 γ per ml	0.2 γ per ml
Thiamine hydrochloride	0.2 γ per ml	0.1 γ per ml
d-Calcium pantothenate	0.2 γ per ml	0.1 γ per ml
Pyridoxine hydrochloride	0.2 γ per ml	0.1 γ per ml
p-Aminobenzoic acid	0.2 γ per ml	0.1 γ per ml
Biotin (free acid)	0.8 mγ per ml	0.4 mγ per ml

solved. While the solution is still hot, filter it through cotton or cloth and add 10 ml to each of 20 culture tubes. Plug the tubes with cotton and sterilize in the autoclave for 20 minutes at 15 lbs. pressure. Cool in an upright position and store in the refrigerator.

11. Culture Medium for Growing Inoculum. To each of a series of culture tubes containing 5.0 ml of basal medium stock solution add 5 ml of a solution containing 0.2 γ of niacin per ml. Prepare this solution by diluting 1.0 ml of standard niacin solution (No. 8) to 500 ml with water. Plug the tubes with cotton and sterilize in the autoclave at 15 lbs. for 15 minutes. The tubes may be stored for several weeks provided evaporation or contamination with toluene, etc., does not take place.

12. Isotonic Salt Solution. Weigh 0.9 g of NaCl and transfer to a 100-ml volumetric flask. Dilute to volume with water and shake until salt has dissolved. Transfer 10-ml quantities of this solution to culture tubes, plug with cotton and sterilize in the autoclave at 15 lbs. pressure for 20 minutes. (Optional, see D-2.)

13. 1 *N* Sulfuric Acid. It is usually advantageous to prepare large volumes of this reagent since it is used in extracting the niacin from most materials. Mark a glass carboy at the 18-liter mark and fill about three-quarters full of water. Add 500 ml of concd. H_2SO_4 and make to the mark with water, giving an approximately *N* solution.

14. 1 *N* Sodium Hydroxide. Since NaOH pellets often contain considerable water, amounts in excess of the calculated NaOH may be used. To prepare 18 liters of 1 *N* NaOH, 720 g of NaOH are needed. From 1 to 15%

in excess of this amount can be used, depending upon the water content of the NaOH pellets. Although this is not a standard solution, it is time-saving to determine the volume which will just neutralize the acid used in the extraction of the samples.

15. 0.1 *N* Sodium Hydroxide. Determine the normality of solution 14 by titration against a standard acid and dilute to a concentration of 0.1 *N* alkali using proportions indicated by the following equation:

$$\text{volume of solution No. 14} = \frac{0.1 \times \text{volume of 0.1 } N \text{ alkali needed}}{\text{normality of solution No. 14}}$$

The alkali solution may also be standardized against potassium acid phthalate, H_2SO_4, or other acid of constant and known concentration.

This reagent is used in titrating the acid produced by *L. arabinosus*. It need not be exactly 0.1 *N*, but it is well to have a standardized solution so that results between laboratories can be compared.

16. Bromothymol Blue Indicator Solution. Weigh 0.1 g of bromothymol blue indicator into a small beaker. Add 3.2 ml of 0.05 *N* NaOH and triturate with a stirring rod until the powder has dissolved. Dilute with water to 250 ml. The solution may also be made up by dissolving in a few ml of 95% ethanol, adding 3.2 ml of 0.05 *N* NaOH, and diluting to 250 ml with water.

D. Procedure

The microbiological method described in this chapter is similar in principle and detail (exceptions noted) to the U.S.P. (*21*) and to the 1945 A.O.A.C. tentative methods for niacin assay which are based on the original method of Snell and Wright (*14*).

Certain time relations exist in microbiological procedures. Active stabs of the stock culture, from one to fourteen days old, must be available for the preparation of the inoculum. The inoculum must be started 16 to 24 hours before it is used in the assay. The time of inoculation of the assay tubes should be chosen so that the titration can be carried out three days later. However, the samples may be extracted and the standard and assay tubes set up and sterilized several days prior to inoculation provided contamination and evaporation are prevented.

1. Preparation of the Stock Culture

(a) Prepare stab cultures in two or more agar stock culture tubes (Reagent 10) using a pure culture of *L. arabinosus* 17-5.

(1) Pure cultures of *L. arabinosus* 17–5 can be obtained from the American Type Culture Collections, Georgetown University, Washington, D.C.

(b) Incubate for 16 to 24 hours at a constant temperature (±0.5° C) between 30° and 37° C.

(c) Store in the refrigerator under aseptic conditions not longer than two weeks, preferably only one week, before transferring to new stab.

(1) One stab culture should be reserved unopened for use in the preparation of subsequent stock culture stabs. The others can be used as many times as necessary in the preparation of inoculum, provided they are not more than two weeks old and sterile technic is observed.

(2) Whenever the stock culture has not been used for several weeks or months, get new cultures or revive the old one by making daily successive stab transfers for at least three days before preparing the inoculum. Poor growth results from old cultures.

2. Preparation of the Inoculum

(a) On the day prior to use, transfer cells from the stock culture to a sterile tube of inoculum culture medium (Reagent 11).

(1) Do not use an old inoculum for preparing a new inoculum. This practice may alter the nutritive requirements of the test organism making it unsuitable for the assay (*27*).

(b) Incubate this culture for 16 to 24 hours at any selected temperature between 30° and 37° C.

(1) Inoculum incubated longer than 24 hours should not be used as the organism tends to become attenuated and poor growth responses are encountered. In case of emergency, the inoculum may be refrigerated for 24 hours before use.

(2) If necessary, an inoculum can be prepared in 8 hours by adding a drop of active inoculum or inoculated test sample medium not more than 24 hours old to a tube of inoculum medium and incubating at the usual temperature. This is an emergency procedure and should not be repeated without returning to the stock stab culture.

(c) Secure cotton plug with a rubber band, adhesive tape or a pin, and centrifuge.

(1) An angle head centrifuge layers cells so that decantation is easy, but the conventional centrifuge is satisfactory.

(d) Decant the supernatant liquid and resuspend the cells in 20 ml of sterile isotonic salt solution (Reagent 12). This must be done aseptically.

(1) The centrifugation and resuspension of cells may be omitted and the fresh inoculum may be used directly. If the resuspension step is eliminated, it is essential that the niacin level in the inoculum medium be as low as directed in Reagent 11.

(2) Twenty ml of inoculum will inoculate 200–400 tubes.

(e) Fill the sterile syringe with the resuspended cells and use at once.

(1) Sterilize the needle and syringe (plunger removed) by wrapping in paper and autoclaving for 15 minutes at 15 lbs. pressure. By pouring the resuspended cells into

the open end of the syringe before inserting the plunger, the disadvantage of withdrawing the inoculum from a deep culture tube with a short needle can be overcome.

(2) A tube drawn out to a capillary or a pipet can be used in place of a syringe.

3. Preparation of Samples

(a) Weigh sufficient material to contain approximately 0.1 mg of niacin into a 250-ml flask.

(1) In choosing the quantity of material to be extracted, the degree of homogeneity with respect to niacin should be considered. In general, samples smaller than 2.0 g should be avoided. With high-potency material, take 2.0 g and then dilute the extract obtained in step 3-(e) sufficiently to give a niacin concentration of approximately 0.1 γ per ml.

(b) Add 100 ml of 1 N H_2SO_4 and mix thoroughly.

(1) It has been shown that dilute acids and water extract less niacin from cereal products than do dilute alkalies (7). Extraction with N H_2SO_4 gives practically the same values for niacin in cereals, animal proteins and pharmaceuticals as does extraction with any strength alkali from 0.05 to 1.0 N. Weak acids apparently fail to convert niacin precursor in cereals to free niacin. Alkaline extraction of samples containing large amounts of fat gives extracts which are difficult to clarify. For these reasons, extraction with N H_2SO_4 is recommended.

(c) Autoclave the mixture at 15 lbs. pressure for 30 minutes.

(d) Add 1 N NaOH to produce a pH of 6.8 using a pH meter or bromothymol blue as an outside indicator.

(1) The pH of the extract is adjusted to 6.8 so that when added to the basal medium in different amounts, it will not change the pH of the basal medium.

(2) In routine work, it is time saving to use the volume of NaOH solution which will just bring the pH to 6.8 without further adjustment. In some laboratories, dispensers are employed to deliver acid and alkali solutions, particularly when routine samples are extracted.

(e) Quantitatively transfer the solution to a 1-liter volumetric flask, dilute to volume with water and mix thoroughly.

(f) Filter the solution through Whatman No. 40 paper or its equivalent. This is the test solution used in the preparation of the assay tubes. The niacin concentration must be 0.1 to 0.2 γ per ml to obtain valid assays.

(1) The first few milliliters of extract should be discarded as the niacin may be adsorbed on the dry filter paper and also because the first few milliliters may be turbid.

(2) The type of filter paper that is most satisfactory depends upon the test material. Use a paper that filters rather rapidly but gives a relatively clear filtrate. Clear filtrates are necessary when the turbidimetric procedure is used but are not necessary when the titration procedure is used.

4. Preparation of Standard Tubes

(a) To duplicate tubes add 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 ml of the working standard niacin solution (Reagent 8).

(1) If turbidimetric measurements are used, it is desirable to use more levels in the lower range of the standard (0.0 to 0.25 γ of niacin per tube in the standard curve). With niacin levels above 0.25 γ of niacin per tube, the growth response as measured turbidimetrically is not linear.

(2) Some workers do not run duplicates at each level of sample, since each of the four levels already constitutes an independent observation of the same thing. They believe that for most practical purposes, use of a single assay tube for each level will give satisfactory accuracy. The standard tubes, however, are always run in duplicate.

(b) Add sufficient water to bring the volume in each tube to 5 ml.

(c) To each of these tubes add 5.0 ml of basal medium stock solution (Reagent 9).

(1) Adding the basal medium to the extract and water gives better mixing of the two solutions than adding the extract and water to the basal medium. Better mixing can be attained in this step by allowing part of the basal medium to run down the side of the tube and part to fall directly on the liquid surface.

(2) An automatic, 5-ml pipet or pipetting machine is a valuable time saver in this step.

5. Preparation of Assay Tubes

(a) To duplicate tubes add 0.5-, 1.0-, 2.0- and 3.0-ml aliquots of the test solution.

(1) The test solution is used at four levels so that several tests will fall on the standard curve. The best index of the validity of the assay is agreement of the calculated results at different test levels.

(b) Add sufficient water to bring the volume to 5 ml.

(c) To each of these tubes add 5.0 ml of basal medium stock solution.

6. Sterilization

(a) Mix the contents of each tube thoroughly by rotating the tube vigorously in the palm of the hand.

