APPLICATION AND ASSESSMENT OF ENERGY DISPERSIVE SECONDARY TARGET X-RAY FLUORESCENCE SPECTROPHOTOMETRY TO THE DETERMINATION OF TRACE ELEMENTS IN HUMAN BLOOD AND PLASMA

by

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B.Sc.(Hons.) Simon Fraser University, 1974

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE in the Department of Chemistry

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Application and Assessment of Energy Dispersive Secondary Target X-ray Fluorescence Spectrophotometry to the Determination of Trace Elements in Human Blood and Plasma.

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Abstract:

A photon excitation, energy dispersive, secondary target x-ray fluorescence (EDXRF) spectrophotometer has been applied to determine quantitatively the concentrations of copper, zinc, bromine and rubidium in human blood and plasma. Normal concentration distributions were produced for specimens from 37 healthy adult males, 38 adult females and 36 children. Levels of plasma copper and blood lead measured by EDXRF were verified using flame atomic absorption spectrophotometry and anodic stripping voltammetry, respectively.

The samples for EDXRF were prepared by freeze drying one milliliter of blood or plasma and the formation of this freeze dried material into 50 mg self-supporting pellets. Yttrium, as an internal standard, was added to all samples prior to the freeze drying process. The lower limit of detectability for a range of elements was deduced and analyses of the errors involved in applying this technique were performed.

The measured mean concentrations of Cu, Zn, Br, and Rb in adult males and females were found to be in good agreement with the mean values reported in the literature, by other techniques. Female
subjects taking oral contraceptives showed a definite increase in plasma copper concentration. No distinctive differences (determined via standard statistical analyses) were observed between the levels of trace elements in the plasma of 10 children suffering from cystic fibrosis and those of normal healthy children. Advantages and limitations of the EDXRF technique in providing quantitative, multi-elemental information for medical purposes is discussed.

Search for correlations between trace elements and other variates (age, disease condition) were performed. Some significant correlations, such as that between copper concentration and age (4-12 yrs.), were found. Changes in trace element correlations between groups of individuals were also found.
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1. INTRODUCTION

While the study of trace elements in human biological systems has been pursued for many years (Dav 72, Und 71, Sch 71), the increased awareness of their role in many types of diseases (McC 71, Vil 74, San 73, Sch 74) has stimulated the need for additional statistical information on elemental concentrations in samples from all areas of society. Coupled with this need has been the desire to develop a multielemental method of quantitative analysis which can provide large amounts of statistical data quickly, efficiently, and accurately. A relatively new technique, Energy Dispersive X-ray Fluorescence Spectroscopy (EDXRF) has proven capable of providing such data on a variety of different types of samples (Gia 73, Gia 74, Cal 71, Ale 74). The purpose of this thesis is to apply and assess the application of EDXRF specifically to the analysis of trace elements in human biological samples, on a quantitative basis. The limitations and advantages of this application will be explored and attempts made to introduce the concept of correlation analysis to the elemental profiles obtained on samples from patients with specific medical disorders.

1.1 Role of Trace Elements In the Body

Six elements (carbon, nitrogen, hydrogen, oxygen, phosphorous, and sulfur) are the major building blocks of living matter and account for 99.3% of the total mass. A
second group of elements, sodium, potassium, calcium, and chlorine in ionic form, comprise most of the remainder. These remaining elements serve several functions, one of which is the maintenance of the proper charge distribution and osmotic pressure between cell membranes.

The third and last group are the trace elements. The term 'trace' originated from the inability of early workers to measure their concentrations. Despite the fact that this is no longer so, the term is kept for historic reasons. A more apt term would be essential, and non-essential biological elements, for considerable knowledge is now available concerning their roles and modes of action. These elements function primarily as catalysts in enzyme systems to either maintain active sites, bridge ternary compounds or change the electronic structure of substrate molecules (Dav 72). It is not surprising therefore to find that many metabolic disorders in man are accompanied by alterations in the concentration of one or more trace elements in some body fluid, especially blood, serum, or plasma (McC 71). There are two groups of catalysts with metal association: metallo-enzymes in which the metal is an integral part of the enzyme protein, and metal-activated complexes in which the metal ion is simply an external activator of the enzyme.

There are several hundred metallo-enzymes and metal-activated complexes known but only certain of these, such as zinc and copper metallo-enzymes, will be discussed for illustrative purposes. Some representative zinc metallo-enzymes
are carbonic anhydrase, first isolated in 1934, carboxypeptidase, alcohol dehydrogenase, and alkaline dehydrogenase (Li 66). Zinc activated enzymes include arginase, histidine deaminase, lecithinase, α-mannosidase, and glycyl L-leucine dipeptidase (Val 62). Zinc is involved in the synthesis of RNA and thus is essential for the growth and propagation of all cellular material. This element also plays an unknown role in insulin and porphyrin metabolism (Dav 72).

Copper is an essential constituent of several proteins, metallo-enzymes and some naturally occurring pigments. The main proteins for which copper is essential are hepatocuprein, hemocuprein, erythrocuprein, and cerebrocuprein I (Dav 72). The main enzymes for which copper is essential in mammals are ceruloplasmin, tyrosinase, amine oxidase, cytochrome oxidase, uricase, and dopamine β-hydroxylase (Dav 72). The function of the enzymes and proteins which contain copper as an essential constituent has not been elucidated in every case.

This is by no means a complete list of the essential biological metals, indeed, we may expect to discover numerous other metal-mediated reactions and enzymes provided we are armed with fast, reliable and sensitive techniques for analysis of trace elements of interest.

Concomitant with the discovery of the existence of essential elements in minute concentrations in biological material, was the finding of trace elements which were considered non-essential and served no obvious purpose within the body. These non-essential metals, such as lead and
mercury, are not under homeostatic control as are the essential trace elements, and their concentrations in the body appear to be dependent upon the rate of their ingestion which arises from agricultural, chemical, and industrial pollution. At high concentrations, normally below that of the essential elements, the non-essential elements may be extremely toxic. Everyone comes in contact with lead since it can be found in such familiar sources as paint pigments, pottery, ink, and gasoline. Mercury, on the other hand, is used mainly as an active ingredient in fungicidal agents. Before these two elements were recognized as possible hazards to man, numerous deaths and poisonings from these toxic elements did occur (Ano 59).

There are other non-essential trace elements which not only become toxic at high concentrations, but at low concentrations seem to have some capacity to appropriate essential trace elements in the body. As an example, bromine can function in place of chlorine to help maintain electrolytic balance (Und 71). Another element, which can take the place of potassium, and as yet has no known function in the regulation of human health and nutrition, is rubidium. Interest in this element has been focused primarily on its relationship to potassium in influencing the resting potential of nerves and muscles, particularly the heart (Pra 72).
1.2 Metabolism of the Essential Biological Metals: Copper and Zinc

The elements copper and zinc are obtained through the diet in varying concentrations. As each has a vital physiological role, the extent of their absorption, metabolism and excretion is under strict homeostatic control, and therefore their concentration in the body remains relatively constant under "normal" conditions of health.

All areas in the study of absorption and excretion of these elements have not been fully elucidated. For example, although it is assumed that copper enters the portal blood from the stomach and upper small intestine (Eva 73), and zinc enters mainly through the duodenum (Pra 66), little is known in man of the modes of transport of these elements across the intestinal walls or from the portal blood to the liver. The major excretory pathway of copper is that of the bile and urine (Eva 73), whereas zinc is excreted via the small intestine and pancreas (Und 71). In some diseases these systems may malfunction; therefore changes in the concentrations of trace elements, from the relatively constant level during health, may reflect the changes in activity of these systems.

1.3 Selection of Human Biological Material Analyzed

The selection of the human biological material to be used in this study is an important consideration if the versatility of the EDXRF technique for multielemental analysis is
to be properly assessed and demonstrated. Since all living matter contains trace elements, the best choice would be the material in the body which is easy to obtain and analyze and displays some variations in trace element concentrations during disease. Human blood and serum or plasma fulfills some of these requirements.

