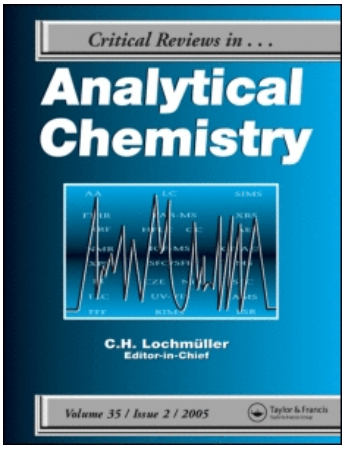

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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To cite this Article Morrison, George H. and Risby, Terence H.(1979) 'Elemental Trace Analysis of Biological Materials', Critical Reviews in Analytical Chemistry, 8: 3, 287 — 320
To link to this Article: DOI: 10.1080/10408347908542713
URL: http://dx.doi.org/10.1080/10408347908542713

ELEMENTAL TRACE ANALYSIS OF BIOLOGICAL MATERIALS

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I. INTRODUCTION

A. Scope and Purpose of the Review

The purpose of this paper is to evaluate the effectiveness of a number of the more popular trace element analytical techniques in meeting the needs of the life scientists. Emphasis is directed towards the determination of trace elements in biological materials using those techniques that are currently being used extensively for this purpose or show promise in the immediate future. Techniques compared include anodic-stripping voltametry (ASV), atomic absorption spectrometry (AAS), electrothermal atomization-atomic absorption spectrometry (ETA-AAS), emission spectrometry (ES), inductively coupled plasma-atomic emission spectrometry (ICP-AES), ion-selective electrodes (ISE), neutron activation analysis (NAA), spark-source mass spectrometry (SSMS), and X-ray fluorescence (XRF) spectrometry. Emphasis is placed on their actual use in clinical analyses as indicated by their appearance in the clinical chemical literature for the past 10 years, rather than conventional analytical sources of information.

Because of the wide variety of biological materials examined for trace element content, this evaluation is limited to the effectiveness of these analytical techniques for the determination of important essential and toxicological elements in serum and/or whole blood. This type of sample was chosen as the most useful clinical diagnostic sample, and also as a biological sample for which substantial trace element data have been published. Since the levels of concentrations of these elements are substantially lower in the blood than most other biological materials, the evaluation assesses the true worth of these analytical techniques to the whole range of biological samples. Criteria used in this comparison include sensitivity, specificity, accuracy and precision, single vs. multielement capability, time of analysis, and general applicability to biological problems.

B. Role of Trace Elements in Biology

During the past decade, there has been an increasing realization of the importance of trace element chemistry in biological systems. This awareness has been stimulated by the rising concern in industrialized nations of man's impact on his environment and its biological effect on him. Of primary importance is the role played by trace elements and their beneficial or harmful effects on the biochemistry of man.

Of the 90 chemical elements that occur in the earth's crust or atmosphere, only 12 (O, C, H, N, Ca, P, K, S, Na, Cl, Mg, and Fe) are present in large amounts in the human body. Of these, the first four account for 96% of the total mass of the organism, with the others making up about 3.6%. The remaining 70-odd elements comprising the remaining 0.4% are the trace elements. Of these, 14 are generally considered to be essential to human metabolism.

Underwood[1] classifies the trace elements into three groups: (1) those essential for higher animals, (2) those possibly essential, and (3) the nonessential elements. According to Cotzias,[2] in order for a trace element to be considered essential, it should meet the following criteria:

1. It should be present in all healthy tissues of all living things.
2. Its concentration in these tissues must be fairly constant.
3. Its removal produces similar structural and physiological abnormalities in different species.
4. Its addition either prevents or reverses these abnormalities.
5. The abnormalities induced by deficiency are always accompanied by pertinent, specific biochemical changes.
6. These biochemical changes can be prevented or cured when the deficiency is corrected.

The elements fulfilling these requirements are Cr, Co, Cu, F, Fe, I, Mn, Mo, Ni, Se, Si, Sn, V, and Zn. In addition, B is an essential element for plants, but not for animals. Table 1 lists the chronology of the discovery of essentiality of these elements.[1,3] Iron was the first element to be recognized as essential back in the 17th century, while the demonstration of essentiality for Sn, V, F, Si, and Ni has only come in the last 7 years. In addition, several other elements are still under consideration as possibly essential. These include Ti,[3] Pb,[3] As,[4,5] and Cd.[5,6]

The essential elements have specific metabolic functions in animal organisms. Several form integral parts of enzyme systems, e.g., Zn alone controls the chemical activity of over 90 enzymes.[7] Some nonhuman species require quite different essential elements for their survival.

In addition to the essential elements, several other elements are always found in body tissues. Some of these enter the body from the environment (food, air, water) in which the organism grows. For a large number of these elements, no biochemical function is known. However, there is another group of elements which are known to be harmful to animals; some through metal carcinogenesis and others by their toxic nature. The list of harmful elements includes: As, Ba, Be, Bi, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Sb, Se, Sn, Te, Tl, V, and Zn. Many of these have been implicated as either tumorogenic or carcinogenic vehicles. Furst[8] points out that carcinogenic substances have the property of readily forming compounds with metals, or at least being metabolized in such compounds. It has been observed that the concentration of many elements are significantly lower in tumors than in the surrounding healthy tissues. This is in agreement with the suggestion that the growth of tumors is related to the change from aerobic to anerobic metabolism.[9] The oxidases are metallo-enzymes, and a change in the trace element concentration level may deactivate the enzyme, which may in turn, bring about metabolic changes.[10] The essential and toxic elements are summarized in Figure 1.

In the above lists of essential and toxic elements, several are common to both categories. Whether an element is essential or toxic will depend to a large extent on its concentration level in the organism. Thus, elemental levels and cause-effects will range from deficiency state, to functioning as a biologically essential component, to an imbalance where an excess of one element interferes with the function of another, to pharmacologically active concentration, and finally, to toxic and even lethal concen-

TABLE 1

Discovery of Trace Element Essentiality for Animals[1,3]

Element	Year	Investigators
Fe	17th Century	—
I	1850	Chatin
Cu	1928	Hart, Steenbock, Waddell, and Elvehjem
Mn	1931	Kemmerer, Todd, Elvehjem, and Hart
Zn	1934	Todd, Elvehjem, and Hart
Co	1935	Underwood and Filmer; Marston; Lines
Mo	1953	DeRenzo, Kaleita, Heytler, Oleson, Hutchings, and Williams; Richert and Westerfield
Se	1957	Schwarz and Foltz; Patterson, Milstrey, and Stockstad
Cr	1959	Schwarz and Mertz
Sn	1970	Schwarz, Milne, and Vinyard
V	1971	Schwarz and Milne; Hopkins and Mohr
F	1972	Schwarz and Milne; Messer, Armstrong, and Singer
Si	1972	Schwarz and Milne; Carlisle
Ni	1973	Nielsen

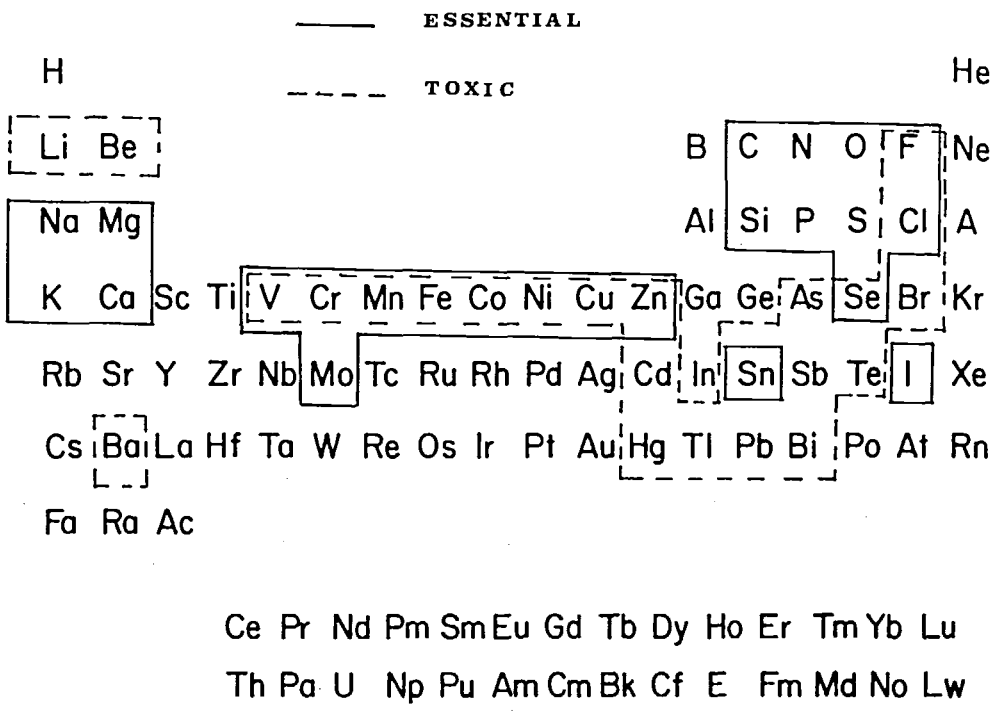

FIGURE 1. Essential and toxic elements in animal and human metabolism.

tration.[11] Thus, the well-known toxicity of an element is no bar to its being essential. Some examples of this are Se, I, and F, all of which are essential or toxic depending upon their concentration levels. In view of this fact, it has been suggested that the concept of beneficial vs. harmful elements is unscientific and should be replaced by the concept of the universal biological dose-response, which postulates that both beneficial and toxic effects are primarily a function of the concentration, but not necessarily of the nature of the element.[5,7,12,13]

II. ANALYTICAL CONSIDERATIONS

A. General Introduction

The limiting factor in the study of the role of trace elements in biochemical metabolism has been the state-of-the-art of trace analysis of biological systems. Three types of information are required, i.e., concentration, distribution, and speciation. Very little progress has been made on the latter two; however, even the accurate determination of many elements present at extremely low levels requires a major effort before unambiguous associations and correlations can be made.

Two different approaches are being employed in the study of trace elements in biological systems. First, in research problems, complex analytical techniques can be employed regardless of time and cost of analyses. Also, samples of specific organs are available from autopsies. On the other hand, the clinical approach is guided by the goals of speed, low cost per analysis, and accuracy. In addition, one is limited to living-population sampling involving only blood, urine, hair, nails, and restricted biopsy samples. Bowen[14] estimates that in Great Britain alone a total of 6×10^9 elemental analyses are performed per year in hospitals.

The rapid development of analytical methodology during the past 2 decades has created powerful tools for trace element research. However, these tools can be danger-

ous if their technical limitations are not clearly understood when applied to biological systems. In view of the ever increasing demands on analytical chemists, it is essential to appreciate these limitations, not only in technology but also with regard to interpretation of results.[13] These demands have been created by the discovery of new essential trace elements, by large international trace element surveys of populations, and by the increasing recognition of public health problems resulting from trace element imbalances.

In view of the increasing use of trace analyses and the wide variety of techniques employed, it is constructive to evaluate the applications of a number of the more popular analytical tecniques in meeting the needs of the life scientists. The techniques compared here are either currently extensively used in trace element analysis of biological materials or show promise for use in the immediate future. They are summarized in Table 2. Emphasis is placed on their actual use in clinical analyses as indicated by their appearance in the clinical chemical literature for the past 10 years, rather than the conventional analytical sources of information. Before comparing the specific techniques, however, a number of general aspects of analyses of biological samples have to be considered such as collection, drying, and storage.

B. Sampling

1. Collection

Collection of meaningful and representative samples is often the most critical step in an analytical procedure. In trace element analysis, extreme care must be taken to avoid contamination of the sample during the sampling step and in all subsequent steps of the analysis. Anand et al.[15] have discussed in detail the problem of contamination of biological samples.

In gathering autopsy samples, care should be taken not to pierce the tissue with metal tools. The sample should preferably be processed in a glove box or a clean area using metal-free plastic forceps and knives or silica knives. The plastic tools should be cleaned by rinsing with dilute nitric acid and doubly distilled water. If a tissue is suspected of having come in contact with metals during handling, the contaminated surface should be cut away and only the inner portion used for analysis.