(1) Mixing without spilling is difficult and time-consuming, but by adding the ingredients as indicated in 4-(c)-(1), sufficient mixing may be attained.

(b) Plug with cotton or cover with caps.

(1) Glass or aluminum caps are satisfactory substitutes for cotton plugs. Metal covers (rustless, such as aluminum, copper or stainless steel) for assay tubes have

been used with success. Caps and covers have been reported to be less successful than cotton plugs when a water bath is used instead of an incubator, since water tends to condense on the cover and may run back into the tubes.

(c) Autoclave at 15 lbs. pressure for 15 minutes.

(1) The minimum time in which sterilization, without undue caramelization, can be accomplished is desired. Pressures above 15 lbs. and periods longer than 15 minutes increase caramelization of the sample. In some autoclaves sterilization is accomplished in considerably less than 15 minutes at 15 lbs. pressure.

7. Inoculation and Incubation

(a) Cool all the tubes to the incubation temperature or below.

(1) All tubes must be the same temperature all the way through the rack. Allow the culture tube rack to stand in a water bath or at room temperature until there is no question that the temperature is uniform. This precaution is especially necessary when turbidimetric measurements are made, since in that procedure the rate of growth, rather than the extent of growth, is being measured. Small differences in temperature, especially at the start of the assay, influence the rate of growth much more than they influence the extent of growth over a long period of time.

(b) Aseptically inoculate each tube with one drop of inoculum.

(1) A 10-ml syringe fitted with a 20- to 24-gage needle is satisfactory for delivery of drops of inoculum into the culture tubes. If the plunger is removed from the barrel of the syringe after starting the inoculum through the needle, uniform drops will continue to fall from the needle. By mounting the syringe directly above a wire or block "stop," both hands are freed to remove the plug and rapidly place the culture tubes into position against the "stop" so that the inoculum falls directly onto the surface of the basal medium.

(c) Incubate for 72 hours at any selected temperature between 30° and 37° C.

(1) Good assay results have been reported with incubation periods as short as 24 hours and as long as 96 hours. The 72-hour period is recommended since there is very little acid production after this time.

8. Titration

(a) Transfer the contents of each tube, successively, to a 50-ml Erlenmeyer flask and rinse with 10 ml of water.

(b) Titrate the contents of the flask with 0.1 *N* NaOH (Reagent 15) to a pH of 6.8 using bromothymol blue (Reagent 16) as an internal indicator.

(1) Two to three drops of indicator should be used. The end point may be taken where the yellow color changes to green or the green changes to blue providing that all tubes are titrated to the same end point. In some laboratories the indicator is

added to the wash water thus ensuring an equal amount of indicator in all tubes when 10 ml of wash water is used. One part of indicator solution for 100 parts of water gives about the right concentration.

(2) Colored extracts may be encountered in the assay of certain foods. This color may interfere by obscuring the end point and necessitate the use of another indicator or an electrometric titration.

(3) In order to compare results between laboratories, it is advisable to plot the standard curve using ml of 0.1 *N* NaOH although assay results can be obtained with other strengths of alkali.

(4) Titration values in excess of 2.0 ml for the tubes containing 0.0 ml of the standard niacin solution indicate an excessive amount of niacin in the basal medium and invalidate the assay.

(5) The growth response of the test organism to niacin can be measured by determining the turbidity developed in the culture tubes. The essential differences in the procedure when turbidimetric measurements are made in place of acid titration are:

The range of the standard curve is reduced.

The contents of the culture tubes after incubation are mixed by shaking and transferred to the cuvet of a photometric colorimeter without rinsing or dilution, and the optical density of the solution is determined using a red filter. By centrifuging out the suspended material in some incubated culture media, a clear solution is obtained which can be tested against water in the colorimeter in the selection of a filter. Select the filter or wavelength which shows the least difference in optical density between the water blank and the clear extract.

9. Calculation

(a) Draw a standard curve for the assay by plotting ml 0.1 *N* NaOH [titer values from 8-(b) above] against γ niacin per tube in the standard series.

(b) Determine the niacin content of the tubes in the unknown series by interpolation of the titer values on the standard curve.

(c) Discard any values which show more than 0.4 or less than 0.05 γ of niacin per tube. Calculate the niacin content of each ml of test solution for each of the duplicate sets of tubes.

(d) Calculate the niacin content of the test material from the average of the values for 1 ml of test solution, obtained from not less than three sets of these tubes which do not vary by more than 10% from the average, using the following formula:

$$\gamma \text{ per g} = \frac{\text{average } \gamma \text{ per ml} \times \text{volume}}{\text{weight of sample}} \times \text{dilution factor}$$

(1) When the above directions are followed there is no dilution factor and the volume is 1000 ml.

(2) A typical example of the calculation of the results of an assay is given on page 140.

Standard Curve Series:

Niacin standard, ml	γ Niacin per tube	0.1 *N* NaOH (in duplicate), ml	
0.0	0.00	0.1	0.1
0.5	0.05	1.6	1.6
1.0	0.10	3.2	3.2
1.5	0.15	4.7	4.9
2.0	0.20	6.1	6.2
2.5	0.25	7.4	7.5
3.0	0.30	8.7	8.7
4.0	0.40	10.8	10.8

Unknown Test Series:

In this example, a 2.00-g sample of whole wheat flour was extracted, neutralized, diluted to 1000 ml, filtered and 1-, 2-, 3- and 4-ml aliquots were tested as described in the procedure.

Tube No.	ml per aliquot	0.1 *N* NaOH, ml	γ Niacin per tube	γ Niacin per ml
1	1.0	2.6	0.080	0.080
2	2.0	5.2	0.165	0.083
3	3.0	7.5	0.253	0.084
4	4.0	9.5	0.336	0.084
5	1.0	2.7	0.084	0.084
6	2.0	5.4	0.172	0.086
7	3.0	7.4	0.248	0.083
8	4.0	9.6	0.340	0.085
				Avg. = 0.0836

The values in column 4 above were found by interpolation of the values shown in column 3 on a standard curve.

Sample Calculation:

$$\frac{0.0836 \times 1000}{2} = 41.8\ \gamma \text{ niacin per g sample}$$

APPLICATION OF METHODS

Those products which have been assayed by the microbiological method or by reasonably similar modifications are listed below. The omission of any product from the list does not necessarily imply that the method is not applicable. Rather, it merely indicates that the committee and reviewers have not applied this method to the product.

Type of material
Milk, cream, ice cream and cheese
Milk, fresh, whole or skimmed
Milk, evaporated, condensed or dried
Eggs
Whole, fresh, frozen, or dehydrated
Meat, poultry and fish
Muscle cuts, fresh or frozen, beef, pork, veal, lamb or poultry
Muscle cuts, cooked, canned, cured and dehydrated
Sausage, bologna, frankfurters, Braunschweiger and salamis
Heart
Liver
Kidney
Dehydrated vegetables
Cabbage
Carrots
Beans
Grain products
Corn, white, yellow
Wheat
Barley
Rice
Oats
Flour
Buckwheat
Rye
White patent
White patent, enriched
Whole wheat

Type of material
Grain products (continued)
Baked products and bread
Rye
White
White, enriched
Breakfast cereals
Corn cereals
Wheat cereals
Oat cereals
Macaroni and spaghetti
Animal feeds
Mixed feeds
Meat scraps and meat meals
Liver meals
Alfalfa
Whey, dried and condensed
Distillers' dried grains
Distillers' dried solubles
Molasses residues
Miscellaneous
Bouillon cubes
Nuts
Wheat germ
Yeast
Pharmaceuticals
Vitamin tablets or capsules
Malt preparations
Yeast preparations
Liver preparations
Preparations containing large amounts of iron
Preparations containing absorbents

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CHAPTER 7

Ascorbic Acid

PRELIMINARY CONSIDERATIONS (*1–5*)

The vitamin C activities of foods and biological materials are associated with their *l*-ascorbic acid contents.* By definition, one International Unit (I.U.) or one U.S.P. XII unit of vitamin C is the antiscorbutic activity of 0.05 milligram of ascorbic acid; hence one gram of ascorbic acid is equivalent to 20,000 International Units.

Some of the more important sources of ascorbic acid in the American dietary are potatoes, citrus fruits, leafy vegetables, tomatoes and other types of fruits and vegetables. The vitamin C activity of a specific sample of a fruit or vegetable product is influenced by a variety of factors such as maturity, conditions under which it was grown, treatment between harvesting and consumption, etc.

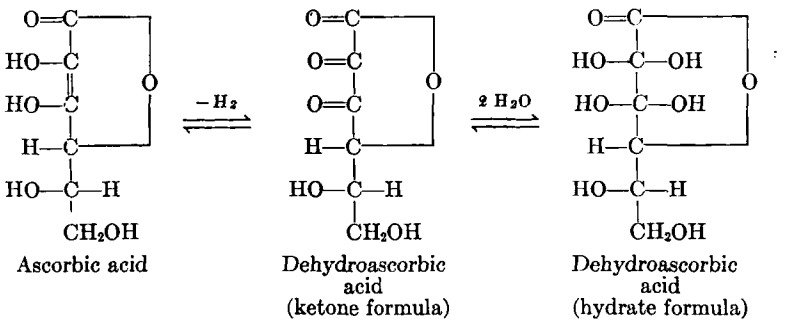
Certain of the physical and chemical properties of *l*-ascorbic acid are:

Empirical formula	$C_6H_8O_6$
Molecular weight	176
Melting point	192° C
Acidic dissociation, pK_1	4.2
Acidic dissociation, pK_2	11.6
Solubility	Soluble in water, methyl alcohol, ethyl alcohol Insoluble in ether, xylene, benzene, chloroform, petroleum ether, etc.
Specific rotation in water	24°
Specific rotation in methyl alcohol	48°
Absorption maximum in water	265 mμ; small band between 350 and 400 mμ
Oxidation-reduction potential, E_0^1	0.166 V at pH 4.0 and 35° C

As is evident from the structural formula, ascorbic acid contains two enolic groups. Therefore, when it is treated with a suitable oxidizing agent, two atoms of hydrogen are lost giving dehydroascorbic acid. On

*Hereafter, ascorbic acid will refer to *l*-ascorbic acid unless otherwise indicated.

treatment with reducing agents such as hydrogen sulfide, one molecule of dehydroascorbic acid takes on two hydrogen atoms to form ascorbic acid.



Ascorbic acid — Dehydroascorbic acid (ketone formula) — Dehydroascorbic acid (hydrate formula)

Dry crystals of ascorbic acid are stable on exposure to air and daylight at ordinary room temperature for long periods of time. In aqueous solutions below pH 7.6, ascorbic acid is not oxidized on exposure to air unless traces of copper or other materials that catalyze the reaction are present. In the presence of air and a suitable catalyst, ascorbic acid is readily oxidized to dehydroascorbic acid. Below pH 4.0, dehydroascorbic acid is fairly stable. However, above pH 4.0, dehydroascorbic acid readily undergoes an irreversible rearrangement to biologically-inactive material.