Each organ within the human body has the capacity to store and utilize elements to varying degrees. As an indication of the variability of the levels of the trace elements in the body, Anspaugh (Ans 71) has compiled the average concentration levels of seventy-three elements in forty-five healthy organs and tissues in the human body. He has then compared these levels with the concentrations found in patients with one hundred forty-three diseases, syndromes and conditions. Since definite correlations do exist, determinations of the levels of trace elements in specific organs may yield information on the status or identification of the associated disease or the degree of damage to that particular organ as a result of the disease. Since neither time nor circumstance normally allow analysis of living tissue in an otherwise healthy organ, the more easily accessible and routinely examined bodily fluids such as blood, plasma or serum, and urine are obvious choices for consideration. Although blood may not represent the ideal medium, since the levels of trace elements are generally lower than the levels in other organs, e.g., the liver, it can still yield considerable information.
Blood is the central homeostatic exchange fluid between all organs in the body and variations of the metabolic balance between the internal organs and systems can be observed from trace element determinations (compare with Section 1.2).

Whole blood is composed of two major fractions, cellular (erythrocytes and leukocytes) and extracellular (known as plasma). There are some distinct differences in trace element levels for these two fractions because of the differences in function discharged. Thus, the concentration of potassium, the major element which maintains osmotic pressure and the proper charge distribution within the cells, is 22.7 times greater inside the cell than outside. As potassium shares some of the properties of rubidium, rubidium levels in the red cells are concentrated up to eight times those found in plasma (Ans 71, Woo 70, Und 71). The opposite situation exists for sodium, for which the cell levels are one-tenth those of the plasma. Two of the essential biological metals, iron and zinc, function primarily within the red cell where they maintain oxygen and pH balance. The concentrations of these elements (Fe and Zn) are greater within the cells than in the plasma. The red cells are dynamic, living systems, which maintain constant levels of metabolites and elements, so therefore, variations in the levels of trace elements in the cells are generally much less than those in plasma.

The protein-salt solution which carries the red cells in the blood stream is known as the plasma. Proteins occur in the plasma in concentrations ranging from 6.8 to 8.8 grams
per one hundred milliliters of solution (Tie 70). In serum, which is plasma lacking fibrinogen, the protein levels are decreased by 0.2 to 0.4 grams percent. The plasma proteins play a role in nutrition as they represent a storage for amino acids. In addition, these proteins also act as important transport agents; many vital metabolites (metal ions, hormones and lipids) are transported throughout the body, bound to specific carrier proteins. For example, transferrin transports the majority of iron in the plasma (Und 71) and ceruloplasmin transports 60-99% of the total plasma copper. Further, zinc has been discovered to exist in the plasma both loosely bound (34%) and firmly bound (66%) (Pra 66); the latter presumably associated with carrier protein in plasma.

Urine may also be a potentially valuable biological fluid as an aid in diagnosis and treatment of numerous trace element related disorders. However, urine was not examined in this study. Since most elements in urine are found in about one-tenth the concentration of that in plasma, different sample preparation methods than those used for blood are probably necessary. Many diseases with manifested changes in trace element concentrations in the body, which are reflected in the serum or plasma, are not necessarily reflected as changes in the levels of excretions of these elements in the urine. Thus, in order to undertake a thorough study of XRF applied to trace element levels in urine, a completely separate study would be necessary.

Because blood is a complex mixture of components with
different trace element contents, it is often desirable to measure separately trace element levels in both blood and plasma from the same individual. In order to do this, anti-coagulants must be added to the specimen immediately after it is drawn. Several anti-coagulants are available and final selection for their use in trace element analysis is based upon their modes of action, quantity necessary to inhibit clot formation, and possible extent of contamination by trace elements. The most common anti-coagulant, oxalate, inhibits coagulation by forming insoluble complexes with calcium ions (Tie 70). Therefore, this anti-coagulant cannot be used for analysis of divalent cations such as calcium. Another anti-coagulant, ethylenediaminetetraacetic acid (EDTA), acts in a similar manner to the oxalates, by complexing calcium ions in blood; however, the probability of this chemical complexing other free metal ions in blood is rather high. In addition, "practical" and "technical" grades of anticoagulants are available and sometimes contain significant amounts of trace cations (Rob 72). The anticoagulant of choice for trace metal determinations in this study is heparin, a naturally occurring anticoagulant which acts as an antithrombin. Heparin prevents the formation of prothrombin and thus prevents the formation of fibrin from fibrinogen. Heparin has been chosen for use in trace element analysis because it is available in a very pure form and is more potent than the other anticoagulants.
Trace Element Concentration Levels in Blood and Serum/Plasma: Clinical Significance

It is important for a successful completion of the primary aim of this study to have knowledge of the "normal" trace element concentration levels expected in blood and plasma/serum. Further, it is also of considerable interest from a clinical diagnostic point of view to be aware of the variations of these levels that have been observed as a function of particular medical disorders. For these reasons Table I has been prepared from information available in the literature for the elements iron, copper, zinc, bromine, rubidium, and lead. The choice of only these elements was predicated by the fact that these were observable with the EDXRF technique employed herein. It should be emphasized that there are other elements of importance from a clinical point of view, and some of these, such as chlorine, potassium and calcium can also be observed with the assessed technique. These other elements were not determined quantitatively because they are done routinely and easily by other methods.

Elemental concentrations, generally measured in serum or plasma where the major transport proteins occur, can vary significantly from the normal concentrations, and in different amounts, depending on both the type and severity of the disease or condition. Tietz (Tie 70) defines 'normal value' or 'normal concentration' as "that amount of the constituent
TABLE I

Trace Element Levels in Various Disorders*

<table>
<thead>
<tr>
<th>Element</th>
<th>Condition</th>
<th>Whole Blood</th>
<th>Serum (s) Blood</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>Normal male</td>
<td>127(67-191)</td>
<td>Und 71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal female</td>
<td>113(63-202)</td>
<td>Und 71</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>Normal adults</td>
<td>98±13</td>
<td>109±17 (p)</td>
<td>Und 71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100(77-106)</td>
<td>97 (s)</td>
<td>Ans 71</td>
</tr>
<tr>
<td></td>
<td>Adult male</td>
<td>123±23  (s)</td>
<td>Sin 70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult female</td>
<td>113±19  (s)</td>
<td>Yum 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
<td>113±20  (s)</td>
<td>Yum 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>169</td>
<td>Und 71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oral contraceptive</td>
<td>204±11 (s)</td>
<td>Sin 70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Children 6-12 years</td>
<td>132±23 (s)</td>
<td>Tes 73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acute leukemia</td>
<td>195</td>
<td>236 (p)</td>
<td>Und 71</td>
</tr>
<tr>
<td></td>
<td>Chronic leukemia</td>
<td>148±30    (s)</td>
<td>Sin 70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hodgkins disease</td>
<td>142</td>
<td>171 (p)</td>
<td>Und 71</td>
</tr>
<tr>
<td></td>
<td>Diabetes mellitus</td>
<td>155±44    (s)</td>
<td>Sin 70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arteriosclerotic dis.</td>
<td>156±49    (s)</td>
<td>Sin 70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe deficiency anemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>149±43    (s)</td>
<td>Sin 70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infants</td>
<td>168</td>
<td>Und 71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wilson's disease</td>
<td>79</td>
<td>55 (p)</td>
<td>Und 71</td>
</tr>
<tr>
<td></td>
<td>Nephrosis</td>
<td>70</td>
<td>80 (p)</td>
<td>Und 71</td>
</tr>
<tr>
<td></td>
<td>Menkes Kinky Hair</td>
<td>27 (s)</td>
<td>Eva 73</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>Normal adults</td>
<td>740(480-906)</td>
<td>104(80-128)</td>
<td>Ans 71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>880±20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal male</td>
<td>107±14</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young males</td>
<td>142±24</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal female</td>
<td>95±15</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young female</td>
<td>132±21</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnant</td>
<td>103±36</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pernicious anemia</td>
<td>98±15</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>84±26</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>64±19</td>
<td>Pr 66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetes mellitus</td>
<td>117±47</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decubitus ulcer</td>
<td>74±22</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downs Syndrome</td>
<td>98±16</td>
<td>McB 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acrodematitis</td>
<td>35 (s)</td>
<td>Str 75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enteropathica</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br</td>
<td>Normal adults</td>
<td>385(372-392)</td>
<td>478(412-491)</td>
<td>Ans 71</td>
</tr>
<tr>
<td>Rb</td>
<td>Normal adults</td>
<td>150</td>
<td>&lt;28 (s)</td>
<td>Ans 71</td>
</tr>
<tr>
<td>Lead</td>
<td>Normal adults</td>
<td>&lt;50</td>
<td></td>
<td>Tie 70</td>
</tr>
</tbody>
</table>

* in some cases only the mean concentration has been reported
** Based on 9 year old children from regression equation

mean concentration = (159 - 2.96 x age) µg/100 mls.
of interest which is found in the body fluid or excretions of a group of clinically normal (apparently healthy) persons". 'Apparently healthy' individuals are those considered to be in a state free of any obvious or overt abnormalities.

