QUANTITATIVE ULTRAMICROANALYSIS

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PREFACE

Ultramicroanalysis is a field of analytical chemistry in which progress has to some extent paralleled the tremendous advances made in other phases of that subject, such as instrumental analysis. The remarkable extent of its utilization by the Manhattan Project and the Atomic Energy Commission in both research and production phases of nucleonics has not been appreciated in detail by the scientific public because of the restrictions of security and the limited release of information. For the same reasons, it must suffice in this volume to state that the contributions made by the use of ultramicrotechniques were unique and virtually indispensable to the development of the atomic bomb and the subsequent nuclear investigations.

In the long range, the biochemist, clinician, and biological research investigator stand to profit most from the use of very small-scale methods, and it is to them primarily that this volume is directed. Not only does the field suffer from a scattered and partially inaccessible literature, but the deviations from the accepted standard methods are such as to generate in the minds of some a hesitancy or even a mistrust of methods designed for so great sensitivity.

Teaching of ultramicrotechniques for a considerable period of years has demonstrated completely that they are grasped at least as easily as are standard microchemical methods and that they often yield better results in the hands of students. It is believed that they offer unique value as laboratory training methods and as technical discipline. In many instances they demonstrate the principles of analysis better than could be accomplished by conventional macro- or even micromethods. With them, the research investigator can often by-pass the difficult sampling problems of biological systems by isolating the small and homogeneous system with which sampling errors are best controlled. By so doing he can also obtain information with a wealth of detail which is unique in biochemical analysis.

This volume is not intended to include every aspect of the

subject nor to exhaust the bibliographical possibilities. Many methods and techniques have been omitted intentionally, not only because of their deficiency but because they did not fit smoothly into the generalized technical scheme which was followed. Errors of omission have undoubtedly occurred, and, however regrettable, it is inevitable in so scattered a literature that not every method should receive detailed scrutiny. It is also true that many of the highly sensitive methods published fall below the standards of accurate analytical requirements which this author feels must be maintained.

I am indebted particularly to the many students and associates whose labors have contributed so greatly to the development and continued interest in this field and without whom this volume could not have been written. Facilities and financing for much of the work reported was generously provided by the Rockefeller Foundation, the American Cancer Society, and the United States Public Health Service, to all of whom a debt of gratitude is due. To many other workers in the field of ultramicrochemistry, particularly Dr. K. Linderstrøm-Lang, Dr. H. Holter, Professor E. J. Conway, and Dr. R. Craig, the author is indebted for voluntary cooperation and much unintentional assistance. Appreciation is due Dr. O. H. Lowry, Dr. P. F. Scholander, Dr. C. B. Anfinsen, and particularly Dr. C. L. Claff for their willing assistance in making available much material included in this volume. Acknowledgment is due to authors, publishers, and editors who have willingly granted permission for reproductions, and special thanks are due the Microchemical Specialties Co. for photographs and access to the latest models of various pieces of equipment described.

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CHAPTER 1

Introduction

During the first quarter of the present century, the development of microchemical methods and their adoption by chemists, biologists, and others was very extensive. During the following ten years, microtechniques were recognized by a majority of analytical chemists and became an accepted part of their practice. Despite the wide use of various forms of microchemical methods in many fields, there is still no generally accepted definition of the limits which should be termed "micro." The common understanding of the term is certainly that employed in such standard works as Pregl (1), Niederl and Niederl (2), Emich (3), and others (4), in which the sample size is of the order of 1 or a few milligrams. For convenience, this scale of operations will henceforth be termed *milligram analysis*, a term which is relatively unambiguous.