The vitamin C activity of dehydroascorbic acid is similar to that of ascorbic acid. However, the relatively labile nature of dehydroascorbic acid suggests that once ascorbic acid in a food has been oxidized to this compound, the value of the product as a source of vitamin C has been impaired. Unless this is considered, measurement of both ascorbic acid and dehydroascorbic acid may give misleading biological values for foods that are not to be consumed immediately.

The oxidation-reduction properties of ascorbic acid are widely used as the fundamental reaction in the measurement of vitamin C. In such methods, acid extracts of the food or other materials being assayed are prepared. The reducing capacity of the extract is measured by treatment with a suitable oxidizing agent such as 2,6-dichlorophenolindophenol, iodine, ferricyanide, methylene blue, etc. Of these, 2,6-dichlorophenolindophenol has generally been found to be the most satisfactory.

Ascorbic acid and dehydroascorbic acid possess certain chemical properties characteristic of sugars, such as formation of osazones and conversion to furfural. Colorimetric determination of the furfural as an aniline derivative has been used to a limited extent for the estimation of

ascorbic acid in certain materials (*6*). Such methods, however, have been found generally unsatisfactory for the measurement of ascorbic acid in foods.

Dehydroascorbic acid or one of its derivatives [probably 2,3-diketo-*l*-gulonic acid (*8*)] reacts with 2,4-dinitrophenylhydrazine to form a characteristic osazone. Upon treatment with sulfuric acid, this osazone is converted into a soluble red pigment. The Roe and Oesterling method (*7*) for determining ascorbic acid is based upon measurement at 540 mμ of the red color produced by this series of reactions under carefully controlled conditions.

Measurement of light absorption in the region of 265 mμ is used to a limited extent as the basis of physical methods for estimating vitamin C. Such methods have not found extensive application in determinations of the ascorbic acid contents of biological materials.

Methods Available

A reasonably satisfactory bioassay method based upon a determination of the minimum quantity of product required to protect a guinea pig from scurvy was described by Sherman, LaMer, and Campbell in 1922 (*9*). Since that time a variety of bioassay and chemical methods for the determination of vitamin C activity or ascorbic acid content have been developed (*1–3,10–12*).

Bioassays are time-consuming, expensive, and leave much to be desired insofar as precision is concerned. However, bioassays have the advantage of measuring the summation of chemical entities that possess vitamin C activity but exclude materials devoid of vitamin C activity. Since the development of chemical methods for determining ascorbic acid, bioassays have been largely confined to use in comparative studies to establish the specificity of chemical methods for determining ascorbic acid in individual products. The vitamin C activity determined by a satisfactory chemical method should not differ by more than ± 20% from the value obtained by bioassay of the product; such a tolerance is allowed to compensate for the lack of precision in the bioassay.

Oxidation of ascorbic acid with the dye 2,6-dichlorophenolindophenol has found extensive use as the basis of technics for determining ascorbic acid. Adaptations of visual titration methods as described by Bessey and King (*10*) are relatively simple technics that require the use of a minimum amount of equipment and chemicals. Such methods, however, are not applicable to the study of products containing water- or acid-soluble pig-

ments or reducing agents other than ascorbic acid which react with 2,6-dichlorophenolindophenol at a rate sufficiently rapid to influence the end point of the titration.

Photometric methods (*11,12*) based on the 2,6-dichlorophenolindophenol reagent avoid certain of the limitations of the visual titration technics. Difficulties encountered in determining the end point of moderately turbid or pigmented acid extracts are partially overcome by instrumentation. With the photometric method, it is also possible to determine the extent to which the oxidation-reduction reaction has progressed at any time interval. By limiting the time of the reaction and correcting for the influence of nonspecific reducing agents during a definite time, it is possible to avoid to some extent the influence of certain reducing agents other than ascorbic acid.

In the case of highly pigmented or turbid acid extracts, the photometric methods are not entirely satisfactory for measuring the degree of reduction of 2,6-dichlorophenolindophenol. The fact that this dye is readily soluble in xylene makes it possible to use a xylene extraction technic (*13*) to eliminate interference from acid-soluble pigments and turbid solutions.

In some materials, the value of the 2,6-dichlorophenolindophenol reagent for measuring ascorbic acid is limited by the presence of reducing substances such as "reductones" and "reductic acid" (strong reducing compounds formed during prolonged storage of biological materials under some conditions or by heating certain carbohydrates in an alkaline medium), ferrous iron, etc. Several procedures have been devised to minimize these interferences. The differences in rates at which ascorbic acid, "reductones," and ferrous iron couple with formaldehyde have been reported to provide a means of differentiating ascorbic acid from these substances. However, the specificity of such methods for the determination of ascorbic acid is not well established (*14,23*). The use of ascorbic acid oxidase to effect the specific oxidation of vitamin C also has been proposed (*15*). The ability of an enzyme to effect selective oxidation of ascorbic acid in the presence of certain other reducing materials has been questioned. Recently, the use of enzymatic oxidation of ascorbic acid to dehydroascorbic acid, followed by selective microbiological reduction of dehydroascorbic acid has been suggested (*16*), as a basis for estimating ascorbic acid.

Reaction of derivatives of ascorbic acid with 2,4-dinitrophenylhydrazine permits the determination of ascorbic acid by methods not based upon oxidation-reduction properties. A technic described by Roe and Oesterling

for total and dehydroascorbic acid (7), appears to avoid interference from certain types of reducing materials. Although osazones result from the reaction of sugars with 2,4-dinitrophenylhydrazine, their properties and rates of formation are sufficiently different from those of ascorbic acid derivatives to prevent interference. This method may not avoid the interference from "reductones" and "reductic acid"; however, it has been modified (*23*) to indicate the presence of "reductones" or "reductic acid" in the sample. In addition, a means of correcting for these substances has been reported (*23*).

Sampling

The importance of the proper handling of samples for ascorbic acid determinations cannot be overemphasized. The sampling and extraction of the material under examination must be carried out with minimum delay so that no significant change in ascorbic acid takes place prior to analysis. After raw materials are cut, bruised, or chopped, they must be handled very rapidly and blended with stabilizing acid as quickly as possible to prevent undue oxidation of the vitamin. This is particularly true of raw products high in ascorbic acid oxidase activity. As much of the comminution as possible should be conducted in the presence of a stabilizing acid such as 6% metaphosphoric acid which retards the oxidation of ascorbic acid by inactivating the catalytic effect of ascorbic acid oxidase and copper. Metaphosphoric acid also precipitates interfering substances such as protein and thereby facilitates subsequent clarification of the extract.

Metaphosphoric acid is used as the extracting acid for most products. However, if iron is present, as it may be in canned foods which have been stored for a long period of time or in certain pharmaceuticals, then 8% acetic acid may be used. This extracting medium has the particular advantage of minimizing ferrous iron interference.

Dehydrated foods require a slight variation from the handling of other samples. A definite weight of the sample is first rehydrated with 100 ml of 3% metaphosphoric acid for approximately 15 minutes, after which it is blended and treated by the usual procedure for analysis. Foods that were sulfited during dehydration should be treated with a suitable reagent such as acetone (*21*), to remove sulfur dioxide prior to measurement of ascorbic acid.

Urine samples are assayed in much the same manner as other substances. In the case of blood, however, the sample must be transferred from the syringe into a flask which contains lithium oxalate to prevent the

blood from clotting. The plasma obtained from the oxalated blood is promptly deproteinized with metaphosphoric acid and assayed.

2,6-DICHLOROPHENOLINDOPHENOL VISUAL TITRATION METHOD

A. Principle

The visual titration method is based upon the reduction of the dye (2,6-dichlorophenolindophenol) by an acid solution of ascorbic acid. In the absence of interfering substances, the capacity of an extract of the sample to reduce a standard solution of the dye, as determined by titration, is directly proportional to the ascorbic acid content.

B. Equipment

The equipment listed is adequate for one sample. The number of samples that can be handled simultaneously by one operator is conditioned by the nature of the sample. In the case of juices or other products that do not require blending, samples can be analyzed quite rapidly.

No. of pieces	*Item*
1	*Balance*, with weights, capacity 1000 g, accuracy of 0.1 g (to weigh a 200- to 300-g sample)
1	*Balance*, with weights, capacity 100 g, accuracy of 10 mg (to weigh a 5- to 20-g sample)
1	*Weighing Pan*, 50-g capacity
1	*Waring Blendor*, or its equivalent
1	*Beaker*, 500 ml
1	*Funnel*, 3-inch diameter, short stem
2	*Volumetric Flasks*, 100 ml
2	*Funnels*, 2- to 3-inch diameter
2	*Filter Papers*, Whatman No. 12 fluted, 12.5 cm, or equivalent
1	*Funnel Rack*, 6 openings
2	*Receiving Vessels*, 100 ml (flasks or beakers)
1	*Volumetric Transfer Pipet*, 10 ml
2	*Erlenmeyer Flasks*, 50 ml
1	*Microburet*, 10 ml, graduated to 0.05 ml
1	*Buret Stand*
1	*Reducing Valve*, for nitrogen cylinder
—	*Volumetric Transfer Pipets*, assorted sizes including 1, 5, 10, 20 and 25 ml

C. Reagents

All chemicals should meet A.C.S. specifications or be of Reagent Grade. The distilled water used in making acid solutions for extraction of samples should be "copper-free." For this reason it may be desirable to redistil certain lots of distilled water from an all-glass still.

1. 6% Metaphosphoric Acid Solution. Without heating, dissolve 60 g of reagent grade HPO_3 sticks in 900 ml water, redistilled from glass. Dilute to one liter and store at 3° C when not in use. On standing in solution, HPO_3 is slowly hydrolyzed to H_3PO_4; hence, a fresh solution should be prepared weekly.

2. 3% Metaphosphoric Acid Solution. Dilute 500 ml of the above 6% HPO_3 solution to 1 liter with redistilled water.

3. Ascorbic Acid Standard. Dissolve 100 mg of ascorbic acid (preferably U.S.P. Reference Standard obtainable from E. Fullerton Cook, United States Pharmacopoeia, 43rd. St. and Woodland Ave., Philadelphia, Pa.) in 3% HPO_3 solution and dilute to 500 ml with the same solvent. As this solution is unstable, use immediately to standardize the dye.