An analytical technique must be sufficiently sensitive to allow determinations over the wide range of expected variations from the 'normal' concentrations. Of particular importance to the EDXRF system, which is a multielemental method, is that certain diseases may be characterized by different concentration changes for the elements studied. For example, different relative increases in both the zinc and copper levels in serum from patients with diabetes mellitus has been observed (Ros 68), and there is an apparent correlation between serum copper levels and Hodgkins' disease (Hrg 73). The absence of measurements, especially for whole blood, should also be mentioned as a potential area for future studies.

In general, changes in the normal levels of trace elements in the body reflected in the blood system may also originate from changes in diet and environmental exposure, as well as a function of the sex and age of the individual. Zinc deficiency can be caused in some cases by high intake of calcium which apparently promotes an abnormal excretion of zinc (For 60). Levels of copper in plasma/serum on the other hand, which generally increase for many diseases, have been observed to increase significantly in subjects employing oral contraceptives (Sch 72, Hor 75).

As displayed in Table I, there have also been
differences observed for certain elemental concentrations between male and female subjects as well as changes that occur with age. Children of ages from 6 to 12 years have been shown to exhibit a decreasing copper concentration in serum with increasing age (Tes 73). Some data also exist showing a significant increase in serum copper concentrations with age in adult males but not adult females (Yum 74). Conflicting information exists for zinc levels although recent reports (Yum 74) find indistinguishable changes. Clearly this area of possible correlations with age is one which could lend itself well for EDXRF studies. In any event it should be emphasized that although some correlations have been observed for trace element levels and age (Tes 73, Del 73), this information can be difficult to define unambiguously.

The last two elements of interest in this study, bromine and rubidium, being considered non-essential, have had few clinical correlations demonstrated in various conditions in the human body. They are also difficult to measure experimentally. The levels of bromine in human blood and plasma are surprisingly uniform and average 385 (372-395 μg/100 mls) and 478 micrograms per one hundred milliliters, respectively (Ans 71). Apparently, the determination of bromine levels was undertaken only in problems related to drug abuse since bromine containing drugs were used in some cases for the purposes of sedation.

Rubidium, as RbBr, was introduced into human therapy
in the 1880's as a treatment for epilepsy. Recently, some interest in rubidium therapy on depressed patients has developed (Fie 73). No clinical correlations involving this element in blood and plasma have yet been observed, however.

1.5 Analytical Methods for Trace Element Analysis

In the past two decades there have been several methods developed which are capable of analysis of trace elements in concentration ranges of parts per billion and parts per million. These methods, which include atomic absorption spectroscopy (AAS), anodic stripping voltammetry (ASV), mass spectrometry, energy dispersive x-ray fluorescence (EDXRF), neutron activation analysis (NAA) and wavelength dispersive x-ray fluorescence (WDXRF), have been used in a range of analytical applications as varied as: detection of mercury in air, vegetables and animals; lead in canned food; arsenic in semiconductors; rubidium in rocks; and copper in the serum of diseased subjects. Such analyses have become important in the detection and assessment of contaminants in pollution studies and the detection of trace elements in connection with human health, nutrition and disease.

Since the development of the high resolution semiconductor detectors, EDXRF has found exceptionally wide applications (Tac 73, Gia 74, Bil 72, Gre 73, Leo 74, Rho 72, Coo 73, Cam 74, Cal 71, Ale 74). XRF measurements have advantages over other analytical devices because XRF is non-destructive, fast, and multielemental and could therefore easily be used
for the production of trace element profiles for assessment of numerous diseases (Wol 73).

This study is devoted primarily to the application and assessment of EDXRF to the quantitative determinations of trace elements in human biological specimens. The principles of this technique will be presented followed by a general discussion of the other techniques utilized to verify the results obtained by FDXRF.

1.51 X-Ray Fluorescence
Basic Principles

There are many excellent reviews available which discuss the principles of XRF (Gia 73, Gou 74, Rus 72, Ber 75, Por 73). Let it suffice here to delineate some of the major features of this technique.

Elemental analysis in the XRF technique relies on the measurement of the energies and intensities (for quantitative analysis) of the emitted characteristic x-rays when vacancies are created in the inner electron shells (primarily K and L shell) of the elements in the sample of interest. These vacancies are created through the interaction of either x-rays, low energy gamma-rays, or charged particles (alphas and protons).

In most commercial XRF systems and in the system available at S.F.U., a primary beam of continuous energy x-rays is generated through the bombardment of an anode with an electron beam of variable current. This generated continuum is then allowed to excite the sample directly or to strike an
intermediate target of choice. This latter method, which is preferred when low backgrounds are necessary, leads to a production of nearly monochromatic radiation. Often an appropriate metallic foil is placed between this secondary target and the sample to act as a filter. This secondary beam is then allowed to interact with the specimens.

The choice of an appropriate secondary target is predicted by the need to have the energy of the exciting photon radiation only slightly greater than the absorption-edge energy, $K_{\alpha_b}$, of the elements of interest. For this analysis of biological specimens a Mo target was used since its $K_{\alpha}$ radiation (17.5 keV) was above the $K_{\alpha_b}$ energy of iron (7.1 keV), copper (9.0 keV), zinc (9.7 keV), bromine (13.5 keV), rubidium (15.2 keV) and other elements of potential medical interest. The highest number of vacancies occurs as the energy of the $K_{\alpha_b}$ approaches the excitation energy (Wol 73a).

Given appropriate excitation conditions, vacancies in the inner electron orbits can lead either to the emission of x-rays characteristic of the element (fluorescence) or to the emission of Auger electrons. These competing processes vary as a function of the atomic number, $Z$, of the element, with the fluorescence process dominating at higher values of $Z$. These have been well studied in the past, and the reader is referred to any standard text on atomic physics for a more complete discussion (Ber 75).

For elements whose $K_{\alpha_b}$ energy does lie lower than the excitation energy, the most intense x-rays emitted correspond
to electronic transitions between the K and L shells (labelled $K_{\alpha}$ lines) and between the K and M shells (labelled $K_{\beta}$ lines). The observed relative intensities ($K_{\alpha}:K_{\beta}$) are a function of the Z of the element and are of the order of 25:1 for aluminum and 5:1 for copper (Lie 72). For elements whose $K_{ab}$ energy lies above the energy of the excitation source, the intensely observed x-rays correspond to transitions between $M_{V}$ and $L_{III}$ levels ($L_{\alpha_{1}}$), $M_{IV}$ and $L_{II}$ levels ($L_{\beta_{1}}$), and $N_{IV}$ to $L_{II}$ levels ($L_{\gamma_{1}}$); the relative intensities of these lines is approximately 5:4:2. In summary, elements of middle and low Z are identified through the observation of their K lines while elements of high Z through their L lines.

Energies of the x-rays, expected from typical trace elements in a biological specimen are presented in Table II (Ber 75).