2. Drying and Ashing

Drying of the samples is usually accomplished by freeze-drying, air-oven drying, or vacuum drying at room temperature. All of these methods are subject to loss of certain elements and should be carefully studied for the particular element under consideration. Freeze-drying is the method considered least susceptible to elemental loss. However, Fourie and Peisach[16] have shown that even here losses of up to 15% occurred

TABLE 2

Analytical Techniques Surveyed

Anodic-stripping voltametry (ASV)
Atomic absorption spectrometry (AAS)
Electrothermal atomization-atomic absorption spectrometry (ETA-AAS)
Emission spectroscopy (ES)
Inductively coupled plasma-emission spectrometry (ICP-ES)
Ion-selective electrodes (ISE)
Neutron activation analysis (NAA)
Spark-source mass spectrometry (SSMS)
X-ray fluorescence spectrometry (XRF)
Proton-induced X-ray excitation spectrometry (PIXE)

for Se, Cd, and Pb in oyster samples. New methods involving freeze drying and homogenizing the sample in a teflon ball mill at liquid nitrogen temperature need to be explored further.

Air-oven drying of biological materials is perhaps most widely practiced. Conclusions as to the extent of trace element losses are conflicting. Strohal et al.[17] showed that Ce, Co, Mn, Pa, Ru, and Zn were partially (15 to 20%) volatilized even at 100°C and lower with mollusk samples. However, Koirtyohann and Hopkins[18] found no losses of Cr, Fe, Zn, or Cd on drying at 110°C or ashing up to 600°C. Chromium was lost from blood samples, but not from liver samples heated to 700°C. Raaphorst et al.[19] also found no losses of Zn or Co from marine mussels and seaweed during dry ashing up to 1000°C. Fourie and Peisach[16,20] found no losses of Cr, Zn, Fe, or Co from oysters upon oven drying up to 120°C, but losses up to 20% of Se, Cd, and Pb were observed.

Drying samples under vacuum at room temperature, though safer for most elements, probably leads to losses of such volatile elements as Hg, As, and Se.

Wet ashing of biological materials using mixtures of acids has been thoroughly studied by Gorsuch.[21] A nitric acid-perchloric acid mixture is recommended, with only Hg and Se resulting in low recoveries. He found dry ashing to be unsatisfactory for As, Cu, Ag,nCd, and Pb.

During the past few years, low-temperature ashing (LTA) has been increasingly used. Here the biological sample is ashed at about 100°C in an electrodeless radiofrequency (RF) field in an excited-oxygen atmosphere. The method is time-consuming for large samples, but recoveries of nearly 100% for most elements have been reported.[22,23] Only Hg and Os were lost during LTA ashing. The technique is capable of preserving the morphological features of some specimens. The resultant ash is readily soluble in acids for subsequent analysis by atomic absorption spectrometry or radiochemical methods. It is also suitable for pelletizing for XRF, ES, or SSMS, or for irradiation in NAA.

3. Storage and Contamination

During sample preparation and analysis, great care must be taken to avoid contamination of samples by storage vessels, reagents, air, etc. The problem is particularly acute because of the very low concentrations of trace elements in many biological tissues. Robertson[24] has evaluated common laboratory glassware, reagents, and miscellaneous equipment for their content of ten trace elements, and the results are dramatic. For example, Kimwipe® tissues commonly used in most U.S. laboratories for wiping pipettes and other laboratory items contains 48 ppm of Zn and could be a potential source of contamination for this important element. A recent monograph by Zief and Mitchell[25] is quite instructive in the control of contamination in trace element analysis.

The impact of contamination is strongly related to the concentration level of the trace element present in the sample, Thus, Fe, Cu, or Zn, which are present at ppm levels in blood serum, give accurate results, whereas Mn, Cr, or Co, present at the ppb level, reveal wide scatter in the literature. Bowen[26] and Comar[27] have shown how literature values for Mn in blood have come down over the years. In 1940, it was reported as high as 10 ppm, but recent reports indicate only 0.06 ppm. It is not that the Mn level in blood has decreased, but rather that more accurate analyses resulting from minimization of contamination have been used. Speecke et al.[28] have compiled Cr values in human blood serum for the past 16 years and have found a spread from <1 to 345 ppb, although the most likely value is about 0.2 ppb. Heparin, often added to blood samples to prevent coagulation, has been shown to contain at least six trace elements at levels several orders of magnitude higher than present in blood (see Table 3). According to Bowen[29] the majority of the early determinations for Mn in blood

TABLE 3

Trace Element Contamination of Human Blood by Heparin

Element (ppm)	Heparin	Blood
Ba	2.5—12	0.069
Ca	300—2900	62
Cu	0.65	1.1
Mn	3.6	0.026
Sr	5—92	0.039
Zn	28	6.5

From Bower, H. J. M., *Trace Elements in Biochemistry*, Academic Press, London, *1966*. With permission.

were actually determinations of Mn in dilute solutions of heparin. Other examples of discrepancies in trace element analysis of biologicals include Mn in human bone ash (<0.5 to 273 ppm by spectrophotometry and NAA), Ag in human blood by DC arc emission spectroscopy (3 to 190 ppb), and Co in human blood by spectrophotometry (0.3 to 110 ppb).[14] True values in the first two cases are on the order of 1 ppm Mn and a few ppb of Ag. The true mean value for Co in blood is uncertain since other techniques also result in a wide scatter of values.

Among the various biological samples, serum is particularly susceptible to changes in trace element levels since it is a liquid. Fisher et al.[30] have shown that the container composition had little effect on mineral levels of serum stored in Pyrex®, polypropylene, polycarbonate, vycor, or Teflon® flasks. Temperature effects were not significant in refrigerated and frozen samples provided the serum was equilibrated to room temperature before aliquots were taken for analysis. The effect of storage time was highly significant for all elements tested. Zinc decreased 20% after 2 days; Ca, Mg, and Cu had a 10% decrease after 2, 4, and 8 days, respectively; Na and K had maximal decreases of only 3.5 and 2% with storage time. Factors that affect serum pH levels appear to influence the elemental concentrations. Thus, if serum samples are to be stored, they should be quickly frozen after collection with a minimum of air space above the serum. Before analysis, the frozen serum should be allowed to come to room temperature and then thoroughly mixed since concentration gradients have been observed to form in refrigerated serum.[31]

C. Human Serum and Blood Studies

To obtain useful information from a trace element analysis, a meaningful tissue must be selected. It must be one that reflects deficient or excessive environmental exposure, or alternatively, one that is practically linked to a disease process under investigation. Some knowledge of the metabolism and site of action of the trace elements is helpful in the selection of a meaningful tissue. According to Mertz,[13] tissues fall into four classes: (1) regulatory sites, (2) tissues in which an element has an essential function, (3) tissues related to transport, storage, and excretion, and (4) tissues which sequester trace elements after excessive exposure.

In view of the large number of tissues examined for trace elements, this evaluation will be largely limited to the body fluids which fall into the third category. These fluids are readily available and are commonly used specimens when trace elements are being determined. They include whole blood, serum, plasma, urine, and cerebrospinal fluid.[15] These fluids are presumed to reflect the distribution of the elements in the body at the moment the specimen is collected. Serum, promptly separated to minimize the

extracorporeal exchange of ions with erythrocytes, appears to be the specimen of choice in most cases and is the most frequently analyzed sample. Levri and Angel[32] have shown that, at least for Mn, Cu, Fe, and Zn, the concentrations in serum and urine accurately reflect the imbalance in the body in the case of diseases such as Wilson's disease, cirrhosis, and viral hepatitis. Therefore, for the purpose of this review we will concentrate mostly on the analysis of serum and/or whole blood in order to compare the effectiveness of the various analytical techniques in determining the important essential and toxicological elements.

In most clinical laboratories, several inorganic elements (Ca, Na, K, P, Cl) are routinely determined in every specimen of blood taken from the patients. Bowers[33] lists 13 elements (Ca, P, Na, K, Cl, Br, Mg, Li, Pb, Cu, Zn, Cd, and Hg) that are routinely determined in serum in addition to a host of other tests carried out at the Hartford Hospital in Connecticut. In most clinical laboratories, Na and K are usually determined using flame photometry; Ca, Mg, Cu, and Zn are determined by AAS; Pb is determined in serum by ETA-AAS; Hg by cold vapor AAS; and As colorimetrically.[7]

To appreciate the magnitude of the analytical problem, Figure 2 shows the concen-

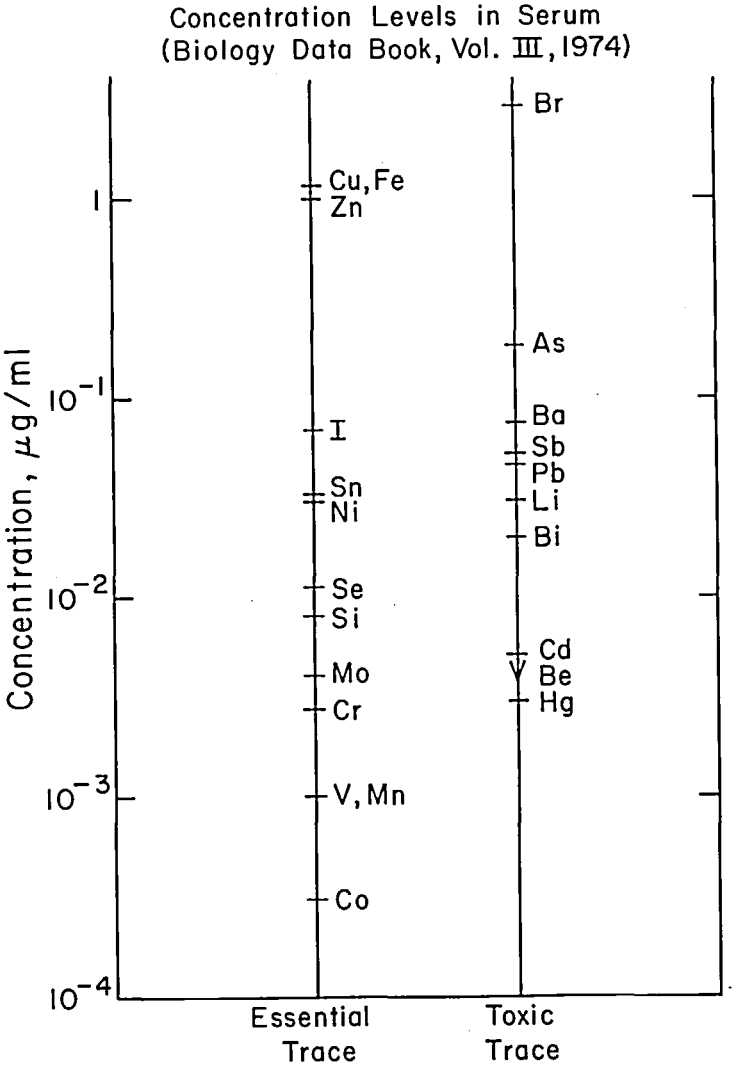

FIGURE 2. Concentration levels in human blood serum.[34]

tration levels for the essential and some toxic trace elements in human serum. The data are taken from the Biology Data Book[34] and represent one of the most reliable estimates of this complex problem. The concentrations for the essential trace elements range from a low of 0.3 ng/mℓ for Co to about 1 μg/mℓ for Cu and Zn. The corresponding range for the toxic elements is from 3 ng/mℓ for Hg to 3 μg/mℓ for Br. These concentrations in serum are lower than in most tissues and present a formidable challenge for the analyst.

III. EVALUATION OF METHODS

A. Criteria for Evaluation

The criteria used for the comparison of trace element techniques include sensitivity, specificity, accuracy and precision, single vs. multielement capability, time of analysis, and general applicability to biological problems. There have been a few recent reviews on similar aspects, but none have critically evaluated the individual techniques for biological analyses.[35-37]

Based on the capability of the methods for the determination of one or more elements, they may be classified as single-element techniques (AAS, ETA-AAS, ASV, NAA, and ISE), and multielement techniques (ES, ICP-ES, NAA, SSMS, XRF, and proton-induced X-ray excitation [PIXE]. When information on a large number of elements is desired, multielement methods are preferred in order to save time; however, where the highest accuracy is necessary, single element techniques are employed.