4. 0.025% 2,6-Dichlorophenolindophenol Solution. Dissolve approximately 50 mg of the sodium salt of 2,6-dichlorophenolindophenol (sodium 2,6-dichlorobenzenonindophenol, Eastman Kodak Co., Rochester, N.Y. or equivalent) in approximately 150 ml of hot water containing 42 mg $NaHCO_3$, cool and dilute with water to 200 ml. Place in a brown bottle and store at 3° C, renewing once a week. Standardize daily as follows:

Dilute a 5-ml aliquot of the standard ascorbic acid solution (containing 1 mg ascorbic acid) with 5 ml of 3% HPO_3. Titrate with the dye solution to a pink color which persists for 15 seconds. Since this volume of dye represents 1 mg of ascorbic acid, the ascorbic acid equivalent (T) of 1 ml of dye solution is equal to 1 divided by the volume in ml of the dye solution used in this titration.

5. Source of Nitrogen. A cylinder of N_2 with facilities for saturating the gas with moisture. Water-pumped N_2 is preferable to oil-pumped N_2.

D. Procedure

1. Extraction

(a) Blend equal weights (200 to 300 g) of the sample (W_1) and 6% HPO_3 (W_2) to yield a homogeneous slurry.

(1) The procedure described is applicable to food products, but it may be modified for use in the analysis of blood, etc.

(2) A large sample is required to obtain representative sampling of a heterogeneous mass of the material being analyzed.

(3) A solution of 3% HPO_3 is not adequate for inactivating the enzymes in certain fresh vegetables. Therefore, 6% HPO_3 is preferred (*17*). A technic for sampling products to prevent the oxidation of ascorbic acid has been described (*12*).

(4) An 8% acetic acid solution has been recommended as an extracting medium for processed materials that may contain large amounts of Fe^{++}. This condition may occur when canned foods have been stored for long periods of time prior to opening. When HPO_3 or oxalic acid is used as an extracting medium, Fe^{++} will reduce 2,6-dichlorophenolindophenol, resulting in erroneously high values for ascorbic acid. If acetic acid is used, Fe^{++} will not react with the dye at a rate sufficiently rapid to affect the titration. The theoretical explanation advanced for this is that HPO_3 and oxalic acid react with Fe^{+++} and upset the equilibrium of $Fe^{++} \rightleftharpoons Fe^{+++}$. This permits the Fe^{++} to reduce the dye. When acetic acid is the extracting medium, Fe^{+++} is not removed from the equilibrium condition and thus reduction of the dye by Fe^{++} does not occur (*18,19*). It should be emphasized, however, that when oxidative enzymes or catalysts such as Cu ions are present, acetic acid will not stabilize the medium. Therefore, the use of acetic acid as an extracting acid should be limited to those products that have been processed and are suspected to be contaminated with an excess of Fe^{++}.

(5) If the material is a liquid with a very low solids content, it is not necessary to perform this step. Begin immediately with 1-(b).

(6) When HPO_3 is rejected for economic reasons or because of difficulty in securing the reagent, 1% oxalic acid may be used as an alternative stabilizing medium (*20*). In this case, a 2% oxalic acid solution should be used as the initial extracting acid, thus resulting in a 1% solution when equal parts of sample and extractant are blended. If large amounts of protein material are present, undesirable turbid extracts may result since this medium is not as efficient as HPO_3 in precipitating proteins.

(7) An inert atmosphere has been suggested for use during the blending of fresh biological material containing large amounts of oxidative catalysts. Such a step would minimize contact with atmospheric oxygen. A simple means of conducting this step is to introduce N_2 through a glass tube extending into the blendor bowl under the surface of the liquid extractant. If N_2 is bubbled into the bowl for 15–30 seconds before starting the blendor, the possibility of oxidation by catalysts or enzymes which are liberated with the breaking of the sample tissues is somewhat lessened. This is particularly true if the extracting medium does not completely inactivate these catalysts.

(b) Weigh 10 to 30 g of this slurry (W_3) (sufficient to yield 1 to 5 mg of ascorbic acid) into a weighing pan, transfer to a 100-ml volumetric flask and dilute to 100 ml (V_1) with 3% HPO_3.

(1) At this point, it is advisable to make the required weighing and dilution of the sample as rapidly as possible to minimize oxidation due to failure of the stabilizing medium to completely inactivate any catalysts present.

(2) On dilution of the blended sample, the presence of foam may cause difficulty in ascertaining the proper liquid level in the flask. The addition of a drop of caprylic alcohol has been found to be quite satisfactory in breaking this foam. This reagent does not appear to have any effect on subsequent steps in the procedure.

(3) A short-stem, 3-inch funnel with a relatively large bore in the stem aids in transferring the slurry from the weighing pan to the 100-ml volumetric flask.

(4) In the case of products that were sulfited during dehydration, the effect of SO_2 can be eliminated readily by adding 20 ml of acetone before dilution to the mark.

(c) Filter the diluted sample, discarding the first few ml of filtrate.

(1) The sample may be centrifuged at this point instead of being filtered, dependent upon the operator. For samples that filter slowly, centrifugation is preferable.

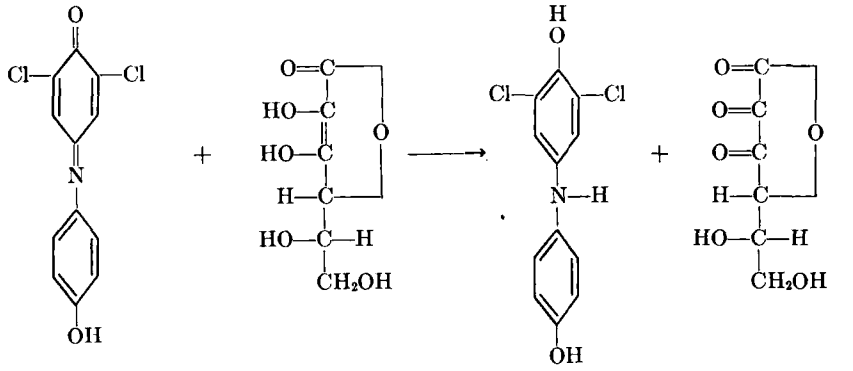
2. Titration of Reduced Ascorbic Acid

(a) Pipet a 10-ml aliquot (V_2) of the filtrate from D-1-(c) into a small Erlenmeyer flask.

(1) In the case of products of low ascorbic acid potency (5 mg per 100 g or less), it is convenient to titrate a 25-ml aliquot. If it is desired to maintain the volume at 10 ml to facilitate detection of the end point, 0.01% 2,6-dichlorophenolindophenol solution may be used.

(b) Titrate immediately with the standardized solution of 2,6-dichlorophenolindophenol (V) to a faint pink end point which persists for 15 seconds.

(1) Titration of reduced ascorbic acid with 2,6-dichlorophenolindophenol dye oxidizes the reduced ascorbic acid to dehydroascorbic acid.



The dye as made up in the dilute $NaHCO_3$ solution is blue, but in an acid medium such as the stabilizing solution used in this determination, it is pink. Therefore, the color change in this method, at the end point, is from colorless to pink. In the titration of the extracts with the dye, it is desirable to add the dye quite rapidly until the

pink color does not immediately disappear. Then as rapidly as possible, add the dye dropwise with constant mixing of the solution until the faint pink color of the solution resulting from the unoxidized dye, persists for 15 seconds. A rapid titration and short-time end point are desirable because of possible interfering action of other constituents of the solution. In general, such materials react more slowly with the dye than does ascorbic acid; therefore, their effect should be kept at a minimum by rapid titration (*10*).

3. Calculation

(a) Calculate the ascorbic acid according to the following formula:

$$\frac{W_1 + W_2}{W_1 \times W_3} \times \frac{V_1}{V_2} \times 100(V \times T) = \text{mg per 100 g sample}$$

W_1 = weight of original material in g
W_2 = weight of extracting acid used in g
W_3 = weight of slurry taken for analysis in g
V_1 = volume to which the slurry (W_3) is diluted in ml
V_2 = volume of filtrate taken for titration in ml
V = volume of dye, in ml, required for ascorbic acid titration
T = ascorbic acid equivalent of the dye in mg per ml of dye (C-4)

(1) If some of the dilutions were omitted, this will be compensated for in the formula. However, if the sample has been handled exactly as described in the procedure, the formula will simplify to

$$\frac{2000\ VT}{W_3} = \text{mg per 100 g}$$

(2) Sample calculation of ascorbic acid content:

W_1 = 300 g
W_2 = 300 g
W_3 = 30 g
V_1 = 100 ml
V_2 = 10 ml
V = 3 ml
T = 0.125 mg per ml

$$\frac{300\text{ g} + 300\text{ g}}{300\text{ g} \times 30\text{ g}} \times \frac{100\text{ ml}}{10\text{ ml}} \times 100 \times 3\text{ ml} \times 0.125 = 25\text{ mg per 100 g sample}$$

(3) The visual titration method, as described, measures reduced ascorbic acid, not total (reduced plus dehydroascorbic acid). Reduction with H_2S has been described as being satisfactory for reducing dehydroascorbic acid to ascorbic acid (*2*). If an acid extract of the sample is treated with H_2S, followed by removal of the H_2S by bubbling N_2 through the solution, the titration with the standard dye solution may be a measure of ascorbic acid plus dehydroascorbic acid. The value of the H_2S procedure as a measure of dehydroascorbic acid is open to question (*3*). Photometric methods employing 2,6-dichlorophenolindophenol may measure total as-

corbic acid (*12*). The Roe and Oesterling 2,4-dinitrophenylhydrazine method (*7*) may also be used for the determination of dehydroascorbic acid.

(4) Duplicate determinations on a single sample should yield values checking within 5%. In many cases, especially with high-potency samples, better checks may be expected.

PHOTOMETRIC METHOD

A. Principle

The photometric method is based upon measurement of the extent to which a 2,6-dichlorophenolindophenol solution is decolorized by ascorbic acid in sample extracts and in standard ascorbic acid solutions. Since reduction of the dye with ascorbic acid is practically instantaneous, but reduction of dye with interfering reducing substances is slow (presumably), determination of the decrease in color intensity with time permits correction for reduction of the dye by substances other than ascorbic acid.

B. Equipment

The equipment listed is adequate for one sample. The number of samples that can be handled simultaneously by one operator is conditioned by the nature of the sample.

No. of pieces	*Item*
—	With the exception of a 10-ml microburet and the buret stand, all of the equipment listed under "Equipment for 2,6-Dichlorophenolindophenol Visual Titration Method" is required. In addition, the following are needed:
1	*Direct-Reading Photoelectric Colorimeter.* This permits selection of light with a wavelength of approximately 520 mμ. The galvanometer should come to rest in less than 5 seconds. The Evelyn Photoelectric Colorimeter (Rubicon Co., Ridge Ave. at 35th St., Philadelphia, Pa.) or an equivalent instrument is satisfactory.
2	*Colorimeter Tubes*
1	*Rack for Colorimeter Tubes,* 12 places
1	*Pipet,* to deliver 5 ml in less than 1 second. The tip may be cut from a volumetric transfer pipet.