There are two basic methods whereby x-rays emitted by a sample are dispersed, so that they may be isolated and measured. The two methods of dispersion are distinguished as wavelength dispersion and energy dispersion. In wavelength dispersive spectrometers the x-rays from the specimen are highly collimated and dispersed according to the Bragg law by a diffracting crystal before being detected at the angle corresponding to the characteristic wavelength to be measured. The usefulness of this method is quite limited as compared to that of energy dispersive XRF because the radiation originating from the sample must be diffracted and collimated after leaving the sample. Further, these processes (diffraction and collimation) ultimately lead to increased absorption and
TABLE II

Emission Energies of Some Elements in keV

<table>
<thead>
<tr>
<th>Element</th>
<th>$K_{\alpha_1}$</th>
<th>$K_\beta$</th>
<th>$L_\alpha$</th>
<th>$L_\beta$</th>
<th>$L_\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>2.308</td>
<td>2.46</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cl</td>
<td>2.622</td>
<td>2.82</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>K</td>
<td>3.313</td>
<td>3.59</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ca</td>
<td>3.691</td>
<td>4.01</td>
<td>0.341</td>
<td>0.344</td>
<td>--</td>
</tr>
<tr>
<td>Fe</td>
<td>6.403</td>
<td>7.06</td>
<td>0.704</td>
<td>0.717</td>
<td>--</td>
</tr>
<tr>
<td>Ni</td>
<td>7.477</td>
<td>8.29</td>
<td>0.849</td>
<td>0.866</td>
<td>--</td>
</tr>
<tr>
<td>Cu</td>
<td>8.047</td>
<td>8.93</td>
<td>0.928</td>
<td>0.948</td>
<td>--</td>
</tr>
<tr>
<td>Zn</td>
<td>8.638</td>
<td>9.62</td>
<td>1.01</td>
<td>1.03</td>
<td>--</td>
</tr>
<tr>
<td>Br</td>
<td>11.92</td>
<td>8.38</td>
<td>1.48</td>
<td>1.53</td>
<td>--</td>
</tr>
<tr>
<td>Rb</td>
<td>13.40</td>
<td>15.0</td>
<td>1.69</td>
<td>1.75</td>
<td>--</td>
</tr>
<tr>
<td>Y</td>
<td>14.96</td>
<td>16.9</td>
<td>1.92</td>
<td>2.00</td>
<td>--</td>
</tr>
<tr>
<td>Pb</td>
<td>74.96</td>
<td>84.9</td>
<td>10.5</td>
<td>12.6</td>
<td>14.8</td>
</tr>
</tbody>
</table>

*The excitation source used in these studies is bichromatic Mo $K_\alpha$ (17.5 keV) and Mo $K_\alpha$ (20.0 keV) radiation.*
scatter. An advantage of wavelength dispersive XRF (WDXRF) over energy dispersive XRF (EDXRF) is that higher resolution for characteristic lines can be obtained.

In EDXRF studies, the system used herein, the recent introduction (Bow 66) of the use of Si(Li) x-ray spectrometers to detect the energies of any incident photons in an integral fashion, has increased tremendously the versatility of the analytical technique. These detectors essentially generate an electrical output pulse whose height is proportional to the energy of an incoming photon. Unlike WDXRF, which is limited to analyzing one line at a time, these solid state detectors allow measurement of many photons in a single determination and have made EDXRF truly a multielemental technique. Thus, this technique is applicable to elements from sodium to uranium, although in most commercially available systems, detector resolution and window thicknesses limit analyses to elements with atomic numbers greater than that of aluminum.

1.511 Matrix Effects and Sample Size

For an understanding of the concept of 'thickness' in XRF analysis, the following discussion is presented.

Two processes which affect XRF spectrophotometric determinations to the greatest extent, especially for quantitative determinations, are the absorption and scatter of incoming and outgoing radiation in the sample. If a perfectly collinear monochromatic x-ray beam of intensity $I_0$ is directed on an absorber at an angle $\theta$ having uniform thickness $x$ (cm)
and density $\rho$ (g/cm$^2$) (see Figure 1A, Appendix I), the emergent beam will have an intensity $I$, given by the Lambert law: (Lie 72)

$$I = I_0 \exp(-\lambda \rho x \csc \theta)$$

(1)

where $\lambda$ is the mass absorption coefficient in cm$^2$/g. Thickness can also be expressed in an area concentration (g/cm$^2$) which is the product of $x$ and $\rho$.

In the above simple case, the incident beam is attenuated on passing through an absorber. In the case where a monochromatic x-ray beam is used to excite trace atoms (e.g., Cu) in a sample, and the characteristic x-rays of these atoms (e.g., $K_\alpha$) are collected on the same side of the source, a more complex relationship results. This relationship has been determined as:

$$\frac{I_d}{I_0} = \frac{\lambda_1 \alpha I_0 (1-\exp(-\lambda \rho x \csc \theta))}{\lambda}$$

(2)

where $I_d$ = intensity of x-ray detected from excited element
$I_0$ = intensity of exciting radiation bombarding sample from same side as that detected
$\lambda$ = total of $\lambda_1$ and $\lambda_2$
$\lambda_1$ = mass absorption coefficient for incoming radiation
$\lambda_2$ = mass absorption coefficient of outgoing radiation
$x$ = thickness of sample (gm)
$\theta$ = angle between collimator and sample plane
$\alpha$ = excitation constant *

(see Appendix I for derivation)

* The excitation constant, $\alpha$, is a complex function of the proportion of x-rays detected from those excited and involves photo-electric cross sections, fluorescence yield, solid angle into the detector, detector efficiency and absorption in the Be window of the detector.
According to this relationship, as the sample thickness increases the Cu Kα x-ray intensity (for example) will increase at a continuously decreasing rate until an 'infinite' thickness is reached where the increase in x-ray quanta reaches a maximum, asymptotically. This infinite thickness can be calculated by placing $x = \infty$ in (2). Thus,

$$\bar{I}_\infty = \alpha \lambda_1 I_0 / \lambda$$  \hspace{1cm} (3)

where $\bar{I}_\infty$ is the intensity of the emitted characteristic line under this condition. Dividing equation (3) by (2), we have the intensity ratio:

$$\frac{\bar{I}_d}{\bar{I}_\infty} = \frac{1 - \exp(-\lambda x \rho \csc \theta)}{\alpha} \hspace{1cm} (4)$$

As $\bar{I}_d/\bar{I}_\infty$ approaches unity, the curve reaches a maximum value, called the infinite thickness. Figure 1 shows a plot of $\bar{I}_d/\bar{I}_\infty$ versus the mass of the absorber assuming that the absorber has a mean atomic number of 6 (i.e., carbon matrix) and the exciting radiation has the energy of the Mo Kα line. The 'infinite' thickness may be deduced from this plot. The mass of absorber for phosphorous, iron and yttrium using a pellet press which produces samples of radius $r = 6.5$ mm, is about 20, 150 and 1200 mg respectively.

The concept of infinite thickness is extremely important in XRF analysis since no increase in signal is observed above this thickness. There is therefore no necessity in preparing samples of greater mass than this limit. A further limitation on sample size is caused by the often small quantity of biological samples available for analysis.

An infinitely thin sample is also impractical.
FIGURE 1

Relationship Between Intensity Ratio and Sample Mass
Although absorption effects are extremely small, and in the infinitely thin region there is a direct relationship between sample thickness and intensity of fluorescent x-rays, the signal is usually so small that extremely long count times are necessary to obtain statistically significant peaks. In addition, infinitely 'thin' samples will be extremely fragile and difficult to handle. The most practical thickness for trace element analysis in terms of maximum fluorescence, minimum absorption, and mass of material available is probably the thickness shown in the intermediate exponential region of Figure 1.

Coupled with the effects of fluorescence and absorption are the effects of the Compton (inelastic) and Rayleigh (elastic) backscatter of the source radiation. As the thickness of the sample increases so do the x-rays and background effects originating from this scattering process. As the flux of x-rays reaching the detector increases, there is an increasing inability of the electronic apparatus to process all of the incoming x-rays at one time with a subsequent decrease in the signal to noise ratio. It follows therefore, that there exists an optimum thickness where signal-to-background ratio is a maximum and absorption and scatter effects are minimized. This optimum thickness can be determined by appropriately designed experiments and is expected to occur in the intermediate region shown in Figure 1.

A striking feature of Figure 1 shows that in the range of thickness that represents the best choice for quantitative analysis, a small variation in thickness produces a large
variation in intensity. In addition, the intensity of the characteristic radiation from separate elements varies in different proportions depending on the thickness of the samples analyzed. Thus, for quantitative analysis, the thickness of the samples must remain constant.

There are two ways to relate the intensities of characteristic lines to the absolute concentrations in the samples analyzed. The first requires the knowledge of initial intensity, absorption, interelement effects, geometry, detector efficiency, fluorescence yield, and photoelectric processes (Gia 73, Jen 67). This method requires determination, experimentally, of some of the above terms and does not account for nor compensate for absorption-enhancement, inhomogeneity and long term instrument drift for all types of specimens.

To avoid the problems associated with calculating concentration directly from intensity, a second method involves the development of response functions by the method of standard additions (Ber 75) with the use of an internal standard. The standard addition method involves the addition of a standard to increase the elemental concentration by known proportions. The intensities of unknown samples are then compared to the intensities observed while using these standards. An internal standard is also added to all specimens during sample preparation and the intensities of the element of interest are reported relative to the intensity of the internal standard. The internal standard method compensates for absorption, instrument drift, and homogeneity effects since the x-rays from
the standard will vary in direct relation with the variation of the characteristic x-rays. An intensity of about 100,000 counts per minute is necessary to fluoresce a trace element such as zinc at the rate of about 60 counts per minute. Thus, interelement effects, such as enhancement, may safely be ignored while analyzing trace elements which produce photopeaks at this low rate.