B. Sensitivity

Perhaps the most crucial criterion in trace element analysis is sensitivity. Unless the element can be detected, all other considerations are inconsequential. As mentioned earlier, because of the wide variety of biological materials examined for trace elements, the evaluation of sensitivity for the determination of the important essential and toxicological elements is restricted here to serum and/or whole blood. This type of sample was chosen as the most useful clinical diagnostic sample and also as a biological sample for which substantial trace element data have been published. Therefore, the ability to detect and determine trace elements at these low levels (Figure 2) is vital to trace element analysis of biologicals.

As a first approach, one could examine the published detection limits for the various techniques for the elements of interest. These limits are idealized in that they assume no spectral or other interferences, matrix effects, and other analytical complications which result from the analysis of real, complex samples. Also, these limits are assumed to be direct so that preconcentration techniques would improve the detection limits for all measurements methods. Realizing the obvious limitations of comparing these generalized detection limits, it is still informative to examine each of the techniques and their capability of reaching the levels of interest in human serum.

Table 4 summarizes the sensitivity and/or detection limits for the essential and toxic elements of interest for the different analytical techniques. Trace element levels in normal human serum are included for comparison. Little data are available for ASV so far; however, for the few elements studied, they are compatible with concentration levels in serum.[36,38,39]

AAS has had a strong impact on trace analysis in general and has been extensiyely used for biological analyses. Figure 3 is a plot of the sensitivity limits[40] for the elements of interest using AAS vs. their concentration levels in serum. The elements lying above the 45° line are not of high enough concentrations to be determined using AAS; only Cu, Fe, Zn, As, and Li lie above the required limits in serum. However, many more elements have been determined in serum by AAS using preconcentration. Sensitivity in AAS is defined as that concentration of an element in aqueous solution which ab-

TABLE 4

Sensitivities and Detection Limits of Analytical Techniques

Element	Normal serum (ppm)[a]	ASV detection Limits[b] (ppm)	AAS sensitivity (ppm)[c]	ETA-AAS sensitivity (ppm)[d]	DC arc ES detection limits (ppm)[e]	ICP-ES detection limits (ppm)[f]	NAA sensitivity (ppm)[g]	SSMS detection limits (ppm)[h]	XRF detection limits (ppm)[i]
Major essentials									
Na	140	—	0.003	0.000004	0.5	0.0002	0.004	0.0002	1
K	4.4	—	0.01	0.0001	3	—	0.2	0.0003	2
Mg	1.85	—	0.003	0.000007	0.1	0.000003	0.5	0.0002	—
Ca	5	—	0.02	0.0004	0.1	0.000001	4	0.0003	0.5
P	114	—	—	—	—	0.02	—	0.0003	4
Trace essentials									
Co	0.0003	—	0.07	0.00006	0.1	0.0001	0.01	0.0005	0.2
Cr	0.0028	—	0.06	0.00004	1	0.00008	0.3	0.0005	0.7
Cu	1.14	0.000005	0.04	0.00004	0.1	0.00 04	0.002	0.002	0.4
F	0.0028	—	—	—	100	—	—	0.0002	1
Fe	1.15	—	0.06	0.000008	1	0.0005	2	0.0005	0.3
I	0.07	—	—	—	—	—	0.002	0.001	0.2
Mn	0.0014	—	0.02	0.00001	0.1	0.00001	0.001	0.0004	0.2
Mo	0.004	—	0.33	0.0002	1	0.0002	0.1	0.003	1.5
Ni	0.03	—	0.07	0.0001	0.2	0.0002	0.7	0.002	0.2
Se	0.011	—	0.5	0.0001	100	0.03	0.01	0.002	—
Si	0.08	—	2	0.007	0.5	0.01	—	0.0002	11
Sn	0.033	0.002	1	0.0004	0.5	0.003	0.03	0.004	—
V	0.001	—	0. 9	0.0005	0.5	0.00006	0.002	0.0004	0.15
Zn	1.0	0.00004	0.009	0.000002	5	0.0001	0.1	0.002	0.2
Trace toxic									
As	0.19	—	0.1	0.0002	10	0.002	0.05	0.0006	—
Ba	0.079	—	0.2	0.00006	0.1	0.00002	0.02	0.002	0.4
Be	<0.004	—	0.02	0.000001	0.05	0.0004	—	0.00008	1
Bi	0.02	0.00005	0.22	0.0003	0.2	0.05	—	0.002	2
Br	3 (0.7-13)	—	—	—	—	—	0.003	0.002	1
Cd	0.005	0.000005	0.01	0.000003	2	0.00006	0.005	0.007	2.5
Cl	103 meq/*l*	—	—	—	—	—	0.05	0.0004	6
Hg	0.003	0.001	2.2	0.0005	2	0.001	0.003	0.007	1
Li	0.031	—	0.02	0.0006	0.1	—	—	0.00006	2
Pb	0.046	0.00001	0.1	0.00005	0.02	0.001	0.5	0.003	—
Sb	0.054	0.0001	0.3	0.0002	2	0.2	0.007	0.002	—
Tl	—	0.00001	0.3	0.00007	2	0.2	—	0.004	—

[a] Reference 34,
[b] References 36, 38, 39.
[c] Reference 40.
[d] Reference 41.
[e] Reference 42.
[f] References 43,44.
[g] Reference 45.
[h] Reference 46.
[i] References 42, 46.

sorbs 1% of the incident radiation intensity passing through a cloud of atoms being determined.

At the present time, the most powerful technique for trace element analysis of biologicals is electrothermal atomization-atomic absorption spectrometry. Flameless sampling devices, which have become available during the past 5 years, have greatly extended the capabilities of AAS. Figure 4 compares the sensitivity limits of ETA-AAS[41] with the concentration levels in serum. The definition of sensitivity limits is the same as with AAS. All the elements of interest lie above the sensitivity limits of this technique, so it is potentially one of the most sensitive techniques for serum analysis. Only the halogens cannot be determined.

Historically, emission spectroscopy was the most sensitive trace technique for many

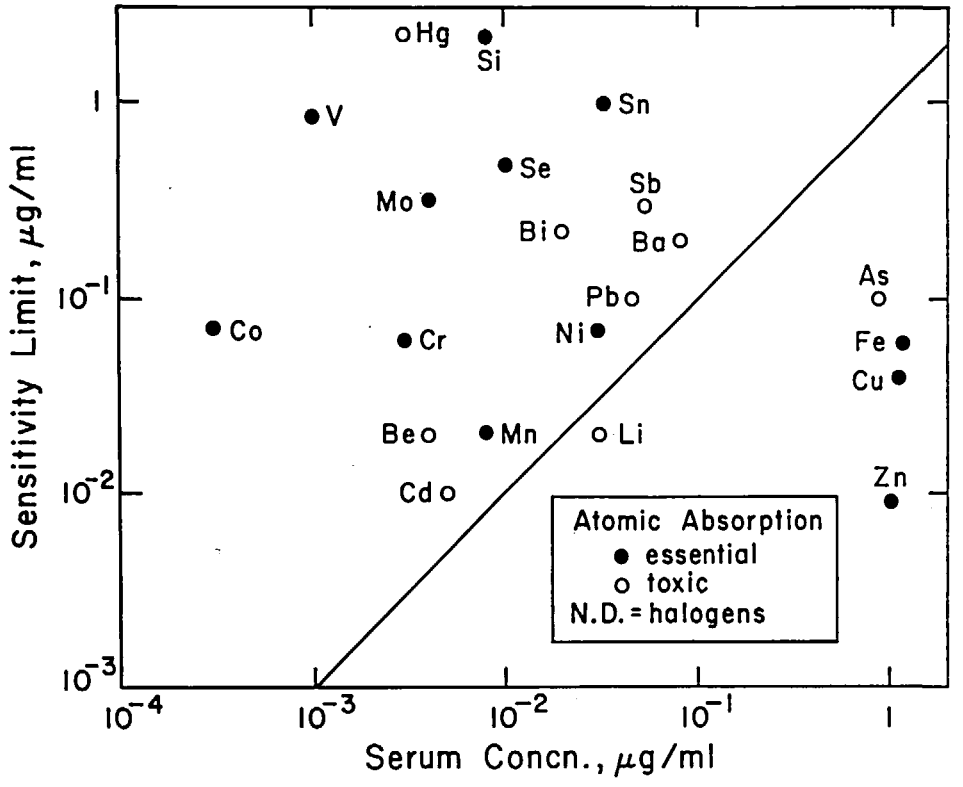

FIGURE 3. Comparison of sensitivity limits of AAS[40] and the concentration levels of trace elements in human blood serum.

years. Figure 5 plots the ES detection limits[42] against the serum levels. Only Cu, Fe, and Pb have detection limits low enough to be applicable, so without preconcentration this technique is inadequate for serum analysis.

A recent entry into the family of trace element analysis techniques is the inductively coupled plasma source in atomic emission spectrometry. Preliminary results indicate this technique is one of extreme sensitivity with detection limits rivaling those of ETA-AAS. In addition, this technique provides multielement determination. Figure 6 shows that, in principle, detection limits for all of the essential and toxic trace elements except Sb, Bi, Se, and Si lie well below the concentration levels in serum.[43,44] However, the halogens are not determinable. The detection limit in this case is defined as the concentration required to produce a line signal twice as large as the standard deviation of the background scatter.

A technique that has shown great sensitivity in general trace element analysis for many years is neutron activation analysis (NAA). Figure 7 indicates that the sensitivity limits[45] of a majority of the elements of interest, defined as twice the background, lie at or below the levels in serum so that this technique can be quite useful. These limits for NAA, using a thermal neutron flux of 10^{13} neutrons cm^{-2} sec^{-1}, can be lowered by employing radiochemical separations after irradiation. Thus, at least 36 elements have been determined in serum by NAA. Only Be, Si, Tl, Pb, Bi, and F among the elements of interest have not been determined.

Another technique that has had considerable success in general trace element analysis is SSMS. Figure 8 shows that SSMS detection limits are sufficiently low so that almost all of the elements lie at or below the serum levels.[46] Literature data show that at least 54 elements have been determined in human blood by SSMS. Only Co and Be among the elements of interest have not been determined.

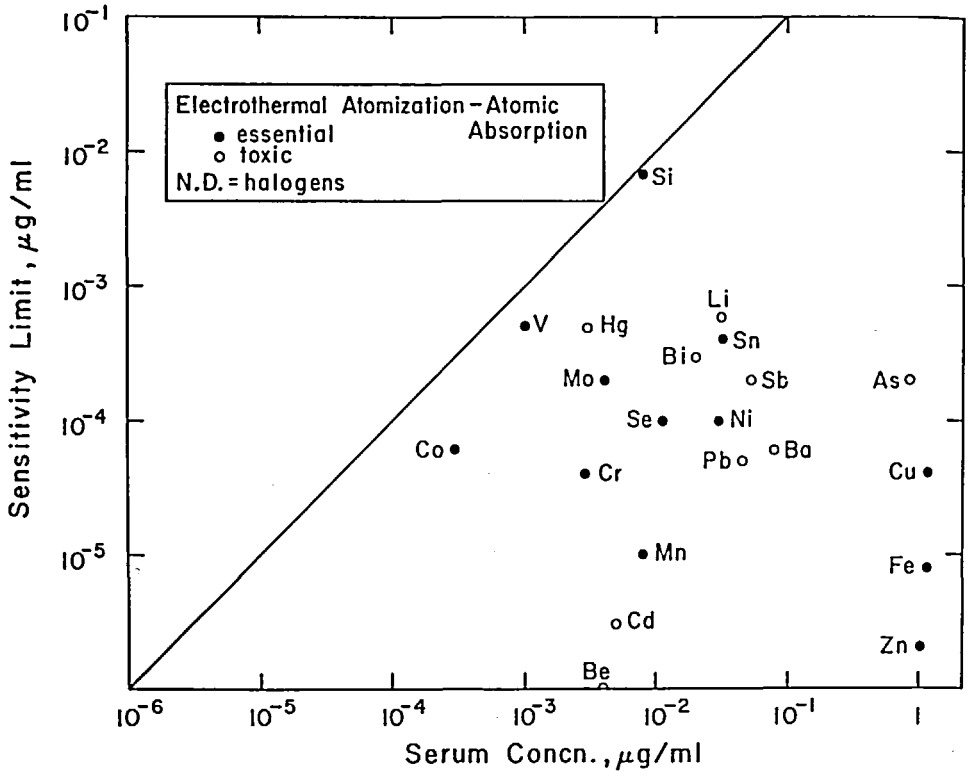

FIGURE 4. Comparison of sensitivity limits of EA-AAS[41] and the trace element concentration levels in human blood serum.