C. Reagents

All chemicals should meet A.C.S. specifications or be of Reagent Grade. The distilled water used in making acid solutions for extraction of samples should be "copper-free."

1. 6% **Metaphosphoric Acid Solution.** Without heating, dissolve 60 g of reagent grade HPO_3 sticks in 900 ml water, redistilled from glass. Dilute to 1 liter and store at 3° C when not in use. Prepare solution weekly since HPO_3 in solution is slowly hydrolyzed to H_3PO_4.

2. 3% **Metaphosphoric Acid Solution.** Dilute 500 ml of the above 6% HPO_3 solution to 1 liter with redistilled water.

3. **Citrate Buffer.** Dissolve 29.4 g of citric acid in 140 ml of 2.0 *N* NaOH (carbonate-free) and make to 250 ml with redistilled water.

4. **Ascorbic Acid Standard.** Dissolve 100 mg ascorbic acid (preferably U.S.P. Reference Standard obtainable from E. Fullerton Cook, United States Pharmacopoeia, 43rd St. and Woodland Ave., Philadelphia, Pa.) in 3% HPO_3 solution and dilute to 100 ml with the same solvent. Pipet 5 ml of this solution into a 100-ml volumetric flask and dilute to volume. Pipet 2 ml of the second dilution into a 250-ml volumetric flask, add 5 ml citrate buffer, 12.5 ml 6% HPO_3 and water to volume. This buffer brings the pH of the solution to 3.5 to 3.7. The buffered solution contains 4 γ ascorbic acid per ml. It is unstable and should be used at once to establish a constant for the instrument [D-3-(a)].

5. **2,6-Dichlorophenolindophenol Solution.** Dissolve 200 mg of the sodium salt of 2,6-dichlorophenolindophenol (sodium salt of 2,6-dichlorobenzenonindophenol, Eastman Kodak Co., Rochester, N.Y., or equivalent) in approximately 150 ml of hot redistilled water containing 42 mg $NaHCO_3$, cool and dilute with redistilled water to 200 ml. Keep in the refrigerator when not in use. Pipet 4 ml of the stock solution into a 250-ml volumetric flask and dilute to volume with redistilled water to produce the dye solution used for the colorimetric readings. This diluted dye solution is stable at least 5 days at 3° C. The stock solution is stable up to 5 weeks at 3° C.

D. Procedure

1. Extraction

As an alternative and simplified procedure, the method of Loeffler and Ponting (*22*) may be used. See D-4-(a)-(4).

(a) Blend equal quantities of the sample (W_1) and 6% HPO_3 (W_2) to yield a homogeneous slurry using a Waring Blendor, or equivalent.

(1) See Visual Titration Method Procedure [D-1-(a)]. HPO_3 inactivates the ascorbic acid oxidase and forms a nonionizable copper complex (*11*).

(b) Weigh 2 to 4 g (W_3) of this slurry, sufficient to yield 200 to 400 γ of

ascorbic acid into a weighing pan. Transfer to a 100-ml volumetric flask. Add 20 ml citrate buffer and 50 ml 6% HPO_3 and dilute to 100 ml (V) with water. Mix thoroughly.

(1) The addition of the citrate buffer to the solution at this point brings the pH to 3.5 to 3.7. It is important that the pH should be within this range and never more than 4.0.

Below a pH of 4.0, ascorbic acid oxidase is inhibited (*15*), the dye is stable and the rate of reaction between ascorbic acid and the dye is maximum (*12*). See Visual Titration Method [D-1-(b)] for additional information.

(c) Filter the diluted sample, discarding the first 10 ml.

(1) The first 10 ml should be discarded since there may be some initial adsorption on the filter paper. Also, the first 10 ml of filtrate may be more turbid than the subsequent portions.

(2) In case it is desirable to determine total ascorbic acid (reduced plus dehydro), H_2S is bubbled through an aliquot of the acid extract [D-1-(b)] for 15 minutes and the sample is held at room temperature for 2 hours. The H_2S is completely expelled by bubbling wet N_2 through the extract for 1 hour, or until the N_2 coming from the tube gives a negative test for H_2S. From this point on, the procedure is the same as that for reduced ascorbic acid. The photometric method using 5- and 10-second readings assists in minimizing the effect of small residual amounts of H_2S (*12*).

(d) Prepare a reagent blank by diluting 12.5 ml of 6% HPO_3 and 5 ml of citrate buffer to 25 ml with water.

2. Photometric Determination of Ascorbic Acid

The following directions apply specifically to use of an Evelyn Photoelectric Colorimeter.

(a) With 10 ml of water in a colorimeter tube, set the galvanometer at 100 using a 520-mμ filter. Place a colorimeter tube containing 5 ml of dilute dye solution (C-5) in the instrument and run 5 ml of the buffered sample extract [D-1-(c)] rapidly into the dye. Place the pipet well down into the colorimeter tube (below the surface of the dye solution) and begin to time the reaction when three-fourths of the volume of the solution has been delivered. No stirrer is necessary. Record the galvanometer readings at 5 seconds (Gs_1) and 10 seconds (Gs_2). Add a crystal of ascorbic acid to completely reduce the dye and record the galvanometer reading (Gsr).

(1) The reaction between ascorbic acid and 2,6-dichlorophenolindophenol is presumed to be instantaneous. In order to obtain the galvanometer reading of the unreduced dye at zero time, the difference between the galvanometer readings at 10 seconds and 5 seconds is subtracted from the reading at 5 seconds. This is more

nearly correct than the procedure which recommends 15-second and 30-second intervals, since with a longer period of time, other reducing agents present react with the dye to a greater extent. It will be observed that the difference between the 5-second and 10-second readings of a pure ascorbic acid solution is very slight.

In samples containing other reducing substances, the difference may be large. These other reducing substances react at a much slower rate than does ascorbic acid. The longer the interval between (Gs_1) and (Gs_2) readings, the more opportunity the other reducing substances have for affecting the reduction of the dye (*12*).

(2) Determination of (Gsr) permits correction for the color or turbidity in the extracts of most products. However, in the case of certain products containing large amounts of acid-soluble pigments or those that yield highly turbid filtrates, the photometric method as described may not be satisfactory. For such materials, the xylene extraction technic described by Pepkowitz (*13*) may be more satisfactory.

(b) To 5 ml of diluted dye solution (C-5) in a colorimeter tube, add 5 ml of reagent blank [D-1-(d)] and a crystal of ascorbic acid. Place the tube in the instrument and adjust so that the galvanometer reading is the same as (Gsr) [D-2-(a)]. Place another colorimeter tube in the instrument, add 5 ml of the diluted dye solution (C-5) and 5 ml of reagent blank [D-1-(d)], and record the galvanometer reading after 5 seconds (Gb).

(1) This determines the color due to the dye and other reagents. Setting the instrument to (Gsr) eliminates the effect of color or turbidity in the sample extract.

3. Determination of Instrument Constant (K)

K should be determined frequently but need not be determined daily. If acetic acid is used as the diluent, a value for K must be determined substituting 8% acetic acid for 3% HPO_3 and omitting the buffer.

(a) Repeat steps described under 2-(a) and 2-(b), using 5 ml of buffered ascorbic acid solution (C-4) in place of sample extract.

(1) This procedure as outlined, establishes a K value for only one ascorbic acid concentration (4 γ per ml). However, K values should be determined for a series of ascorbic acid standards (1.0 to 10.0 γ per ml). In calculating ascorbic acid values of samples, only those galvanometer readings obtained in the range for which K values were found to be constant within plus or minus 5% should be used.

(b) Calculate K from the following equation:

$$Ca = K (\log [Ga_1 - (Ga_2 - Ga_1)] - \log Gb)$$

Ca = γ ascorbic acid per ml of buffered ascorbic acid solution (C-4)
K = instrument constant
Ga_1 = 5-second galvanometer reading [D-3-(a)]
Ga_2 = 10-second galvanometer reading [D-3-(a)]
Gb = galvanometer reading for blank [D-3-(a)]

(1) Sample calculation of K using data obtained with the Evelyn Photoelectric Colorimeter:

Ca = 4 γ per ml	4 γ per ml = K (log [65.3 − (66.8 − 65.3)] − log 40.5)
Ga_1 = 65.3	4 γ per ml = K (log 63.8 − log 40.5)
Ga_2 = 66.8	4 γ per ml = K (1.8048 − 1.6075)
Gsr = 97.0	4 γ per ml = 0.1973 K
Gb = 40.5	K = 20.3 γ per ml

4. Calculation

(a) Calculate the ascorbic acid content of the sample from the following equation:

$$C = K\,(\log [Gs_1 - (Gs_2 - Gs_1)] - \log Gb)\,\frac{W_1 + W_2}{W_1} \times \frac{V}{W_3} \times \frac{100}{1000}$$

C = ascorbic acid content of product expressed as mg per 100 g
K = instrument constant expressed as γ per ml [D-3-(b)]
Gs_1 = 5-second galvanometer reading for sample [D-2-(a)]
Gs_2 = 10-second galvanometer reading for sample [D-2-(a)]
Gb = galvanometer reading for blank [D-2-(b)]
W_1 = weight (g) of original product used [D-1-(a)]
W_2 = weight (g) of extracting acid [D-1-(a)]
W_3 = weight (g) of slurry used [D-1-(b)]
V = volume (ml) to which the slurry (W_3) is diluted [D-1-(b)]

$\frac{100}{1000}$ = factor for converting the calculation from γ per g to mg per 100 g.

(1) If the procedure is followed exactly, the expression $\frac{W_1 + W_2}{W_1} \times \frac{V}{W_3}$ becomes $\frac{20}{W_3}$. If many assays are to be calculated, this may be combined with K, giving $\frac{20\,K}{W_3}$ or, for the illustration above $\frac{406}{W_3}$. The formula then becomes $C = \frac{406}{W_3}$ (log [Gs_1 − (Gs_2 − Gs_1)] − log Gb)

(2) Sample calculation using data obtained for raw peas; readings from an Evelyn Photoelectric Colorimeter:

C = mg ascorbic acid per 100 g raw peas
K = 20.3 γ per ml. Calculated under [3-(b)]
Gs_1 = 58.0
Gs_2 = 58.8
Gsr = 95.0
Gb = 41.0
W_1 = 300 g
W_2 = 300 g
W_3 = 3 g
V = 100 ml

$$C = \frac{20.3\,\gamma}{\text{ml}} (\log 58.0 - (58.8 - 58.0) - \log 41.0) \times \frac{300\text{ g} + 300\text{ g}}{300\text{ g}} \times \frac{100\text{ ml}}{3\text{ g}} \times \frac{100}{1000}$$

$$C = \frac{20.3\,\gamma}{\text{ml}} (\log 57.2 - \log 41.0) \times \frac{600\text{ g}}{300\text{ g}} \times \frac{100\text{ ml}}{3\text{ g}} \times \frac{1}{10}$$

$$C = \frac{20.3\,\gamma}{\text{ml}} (1.7582 - 1.6128) \times \frac{20}{3}$$

$$C = \frac{20.3\,\gamma}{\text{ml}} \times 0.1454 \times \frac{20}{3}$$

C = 19.8 mg per 100 g of raw peas

(3) Total ascorbic acid may also be determined. See D-1-(c)-(2).