1.512 Sample Preparation Methods

The method used for preparing blood and plasma/serum specimens for analysis by XRF is obviously quite important, and a final selection must be guided by such criteria as ease of handling, minimization of introducing contaminants, increasing the concentration of the trace elements in the samples, minimizing sample thickness to reduce matrix effects, allowing for the possibility of re-analysis, storage capabilities, and utilizing as small an initial specimen as possible, etc. A variety of different techniques, both for blood samples and related liquid specimens, have potential, but the general techniques evaluated and utilized in this study were direct spotting onto filter paper, freeze-drying and pelletizing the lyophilized powder, and dry ashing or wet ashing of the wet specimen.

In general, these techniques are in essence concentrating part of the specimen by removing water which constitutes about 50 to 90% of the mass. Filter paper spotting (Pfe 54) was included for its obvious simplicity although this method may prove to be inadequate as the elements may not be sufficiently concentrated to be detectable. Lyophilization has been attempted elsewhere (Lub 72) to a limited extent
while the additional step of pellet formation was incorporated by Woldseth (Wol 73) and Giaque, et al. (Gia 73). This technique appears to have great promise although extensive analytical studies were not performed. The use of ashed samples is not new as this has been incorporated in both proton induced (Bea 74) and photon (Ong 71) induced XRF studies but these again were not thoroughly assessed nor were the results thoroughly verified.

In general, pelletizing a sample does concentrate the trace elements in a specimen, however, this process also concentrates the sample matrix (carbon). This in turn leads to an increase in the background scatter radiation and through absorption, a reduction of the signal. These processes ultimately effect a net reduction in the signal to noise ratio. Removal of such interferences can be accomplished by removal of the matrix through an ashing procedure, however such methods are known to volatilize certain elements (Gor 70) and possibly introduce contamination.

As mentioned earlier, a prior separation or concentration step may prove to be necessary. Sometimes these manipulations can lead to a loss of sample and again introduce contaminants. In general, contaminants can be present in the standard samples used in calibration, reagents used for sample preparation prior to analysis and in the apparatus, such as glassware. Because of the rough surface structure of glassware, it is particularly prone to contamination. Some elements, such as lead, may be leached from its surface and some elements
in very low concentration may be adsorbed to varying proportions on its surface.

In order to develop a reliable and accurate method for trace element analysis, the utmost care, both in handling and choice of materials must be made. In addition, the manipulative procedures used in preparing the samples must be kept to a minimum. Choice of the preparation method depends to a large extent on the analytical capabilities of the instrument. Since energy dispersive XRF is a relatively new addition to the trace element field, an assessment of the best sample preparation method, while considering the basic principles of XRF analysis, must be made.

1.513 Limits of Detection

In the development of a new trace element analysis technique one of the most important determinations for comparison against other established techniques is evaluation of the lower limit of detectability (LLOD). The LLOD may be defined in several ways, but the most generally accepted is the amount of analyte that gives a net peak intensity equal to 3 times the standard counting error of the background intensity (Gou 74, Rho 72). Since the counting error is approximately equal to the square root of the number of counts in a peak, the LLOD is simply:

\[ \text{LLOD} = \frac{(3\sqrt{N_b})C}{N_C} \]  

(5)

where \( C \) is the amount of analyte which gives a total of \( N_C \) counts in the spectral peak. \( N_b \) is the total counts in the
background below the spectral peak.

When a distinct spectral peak occurs in the background spectrum, this must also be taken into account in the evaluation of LLOD. To achieve this, the observed area of such background peaks, \( N_a \), and the statistical fluctuation of such peaks (\( 3\sqrt{N_a} \)) are included in the LLOD as:

\[
\text{LLOD} = \left( N_a + 3\sqrt{N_a} + N_b \right) \frac{C}{N_C}
\]  

(6)

1.514 Precision

Precision, which can be considered the closeness of approach of replicate values to a common value, is one indication of a technique's value as a practical analytical method. If a normal distribution of random errors occurs, the standard deviation can be used to express the precision of the set of measurements. The standard deviation observed is often made up of individual variation and errors from various sources which should be evaluated separately in order to determine the precision of the technique under study (Buc 74). So long as the individual variables are independent and distributed according to a Gaussian distribution, the standard deviation \( S \) can be combined in quadrature as follows:

\[
S_{\text{observed}} = \left( S_i^2 + S_{ii}^2 + \ldots S_n^2 \right)^{1/2}
\]  

(7)

In the methods used in XRF spectrometry these variations were considered to arise from:

i) counting statistics, \( S(\text{count}) \):

The error associated with the statistical
fluctuation of the number of counts in the peak.

ii) drift, $S(\text{drift})$:

These errors are due to variations in voltage and current settings in the x-ray tube and electronic components of the apparatus.

iii) sample preparation, $S(\text{prep})$:

Variations in any stage of the manipulative procedures which make up the sample preparation steps may result in wide variation in the results:

iv) placement, $S(\text{place})$:

Since the fluorescent and scattered x-rays from the sample are collimated to an area much smaller than the sample itself, slight inhomogeneities in the sample may introduce a measurable error.

Applying equation (7), the total observable error, which is a composite of these individual independent errors, can then be represented as:

$$S_{\text{obs}} = (S(\text{count})^2 + S(\text{drift})^2 + S(\text{place})^2 + S(\text{prep})^2)^{1/2}$$

(8)

For convenience $S$ can be replaced by the relative standard deviation, $\varepsilon (=S/N \times 100$ where $N$ is the mean of a set of measurements). It is useful to the assessment of this technique, as applied to biological samples, to evaluate each individual error above in separate experiments. Unfortunately some of these, e.g., the preparation error, can not be measured in isolation but rather only through experiments involving a combination of errors. Manipulation of equation (8) is thus
necessary to estimate these.

The counting error may be estimated by the square root of the total number of counts accumulated in the spectral peak. In the case where an internal standard is used, as mentioned earlier, and the peak area of this standard used for normalization, the relative counting error will involve the combination of errors for both peaks. When a background peak exists and is stripped out of the spectra after the normalization process, the relative standard counting error is a composite of the errors associated with the peak area of the standard, analyte and background as follows:

\[ \varepsilon_{\text{count}} = \left[ (N_u + N_b)(N_u - N_b)^{-2} + (N_{ST})^{-1} \right]^{1/2} \]  

(see derivation in Appendix IV)

\( N_u \) denotes the total peak area of the analyte; \( N_b \) background area and \( N_{ST} \) the area of the standard.

The accuracy of the obtained final concentration levels will be estimated by verification of some of the measurements using other analytical techniques and will be discussed below.

1.6 Verification (Accuracy)

This study is devoted primarily to the application and assessment of the XRF technique to the quantitative determination of trace elements in biological material. However, other established techniques are of interest for the comparison of results obtained by XRF. These methods differ substantially in their basic principles and are each suited to measurement of certain elements in particular matrices and in specific
concentration ranges. For example, a particularly sensitive analytical instrument, the mass spectrometer, has been eliminated as a potentially useful comparative method because of the organic interferences which result when analyzing biological material. Probably one of the most sensitive techniques for a large number of elements is neutron activation analysis. It too has its limitations and was not further considered in this study. The product is radioactive, and in some cases such as the analysis of lead, the sensitivity is too low (due to low probability of neutron capture leading to radioactivity production) for useful measurements in trace concentration ranges around those found in biological systems (about 0.1 to 0.5 ppm). Atomic absorption and anodic stripping voltammetry, on the other hand, are particularly well suited for analysis of trace elements in biological fluids. These methods are very sensitive for many elements, very small sample sizes can be used, and matrix interferences can be minimized. Thus, these two techniques were used to check the results of XRF measurements in blood plasma. The principles of these techniques are to be covered separately.

1.61 Atomic Absorption

The atomic absorption spectrophotometer (A.A.) has proven to be the most useful and valuable instrument so far for trace element analysis in a wide range of materials. Analysis of trace elements via A.A. is based on the measurement of the absorption of resonance radiation by free atoms
in the ground state.