Figure 9 shows the corresponding comparison for the detection limits for XRF.[42,46] The technique is not sensitive enough for most elements in serum. Only Cu, Fe, Zn, and Br have detection limits below the concentrations found in serum.

To summarize the evaluation of sensitivity for serum analysis, Figure 10 indicates the number of essential and toxic elements whose detection or sensitivity limits are equal to or below the concentration levels in serum for the various techniques. For the 14 essential and 12 toxic trace elements in serum, ETA-AAS offers the most potential at this time, with ICP-ES and SSMS coming very close; NAA is next, followed by AAS. XRF and DC arc ES appear to be of limited capability. It must be strongly emphasized that this comparison indicates only potential sensitivity for direct determinations without preconcentration. As will be apparent below, other factors may overrule this sensitivity capability.

Table 5 indicates the number of published, analytical and clinical serum studies using the various techniques in the last 7 to 10 years, and as might be expected, AAS and ETA-AAS dominate the picture. Next in frequency is NAA, including both instrumental methods and those with radiochemical separations. Other techniques have been only infrequently used.

C. Comparison of Techniq

Other criteria for the choice of an analytical method, namely specificity, accuracy and precision, single vs. multielement capability, time of analysis, and general applicability to biological problems, will be considered below under individual techniques.

1. Anodic Stripping Voltametry

Polarographic and ASV techniques are based on the fact that before different metals

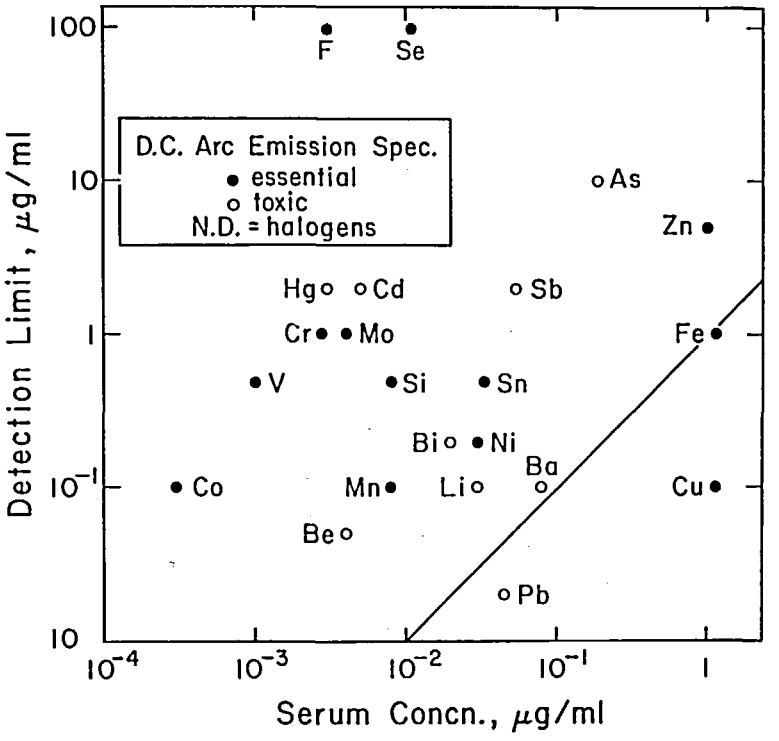

FIGURE 5. Comparison of detection limits of emission spectroscopy[42] and the trace element concentration levels in human blood serum.

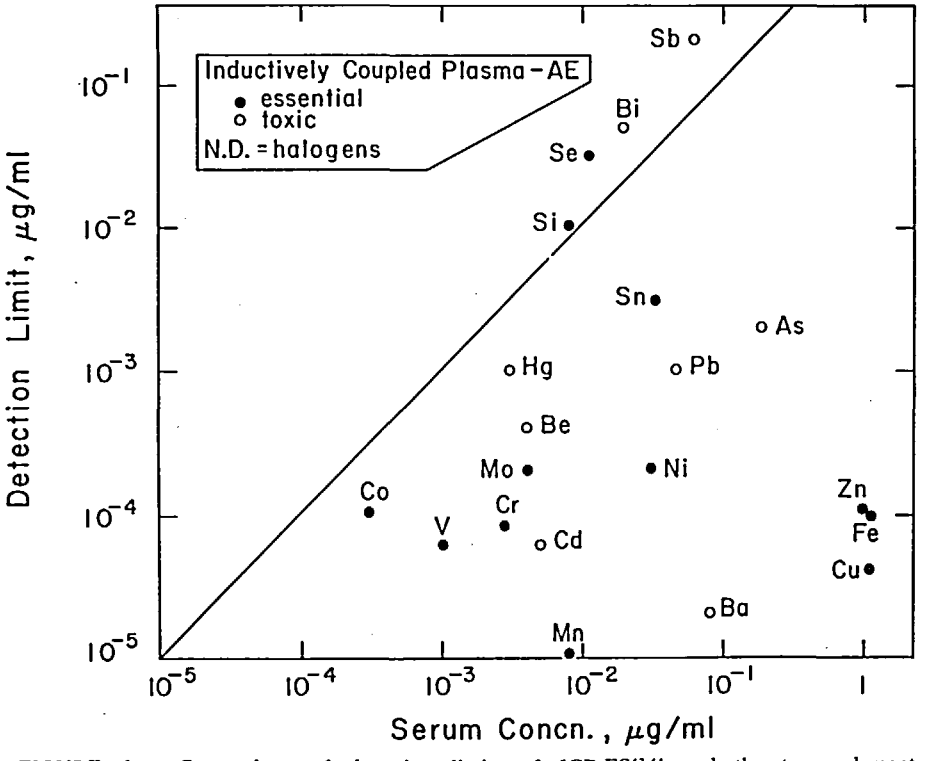

FIGURE 6. Comparison of detection limits of ICP-ES[43,44] and the trace element concentration levels in human blood serum.

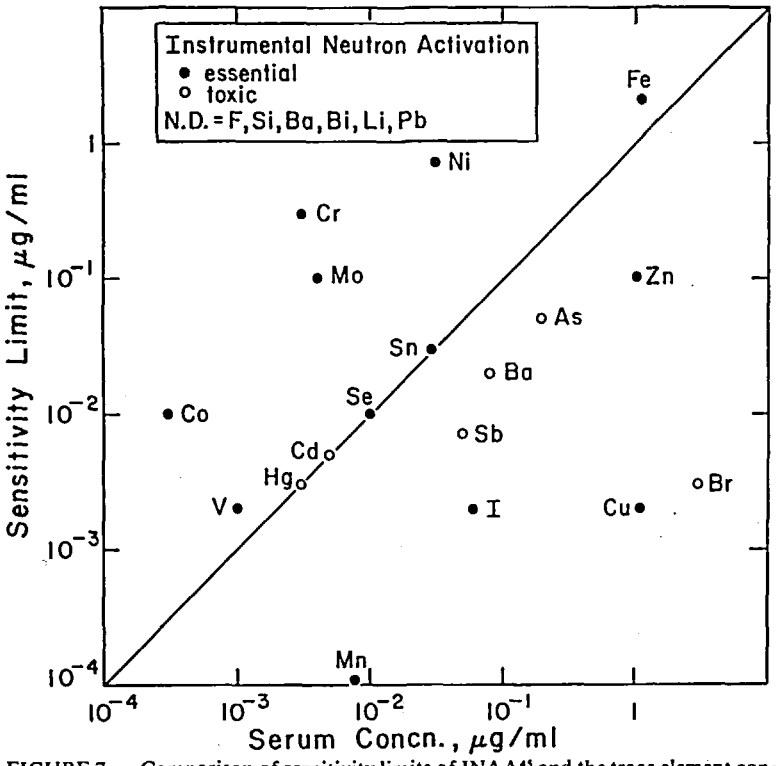

FIGURE 7. Comparison of sensitivity limits of INAA[45] and the trace element concentration levels in human blood serum.

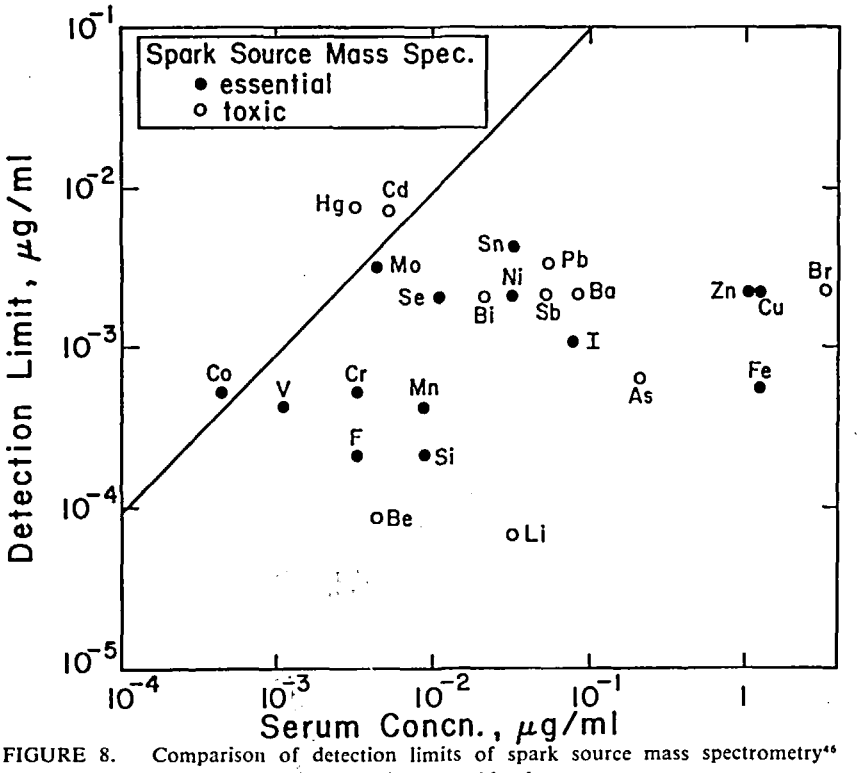

FIGURE 8. Comparison of detection limits of spark source mass spectrometry[46] and the trace element concentration levels in human blood serum.

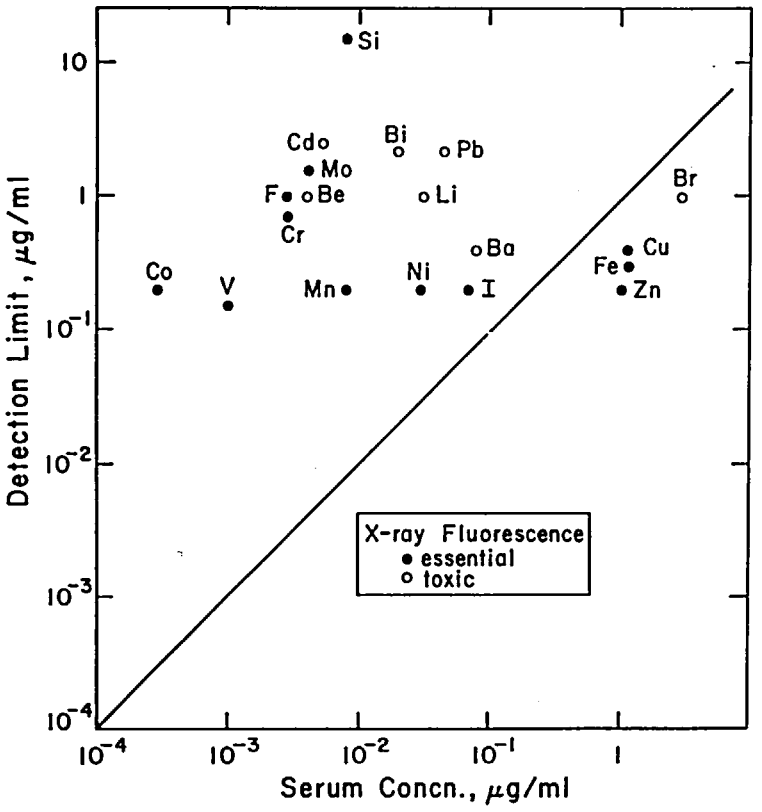

FIGURE 9. Comparison of detection limits of XRF spectrometry[42,46] and the trace element concentration levels in human blood serum.