In recent years, the research microchemist has increasingly turned his attention toward a much smaller scale of analysis, and indeed, many and far-reaching applications of smaller-scale analysis have been made. In general, these may be termed *microgram analysis* since the sample size is such as to contain an amount of the material determined of the order of 1 or a few micrograms. Such procedures are generally applied either (*a*) to analysis of very small samples or (*b*) to analysis of traces of material in larger samples. In the former, volumetric procedures have in general been applied; in the latter, the most common methods are colorimetric. These methods are capable of much further reduction in amount of material analyzed than is indicated by the term "microgram analysis" and indeed may in many instances be applied to the analysis of traces of materials contained in very small samples. This volume is intended to summarize the present status of microgram procedures for analyses of all types that have been proved practical and to include the smaller-scale procedures of colorimetry which have been tested. The field is still to be considered as in the developmental

stage, and many practical and desirable methods have not been developed or tested. These omissions should be readily noted from the context.

Magnitudes

Confusion frequently arises in the use of microchemical units, particularly as to their designation as "micro-," "ultramicro-," and "semimicro-." As indicated above, most of this ambiguity and confusion may be eliminated by the terms "milligram analysis" and "microgram analysis." The smaller-scale colorimetric procedures may in certain instances be applied to analysis of amounts of the order of 1 millimicrogram which might well be termed "submicrogram analysis." Table 1 indicates the *approximate* ranges of various scales of analysis and the reductions achieved at each scale.

TABLE 1. SCALE OF MICROMETHODS

	Sample Size	*Reduction*
Macromethods	100 mg.	
Semimicromethods	10–20 mg.	1/5–1/10 from macro
Micromethods (milligram)	1 mg.	1/100 from macro
Ultramicromethods (microgram)	0.001 mg.	1/1000 from micro; 1/100,000 from micro
(Submicrogram methods)	0.000001–0.00001 mg.	1/100,000 to 1/1,000,000 from micro

Semimicromethods are less well defined and developed than the other categories with the exception of the last. No well-defined set of methods has been described between milligram and microgram methods, but the two types of procedures may usually be extended to fill the intervening gap. It is interesting to note that microgram methods may be more readily extended upward than may milligram methods downward.

The *units* (5, 6) commonly employed in microgram and other small-scale analyses may be defined as follows:

$1\ \gamma = 1\ \mu g. = 0.001$ mg. $= 10^{-6}$ g. $= 1$ microgram
$1\ \lambda = 1\ \mu l. = 0.001$ ml. $= 10^{-6}$ l. $= 1$ microliter
$1\ \epsilon = 1\ \mu$equiv. $= 0.001$ m. equiv. $= 10^{-6}$ equiv. $= 1$ microequivalent

These terms are all analogous to the common designation of microscopic lengths by the use of μ, where

$$1\ \mu = 1\ \mu\text{meter} = 0.001\ \text{mm.} = 10^{-6}\ \text{m.}$$

It is convenient and desirable to make routine use of these symbols in calculation rather than small decimal fractions of the common units for mass, volume, and equivalents. The following simple relationships show that the calculations are of equal simplicity:

$$\text{ml.} \times N = \text{m. equiv.}$$

$$\lambda \times N = \epsilon$$

$$\frac{\epsilon}{\lambda} = \frac{\text{m. equiv.}}{\text{ml.}} = \frac{\text{equiv.}}{\text{l.}} = N$$

$$\frac{\gamma}{\lambda} = \frac{\text{mg.}}{\text{ml.}} = \frac{\text{g.}}{\text{l.}}$$

General Uses of Microgram Analysis

In the field of biology particularly, there are innumerable instances in which the available sample is too small for conventional analysis, even by micromethods. An extreme example is the field of insect physiology, but almost as striking is that of invertebrate biochemistry. Many applications of microgram chemical analysis may be made in both fields, as well as in tissue culture study, protozoology, and other allied sciences. Cytology and cellular chemistry may expect great advances from consistent application of the smallest-scale chemical analysis.

Very important and apt to be underestimated is the possible application to ordinary biochemical studies. Most animal experimentation requires comparatively large amounts of blood, such as may be obtained without slaughter only from dogs or animals of similar size. The equivalent information may be obtained by analysis of tail blood from mice or rats in many instances, without slaughtering the animals to obtain enough blood for ordinary scale analysis.