The atomic absorption spectrophotometer is capable of analyzing at least 14 elements of biological interest at concentrations in aqueous media of 0.5 to 0.003 μg/gram and sensitivities of 0.2 to 0.01 μg/gram per 0.01 absorbance. Reproducibility is reported to be of the order of 5% (Sch 71). As with other analytical techniques which attempt to determine elements in trace quantity with reasonable accuracy, it too has its disadvantages. For example, using commercially available apparatus, atomic absorption spectrometry may be used to measure only a single element per analysis. Also, A.A. is destructive, and some ionic, elemental, and spectral interferences exist which can decrease the reliability of the determinations. These interferences may involve light scattering by unevaporated salt particles, absorption by foreign atoms, and ionization of the atoms to be analyzed. Most interference effects can be decreased or eliminated by imitating the sample composition when analyzing standards, or utilizing the method of standard additions to develop the standard curves.

In order to measure atomic absorption, free atoms, in the gaseous state, must be produced. A flame burning in air represents a good method for converting a solution into an atomic vapor. In order to use this method, however, the samples must be in solution. The greatest problems in using the flame are the turbulence caused when the gases are mixed inducing noise, and some metal atoms are lost by the formation
of oxides. Another method, in commercial use, for producing free atoms, is the electrically heated graphite furnace. Considerably smaller sample sizes can be used and the problems of interference in the flame can be eliminated. Although much higher sensitivity can be obtained with the use of the furnace, there are problems involving quantitative sample transfer and carbide formation. Carbide formation decreases the thermal contact with the walls of the furnace and the sample (Sme 76). These problems sometimes outweigh the advantages of increased sensitivity.

Since blood and plasma are liquid mixtures, these samples can be aspirated directly into the flame or pipetted into the furnace. There are few sample preparation methods available using A.A. for the analysis of blood, probably because of its complex organic nature. On the other hand, there exist numerous techniques for the analysis of elements such as iron, nickel, copper, zinc and chromium in serum and plasma (Sun 73).

Using the review by Sunderman (Sun 73) as a starting point, the literature was surveyed to determine an appropriate element to analyze and a reliable method for preparation of the samples for which to verify the results of x-ray fluorescence. Of the four elements analyzed via XRF the determinations of copper present the least difficulty in atomic absorption spectroscopy, since this method is quite sensitive to copper and the reported analytical curve is reasonably linear up to high levels of absorbance. In addition, few spectral
interferences exist at its resonant wavelength of 327.4 nm and the spectral slit width has little influence on copper absorption up to a spectral slit width of about 2.0 Å (Sla 68). Contamination by copper, which can decrease reliability of an A.A. measurement immensely, is generally of much smaller magnitude than the contamination by other elements such as iron and zinc.

The sample preparation techniques which required a minimum of manipulation were investigated. Concentration methods were abandoned because of the possibility of contamination and sample loss. In addition, concentration is no longer necessary because of the increased sensitivity of the new A.A. spectrophotometers. The methods which do not involve concentrating steps involve direct aspirations into a flame, direct introduction into a furnace, and aqueous dilution and deproteinization with trichloroacetic acid and hydrochloric acid. Standards are normally prepared with the same concentrations of acid or salts normally found in blood or serum since it has been found in some cases that these ions affect the analytical sensitivity of the instrument (Mat 71, Sun 67, Kur 72, Sun 73, Sme 76). When aspirating plasma either directly or diluted by water, a viscosity difference exists between samples and standards. To reduce this viscosity difference, dextran or glycerol can be added to the standards.

One of the most reliable methods for copper analysis in serum involves the use of 1.4 N HCl to dissociate protein bound copper, followed by 20% trichloroacetic acid which is
added to deproteinize the serum (Sun 73). In this method, the supernatant is then aspirated directly into the flame. It has been indicated since, that the HCl is probably unnecessary when using 20-25% trichloroacetic acid (TCA) (Pip 67) as TCA at this strength can serve both purposes. After deproteinization, the serum contains 12.5% TCA and inorganic ions. There have been conflicting reports in the literature on the interference of copper absorbance with these ions. For example, Meret (Mer 71) suggested that NaCl enhanced copper absorption, whereas Sinha (Sin 70) found no effects of foreign ions on flame A.A. copper determinations.

Flameless methods have also been applied to copper determinations in serum or plasma. The increased sensitivity of the graphite furnace over that of the flame-nebulizer system makes it necessary to dilute plasma with distilled deionized water prior to analysis and transferral into the furnace. There also exists conflicting reports concerning the nature of the inorganic ion interferences while measuring copper. Matousek (Mat 71) simply added a normal complement of these ions to the standards without assessment of their effects. Glenn (Gle 71), on the other hand, found phosphate, sodium, potassium and magnesium to diminish the response of copper, and calcium to enhance it. The conclusion he reached was that the total effects cancelled and he therefore prepared aqueous standards using the method of standard additions to create analytical curves.
1.62 Anodic Stripping Voltammetry

The measurement of elemental concentrations by anodic stripping voltammetry (A.S.V.) requires two discrete steps. The species to be analyzed is plated (usually reductively) onto an electrode and then stripped (usually oxidatively) back into solution by means of a forward and reverse 'ramp' voltage. The current pulse produced, at the oxidation potential of the species analyzed, is proportional to the concentration of the metal in solution. As the newer, commercially available A.S.V. instruments utilize the Mg-C electrode, only those metals with standard electrode potentials less than that of mercury (-0.789 volts) can be analyzed.

The new developments in the instrumentation and techniques associated with A.S.V. have made this a valuable micro-method in determining minute concentrations of trace elements such as lead, copper, zinc, and cadmium in a wide range of materials. This method has recently been applied successfully to the determination of lead in blood (Sea 73, Mor 76) with good correlations between A.S.V. and A.A. It was decided then to assess the results from XRF analysis of lead in blood using an A.S.V. system.
2. EXPERIMENTAL TECHNIQUES

2.1 X-Ray Fluorescence Studies

2.11 XRF - Instrumentation

A secondary target photon excitation, energy-dispersive XRF spectrometer was used in all XRF studies. A schematic representation of this system is shown in Figure 2. The Siemens x-ray tube, equipped with a gold anode, was used to generate the source of priming Bremsstrahlung radiation for excitation of the secondary target. A Kevex Si-Li semiconductor detector, along with standard nuclear electronics, was used to detect the emitted radiation and shape the pulses emitted by the samples. During all analyses, the sample chamber was evacuated and the samples rested upon a Mylar foil, 4 microns thick. Two different sample chambers were used on separate occasions. A stainless steel single sample changer (SSC) which was provided with the system was utilized in a number of initial studies. As the work progressed, an aluminum multiple sample changer (MSC) was designed and incorporated in the subsequent studies. This permitted the analysis of up to sixteen specimens, consecutively.

As discussed earlier, molybdenum was selected as the secondary target because it was available and the energy of the Mo Ka excites most efficiently the elements we were interested in. The resultant photon spectrum following excitation was stored in 400 channels in a Victoreen Scipp Model 104TP multichannel analyzer. These data, in digital form, could
FIGURE 2

A Schematic Representation of the Photon Excitation, Secondary Target X-Ray Fluorescence System with Multiple Sample Chamber in Place
then be transferred onto magnetic tape for further detailed analysis on the IBM 370 computer using the computer code Sampo, (Rou 69). This code, used to fit the observed spectrum, yields information on the energy and area of the observed spectral peaks. Energy and peak area calibration was achieved by fluorescing both pure and mixed samples composed of elements of choice. Since many of the spectra obtained in this study are quite similar, a typical spectrum with appropriate peak identification is presented in Figure 3. A list of energies was given in Table II.

2.12 XRF - Materials

Of primary concern in the application of XRF to trace element analysis is that all the apparatus be scrupulously clean. Prior to use, all glassware was washed in reagent grade nitric acid and stored with distilled-deionized (d.d.) water. All plastic bottles were cleaned with 0.1 N nitric acid and subsequently stored in d.d. water. All sterile polypropylene and polystyrene plastic tubes were used without prior cleaning since they were found to be essentially trace metal free. A 3400 ppm Yttrium stock solution prepared from Y(NO₃)₂·6H₂O (BDH technical) was used as the internal standard for purposes of normalization. Yttrium has been chosen because its K absorption edge is just below the energy of the exciting radiation, the Kα x-rays are absorbed to a very small extent in 'thin' samples and because Yttrium is not found to any appreciable extent in biological material.
FIGURE 3

Typical XRF Spectrum
Stock solutions (1 mg/l) of various elements were prepared from the following salts:

- Calcium: \( \text{Ca(NO}_3\text{)}_2.4\text{H}_2\text{O} \)
- Chromium: \( \text{Na}_2\text{Cr}_2\text{O}_7.2\text{H}_2\text{O} \)
- Iron: 99% Fe metal
- Copper: \( \text{Cu(CH}_3\text{COO)}_2.\text{H}_2\text{O} \)
- Zinc: \( \text{ZnSO}_4.\text{H}_2\text{O} \)
- Bromine: KBr (dried)
- Lead: \( \text{Pb(C}_2\text{H}_3\text{O}_2).3\text{H}_2\text{O} \)
- Rubidium: RbCl

Since water soluble iron, copper, and zinc salts are particularly hydrophilic, these three solutions were also prepared using pure metals dissolved in Ultrex (J.T. Baker) nitric acid.