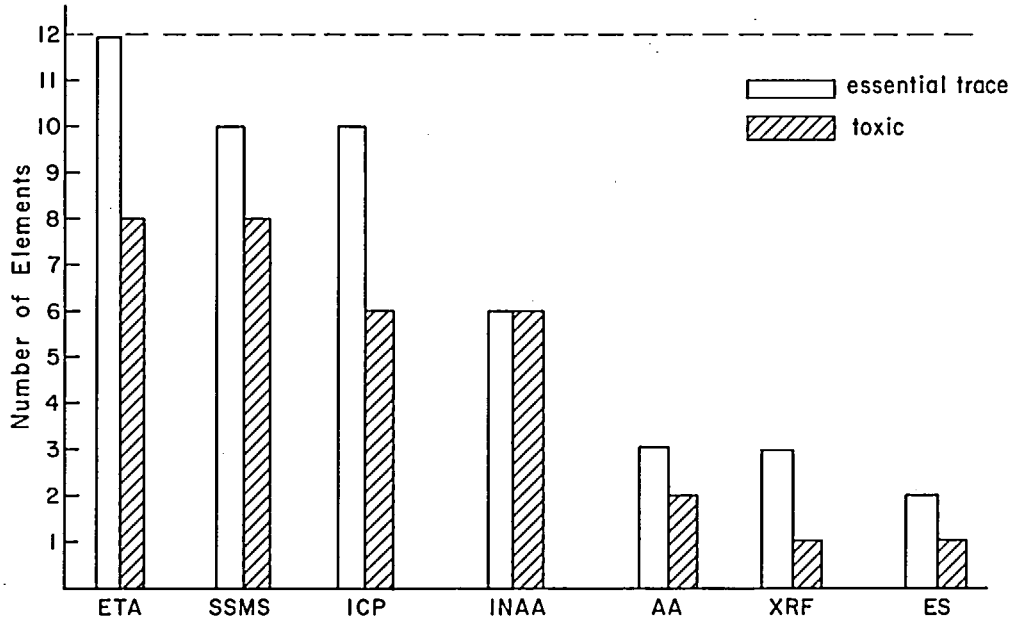

FIGURE 10. Comparison of capabilities of some analytical techniques for trace element determination in human blood serum.

TABLE 5

Published Serum Studies in Last 10 Years

Technique	Number of Papers	Number of different elements determined
AAS	>100	22
ASV	6	11
ETA-AAS	60	19
ICP-ES	4	10
ISE	22	5
INAA	>20	36
RNAA	>35	30
SSMS	6	54
XRF	6	23
PIXE	7	17

are deposited from solution onto a cathode they require the application of varying electrical potentials. Resulting characteristic half-wave potentials can be used for qualitative identification and quantitation of the metals. In ASV, after the metal ions have been preconcentrated from solution onto an amalgamated stationary hanging mercury-drop electrode using a more negative potential than the reduction potentials of species to be determined, the process is reversed, and the metals are oxidized and stripped anodically using a slowly increasing positive potential. The concentration of each metal is a direct linear function of the current measured during the stripping step.

The basic instrumentation required for ASV is relatively inexpensive and readily available commercially. A complete and relatively versatile ASV system including cell and electrodes generally costs about $3000 to $4000. Copeland and Skogerboe[38] have reviewed the theory and applications of ASV. The method is applicable only to a limited number of elements (about 20), since it can be used only for those elements that are easily reduced to the metallic state and reoxidized. The technique is extremely sensitive with detection capabilities of 0.001 ppb and is well suited for analysis of natural waters. Samples as small as 100 μl can be analyzed, though typical samples are in the 1 to 50 ml range. ASV requires samples to be brought into solution before analysis. It may be possible with ASV to differentiate between various oxidation states or between such categories as free and bound metals.

ASV is a very matrix-sensitive technique, e.g., when using an acetate electrolyte for Zn, Cd, Pb, and Cu analysis, the size of the waves are highly pH dependent. Since it is often difficult to duplicate exactly the same conditions in both samples and standards, standard addition methods are used to correct for this variability. Although this calibration takes longer, simultaneous determinations of up to four to six elements can often be done with no additional plating time, equipment, or sample requirements. Interferences in ASV analyses include problems such as overlapping stripping peaks, intermetallic compound formation, and electrode surface film formation. For trace analysis, the cell and all other parts in contact with analyte solution should be of a material chosen to reduce contamination from leaching and/or loss due to adsorption. Teflon® or acid-leached quartz and Pyrex® are commonly used. The use of solid electrodes requires that precise electrode cleaning, polishing, and pretreatment procedures be used to obtain reproducible results.

Typical analysis time in ASV is about 10 min. Accuracy and precision on the order of 3 to 10% are obtained for typical trace concentrations of 10^{-5} to 10^{-8} M. There have been only a few applications of ASV in biological analysis. These include determinations of Pb, Cu, Ti, Bi, and Zn in blood serum,[47] Au in serum,[48] Pb and Cd in blood, plasma, and urine,[49] Pb in blood[50,51] and urine,[51] and Hg, Bi, Sb, Cu, Pb, Sn, Tl, In, Cd, and Zn in urine, plasma, and whole blood.[52]

2. Atomic Absorption Spectrometry

Perhaps the most widely employed technique in biological analyses is AAS. Basically, the technique involves aspiration of the sample solution into a flame whose high temperature converts the metal ions into atoms in the vapor state. Most of this atomic vapor exists in the ground electronic state and can therefore absorb resonant radiation of an appropriate wavelength. A hollow cathode lamp is used as the source. This is a sharp line source consisting of a cathode of the element of interest and a tungsten anode enclosed under reduced pressure and filled with an inert gas. When a voltage is applied across the electrodes, the filler gas is positively ionized at the anode and is accelerated towards the cathode, which under this bombardment sputters to form an atomic vapor in an excited electronic state. On returning to the ground state, lines characteristic of the element are emitted. These pass through the flame where certain ones are absorbed. This absorption is measured with a photomultiplier tube after passing through a monochromator. Since usually only the test element can absorb this radiation, the method becomes very specific.

Over 70 elements can be determined by AAS. Some difficulties are encountered with certain refractory metals. Nonmetals such as S, P, and halogens can be determined by indirect procedures which are only infrequently used. Usually the sample has to be brought into solution before measurement. Aqueous as well as some organic solvents can be used. Preconcentration steps such as ion exchange or solvent extraction are often employed to enhance the sensitivity. Standardization is achieved by using either calibration curves or standard addition methods. Reviews pertinent to biological analyses include those by Christian,[53] Stevens,[54] and Berman.[55] The precision of the results is often on the order of 1 to 5%. Once the sample has been brought into solution, the analysis can be completed in a few min. The cost of the instrument including background corrector, digital absorbance and/or concentration display, and automatic gas-flow controller runs up to $15,000, although much simpler models start from about $4000. The price of hollow cathode lamps (each about $150 to $200) for each element must be added to this estimate.

Simplicity of operation and relatively low cost have contributed to the immense popularity of this technique in clinical analysis. At first it was thought that the AAS technique was free from all interferences. However, experience gained over the last two decades has shown that though it may be mostly free from atomic spectral interferences, there are a large number of other problems such as molecular spectral interferences, ionization interferences, mutual chemical interferences, and matrix interferences because of differing viscosity and surface tension between sample and standard solutions. Air-acetylene is the most commonly used flame; however, for refractory elements and to overcome many chemical interferences, a nitrous oxide-acetylene flame is often employed. Only one element is determined at a time, although recently one manufacturer has made available instruments capable of determining two elements simultaneously.

Samples such as blood, serum, or urine can be directly analyzed for certain elements by AAS. Where sensitivity for an element is very high, e.g., Mg, the sample can be simply diluted 10 to 100-fold with distilled water and measured. Since serum has a high viscosity, protein or similar high-molecular-weight substances should be added in equivalent proportions to the aqueous standards used. Sometimes adding an ionization buffer eliminates some chemical interferences, e.g., serious interference by phosphate in the determination of Ca in blood and urine can be suppressed by addition of Sr^{+2} or La^{+3} salts to the solution, which preferentially combine with phosphate ions and thus release Ca.

Solvent extraction has been widely employed as a preconcentration step in AAS. This frees the metal from other possibly interfering elements and concentrates the element of interest into a much smaller organic solvent volume, which usually produces

an enhancement of the signal in the flame. A commongy used solvent extraction system has been ammonium pyrolidine dithiocarbamate (APDC) into methyl isobutyl ketone, although many other chelating agents have been used.

Published applications of AAS pertaining to the analysis of serum in particular, and other biological materials in general are abundant. It is beyond the scope of this review to list all of them. Only a few typical and recent papers are cited here. Reviews by Stevens[54] and Berman[55] and periodic bibliographies compiled by the Perkin-Elmer Corporation and published in the Atomic Absorption Newsletter should be consulted for complete coverage. Published AAS papers roughly fall into two equal categories: those employing chemical separations and those without. Most of the essential as well as toxic and many other elements have been determined in biological materials by AAS. Delves et al.[56] used as little as 1 m*l* sample of blood to determine eleven metals — Fe, Cu, Bi, Zn, Cd, Pb, Co, Ni, Mn, Sr, and Li — by sequential solvent extraction employing various chelating agents. Other chemical separations used in blood and serum analysis include Mn with oxine[57] and Na-diethyldithiocarbamate (NaDDC);[58] Cd,[59] Cd, Pb, Tl, and Ni,[60] Pb,[61] Tl,[62] and Cd, Pb, and Zn[63] with APCD; Fe and Zn with a tridentate ligand, PAPHY;[64] and Co with PAN.[65] Direct determinations of trace metals in serum, blood, and urine include Cd,[66-68] Ca and Mg,[69] Li,[70] Tl,[71] Cu and Zn,[72] and Pb.[68,73]

3. Electrothermal Atomization-Atomic Absorption Spectrometry

Perhaps the fastest growing technique in its application to biological anaysis is ETA-AAS. Electrically heated graphite tubes or rods reaching temperatures up to 3000K replace the regular flame in this modification of AAS. Except for the flameless environment, the principles of ETA-AAS are the same as in the case of AAS. The electrical power to the furnace is applied in three steps — drying to remove the water and acid vapor, ashing to remove organic and more volatile compounds in the sample, and finally atomization of the metal species of interest. Some attempts have been made to analyze solid samples directly by ETA-AAS; however, there are at least two difficulties encountered in this approach. Since generally only mg amounts are analyzed, it is difficult to obtain a representative sample. Secondly, it is problematic to find a calibration procedure which accounts for a solid matrix.

Like AAS, ETA-AAS is a single-element technique and is specific for the element being determined. Two major advantages of ETA-AAS over standard AAS are small sample volume and high sensitivity. Usually 5 to 10 μl of sample is all that is needed for ETA-AAS vs. several m*l* needed for aspiration in AAS. Sensitivity of ETA-AAS is 100-fold or more improved over AAS (Table 4). Measurement time is slightly more than in AAS, since the sample has to go through the stages of *in situ* drying, ashing, and atomization, and then cooling of the carbon furnace. The cost of an ETA-AAS system including graphite furnace and power supply is about $4000 in addition to the base price of an AAS unit and hollow cathode lamps.

When first introduced, the ETA-AAS system was hailed as extremely sensitive and completely interference free. The sensitivity claim is certainly true, but it has now been realized that a variety of interferences plague the technique, and hence, it has been recommended even by the manufacturers[41] to use ETA-AAS only when flame AAS cannot meet the demands of sensitivity and/or sample volume limitation. The precision of ETA-AAS is normally slightly less than for flame AAS, on the order of 2 to 5%. Because of its sensitivity, extreme care has to be taken to observe cleanliness in the laboratory when using ETA-AAS. Contamination from dust, water, air, pipette tips, and Kimwipes® very often results in mystifying ETA-AAS results. The graphite rods and tubes have a finite lifetime and usually have to be replaced after 150 to 200 firings.