(4) *Modification of Loeffler and Ponting (22).*

This technic, originally intended for fresh, frozen, or dehydrated fruits and vegetables, has been applied to many foods. The method involves extraction of a sample with a fairly large amount of 1% HPO_3, in a high-speed cutter such as a Waring Blendor, and measuring with a photoelectric colorimeter the extent to which the extracted ascorbic acid decolorizes a standard dye solution.

A solution of 1% HPO_3 may not be adequate to protect ascorbic acid in certain foods from oxidation during extraction. In such cases, stronger solutions of HPO_3 should be used for the original extraction. Under these conditions a buffer should be employed in a later dilution to bring the pH into the range 3.5 to 3.7 prior to photometric reading of the sample.

The dye solution is made by dissolving 13 to 17 mg of 2,6-dichlorophenolindophenol in water and diluting to one liter. This solution is standardized by setting the instrument to 100% transmission (filter 520) with 10 ml of distilled water and determining the 15-second reading given by a tube containing 1 ml of 1% HPO_3 and 9 ml of the dye solution. This reading is G_1 and should be about 30. The reaction between ascorbic acid and stronger dye solutions, i.e., those giving readings below 30, is not a linear relationship. A calibration curve rather than a constant factor must be used with such solutions.

In practice, a representative sample of material is extracted in a blendor with sufficient HPO_3 (1%) to give an ascorbic acid concentration of 5 to 50 γ per ml. A ratio of 7 parts of HPO_3 solution to one of vegetable or fruit tissue is usually satisfactory. If the filtrate is too concentrated, the solution can be diluted with 1% HPO_3; if too dilute, a larger sample can be taken or less extracting solution used. Buffering is unnecessary since 1% HPO_3 yields a pH sufficiently low to retard losses during blending and yet sufficiently high to prevent fading during the reaction with the dye.

1-ml aliquots of the filtrate are measured into each of two matched colorimeter tubes. To one is added 9 ml of water. This tube is shaken and with it in place, the instrument is adjusted to 100% transmission, thus correcting for any color or turbidity which may be present in the same filtrate. To the other tube, 9 ml of the standardized dye solution is added from a rapid-delivery pipet (emptying in less than 5 seconds). The tube is shaken gently and a reading taken 15 seconds after the start of the dye addition. The reading is G_2. In cases where interfering substances occur, readings are taken at 15- and 30-second intervals, and the difference between

the two is subtracted from the 15-second reading to correct for these slower reducing substances. 10- and 20-second readings have been used with equal success.

Because of the thorough disintegration of the sample in the blendor, the ascorbic acid is distributed uniformly through the entire liquid phase present. By determining the ascorbic acid concentration in the filtrate and knowing the total volume of liquid, the sample concentration is easily calculated. The total volume of liquid is simply the sum of the volume of HPO_3 solution plus the liquid originally present in the sample, i.e., the per cent moisture times the sample weight.

The ascorbic acid concentration in mg per 100 g of tissue is, therefore,

$$K\,(L_1 - L_2) \times \frac{\text{ml acid extractant} + (\%\ \text{liquid in sple})\ \text{wt. of sple}}{\text{wt. of sample}} \times 100$$

where

L_1 = log 100 − log G_1, or color density corresponding to galvanometer reading of dye

L_2 = log 100 − log G_2, or color density corresponding to galvanometer reading of sample

K = instrument constant relating color densities to milligrams of ascorbic acid. This is obtained by determining the galvanometer readings for solutions of ascorbic acid varying in concentration from 5 to 50 γ per ml and substituting in the above formula. For this purpose, the formula reduces to

$$\text{mg ascorbic acid per ml} = K\,(L_1 - L_2)$$

The constant should be determined for each instrument and checked often, but not daily. Only that range of galvanometer readings which gives a constant K value may be used in assay work unless a calibration curve is established.

2,4-DINITROPHENYLHYDRAZINE METHOD

A. Principle

This procedure is an adaptation (*24*) of the method described by Roe and Oesterling (*7*) and is based on the oxidation of ascorbic acid to dehydroascorbic acid followed by coupling with 2,4-dinitrophenylhydrazine under controlled conditions to give red-colored osazones. A comparison of color produced in samples and ascorbic acid standard solutions is used as a means of determining ascorbic acid contents of biological materials.

B. Equipment

The equipment listed is adequate for one sample, although the number of samples that can be handled simultaneously by one operator is conditioned by the nature of the sample and equipment used.

No. of pieces	*Item*
1	*Analytical Balance*, with weights, accuracy of 0.2 mg
1	*Balance*, with weights, capacity of 1000 g, accuracy of 0.1 g
1	*Balance*, with weights, capacity of 100 g, accuracy of 10 mg
1	*Waring Blendor*, or its equivalent
1	*Centrifuge*, large enough to accommodate 50-ml centrifuge tubes
1	*Water-Bath*, constant temperature, ± 0.5° at 37° C
1	*Beaker*, 500 ml
1	*Weighing Pan*, 50-g capacity
3	*Funnels*, 2- to 3-inch
2	*Volumetric Flasks*, 100 ml
2	*Centrifuge Tubes*, 50 ml
2	*Filter Papers*, Whatman No. 12 fluted, 12.5 cm or equivalent
1	*Funnel Rack*, 8 openings
2	*Receiving Vessels*, 100 ml (flasks or beakers)
—	*Assorted Volumetric Transfer Pipets*, 1, 2, 5, 10 and 25 ml
2	*Mohr Pipets*, 5 ml (Normax)
1	*Spectrophotometer or Photoelectric Colorimeter*, with cuvets and filters or other means of selecting a light band in the region of 510 to 540 mμ
1	*Reducing Valve*, for nitrogen cylinder
24	*Test Tubes*, ¾ inch × 6 inches
4	*Erlenmeyer Flasks*, 250 ml

C. Reagents

All chemicals should meet A.C.S. specifications or be of Reagent Grade.

1. 9 *N* Sulfuric Acid. Add cautiously 250 ml of concd. H_2SO_4 (sp. gr. 1.84) to 700 ml of water, cool and dilute to 1 liter with water.

2. 2% 2,4-Dinitrophenylhydrazine. Dissolve 2 g of 2,4-dinitrophenylhydrazine (Eastman Kodak or equivalent) in 100 ml of 9 *N* H_2SO_4 and filter. Keep under refrigeration when not in use and prepare a fresh solution after two weeks.

3. 10% Metaphosphoric Acid. Without heating, dissolve 200 g of reagent grade HPO_3 sticks in 1800 ml distilled water of low copper content (redistilled from glass). Store at 3° C when not in use. On standing in solution, HPO_3 is slowly hydrolyzed to H_3PO_4; hence, a fresh solution should be prepared weekly.

4. 5% Metaphosphoric Acid. Dilute 500 ml of 10% HPO_3 to 1000 ml with water.

5. 1% Thiourea in 5% Metaphosphoric Acid Dissolve 5 g of thiourea in 500 ml of 5% HPO_3.

6. 85% Sulfuric Acid. Add cautiously 900 ml H_2SO_4 (sp. gr. 1.84) to 100 ml water.

7. Bromine. Bromine is very poisonous and must be handled with great care. This reagent should always be used under a chemical hood.

8. Nitrogen. A cylinder of N_2 with facilities for saturating the gas with moisture. Water-pumped N_2 is preferable to oil-pumped N_2.

9. Ascorbic Acid Standard—(1 mg per ml). Dissolve 100 mg ascorbic acid (preferably U. S. Pharmacopoeia Reference Standard) in 100 ml of 5% HPO_3.

D. Procedure—Total Ascorbic Acid

The procedure describes the determination of total ascorbic acid (reduced plus dehydro). With slight modifications, dehydroascorbic acid may be determined. The method is applicable to biological materials and to many fruits and vegetables.

1. Extraction

(a) Blend 200 g of sample (W_1) and 200 g of 10% HPO_3 (W_2) for 2 to 5 minutes to a homogeneous slurry.

(1) A sample of 200 g is used to compensate for variations within the sample material.

(b) Weigh 10 to 40 g of this slurry (W_3), estimated to contain 1 to 2 mg of ascorbic acid, into a 100-ml volumetric flask. Dilute to 100 ml (V_1) with 5% HPO_3 solution and mix thoroughly.

(1) If dehydroascorbic acid is to be determined, weigh 40 g of this slurry into a 100-ml volumetric flask and dilute without delay to volume (V_1) with 1% thiourea in 5% HPO_3. Most foods are low in dehydroascorbic acid and 40 g is about the maximum amount which can be handled. By adding thiourea at this point, oxidation of ascorbic acid to dehydroascorbic acid is inhibited. However, small amounts of dehydroascorbic acid may be formed during the blending of a few exceptional samples with equal weights of 10% HPO_3. In such cases, the original sample [D-1-(a)] should be blended in 10% HPO_3 containing 1% thiourea. Filter or centrifuge as in D-1-(c).

(2) Dilute to volume by proceeding directly to D-3-(a). From this step the procedure is the same as that for total ascorbic acid except that the calculation gives dehydroascorbic acid rather than total ascorbic acid.

(c) Remove the suspended solids by centrifuging and decanting the supernatant liquid, or by filtering through a No. 12 fluted Whatman paper.

(1) The choice between filtering and centrifuging is influenced by the type of product being analyzed. For example, slurries of corn and lima beans may filter slowly.

2. Oxidation to Dehydroascorbic Acid

(a) To the solution from D-1-(c) add 2 or 3 drops of bromine from a medicine dropper (*24*). Handle bromine with caution and use it *only* under a good chemical hood. Shake gently until the solution is slightly yellow, decant from excess bromine and pass air or nitrogen (saturated with water) through the solution until the dissolved bromine is expelled.

(1) With the exception of some pigmented samples, the solution becomes colorless when the bromine is expelled. A trace of residual bromine will be taken care of by thiourea added in a later step.