Another very large field for microgram techniques is in the clinical laboratory where the advantages of analyzing finger or ear blood rather than the much larger amounts usually taken from the arm veins are very evident. The pediatrician is in

position to profit especially from this substitution, because of the well-known difficulties of withdrawing large samples of blood from infants. Probably the most extensive application to the field of biology up to date is in the field of enzymic histochemistry, which has been recently reviewed by Glick (7) and earlier by Kirk (8).

The widest field of application so far found for microgram chemistry was in connection with the studies of the fundamental chemistry of plutonium, most of which was elucidated before as much as a milligram of the element was in existence. It is to be expected that the new subject of isotope chemistry will give many fruitful applications of these techniques because it is frequently possible to obtain only the most minute amounts of special isotopes without great difficulty. Moreover, since most pure isotopes which become available are the result of formation by neutron or deuteron bombardment in a cyclotron or pile, they are nearly always quite radioactive and should be used in very small quantities if the operator is not to be exposed to the hazards of high levels of radioactivity. Thus, it was necessary to conduct all control analyses in the Hanford plutonium plant on a microgram scale in order to diminish the level of gamma activity to an amount considered safe for the analysts. In this case, it was not deficiency of analytical material which was the determining factor in choosing microgram analytical methods. All control analyses were based directly on the techniques described in this volume.

Types of Method

The analytical principles which have proved useful for microgram analysis are not inherently different from those long employed with the conventional size of sample. These principles are detailed in numerous standard textbooks. The techniques which must be employed are considerably different. If very small amounts of material may be weighed, measured, filtered, ignited, stirred, etc., the more satisfactory analytical methods which ave been long employed may usually be adapted to microgram quantities by making the appropriate changes in the technique of each operation. The following outline indicates the present status of various types of procedure.

1. *Gravimetric.* No complete procedure for analysis on a

microgram scale by gravimetric procedures has so far been published. This omission is due to the previous lack of reliable balances that will weigh conveniently to small fractions of a microgram. A number of balances of sufficient sensitivity have been described, but they were all highly specialized, difficult to construct and use, and consequently rather impractical for general analytical application. It is now possible to obtain quartz fiber balances capable of weighing rapidly and reliably to $\pm 0.005\ \gamma$ and probably to $\pm 0.001\ \gamma$ (9), and it is anticipated that gravimetric microgram methods may now be developed as needed.

2. *Titrimetric.* The most accurate and precise procedures so far described belong in this category. Liquid volumes may be reliably dispensed and measured in very small volumes, so that this basic method is one of the most favorable for general application. The limitations of microgram titrimetric procedures are similar to those of larger-scale titrimetric methods, viz., a frequent lack of specificity which may demand the use of separation procedures, and the errors which are characteristic of all titration procedures.

3. *Colorimetric.* Microgram colorimetric methods are numerous (10), though the lower limits of most of them as currently used lie somewhat higher than is typical of the methods described in this volume. They are inherently less accurate than titrimetric methods, and they may or may not be more rapid or specific.

When the colorimetry is performed with the spectrophotometer, the reliability is greatly increased over simpler color measurements, and the errors are correspondingly less, though the precision may not be any greater. In general, the error of spectrophotometric analysis may be readily maintained within limits of about ± 2 to 5%, and under favorable circumstances may be reduced to less than 1%. In the biological field, particularly, these methods are appealing because of the lack of other types of procedure for many of the important organic constituents of the biological system. It is unfortunately true that methods of great practical interest are frequently not well studied and may be of a very low order of dependability. This is equally true of the larger-scale colorimetric methods for these constituents.

4. *Gasometric* (exclusive of gas analysis). Methods based on the measurement of gas volumes are rarely used for microgram

analysis. They are inherently less sensitive than titrimetric methods and much less so than colorimetric. There is no necessary reason for the lack of microgram gasometric methods for numerous constituents, and it is to be expected that certain ones will be developed. In some instances, such methods should be very favorable as compared with other types of procedure.

5. *Physical.* Specialized physical methods for respirometry and for enzyme studies, such as the ingenious Cartesian diver micromanometer of Linderstrøm-Lang (11), have received considerable attention and are widely employed.