Within the last 2 to 3 years, there has been a mushrooming growth of applications of ETA-AAS to biological materials. Sunderman[74] has reviewed this field. We will cite

only a few typical papers. At least 19 essential and toxic trace elements have been determined by ETA-AAS in blood and serum. The overwhelming majority of papers analyze serum or blood directly without any chemical preconcentration, a tribute to the extreme sensitivity of this technique. Often the sample has to be diluted for analysis. Among the elements determined, nearly half of the papers concern determination of Pb, attesting to the concern over Pb pollution in our environment.[75-82] Other trace elements determined in blood, serum, and urine include Mg,[82] Fe, [82,83] Cu,[82-88] Zn,[80,82,88,89] Li, [90,91] Au, [91-94] Cd,[88,99-97] Mn,[86,87,98-100] Ni, [101, 102.] Co,[86,87,91] Pt and Pd,[103] Sr,[100] Tl,[80] Cr,[104-106] Hg,[107] and Al.[108] A few papers utilize chemical preseparation such as APDC extraction of Pb[75,97] and Cd[97] from blood, Ni extraction as dimethylglyoxime[109] or furildioxime[102] complexes, and Mn extraction as the cupferron complex,[110] all in methyl isobutyl ketone.

4. Emission Spectroscopy

The multielement capabilities of ES coupled with its selectivity and sensitivity as an analytical tool for trace element research has been recognized by many investigators. Up to 70 elements at levels down to 10 ng can be analyzed by ES without excessive pretreatment of sample. Sensitivity varies widely from ppm levels for simpler low atomic number elements to 0.1% for some of the transition or semimetal elements. ES is based on light emission at characteristic wavelengths by the elements. When heated to a high enough temperature, every element emits discreet and characteristic spectral lines. The number of lines of an element varies inversely with its sensitivity. Thus, Na with a simple spectrum of a dozen or so lines widely dispersed from the UV to IR regions is highly sensitive in ES. Uranium, on the other hand, with thousands of lines is very insensitive. However, by taking advantage of characteristic wavelengths, specific identification and quantitation of elements is possible. In practice, powdered samples are homogenously mixed with high purity graphite, packed into graphite electrodes, and are subjected to a DC arc or spark for a few sec. The resultant light emission is dispersed by a prism or grating spectrograph and can be either recorded on a photoplate or electronically detected.

Our laboratory has developed a procedure for the direct determination of elements in biological materials without appreciable alteration of the sample.[111] The high-current-density DC arc method utilizes 25 to 50 mg of dried material and provides detection limits of 1 ppm or less for 26 elements. Analyses of bone, blood serum, animal tissue, and plant samples utilizing only the lower wavelength region produced line intensities suitable for quantitation. A precision of 15% can be generally expected. Sensitivity could be increased by using the ashed samples. Using 5 to 10 mg of ash, detection limits between 1 and 150 ppb were obtained for 22 elements in whole blood, ash samples with a precision of 20% and an ash concentration factor of 25.[112]

Emission spectroscopy has been the method of choice in the classical "standard man" studies of Tipton et al.,[113-116] who determined 19 trace metals and 5 major elements in 29 different human tissues from 150 adult subjects. Other applications of ES to biological analysis include those of blood and serum,[117-120] animal tissues,[121-127] and plant samples.[128,129] In spite of its multielement capability, ES cannot be easily used for analysis of serum because of its low sensitivity compared with the normal levels of trace elements present serum (Table 4). The major elements in serum — Na, K, Mg, and Ca — can be more accurately determined by AAS or flame emission spectrometry. In contrast to the use of ES in analysis of geological or metallurgical materials, considerable time-consuming sample preparation is required for biological samples. Usually, the samples are either wet-ashed in acid[119-121] or dry-ashed in a muffle furnace.[111,112,129] The chemical composition of the inorganic matrix present in the samples analyzed by ES affects the spectral line intensity by changing the physical properties of samples, changing the relative rates of volatilization of sample constituents, and varying the arc

temperature. Thus, when the matrix composition of standards and samples differs appreciably, analytical errors are to be expected. Niedermeier et al.[122] have found that the presence of as little as 1mg% Fe, Mg, Ca, K, or P had a marked influence on the spectral response of 14 trace metals in animal tissue samples. These matrix effects are not generally a linear function of concentration of matrix element, nor are effects of several elements in the matrix additive. In short, prolonged time for the sample preparation, difficulties with accurately reproducing the excitation conditions, and inability to utilize extremely small samples are some of the difficulties encountered in applying ES to biological materials. The DC arc is prone to wandering, has high background, and has a tendency to volatilize elements selectively, all giving rise to poor reproducibility. Use of Stallwood Jet® has corrected many of these faults. With very careful control of various parameters, relative standard deviations of 10% can be routinely obtained in the ES analysis of biological materials.[112,121,128,129]

The cost of instrumentation is fairly high at $50,000. The time for a complete multielement analysis by ES would vary from 1 to several hr including sample preparation, arcing, photoplate developing, and reading of the lines on a microdensitometer.

5. Inductively Coupled Plasma Emission Spectrometry

A most promising analytical technique in recent years has been the development of plasma sources for ES. ICP derives its sustaining power by induction from high-frequency magnetic fields. The plasma is formed and maintained at the open end of a quartz tube assembly. The open end is surrounded by the induction coil, connected to a high-frequency current generator. The plasma is stabilized by a pattern of two or three argon flows. When these flows are properly adjusted, the plasma is readily initiated by tickling the quartz tubes inside the coil with a Tesla discharge. Gas temperatures in the 9000 to 10,000K range are achieved in the plasma. The sample (usually solutions, but sometimes finely powdered solids) is injected into the plasma where the metals undergo atomization and excitation. The emission spectrum of the metal atoms is then analyzed with a spectrograph and electronically read as in the case of ES. The technique is specific for individual elements just as in the case of ES. However, since it is essentially a solution technique where sample solution is nebulized into the plasma, much quicker turnaround time of analysis (a few minutes) is possible as compared with several hrs needed in ES.

ICP-ES is well suited for real-time simultaneous multielement analysis. The high temperatures used result in greater freedom from chemical interferences and increase the range of elements that can be determined. The technique has extremely high sensitivity (Table 4) along with over six orders of magnitude dynamic range. Fassel and Knisely[43] have reviewed the technique. Precisions of about 5% have been achieved. The cost of the instrumentation is in the range of $60,000 to $80,000. In spite of its very impressive sensitivity and multielement capability, the technique has been used in only a few biological applications to date. These include determination of Al, Cu, Fe, Mg, Si, Ag, Pb, and P in blood;[130] Al, Ca, Cu, Fe, Mg, and Mn in serum;[131] Ca, Mg, Fe, Cu, and Zn in serum;[132] about 10 trace elements in blood, tissues, and environmental samples;[133] and 19 major and trace elements in biological materials.[134] The paucity of biological applications is probably the result of newness of the technique as well as its high cost. The next few years should show whether ICP-ES will replace more conventional AAS and ETA-AAS in clinical and analytical laboratories.

6. Ion-Selective Electrodes

In principle, ISE is one of the simplest analytical techniques in use. ISE measure the ion activities or the thermodynamically effective free ion concentrations. The various types of ion-selective glass electrodes available are all members of a continuum

of glass electrodes. The glass electrode comprises a thin walled bulb of cation responsive glass sealed to a stem of noncation-responsive, high-resistance glass. ISE has a membrane construction that serves to block the interfering ions and only permits passage of ions for which it was designed. However, this rejection is not perfect, and hence, some interferences from other ions occur. The electrode potential is measured on a millivolt meter or a modified pH meter. The amount of the millivolt current is proportional to the concentrations of ion being measured.

Electrode calibration curves are good over 4 to 6 decades of concentration. The typical time per analysis is about 1 min, though some electrodes need 15 min for adequate response. Although a single-element technique, many elements can be determined sequentially by changing electrodes, provided calibration curves are prepared for all ions. Also, the instrument is portable and is thus useful for field studies. Sample volumes needed are typically about 5 ml, although 300 μl or less can be measured with special modifications. An accuracy of 2 to 5% is achieved.

ISEs are subject to two types of interferences: method interference and electrode interference. In the first type, some property of the sample prevents the electrode from sensing the ion of interest, e.g., in acid solution fluoride forms complexes with H^+, and the fluoride ISE cannot detect the masked fluoride ions. In the electrode interference, the electrode responds to ions in solution other than the one being measured, e.g., bromide ion poses severe interference in using chloride ISE. The extent of interference depends on the relative concentration of analyte to interfering ions. The interfering ions can be complexed by changing pH or adding a reagent to precipitate them. However, finding the right chemistry is not always easy.

At least 20 elemental ISEs are commercially available, although only a few have been used in biological analysis. They have been more extensively used for water and agricultural problems. Sensitivities are of the order of 1 ppm, but the electrodes are inexpensive ($100 to $200). Nelson[135] has reviewed the technique of ISE. Orion Research Inc. has published an extensive bibliography of ISE applications.[136] Out of the 25 publications in the last 10 years in the clinical field that we found, 9 were on the determination of Ca, 6 each on the determinations of F and K, and the rest on Na and Cl. Obviously, poor sensitivity to most of the trace elements prevents its use in determination of other essential and toxic trace elements in blood or serum. A few typical references are: K, [137-140] Na, [138-140] Ca,[141-144] Cl,[145] and F. [146-148]

7. Neutron Activation Analysis

One of the most sensitive trace element techniques is NAA, where the danger of contamination and loss of elements is eliminated. In NAA, the samples and standards containing the elements of interest are irradiated in a nuclear reactor with a beam of thermal neutrons. The resultant daughter isotopes are measured after suitable decay periods on either a gamma-ray spectrometer or on a beta counter. Usually, the amount of radioactivity recorded at particular energies is a direct measure of the elemental concentrations in the sample. The sensitivity of NAA is only slightly less than that of SSMS, ETA-AAS, or ICP-ES. Accuracy and precision on the order of 1 to 10% can be routinely obtained, although this is a function of counting statistics and cannot always be controlled. A simple Geiger-Muller counter and decade scaler, sufficient for determination of single elements, costs only a few hundred dollars. Multichannel analyzers with sophisticated data reduction capabilities range from under $10,000 to over $30,000. In addition, high resolution Ge(Li) detectors cost around $10,000, depending upon the counting efficiency and resolution of the detector. It is also necessary to have access to a nuclear reactor for irradiation of the samples. Very often the reactor is shared by various departments in a university, or regional reactors serving various industries and institutions over a large area are set up to reduce the cost. The high cost

of NAA has proved to be no deterrent to its extensive application in practically every field as apparent from the tens of thousands of publications using the NAA technique.

The technique is ideally suited for multielement analysis, although determinations of a single element are also quite common. Depending upon the half-life of the isotope produced, an analysis can take from a few min (e.g., ^{28}Al — 2.3 min) to several weeks (e.g., ^{60}Co — 5.23 years).

NAA started as a single-element technique when only Geiger-Muller counters and NaI(Tl) detectors were available. However, with the availability of multichannel analyzers and high resolution Ge(Li) detectors, it has become a truly multielement technique. Radiochemical separations are still resorted to for rapid analysis or for eliminating the interfering activities. Instrumental NAA (INAA) does not involve any chemistry, and the irradiated samples are simply counted over a period of time to get information about the desired elements. The nondestructive nature of INAA is unique amongst all analytical techniques. After INAA measurements are finished, the same sample can be used for determination of other elements by different techniques

An extensive bibliography of NAA up to the end of 1971 has been published by the National Bureau of Standards (NBS).[149] This includes several thousand papers on biological applications of NAA with 147 papers dealing with the analysis of blood alone. A majority of these blood papers deal with the determination of only one to two elements, and where five to six elements are determined, radiochemical separations (RNAA) have been carried out. Since all biological materials contain large amounts of Na, K, Cl, and P, and since these become highly radioactive upon irradiation, their gamma-ray peaks swamp the trace amounts of other isotopes of interest. Several radiochemical schemes have been devised to remove these interfering activities, e. g., use of hydrated antimony pentoxide to remove ^{24}Na, [150] group separation to determine up to 31 elements,[151] automated group separation schemes,[152] etc. Separation schemes employed in RNAA include precipitation, adsorption on inorganic exchangers and ion exchange resins, solvent extraction, and distillation.