(2) This is an unpublished modification (*24*) of the oxidation procedure. By using bromine rather than norite, ascorbic acid and dehydroascorbic acid can be determined on the same original slurry with a single standard curve. Acetic acid or trichloroacetic acid must be present if ascorbic acid is to be oxidized to dehydroascorbic acid with norite.

(3) If the technic described by Roe and Oesterling (*7*) is used to determine total ascorbic acid, the reagents required and the procedure outlined is modified as follows:

1. Acid—Washed Norite. Place 200 g norite in a large beaker and add 1 liter of 10% HCl. Heat to boiling. Filter with suction. Return the norite to the beaker, add 1 liter of water, stir thoroughly and filter. Repeat this procedure until the washings give a negative or very faint test for ferric ion. Dry overnight in an oven at 110° to 120° C. Do not heat to high temperature. Some grades of carbon may not require washing.

2. 10% Metaphosphoric Acid–20% Acetic Acid Solution. Dissolve 200 g of HPO_3 in approximately 1200 ml of redistilled water, add 400 ml glacial acetic acid and dilute to 2000 ml with redistilled water.

3. 5% Metaphosphoric Acid–10% Acetic Acid Solution. Dilute 500 ml of the reagent immediately above to 1000 ml with redistilled water.

4. 5% Metaphosphoric Acid–10% Acetic Acid Solution Containing 1% Thiourea. Add 10 g of thiourea to 500 ml of 10% metaphosphoric acid–20% acetic acid solution, and dilute to 1000 ml with redistilled water.

The following are deviations from the method as described:

Blend equal weights of product and reagent *2*. Ten per cent HPO_3–20% acetic acid is used for extracting the product to assure complete inactivation of ascorbic acid oxidase present in some fresh products.

Weigh an aliquot containing 1–2 mg of ascorbic acid and dilute to 100 ml using reagent *3* and centrifuge or filter. To 50-75 ml of the filtrate, add ½ to 1 teaspoon norite (0.75 to 1.5 g), shake vigorously and filter. Make dilutions the same as

described under D-3-(a) using reagent *4* in place of the 5% HPO_3 containing 1% thiourea. Since norite helps to clarify the solution as well as aiding in the oxidation of ascorbic acid to dehydroascorbic acid, the use of norite in oxidizing turbid or pigmented extracts may be preferable to oxidation with bromine. The vitamin is not oxidized quantitatively using norite unless either trichloroacetic or acetic acid is present.

Calibration Curve. The calibration curve is made the same as described under D-7, except that the standard ascorbic acid solution is diluted to volume with reagent *3*. The reduced ascorbic acid is oxidized to dehydroascorbic acid with norite as described above and the dilutions are made as in D-7-(c) except with reagent *3*.

Since the concentration of acetic acid influences the rate at which dehydroascorbic acid and 2,4-dinitrophenylhydrazine form the osazone, the same concentrations of acetic acid must be used in the final dilutions of ascorbic acid standards and samples.

3. Dilutions

(a) To a 10-ml aliquot (V_2) of oxidized extract [D-2-(a)] add 10 ml of 1% thiourea in 5% HPO_3 and mix thoroughly to yield a diluted sample of 20 ml (V_3) containing approximately 10 γ of ascorbic acid per ml.

(b) To a 5-ml aliquot (V_2) of oxidized extract [D-2-(a)] add 15 ml of 1% thiourea in 5% HPO_3 and mix thoroughly, yielding a diluted sample of 20 ml (V_3) containing approximately 5 γ of ascorbic acid per ml.

4. Formation of Osazone (Red Pigment)

At this point there are two dilutions, that from [D-3-(a)] and that from [D-3-(b)]. From here on, each of these is treated in exactly the same manner. For simplicity, the description will be written for only one, [D-3-(a)]. In practice, both dilutions should be run simultaneously. Each sample is run at two dilutions to serve as a check on possible interference from materials other than ascorbic acid.

(a) Pipet 4-ml aliquots of the diluted sample [D-3-(a)] into each of 3 test tubes.

(b) Set one tube aside to serve as a blank.

(c) To each of the remaining tubes add 1.0 ml of 2% 2,4-dinitrophenylhydrazine reagent.

(d) Place the tubes prepared in the above step in a water bath at 37° C ± 0.5° for exactly 3 hours.

(e) At the end of 3 hours, remove the tubes from the water bath and place them in an ice bath.

(1) The rate of osazone formation is influenced by the temperature and type of acid (acetic, trichloroacetic or HPO_3) in the solution. Therefore, the samples and standards must be treated in the same manner. "Reductones" and "reductic acid" form osazones with 2,4-dinitrophenylhydrazine at much faster rates than does dehydroascorbic acid. Measurement of the color produced after 15 minutes at

25° C has been proposed (*23*) as a test for "reductones" and "reductic acid." A scheme for correcting for certain interfering substances, based on incubation periods of 15 minutes at 25° C and 3 hours at 37° C is described by Penny and Zilva (*23*). They also discuss the use of formaldehyde in connection with the 2,6-dichlorophenolindophenol titration technic for obtaining information regarding the types of materials that may interfere in ascorbic acid determinations.

5. Treatment with 85% Sulfuric Acid (Formation of Soluble Pigment)

(a) Place the tube from D-4-(b) in the ice bath with those from D-4-(e) and add slowly 5.0 ml of 85% H_2SO_4. The addition should be made one drop at a time and should take at least one minute.

(1) The addition of 85% H_2SO_4 may cause a darkening of solutions containing sugars if the H_2SO_4 is added too rapidly or the temperature is allowed to rise.

(b) While the tubes are in the ice bath, add 1.0 ml of 2% 2,4-dinitrophenylhydrazine reagent to the blank. Without removing from the ice bath, shake all three tubes to mix the contents.

(c) Remove the tubes from the ice bath and allow to stand 30 to 40 minutes at room temperature.

(1) The intensity of the color continues to develop for several minutes. The color starts to fade on long standing. A definite schedule for adding 85% H_2SO_4 and reading the samples should be followed.

6. Measurement of Color

(a) Allow the spectrophotometer or photoelectric colorimeter to become stable. Select a light band in the region of 510 to 540 mμ by inserting a suitable set of filters or setting the wave length dial.

(1) It is possible, with certain instruments (the Evelyn Photoelectric Colorimeter or the Coleman Model 6 Spectrophotometer are convenient instruments) to select uniform test tubes that permit reading of the color transmission in the same tube used for formation of osazone and development of color with 85% H_2SO_4, thus avoiding any transfer of the corrosive solution.

(2) The absorption of light by the pigment formed by reaction of 2,4-dinitrophenylhydrazine and dehydroascorbic acid appears to be at a maximum in the range of 510 to 520 mμ. However, Roe prefers to read the samples at 540 mμ to assist in eliminating interference from osazones that may be formed from sugars in the sample.

(b) Transfer the blank [D-5-(c)] to a suitable cuvet. With this in place, set the instrument to read 100% transmission.

(1) Setting the instrument to read 100% transmission for the blank corrects for color in the solution other than that produced from the osazone formed during 3 hours in a water bath at 37° C.

(c) With the instrument set as above, read and record the per cent transmission (G) for the samples.

7. Calibration of Spectrophotometer

(a) To 50 ml of the standard ascorbic acid solution (C-9), add 2 to 3 drops of liquid bromine. Shake and separate from the excess bromine.

(b) Aerate the oxidized solution [D-7-(a)] to remove excess bromine.

(c) Prepare diluted dehydroascorbic acid solutions containing 0.25, 1.25, 2.5, 5.0, 7.5, 10.0 and 12.5 γ per ml by pipetting 0.25, 1.25, 2.5, 5.0, 7.5, 10.0 and 12.5 ml of oxidized solution [D-7-(b)] into seven 100-ml volumetric flasks. Dilute each to the mark with 1% thiourea in 5% HPO_3.

(d) Treat each of the seven standard ascorbic acid solutions [D-7-(c)] in the same manner as samples in steps [D-4-(a)] through [D-6-(c)], using a separate blank for each tube.

(e) Prepare a calibration chart by plotting per cent transmission (G) as ordinate and concentration of ascorbic acid (γ per ml) (R) as abscissa.

(1) A typical calibration chart prepared from data obtained with a Coleman Model 11 Universal Spectrophotometer is shown below:

Ascorbic acid, γ per ml (R)	Per cent transmission (G)
1.25	88.0
2.5	72.8
5.0	59.5
7.5	48.2
10.0	41.0
12.5	37.0

8. Calculation

(a) Calculate the ascorbic acid content of the sample according to the formula

$$C = R \times \frac{W_1 + W_2}{W_1 \times W_3} \times \frac{V_1 \times V_3}{V_2} \times \frac{100}{1000}$$

where

C = mg of total ascorbic acid (ascorbic acid plus dehydroascorbic acid) per 100 g of sample

R = γ of ascorbic acid per ml of solution [diluted sample D-3-(a) or D-3-(b)] obtained by reading from the calibration chart the value corresponding to (G)

W_1 = g of sample [D-1-(a)]

W_2 = g of 10% HPO_3 [D-1-(a)]

W_3 = g of slurry [D-1-(b)]

V_1 = ml diluted to [D-1-(b)]

V_2 = ml of oxidized solution used [D-3-(a)] or [D-3-(b)]

V_3 = ml to which oxidized solution was diluted [D-3-(a)] or [D-3-(b)]

$\frac{100}{1000}$ = factor for converting the calculation from γ per g to mg per 100 g.

When the method is followed exactly, the general formula reduces to:

$$C = \frac{400\ R}{W_3} \text{ when } V_2 = 10 \text{ ml}$$

$$C = \frac{800\ R}{W_3} \text{ when } V_2 = 5 \text{ ml}$$

(1) *Sample Calculation.*

200 g (W_1) of canned tomatoes were blended with 200 g (W_2) of 10% HPO_3 and a 20-g portion (W_3) of the resulting slurry was diluted to 100 ml (V_1), filtered and oxidized with bromine. 5-ml (V_{2a}) and 10-ml (V_{2b}) aliquots were diluted to 20 ml (V_3) with thiourea solution. 4-ml quantities were used for formation of the pigment and the measurement of color intensities. The 5-ml (V_{2a}) dilution gave a galvanometer reading of 72.8 (Ga) and the 10-ml (V_{2b}) dilution gave (Gb) of 59.5. By interpolation upon the calibration curve [D-7-(e)-(1)], the corresponding values were found to be 2.6 γ per ml and 4.9 γ per ml. Then for the 5-ml (V_{2a}) dilution:

$$\frac{2.6}{10} \times \frac{200 + 200}{200 \times 20} \times \frac{100 \times 20}{5} = 10.4 \text{ mg per } 100 \text{ g}$$

and for the (V_{2b}) dilution:

$$\frac{4.9}{10} \times \frac{200 + 200}{200 \times 20} \times \frac{100 \times 20}{10} = 9.8 \text{ mg per } 100 \text{ g}$$

The average value, thus, is 10.1 mg per 100 g.