Methods for microgram analysis that involve the spectrograph, polarograph, conductometric apparatus, and similar specialized physical equipment show great variations in utility and types of application. In general, they have too low sensitivity to be classed along with the more common microgram procedures. They are much more highly specialized and therefore less available. They rarely show any improvement in accuracy over the commoner types of method. Treatment of these special procedures may be found in other books and will receive little discussion in this volume.

Solutions and Standards

Both titrimetric and colorimetric procedures for analysis depend directly on the use of standards, and the possible accuracy of the method never can be better than the reliability of the standard. The accuracy of microgram analysis is limited by the standard in the same manner and to about the same extent as is that of larger-scale analysis.

Standard solutions for microgram analysis need not be made in as large lots as for larger-scale work, but the stability of a large volume toward evaporation and solution of glass from the container is greater than that of a small one. It is advantageous for this reason to make standard solutions for titrimetric procedures in volumes not less than about 250 ml., and for colorimetric methods in volumes not less than 100 ml. if they are to be stored and down to 10 ml. if they are used directly. If the standard material is weighed and dissolved, it is usually necessary to prepare larger volumes than those indicated because the amount of solid weighed would be too small to obtain an accurate weight. It is advantageous to employ the analytical balance which is

usually available and familiar, and not less than 50 mg. of standard substance should be weighed on this instrument in any single sample.

Titrimetric Analysis. *Standard solutions* are made by either of two methods.

1. Approximate weighing and solution of the weighed material, followed by accurate standardization of the solution against primary standards, or

2. Accurate weighing of the solute and dilution to an accurately known volume.

Method 1 is applicable to all types of solution since it requires no accurate determination of the total volume and allows the use of solutes of unknown composition or purity. Acids and bases are ordinarily made this way, and the method can be adopted for any type of material.

Method 2 applies only when the purity and composition of the solute is approximately as well known as would be necessary for a primary standard. It is suitable for silver nitrate, potassium iodate, alkali halides, carefully prepared and equilibrated sodium thiosulfate, and certain other materials. Unless the material is entirely suitable, method 1 is preferable.

Certain reagents such as potassium permanganate, ferrous ammonium sulfate, and ceric sulfate require special precautions or procedures in preparation of solutions. These will be described in connection with their application in oxidimetry. Direct weighing of the solute and dilution to volume may never be employed with these and other materials of unknown purity or composition.

Purity of solvents must receive special care. Water, acids, and other solvent materials employed in making solutions must usually be purer than is necessary on larger-scale analyses. It is a desirable precaution to redistill all water, since distilled water as it is commonly dispensed from large laboratory stills may be much more impure than is generally appreciated. The amount of water needed is ordinarily small and may readily be provided by a small all-glass distillation outfit. Sulfuric acid, particularly, is frequently too impure to use directly and may also be redistilled from an all-glass still of small capacity. It has been noted that microgram titrations with ceric ion are especially sensitive to small amounts of impurity in the water and sulfuric acid added to the reagent.

Primary standards must meet the general requirements of these materials when used on a larger scale, and the standards need not be better than usual. These general requirements include meeting all or most of the following criteria:

1. Constant composition on standing and during weighing. This includes the composition with respect to water of crystallization.

2. Easily duplicated composition on successive batches or successive crystallizations.

3. Accurate stoichiometric relations, depending on (*a*) absence of measurable impurity and (*b*) absence of side reactions.

4. Sufficiently high equivalent weight to yield satisfactory weighing accuracy.

5. Reasonably wide application to different types of standardizations; e.g., both acidimetry and oxidimetry.

6. Negligible or easily determined titration error.

Standards which meet the greatest number of the above criteria include potassium iodate, potassium biiodate, and sodium oxalate. No universal standard meeting all of them perfectly has so far been found.

Suitable primary standards are indicated in Table 2. The details of obtaining and utilizing these and other definite and pure compounds as primary standards may be obtained from other standard works on quantitative analysis (12–14).