In recent years, purely-INAA techniques have come into vogue. Thus, Nadkarni and Morrison[153] have determined up to 36 elements in biological materials and human serum.[154] Other similar work includes that by Cornelis et al.,[155,156] Zdankiewicz and Fasching,[157] Nadkarni et al.,[158] Budinger et al.,[159] Jurgensen and Behne,[160] Bernhard et al.,[161] Clemente et al.,[162] etc. In INAA, the samples are irradiated once for a few min to produce short-lived isotopes which are counted, and then the samples are irradiated for several hours to produce long-lived nuclides. Advantage is taken of the decay properties of isotopes so that the short-lived nuclides are counted first, and as they progressively decay, longer-lived isotopes are successively measured. Interference-free gamma-ray energies are available for most elements, and where there are two isotopic species at the same energy, their contributions can be computed. However, care has to be taken to consider primary and secondary interferences from competing nuclear reactions towards the reaction of interest. Since ^{24}Na or ^{42}K is not removed in INAA, but simply allowed to decay out, certain short-lived species in trace amounts cannot be accurately determined in samples with large amounts of alkalies such as serum. Similarly,[42] Ca and ^{32}P produce intense bremstrahlung radiation which prevents the determination of several long half-lived but low energy isotopes; this is a problem in bone, milk, etc. Under such conditions, radiochemical separations have to be performed.

8. Spark-Source Mass Spectrometry

SSMS is one of the most sensitive and comprehensive techniques of trace analysis of inorganic systems. It employs a vacuum spark in which a high voltage radiofrequency discharge (20 to 100 kV) is produced between two closely spaced electrodes of

the material to be analyzed. The repetition rate and duration of the radiofrequency spark source is variable in order to meet the various analytical requirements. The design of the instrument employs double-focusing Mattuch-Herzog geometry, which allows simultaneous focusing of all masses. This permits simultaneous photographic recording of nearly all elemental masses and integration over a period of time to provide high sensitivity. In the source region of the SSMS spectrometer, two electrodes made from the material being analyzed are sparked resulting in vaporization and ionization of sample constituents. After acceleration of ions into the slit system, the electrostatic analyzer selects for transmission ions of a certain energy range with no mass separation. The magnetic field then separates the ion beam according to mass to charge ratio, providing the mass analysis at the same time. The ions may be recorded either electrically or photographically. In the latter case, it is desirable to put a series of graded exposures on a photoplate. The resolved ions cause blackening on a photographic emulsion, which is then developed and evaluated.

For SSMS, the sample has to be electrically conductive. This is achieved for nonconducting biological materials by blending them with high purity graphite followed by briquetting to form electrodes that sustain the vacuum spark. A serious problem arises in evaluation of the SSMS biological spectra from spectral interferences. Organic ions of differing complexity are formed in the spark, and these spectral interferences may appear at every mass from 13 to above 200 with considerable overlapping of the trace element lines of interest. The source of these lines is obvious when one considers the many possible combinations of C, H O, N, S, P, etc. that can be obtained from the organic matrix present in a biological material. These organic fragments complicate qualitative analysis and prohibit quantitative analysis of many trace elements. There are varying degrees of organic interference from different materials, so that body fluids and bone samples show less interferences than animal tissues and plant materials, thereby permitting limited trace determinations. However, to take full advantage of the multielement survey aspect of the technique, it is necessary to ash the biological samples before analysis.

Ashing removes the spectral interference from organic species; however, spectral interference from the large number of inorganic elements present in relatively high concentration in the preconcentrated biological ash (O, Na, P, S, K, and Ca) presents a problem. Molecular ions formed from the combinations of these elements and their singly and multiply charged species lead to a complex spectrum causing interference with some of the trace elements.

The technique is capable of providing information simultaneously on about 50 or more elements in the concentration range from 100 ppm to a few ppb with reproducibilities on the order of 10 to 25% and with comparable accuracy when standards are employed. Quantitative analysis can be carried out by comparing the intensity of the element of interest to that of an internal matrix element line, that of the same line in a series of known standards containing the element of interest, or that of an added isotope of the element. Usually the measurements are done using photographic plates, but electrical peak detection techniques are increasingly being used.

From considerable experience in our laboratory, the detectable elements can be roughly classified into three groups:

1. Analytically good elements — Li, B, Al, Si, P, Mn, Fe, Cu, Zn, Rb, Sr, Mo, Ag, Cd, Sn, Ba, and Pb; the accuracy and precision of the determination of these elements are usually good on the order of 10%. Most of these are multiisotopic elements.
2. Analytically marginal elements — Be, V, Cr, Co, As, Cs, Bi, Tl, and Sb; most of these are monoisotopic elements that occur at ultratrace levels in biological mate-

rials. If unresolvable interferences occur, the values may represent only upper limits of concentration.

3. Volatile major elements — Na, K, Mg, and Ca; doubly charged ionic lines of these elements are used in SSMS analysis. Because of their volatility and other factors, the precision of their determinations is usually poor, and the accuracy fluctuates within a wide range. AAS and flame emission spectroscopy and other analytical techniques provide more accurate results for these elements.

Among the elements discussed above, SSMS is one of the few instrumental techniques that can successfully determine B, Si, and P. Approximately 5 to 7 days are required for sparking and data processing, in addition to the ashing of biological samples. Instead of photographic plates, if electrical detection is used, the data can be more rapidly acquired. The acquisition of data using electrical detection lends itself to automation.

The cost of a SSMS is in the range $100,000 to $150,000. Photographic and electrical detection systems are desirable for maximum flexibility, and a minicomputer is necessary for photoplate reading and data processing.

SSMS has been extensively used in materials science programs. Its applications in the biological field have been comparatively restricted. Evans and Morrison[163] evaluated the applicability of this technique to biological analysis. Among its applications have been the analysis of blood plasma,[164] fingernails,[165] a very large number of blood and tissue samples,[46,166-169] mussels,[170] serum and tissues,[171] tobacco,[172] hair,[173] and tissues.[174]

9. X-ray Fluorescence Spectrometry

When electrons accelerated across a highly evacuated tube strike a target, the energy is radiated which ranges in wavelength from the IR to X-rays. When wavelength distribution emerging from the X-ray tube is measured, it is found under certain circumstances to consist of a continuum of wavelength and a superimposed line spectrum consisting of sharp emission lines of high intensity characteristic of the target. If an X-ray photon removes an electron from the K or L shell of an atom, an X-ray characteristic of that atom is emitted as an electron falls from another higher energy level to fill the vacancy. Typically, the K X-rays of adjacent elements in the periodic table with atomic numbers $\gtrsim 15$ are separated by energies of a few 100 eV. Earlier, these energies were measured precisely by using gas-flow proportional counters, and the X-rays were counted efficiently with scintillation counters. With the recent advent of high-resolution solid state Si(Li) detectors, it is possible to do both at the same time. These detectors have resolutions down to 150 eV, and it is possible to determine many elements simultaneously by recognizing their characteristic X-rays emitted from a sample.

The basic components of the X-ray spectrograph are a source of excitation consisting of a high energy X-ray tube, either dispersive or nondispersive X-ray optical systems for selecting characteristic X-rays, and integrating or counting circuits for measurements. X-ray tubes commonly made of Mo or Cu have high energy output, spectral purity, and long-term stability. Of these requirements, spectral purity is of greatest importance in trace analysis. Any impurity present in the target will generate spectral lines which will scatter from the sample and be detected. Such elements as Fe, Ni, and Cu should always be suspected. X-ray tubes which initially emit a relatively pure spectrum of the target in addition to the continuum will in time show contamination. Therefore, frequent checking of the spectrum is important.

The characteristic lines emitted by the X-ray tube will not only interfere at positions corresponding to their primary wavelengths, but at somewhat longer wavelengths as well because of the Compton effect. The problem becomes more serious with decreas-

ing atomic number. As a rule, the presence of a characteristic line in the X-ray tube spectrum can be expected to cause an error in the determination of a low concentration element whose characteristic line is slightly greater in wavelength than that of the target line.

Unless a comparison is made with a standard which is identical in composition to the sample, systematic errors in XRF may be large, (caused by changing absorption of primary and fluorescent energy on account of changes in chemical composition of the analytical material). Particle size and shape are important and determine the degree to which the incident beam is absorbed or scattered. Standards and samples should be powdered to the same mesh size, preferably finer than 200 mesh. Matrix dilution with materials having a low absorption, such as starch, Li_2CO_3, lampblack, or gum arabic, reduces the matrix absorption effects. However, this also reduces the sensitivity for measured fluorescence. Internal standards are often used to eliminate systematic errors.

The XRF technique, inherently very precise, rivals the accuracy of wet chemical techniques in the analysis of major constituents. Precisions of the order of 5 to 10% can be achieved when proper corrections are made.

The technique is applicable to all of the elements after Na/Mg in the periodic table. Simultaneous determination of several elements is possible with automated equipment. Its sensitivity is not compatible with the requirements for serum analysis; nevertheless, some work has been done by using preconcentration. At least 23 elements have been determined in serum by XRF, though many more elements have been determined in other biological materials. Giaque et al.[175] reviewed the factors that affect the sensitivity in applications to biological samples. Applications of XRF to biological analysis include blood and tissues.[46,166,167,176-182]

In XRF, the fluorescence is photon induced. When protons or other heavy-charged particles are used for excitation, the technique is called proton-induced X-ray excitation (PIXE). Campbell et al.[183] have reviewed the technique and demonstrated its application to biological materials. The sensitivity of PIXE is several hundred times higher than that of XRF. However, the elaborate instrumentation, e.g., a Van de Graaff accelerator, necessary for excitation in PIXE does not hold promise for its use in clinical laboratories. PIXE has been used in the analysis of whole blood,[184-186] serum,[187-189] and other human and animal tissues.[186,190,191]

D. Accuracy and Precision

Every analytical chemist knows that the signal obtained from the various instrumental techniques may not be related to the amount of the trace element in the analyzed sample. The determination of an element in a complex biological sample is a difficult task which becomes even more difficult at the very low concentrations of elements present. Figure 11 shows the values reported for Cr in blood since 1948 by various investigators.[13] Obviously, they do not represent a biological distribution from the very high to very low values. They show a recent decline of reported values, suggesting a continuous modification of analytical technique, either because of more careful control of contamination or because more recent methods are more specific.

It is generally recognized that each trace analytical technique is superior for some elements and very poor for others. Each suffers to varying degrees from interferences from other elements and/or the biological matrix. It is necessary with a number of single element techniques to include chemical separations and other pretreatment steps to achieve the required specificity for accurate analyses. The problem is more complex with the multielement techniques such as ES, ICP-ES, INAA, SSMS, XRF, and PIXE.

Evaluation of the accuracy and precision of the techniques being examined here for a specific type of biological sample is, admittedly, a difficult if not impossible task.