All stock solutions were stored in plastic bottles. Lead stock solutions were stored in dark plastic bottles since these solutions tend to form insoluble complexes in the presence of light (Tie 70).

2.13 XRF - Methods of Sample Preparation

2.131 Comparative Studies of Methods of Sample Preparation

The first and simplest method studied for preparing samples was the technique of spotting the biological fluids directly onto filter paper. In separate experiments using the XRF system it was observed that Whatman filters were essentially free of trace elements in the region of interest
(Fe-Y). Whatman filter paper, #42, with a thickness measured to be $0.7 \pm 10.2 \text{ mg/cm}^2$, was used in all studies due to its low ash content (and thus high purity). This thickness classified these samples as "thin" specimens (see Figure 1).

Analytical curves for copper, zinc, bromine and lead aqueous solutions were developed using the method of standard additions of known quantities of the element of interest. Following the addition of a measured amount of the internal standard (yttrium) solution, twenty-five μl was pipetted onto the center of the filter paper; a spot size of 2 cm was formed. Blanks were prepared by substituting water for stock solution. Although the curves prepared with aqueous solution remained linear up to several hundred ppm, the lower limit of detectability (LLOD) for each element, Cu, Zn, Br, and Pb was found to be about 463, 287, 214, and 177 μg/100 ml respectively.

When the standard addition method was employed to prepare analytical curves for blood, the lower limit of detectability was found to be about the same as that calculated above. Figure 4 is included to compare the spectrum obtained from a 'blank' filter paper with that from a filter paper with a 50 μl blood spot. It can be seen that the response for an element such as copper was essentially indistinguishable for the two cases.

Despite the simplicity of the spotting technique, the observation of such high LLOD values appears to eliminate this method for trace analysis of blood. Apparently, there is insufficient amount of sample deposited on the filter paper
FIGURE 4

XRF Spectra of 50 μl Flood Spot Compared With a 'Blank' Sample
(both on Whatman #42)
Counting Time = 20 Mins.
and the need to attempt to concentrate prior to analysis is obvious. Attempts to add more quantity onto a filter paper proved unsuccessful probably due to the manner in which it is absorbed, the resulting uncontrollable thickness, and inhomogeneity of the final spot.

Since the method of spotting blood onto filter paper proved unsatisfactory, procedures which concentrated the samples severalfold were examined as potential sample preparation methods for use in XRF analysis. Of the methods available, freeze drying (lyophilizing), wet ashing, and dry ashing represent the best choices in terms of the degree of concentration desired of the trace elements in the prepared samples. Lyophilized material may be formed into self-supporting pellets of predetermined thickness depending on the capacities of the XRF instrument. Samples of dry ashed material result in a much desired decreased matrix, but problems of support of this dried powder, without the addition of a binder, become apparent. Another problem associated with ashing mixtures such as blood and plasma is that some elements are easily volatilized which could ultimately lead to a decrease in sensitivity and accuracy.

In order to compare these methods (freeze dried, wet ash and dry ash), a 20 ml aliquot of pooled serum was treated with one ml of 3400 ppm Yttrium solution. One-third of this treated solution was freeze dried overnight and formed into a pellet of 13 mm in diameter and mass of 50 mg. A 13 mm diameter pellet was used because this size represents the smallest
KBr pellet press available. Some of the remaining serum was ashed in a muffle furnace at 500°C for four hours and the rest wet ashed by refluxing at 100°C in a mixture of 1:1 nitric acid (J.T. Baker Ultrex) and sulfuric acid (J.F. Smith). The three samples were analyzed by XRF for fifteen minutes and the spectra shown in Figure 5. A serum spot on Whatman #42 paper, from the same treated sample, is included for comparison. It is seen, that relative to the Yttrium peak, the ashing and freeze drying methods yield similar results. The spectrum of wet ashed material, which was analyzed in powder form indicates that a loss of bromine has occurred. Individual peak sizes in the dry ashed sample, which was also analyzed in powder form, are the largest and most well defined of the four spectra. However, in order to perform quantitative analysis using dry ashed material, a constant sample thickness is required and the addition of a binding material would be necessary. The binder would have to be mixed quantitatively to form plastic-ash pellets. This additional preparatory step could ultimately lead to increased contamination and tend to dilute the elements in the sample. The final spectrum may in this case be similar to that shown in the spectrum of freeze dried material.

Coupled to the problem of preparing uniform samples, is the need of having available large quantities of biological material to produce samples by ashing. While one to two grams (10-20 ml wet) of dried serum is required to produce a reasonably sized ashed sample only one-tenth of this amount is
FIGURE 5

XRF Spectra of Plasma Samples
Comparing 4 Methods of Sample Preparation
sufficient to prepare lyophilized samples. Bearing in mind ease of handling, ease of preparation and quantity of material available, the freeze drying method was finally chosen for use in all further XRF studies.

2.132 Optimum Sample Thickness

The final choice for optimum thickness depends upon experimental investigation and some discussion of related factors is called for.

In essence the aim here is to effect the greatest signal to noise ratio for as many trace elements, present in the sample, as possible. Four experimental parameters considered important and interdependent are the following:

a) time for which samples are analyzed
b) intensity of exciting beam of Mo radiation
c) thickness of sample
d) quantity of sample available

A thick sample (see Figure 1) could increase the observed signals of interest but will also yield higher scattering of the incident radiation. The net result would be a higher counting rate and a loss of counts due to electronic effects. To compensate for this, a longer counting time (clock time) would be necessary. Alternatively, a very intense exciting beam would produce similar results, but again the signal pulses from the detector would occur at such a high rate that pulse pile up would occur excessively in the amplification system. After preliminary studies (see Section 3.2 and
Appendix VII) it was decided to use 'thin' samples (30-50 mg in thickness) and the system was operated at a primary electron beam current of 50 mA, which is about two-thirds maximum capacity of the instrument. These appeared to be optimum running conditions.

Although the human body contains seven pints of blood, the quantity of sample available for analysis is generally only a few milliliters or less. Considering that in some cases (samples from pediatric patients for example) the amount of available sample is no greater than one milliliter, the mass of sample, or thickness, is limited by this quantity (approximately 95 mg dried weight). The availability of sample is therefore probably the most important single factor in deciding upon thickness of samples for clinical application.

In order to determine the optimum thickness for analysis experimentally, five pellets of mass 15, 30, 70, 120, and 150 mg were prepared, and analyzed for fifteen minutes under molybdenum exciting radiation. The results are displayed in Figure 6. In this figure the area of the spectral peak of interest is presented as a function of pellet (dia. = 13 mm) mass. The non-linearity observed for high masses (> 60 mg) is a result of increasing loss of pulses (dead time)* and higher background scatter counting rates from the thicker

* It is possible in many systems to have an electrical feedback system which in effect allows the storage analyses to compensate for increases in dead-time. The effect of this is to have much longer real count times.
FIGURE 6

Relationship Between Area of Photopeaks and Mass of Serum Sample
MASS at PELLET (rings, d=13 mm.)

LOG AREA of Kα PHOTOEAK

MASS of PELLET (mgs., d=13 mm.)
pellets, as mentioned. A pellet mass of 50 mg appeared to give the highest signal to noise ratio and was selected as the optimum thickness for trace element analysis of blood and plasma samples via XRF.

The most efficient use of available time (given the number of samples to be analyzed, for example, in a clinical setting) limited the counting time to about twenty minutes. A twenty minute count using a 30 mg plasma pellet yields about 1000 counts in the copper peak. A fifteen minute counting time using 50 mg pellets yields the same results. In order to halve the counting error, a counting time of one hour would be necessary. Since a 1000 count size photopeak has an acceptable relative statistical error, a fifteen minute counting time was selected. Thus, the final set of operating conditions for analysis of 50 mg samples was a counting time of 15 min., and an anode current of 50 mA for the primary electron beam.