Numerous other primary standard materials might be mentioned, either because of certain intrinsic merits or because of widespread application in analytical laboratories. Those that are omitted have some disadvantage such as difficult availability, difficult manipulation, or lack of general utility. It is not implied that they are necessarily not useful or dependable.

Standards for colorimetry have been treated inadequately in most standard books, which is unfortunate in view of the wide employment of colorimetric methods and the accuracy which is often claimed for them. It is self-evident that the analytical values cannot be more accurate than the standards employed for comparison or calibration. It is not uncommon for widely adopted standards to be uncertain by several per cent, and instances of a twofold error have been known to occur because of inadequate standards alone.

Standards for colorimetry may not be treated so systematically

TABLE 2. PREFERRED PRIMARY STANDARDS

Solution	*Standard*	R.E.W.*	*Indicator*	*Remarks*
Acid (HCl, H_2SO_4)	$Na_2B_4O_7 \cdot 10H_2O$	190.61	M.R.†	Requires adjustment of water of hydration
	KIO_3	35.66	M.R.	Special titration, $IO_3^- + 5I^- + 6H^+ = 3I_2 + 3H_2O$, in presence of excess neutral thiosulfate
Base (NaOH, KOH)	$H_2C_2O_4 \cdot 2H_2O$	62.99	P.P.‡	Pure material available commercially
	$KHC_8H_4O_4$	204.06	P.P.	Bureau of Standards material available
	$KH(IO_3)_2$	389.85	M.R.	
Potassium thiosulfate	KIO_3	35.66	I_2-starch	Iodometric
	$K_2Cr_2O_7$	49.012	I_2-starch	Iodometric
Potassium permanganate	$Na_2C_2O_4$	66.98	Reagent	Bureau of Standards material available
	$H_2C_2O_4 \cdot 2H_2O$	62.99	Reagent	
Ceric sulfate	As_2O_3	49.47	Fe-phenanthroline	Requires a catalyst (15)
	$Na_2C_2O_4$	66.98	Fe-phenanthroline	Bureau of Standards material available
	$K_4Fe(CN)_6 \cdot 3H_2O$	422.15	Setopaline C	
Silver nitrate	KCl	74.56	Electrometric or Dichlorofluorescein	

* R.E.W. = rational equivalent weight, defined as the true equivalent weight, reduced by the amount of the correction to weight in vacuum. Thus, it is the nominal weight of 1 equivalent balanced against brass weights.

† M.R. = methyl red.

‡ P.P. = phenolphthalein.

as may volumetric solutions because of the greater diversity of the former and the difficulty of obtaining dependable compounds from which some standards may be made. Solutions against which unknowns are to be compared or instruments calibrated should meet as well as possible the following criteria:

1. A definite, reproducible composition.
2. Availability in pure form.
3. Solubility in water, or in a solvent which is suitable for color development.
4. A reasonably high equivalent weight.
5. Lack of groups or radicals in their structure which will interfere with color development.

Table 3 lists suggested standards for elements and radicals which are commonly determined by colorimetric methods. Alternative standards may be substituted for many of these, and in some cases better compounds may be available. Few of them are ideal in their conformance to the above criteria.

It is important with the standards listed in Table 3 or other similar standards that every effort be made to obtain material that is as pure as possible and always well within the error of the colorimetric method chosen. This is no easy task because common commercial chemicals are frequently more impure than the allowable limits, particularly with respect to water content.

No organic compounds are listed because the standard for determination of an organic constituent must usually be that constituent itself, and the only choice exerted by the analyst is to attempt to obtain material of high purity and reproducibility, which in some instances is very difficult. Physical constant determination, e.g., melting points, boiling points, refractive indexes, etc., should be utilized to check the purity of any doubtful standard when possible. Recrystallization, distillation, or sublimation may often be employed to purify further the commercial product.

The judgment of the analyst is indispensable in determining how far it is necessary to proceed for the particular purpose. It must be noted that many of the analytical data in the literature are highly deficient in this regard.