(2) Ascorbic acid values obtained from assay of a sample at two dilutions should agree within 10%. In case they deviate by more than 10%, the assay should be repeated. If the value obtained at one of the levels is consistently higher, interference from materials other than ascorbic acid is indicated.

APPLICATION OF METHODS

Those products which have been assayed by one or more of the methods described or by reasonably similar modifications are listed below. The omission of any product from the list or failure to check it under a method heading does not necessarily imply that the method is not applicable. Rather, it merely indicates that the committee and reviewers have not applied these methods to the product.

A + under a heading for a method indicates that the method is applicable as described. References are made to notes in Section D or to the references cited if special precautions are necessary or if simplifications in the procedure are permissible. A − sign indicates the method to be un-

successful. Those cases for which both successes and failures have been reported, are marked +, −.

Type of material	Visual titration method	Photometric method	2,4-Dinitrophenylhydrazine method
Apples	+		
Apricots, canned	+		
Asparagus			
Canned	+	+	+
Fresh	−		+
Beans, green, canned, dehydrated, fresh, frozen	+	+	
Beans, lima, canned, fresh, frozen	+	+	+
Beets, canned, dehydrated	−	− D-2-(a)-(2)	
Cabbage, dehydrated	+ D-1-(b)-(4)	+ D-1-(b)-(1)	
Candy		+	
Carrots, canned, dehydrated, fresh	+	+	
Corn, canned, fresh	+, −	+, −	+
Cranberries		+ D-2-(a)-(2)	
Cucumbers, dehydrated	+		
Grapefruit juice, canned, fresh, frozen	+	+	+
Guava, jam, canned pulp	+	+	+
Milk, evaporated, fresh	+		
Onions, dehydrated	+		
Orange juice, canned, fresh, frozen	+	+	+
Peaches, canned, frozen	+	+	+
Pears, canned	+		
Peas, canned, dehydrated, fresh, frozen	+	+	+
Pineapple, canned	+	+	
Pineapple juice, canned	+	+	
Potatoes, sweet, canned, dehydrated	+	+	
Potatoes, white, dehydrated	+		
Raspberries, fresh, frozen	−		+
Rutabagas	+		
Spinach, canned, dehydrated, fresh, frozen	+	+	+
Tomatoes, canned, dehydrated, fresh	+		+
Tomato juice, canned, fresh	+ D-1-(a)-(4)		+
Vitamin preparations			
Capsules, gelatin		+	
Emulsions		+	
Powders		+	
Solutions		+	
Tablets, coated		+	
Tablets, uncoated		+	

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CHAPTER 18

Other Vitamin Methods

The preceding chapters include only such methods as have been successfully applied to a variety of products by members of the Association, and their scope is thus limited by the present development of vitamin knowledge, by the experience of the cooperating committee members, and by the time and effort which the latter could expend in preparing this volume. As research and experience in vitamin methodology leads to additional methods of wide applicability, it is intended that revisions of this volume will be expanded to include these advances.

Until it has become possible to offer critically evaluated methods of wide applicability, it is hoped that the analyst who wishes to assay for one or more of these factors will be able to find in the literature methods which will provide him with at least a working basis. With this in mind the following list of references is presented. These have been selected for the purpose of covering the subjects broadly in the current literature, which will in turn cite earlier publications. Since many of these methods are relatively new and of limited applicability, comments and reports of experience are especially invited from users. Such reports will aid materially in the writing of future editions.

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CHAPTER 9

Use of Check Samples in Control of Vitamin Methods

Vitamin assays are subject to several types of errors. Some inaccuracies are inherent in the methods. They occur because of limitations of the equipment used, the quality of the reagents, and the judgment of the operator. Errors of this type are fairly constant in any given series of determinations and their combined effect on the result usually can be evaluated statistically.

In this connection, it should be noted that the term *error* does not imply the occurrence of a *mistake*. Thus, it is an error for an operator to read and record 6.32 ml as the volume discharged from a buret when the volume actually required for the reaction is 6.31 ml. It would have been a mistake had the operator read 5.32 ml. Likewise, if only 96% of a vitamin is extracted when the operator usually obtains 98% by the same technic, an error occurs. Spilling a portion of the extract would constitute a mistake. In any discussion of errors, it is assumed that mistakes have not occurred.

Even with the most careful workers, variations outside the range of manipulative error occasionally occur. These *unpredictable* and often times unsuspected errors may occur when the analyst unwittingly deviates from the routine procedure, or when unexpected changes occur in reagents, standards or cultures, or for other causes. Such errors become apparent only when repeated analyses of samples of products which have been constant suddenly yield atypical results.

Illustrative of this type of variation is the experience of a laboratory which was conducting niacin assays on a product under storage at several different temperatures. For six weeks the niacin retention appeared to be 100 ± 5%; in the seventh week and for several weeks thereafter the values were 115 ± 5%. Later, normal values of 100 ± 5% were again found. Inasmuch as several temperatures were involved, including one of −30° C, it is difficult to believe that the niacin content varied so much or that the

technic was so greatly in error. Similar difficulties have been experienced by practically all laboratories and have unduly complicated many assay problems. Frequently, increases or decreases in the levels of values occur with no apparent relation to changes in technic or reagents. The substitution of new solutions may not restore the original values but usually, after several assays, the initial levels suddenly recur.

Recognition of these fluctuations has led many laboratories to the use of check samples. Ordinarily, the check sample is a relatively stable material representative of the types of products being assayed. After portions of this material have been assayed a number of times a "normal" value is established. This may or may not be the correct value. Other laboratories assaying the material an equal number of times might find a different "normal" value. Nevertheless, the "normal" value represents the expected assay value and if it is not obtained within reasonable limits the entire series of assays in which the check sample is included may be suspected of being atypical. Thus, in the illustration cited above, when the niacin recoveries changed from 100 to 115%, a check sample simultaneously assayed probably would have shown a 15% increase, thereby indicating a change in the response to the assay method. It is possible, of course, that an abnormal value may be found for the check sample while the results for the remainder of the samples in the series may be correct. Usually the analyst can tell from the general level of values when this occurs.

When confronted with a series of atypical results the only sound procedure is to search for better assay conditions and to repeat the assays until satisfactory evidence of valid data is obtained. A less rigorous procedure is to use the check sample as a secondary standard and to recalculate the other values. This is a questionable procedure and should be used only as a temporary or emergency measure. Used consistently, check samples permit the detection of atypical results, minimize the chance of reporting erroneous values, and insure an evenness and constancy not attainable with pure standards alone. The use of check samples does not necessarily insure accuracy; it merely detects lack of uniformity and consistency which are prerequisite for accuracy. When several laboratories make use of the same check samples, and further when different kinds of methods are employed for the same vitamin, then agreement of the results constitutes the best check on accuracy which is available. Where disagreement occurs, a search for modifications of procedure which will eliminate the disagreement is an important factor in the development of improved vitamin methods.

With these benefits in mind, the Association of Vitamin Chemists has prepared for distribution four types of check samples; liver, tomato juice, enriched flour and dried yeast. These materials were selected because they are:

(1) Representative of many general types of assay material.
(2) Relatively stable.
(3) Of such physical characteristics that a large number of replicates could be prepared.
(4) Common sources of the vitamins.
(5) Representative of a wide range of vitamin levels.

Each product has been finely ground, thoroughly mixed, and then sealed under vacuum in tins. Precautions were taken to achieve homogeneity of the mixtures from which these samples were packaged. The units of liver and tomato juice have been heated sufficiently to destroy all bacteria, but not spores. All of the samples are being held under refrigeration until shipped so that changes in vitamin content should be very slow. The handling of the sample after shipment to the user is beyond central control. However, it is reasonable to assume that special care will be observed and that if the sample is used promptly after receipt, variations due to changes during shipment and use will be insignificant.

To increase the usefulness of the check samples, units from each series have been assayed by a number of members of the Association of Vitamin Chemists and the results tabulated. These results are available to users of Association of Vitamin Chemists check samples and may be used as checks against the reliability of methods or technic.

It is presumed that each analyst who uses an Association of Vitamin Chemists check sample will desire to compare his technic with that of other vitamin chemists and, accordingly, will give careful attention to the details of analysis not only to insure that the sample has been analyzed to the best of his ability but also to make sure that any deviations from the published methods will be recognized and recorded.

In order to obtain the maximum benefit from check samples it is imperative that all who use them report the values found in their laboratories, even if the values do not agree with those expected. Unless this is done conscientiously, much of the value of interlaboratory comparison will be lost.

Where several methods of assay or several modifications of one method have been used on the check sample, reports of all values obtained will be

especially helpful in evaluating different procedures. In this connection it should be stressed that it is as important to report values which seem to be far away from that expected as to report the more reasonable figures. Only by recording the failures as well as the successful applications can the usefulness of a method be evaluated.

To facilitate the tabulation and evaluation of data it is suggested that each report list:

(1) The name and serial number of the check sample.
(2) The date received from the Association of Vitamin Chemists.
(3) The date of the assay and name of the analyst.
(4) The general method used; name of originator with literature reference.
(5) Name and type of instrument used, if any.
(6) The values found for the sample and their average.
(7) Details of the procedure:
    (a) Weight of sample.
    (b) Extractant; amount used; conditions of extraction.
    (c) Purification steps.
    (d) Preparation of standard and calibration or standardization of instrument if other than exactly as in the method cited.
    (e) Any special technics.

Each participating laboratory will be assigned a number. Data from all laboratories will be listed and reported by number. Each laboratory will be informed of the number assigned to it but the identity of the various laboratories will not be published. At intervals, depending upon the number of reports received, the Committee on Collaborative Assays will compile a report for distribution to all collaborators. Upon receipt of data from any laboratory, the most recent compilation will be sent to permit immediate comparison of results.

Check samples may be ordered from The Committee on Collaborative Assay, The Association of Vitamin Chemists, 808 South Wood St., Chicago 12, Illinois,* at the following rates for a *single shipment:*

(a) One or two units, $1.00 each.
(b) Three units, $2.50.

*The Association of Vitamin Chemists has been granted the use of office space and the mailing address of the University of Illinois College of Pharmacy. The permission to use these facilities is greatly appreciated.

(c) Four units, $3.25.

(d) Additional units in the same shipment with (c), $0.50 each.

Shipment will be made upon receipt of a request for one or more units, *accompanied by payment for the order.* If remittance does not accompany the order, an additional charge of $0.50 will be made to cover billing expense. In this case, the order should include the name and address of the person or department to receive the invoice. If desired, samples will be sent by air mail at an additional charge sufficient to cover the cost of such transportation.

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