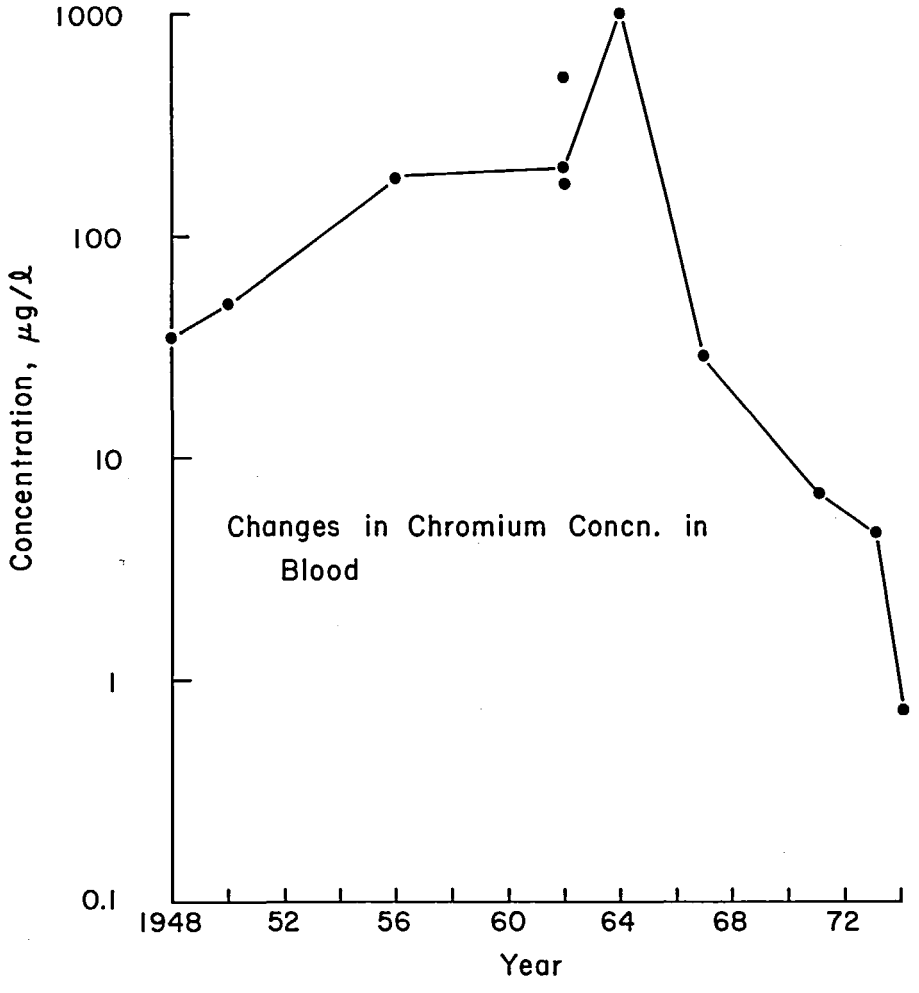

FIGURE 11. Changes in reported chromium concentration in human blood from 1948 to 1974.[13]

Fortunately, a recent intercomparison study was made in Europe[192] for the evaluation of the determinations of Pb, Hg, and Cd in blood. Sixty-six laboratories participated in the analysis of two cow blood samples and one human blood sample. The methods used for Pb and Cd included AAS, ETA-AAS, ASV, polarography, and colorimetry. Mercury was determined using ETA-AAS, colorimetry, polarography, and NAA.

Four identical samples of cow blood C, three identical samples of cow blood D, and one sample of human blood E were sent to the participating laboratories. In addition, identical aqueous samples were also sent. The homogeneity of the samples was periodically checked during the distribution. The results of this "round robin" study are most interesting. Regarding the influence of the technique used, because of the limited number of data available, a meaningful comparison could only be performed between three techniques — AAS, ETA-AAS, and colorimetry — used for Pb determination in cow blood C (Table 6) An f-test showed that there is a significant difference between the distribution of the results obtained by the three techniques. Colorimetry and ETA-AAS exhibit a smaller scatter of results than AAS. It should be noted that the other blood samples, D and E, did not show such a technique effect.

The effect of the laboratory precision is summarized in Table 7 for Pb, Hg, and Cd determinations in the three blood samples. The mean value is given along with the

TABLE 6

Scatter of Methods for Pb in Blood

Method	Level (μg/100 mℓ)	Standard deviation (μg/100 mℓ)	Number of analyses
Colorimetric	15	4.58	9
ETA-AAS	24.1	4.22	14
AAS	23.3	8.57	23

Modified from Lauwerys, R., Buchet, J. P., Roels, H., Berlin, A., and Smeets, J., *Clin. Chem (Winston-Salem, N.C.)*, 21, 551 (1975).

TABLE 7

Effect of Laboratory Precision in Blood Analysis (μg/100 mℓ)

Element	Sample	Mean (number of analyses)	Range	Interlaboratory coefficient of variation (%)
Pb	C	15.0 (51)	2.7—49.0	52.2
	D	24.1 (52)	10.3—87.3	42.9
	E	23.3(55)	1.0—115.0	77.5
Hg	C	4.4 (18)	1.9—9.4	49.7
	D	2.8 (18)	0.8—11.3	86.6
	E	2.6 (18)	0.2—9.0	80.5
Cd	C	1.4 (14)	0.1—9.2	168.0
	D	1.5 (17)	0—7.3	116.0
	E	1.9 (17)	0—11.0	143.0

Modified from Lauwerys, R., Buchet, J. P., Roels, H., Berlin, A., and Smeets, J., *Clin. Chem. (Winston-Salem, N.C.)*, 21, 551(1975).

number of laboratories in parentheses, followed by the range of values obtained and the interlaboratory percent coefficient of variation. All of the techniques were used for these calculations. The important fact to note is that for all of the analyses the range of the reported results is very large. The authors concluded that several laboratories that took part in this intercomparison program (28 to 93% depending on the metal and the biological medium) have not adequately developed the methodology required for precisely measuring Pb, Cd, and Hg in blood.

One can only conclude that accuracy and precision achieved in the determination of trace elements depends on many factors in addition to the technique used. Obviously, an important factor is the laboratory performing the analysis.

E. Use of Standard Reference Materials

The accuracy and precision of an analytical method can be checked by using the standard reference materials (SRM) now available from some government and private agencies. The success of this approach depends to a great extent on the availability of a variety of these SRMs and the accuracy of their elemental concentrations. A reference material (RM) is defined as a well-characterized stable homogeneous material produced in quantity. These RMs are used primarily to check the accuracy and precision of an analytical method and/or interlaboratory comparison. The material is called

a SRM when the elemental concentrations of the RM are known with a high degree of certainty. The U.S. National Bureau of Standards (NBS) calls their materials SRMs to indicate RMs for which the concentration of certain elements have been certified by them using two or more independent analytical techniques or by two different analysts using a standardized method. Meinke[193] has described the SRM program at NBS for clinical measurements. Uriano and Gravatt[194] have recently discussed the role of RMs and reference methods in chemical analysis. The International Atomic Energy Agency (IAEA) and NBS have taken a leading role in issuing biological SRMs over the last several years. Because of the large amount of time and effort involved, only a small number of elements have been certified to date in a small number of biological matrices.

Another approach used by various issuing organizations is to submit the RMs to round robin analyses and to compile a selected list of best values. Experience with a number of RMs over a long period of time has produced enough analytical data to justify confidence in their values to be used as SRMs. However, these secondary SRMs should be used with caution as compared with the primary SRMs, such as those certified by the NBS.

Biological SRMs are of much recent origin compared with the geological and metallurgical SRMs, which have been available for a long time. Professor A. L. Kenworthy of Michigan State University prepared one of the earliest plant material standards. However, its distribution was limited, and it was not extensively analyzed. The first major preparation and distribution of a biological RM was accomplished by Professor H. J. M. Bowen of Reading University in 1964. Bowen's kale has been widely analyzed since then employing a host of analytical techniques, and he has periodically compiled the "most likely" values for various elements in it.[14,195,196] Bowen[14,196] and LaFleur[197] have discussed the approach used in preparing large amounts of homogeneous and stable RMs for trace element certification in materials such as kale, orchard leaves, and bovine liver, respectively.

In addition to the NBS and IAEA, a few clinical SRMs are now available from commercial establishments certified for a limited number of elements. A partial list of available biological and clinical SRMs is included in Table 8. Frequent use of these SRMs to check the accuracy and precision of any analytical procedure should be the integral part of every trace element analytical laboratory.

IV. CONCLUSION

At this juncture, it is helpful to summarize the key points of the evaluation of the various trace element techniques used for biological analysis.

An important consideration is sample size, which is particularly critical in serum analyses. Of the various methods, ETA-AAS, ISE, and ICP-ES require very small samples, followed by AAS and ASV requiring larger samples.

Another consideration in comparing techniques is the amount of sample pretreatment required. In the case of ES, XRF, PIXE, and SSMS (all of which require larger samples), it is necessary to freeze-dry and ash the biological samples. In the case of INAA, only freeze drying is required. When body fluids are being analyzed, ETA-AAS, ICP-ES, AAS, ASV, and ISE can be used to analyze samples directly; however, some pretreatment is often used with AAS. With other biological tissues, ashing and/or dissolution is a necessary step before measurement with these techniques.

With regard to sensitivity, only ICP-ES, SSMS, and ETA-AAS exhibit excellent sensitivities for serum analysis. Generalized accuracy and precision for the techniques indicate that under ideal conditions ICP-ES and ETA-AAS could produce very satis-

TABLE 8

Biological and Clinical Standard Reference Materials

Issuing organization	Standard reference material	Catalogue no.	Ref.
Bowen, H. J. M., Reading University, U. K.	Kale	—	14, 196
Center for Disease Control, Atlanta	Serum	—	—
Dade Chemicals, Florida	Serum	Labtrol, Monitrol	
Eastman Kodak Co., Rochester	Gelatin	TEG-50	198
Fisher Scientific Co., New York	Human serum	Met-E1-S	—
Hyland Inc., California	Urine and serum	—	—
IAEA, Monaco	Sea plant	SP-M-1	
	Copepod	MA-M-1	
IAEA, Vienna	Blood serum	A-2	199
	Animal bone	A-3/1	199
	Fish soluble	A-6	200
	Milk powder	A-7/1	199
	Corn flour	V-1	—
	Wheat flour	V-2/1	199
	Potatoes	V-4	199
	Pig muscle	H-4	—
Proctor and Gamble Co., Cincinnati	Soybean meal	—	201
Technicon Instruments Corp., New York	Serum	—	—
University of Kentucky, Lexington	Tobacco	IR-1	153, 202
U.S. National Bureau of Standards, Washington, D.C.	Oyster meat	SRM-1565	203
	Brewers' yeast	SRM-1569	203
	Spinach	SRM-1570	203
	Orchard leaves	SRM-1571	203
	Tomato leaves	SRM-1573	203
	Pine needles	SRM-1575	203
	Bovine liver	SRM-1577	203
	Albacore tuna	RM-50	203

factory results. Of all the techniques reviewed, only ASV, ISE, AAS, and ETA-AAS are presently limited to single determinations as opposed to the multielement capability of all of the others.

Finally, we must consider the application of the various techniques to biological samples other than serum. The literature has shown that ES, AAS, INAA, SSMS, and ETA-AA have had broad applicability to a wide variety of tissues. Only limited applications of ASV, ISE, and XRF/PIXE have appeared in the biological field. The main problem is the preparation of the sample for analysis. It should also be noted that the levels of concentration of many of the elements in these diverse tissues are often much higher than in serum.

To summarize, we see that while many trace element techniques are available for biological analyses, on the basis of the criteria of sample size, pretreatment, sensitivity accuracy and precision, and cost, the technique of ETA-AAS offers the greatest opportunity. The technique of ICP-ES holds much promise and in addition provides multielement capabilities; however, this technique is still relatively new and requires further study. AAS has been used to great advantage for biological analyses, but suffers from poorer sensitivity unless preconcentration of samples is employed. INAA continues to be a valuable routine technique, but of more limited availability, and SSMS is a good survey technique, but with the limitation of more involved sample preparation.

As we have seen, there are a number of good trace techniques available. The main problems in the analysis of biological materials are concerned with taking a meaningful

sample, the scrupulous avoidance of contamination during the course of the analysis, proper attention to calibration, and statistical quality control. The methods are available, but they are only as good as as the analyst performing the determination. Unless proper care is taken, the data obtained might at best be of doubtful value and at worst result in erroneous correlations between trace metals and various metabolic or disease processes and, in some instances, even lead to clinical interpretations that are totally unwarranted.

As mentioned in the introduction, the scope of this review is limited to methods for the determination of trace element concentrations. With regard to information on the distribution of these elements in biological systems, interesting histochemical studies are increasingly being performed using the electron microprobe, the electron microscope, and the ion microscope. Thus, the distribution of certain elements in specific tissues have been determined *in situ* and correlated with morphology. These techniques are being used at the present time primarily in research studies and could serve as the basis for another review.

In contrast, little progress has been made in providing information on the speciation or chemical form of the elements in biological systems. The development of instrumental methods to determine coordination states and oxidation states of metals in biological tissues provides a unique challenge to analytical chemists.

ACKNOWLEDGMENT

This work was presented in part as a plenary lecture at the 20th Colloquium Spectroscopicum Internationale and 7th International Conference on Atomic Spectroscopy in Prague, Czechoslovakia, August 30 to September 9, 1977. The assistance of R. A. Nadkarni in the preparation of this manuscript is gratefully acknowledged.

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