Operations

Chemical analyses may be considered as a series of operations, each designed to achieve a limited objective such as complete

TABLE 3. STANDARDS FOR COLORIMETRY

Element or Radical	*Standard*
Al	Uneffluoresced potassium alum, $KAl(SO_4)_2 \cdot 12H_2O$
As	As_2O_3, reagent grade, dissolved in NaOH and neutralized carefully
Bi	Pure bismuth metal
Ca	Reprecipitated $CaCO_3$
Cr	Recrystallized $K_2Cr_2O_7$
Co	$CoCl_2 \cdot 6H_2O$
Cu	Electrolytic Cu metal or selected $CuSO_4 \cdot 5H_2O$ crystals showing no effluorescence
Fe	Pure iron wire (for standard) dissolved in 1:3 HNO_3 and boiled to remove oxides of nitrogen
Pb	$Pb(NO_3)_2$ (dry) dissolved in weak HNO_3 solution
Mg	Pure Mg metal dissolved in dilute HCl
Mn	Electrolytic Mn metal in dilute HNO_3 or standard $KMnO_4$ reduced with acid sulfite and with SO_2 boiled out
Hg	$HgCl_2$ (pure)
Mo	Pure MoO_2 in dilute NaOH solution and slightly acidified with HCl after dilution
Ni	Pure Ni metal or uneffluoresced $NiCl_2 \cdot 6H_2O$
K	KCl (pure and dry)
Na	Pure NaCl
Sn	(Stannous) metallic Sn dissolved in concentrated HCl to which is added thioglycolic acid; must be fresh
U	$UO_2(NO_3)_2 \cdot 6H_2O$
Zn	Reagent grade 20 mesh Zn metal dissolved in HCl
Phosphate	Pure, dry KH_2PO_4
Arsenate	As_2O_3, dissolved in NaOH, carefully neutralized, and oxidized exactly with iodine
Silicate	Pure SiO_2, fused with NaOH
Iodine	Resublimed iodine

separation of precipitate, measurement of an exact volume, etc. Assuming a satisfactory chemistry, the accuracy and reproducibility of the analysis will depend on the performance of each of these individual operations with the required accuracy and precision.

Methods for each operation are comparatively standardized for macroanalysis and almost as much so for conventional milligram analysis. In both, the equipment and its corresponding method are designed for the specific operation on its respective scale. Again, if the suitability of the chemical reactions chosen for the microgram analysis is assumed, the development of the method will depend on proper design and manipulation of the equipment so that the operation can be conducted with the neces-

sary expediency, accuracy, and reproducibility. Since the number of necessary operations is limited, many of them being common to a large group of analytical methods, the development of microgram analysis is largely concerned with providing proper types and design of equipment to handle unusually small amounts of material with the same relative dependability as the larger amounts are manipulated in the conventional scale of operations. Also, the limited number of operations allows a considerable degree of standardization to be introduced. If appropriate equipment and methods are available for each operation of a microgram analysis, the method should be practical regardless of other considerations.

The above generalization has been shown by experience to be correct in most instances. Exceptions occur, chiefly due to the following factors which are more important with small amounts than with large.

1. High evaporation loss of solvent from solutions, due to the large surface to volume ratio of small volumes of liquids. This leads to a concentration of solute.

2. High rate of loss of volatile solutes, e.g., iodine, hydrochloric acid, or ammonia, from small volumes of solution. This leads to a lowering of concentration of solute. It may be turned to advantage as in isothermal distillation, or diffusion methods.

3. Surface tension. Manipulation of small volumes of solutions is quite different from that of large volumes in this regard. Surface tension may be a favorable factor in correctly designed apparatus and may lead to much difficulty with improperly designed equipment.

4. Shallow liquid layers, which lead to small light absorption of colored compounds such as indicators. Thus, a greater concentration of indicator is needed with small volumes than with large, leading to a different order of certain errors.

Temperature and pressure changes, absorption, coprecipitation, and similar phenomena have no greater percentage effect on microgram analysis than on other scales of operation.

Errors

Microgram analysis is inherently subject to somewhat different degrees of the common volumetric errors than is milligram