2.14 XRF Studies—Background Effects

2.141 Sources of Background

In trace elemental analysis, when the signals from the elements of interest are small, any peaks occurring in the background spectra can decrease both the sensitivity and precision of the analysis. It is therefore a valuable exercise to attempt to determine their origin and to decrease the contribution of these peaks, which on the analysis of essentially trace element free material (see, for example, Figure 4, Whatman #42) has been found to originate from iron, nickel,
copper, and zinc. To achieve this it is necessary to locate the source of background effects. Of equal importance is the ability to take such effects into account properly once they have been minimized. A considerable length of time and effort was devoted to such studies.

Preliminary efforts using filter paper samples and collimators of different composition from that supplied by the manufacturer indicated quite clearly that the source of background peaks did not originate from the original aluminum covered collimator located between the sample and the detector system. Nor was it found to originate in the material located above the sample in the sample changer.

In order to characterize the background contributions and to determine its features, pellets were prepared from "clean" polyethylene (chromatography grade, Dow Chemical) powder (55.5 mg) and 'Somar blend' (plastic binder supplied by the Kevex Corp., 45.3 mg). The spectra obtained showed that the polyethylene gave slightly smaller background peak sizes than the 'Somar' and thus polyethylene was utilized for the preparation of 'blank' pellets for further background studies. Washing the plastic for 24 hours with concentrated nitric acid, rinsing with water to neutrality and washing the wet plastic with spectroscopic grade acetone did not decrease these spectral peaks. It then appeared that the major portion of the background spectra originated from the steel in the sample chamber or in material on the detector side of the sample (see Figure 2) as was found while using filter paper
samples.

This hypothesis was confirmed by studies of pellets of various thicknesses. The data obtained are displayed in Figures 7 and 8 for equal analysis times. These figures show that the areas of the spectral peaks increase exponentially as a function of sample mass. A similar dependence was also observed for the yield of the Bremsstrahlung scatter radiation (curve C). In all cases the maximum value was obtained for masses around 90 mg which is well below the theoretical estimate of about 700 mg (see Figure 1) expected if these peaks had arisen from elements in the sample itself.

This again suggests strongly that the background spectral peaks originated from elements in the stainless steel sample chamber on the detector side of the sample. These species (Fe, Cu, Ni, and Zn) were fluoresced by the backscattered (Compton and Rayleigh) radiation of the $K_\alpha$ x-rays from the sample. There is probably negligible contribution of Fe, Ni, Cu, and Zn $K_\alpha$ x-rays from the target and filter assembly since double (Compton and Rayleigh) scatter peaks are not observed for these elements. Unfortunately, the source of the background could not be specified more exactly nor could it be eliminated at this time.*

Knowledge of the origin of these background photopeaks

* In order to eliminate or decrease the background spectral peak contributions, an entirely new sample chamber would have to be prepared.
FIGURE 7 AND FIGURE 8

following

Relationship Between Area of Spectral Peak
and Mass of Blank Pellet
A = Iron $K_\alpha$
B = Nickel $K_\alpha$
C = Number of counts in one channel below Iron $K_\alpha$
MASS of PELLET (mg., d=13 mm.)

NUMBER of COUNTS in SPECTRAL PEAK

A = Copper $K\alpha$
B = Zinc $K\alpha$
is not as important as the accurate determinations of their magnitudes. Thus, as long as the peak areas remained relatively constant they could be subtracted when measuring these elements in specimens.

2.142 Determination of Background Levels

It was determined after extensive studies that the range of background effects was critically dependent upon the position of the filter assembly, located between the secondary target and the sample. Table III presents the average observed counting rates (peak areas per unit time) and the range of observed values for studies with the filter in various positions. These include studies with the filter in variable and fixed position, and with the single and multiple sample changers. The decrease in the range of values and the experimentally deduced error for a fixed target assembly position is obvious. It is apparent that changes in position exposed parts of the steel sample chamber leading to increases in observed spectral peaks, particularly iron. The improvement or decrease in the levels of background observed when using the multiple sample changer should also be noted.

2.143 Corrections for Background Effects

There are several methods by which the effects of background could be taken into account. The simplest method involves a direct subtraction of the computed areas in the 'blank' sample from that of unknown samples. However, since
### TABLE III

Determination of Observed Background Levels
Using Polyethylene Pellets

<table>
<thead>
<tr>
<th>CONDITIONS</th>
<th>Counts per min. ± 2<em>S</em> (Range of Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe</td>
</tr>
<tr>
<td>A) S.S.C. (Single Sample Changer)</td>
<td></td>
</tr>
<tr>
<td>Variable Filter</td>
<td>166 ± 70</td>
</tr>
<tr>
<td>Assembly Position</td>
<td>(77-299)</td>
</tr>
<tr>
<td>B) S.S.C.</td>
<td></td>
</tr>
<tr>
<td>Fixed Filter</td>
<td>228 ± 58</td>
</tr>
<tr>
<td>Assembly Position, Consecutive</td>
<td>(216-239)</td>
</tr>
<tr>
<td>Determin's.</td>
<td></td>
</tr>
<tr>
<td>C) S.S.C.</td>
<td></td>
</tr>
<tr>
<td>Fixed Filter, Assembly Position,</td>
<td>90 ± 12</td>
</tr>
<tr>
<td>Over 2 Mo Period</td>
<td></td>
</tr>
<tr>
<td>D) M.S.C. (Multiple Sample Changer)</td>
<td></td>
</tr>
<tr>
<td>Fixed Filter</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>Assembly Position, Consec. Studies</td>
<td></td>
</tr>
<tr>
<td>E) M.S.C.</td>
<td></td>
</tr>
<tr>
<td>Fixed Filter</td>
<td>42</td>
</tr>
<tr>
<td>Assembly Position, 8 Hour Count</td>
<td></td>
</tr>
</tbody>
</table>

*S  Calculated standard deviation for many observations.
** Very low value because peak was often not integrated.
the background has been shown to be a function of backscattered radiation (see Section 2.141 and Figure 7), this method of background subtraction suffers from insensitivity to fluctuations in the background. Generally therefore, it is more useful to subtract the entire 400 channels of data based on the magnitude of the backscattered radiation with a subsequent analysis of the resulting data by the computer fitting routine. For best results it was found that normalization of the background spectrum to the spectrum of interest was best achieved using the Compton scatter peaks. Thus, all observed spectra are subjected to a subtraction of the normalized background spectrum prior to analysis by the computer fitting code.

2.2 Atomic Absorption Studies

2.21 A.A. - Early Studies

In our early studies of the comparison of results obtained with XRF versus those obtained by A.A., the A.A. measurements were performed in other laboratories. The results of one of these studies are provided in Appendix V. Discrepancies were noted and it was then decided to pursue such studies here both for convenience and for a more strict control of the A.A. analytical procedures.

* Another method which has also been attempted, and which was hoped to be sensitive to background fluctuations, was the normalization of the 'blank' spectra to the Ni photopeaks. However, since this peak is small and fit poorly, the procedure was unreliable. Results of using this method are provided in Appendix V.

† These other labs were Essondale Clinical Lab and Vancouver General Hospital Clinical Lab.
2.22 A.A. - Instrumentation

A Perkin Elmer Model 305 Atomic Absorption Spectrophotometer, available at S.F.U., was used to verify copper concentrations in specific samples which had been previously analyzed via XRF. Both the single slot burner for flame A.A. and an HGA-70 graphite furnace for flameless A.A. were used to develop and assess reliable and practical procedures for copper determinations in plasma. An air-acetylene mixture was used to produce the flame when using the single slot burner and the spectrophotometer was operated with the hollow cathode lamp at 25 mA, slit 4, chart expansion 20. For the flame the air flow was set at 50 cfpm and acetylene, 20 cfpm. The initial settings for slit width, damping, and the voltage applied to the graphite furnace were obtained from the Perkin Elmer specifications. The most sensitive absorption line for copper, at 324.7 nm, was employed in all determinations.

2.23 A.A. - Sample Preparation Methods

There are three basic methods available for measuring plasma copper by the flameless A.A. technique, which require little sample manipulation. They are standard additions to aqueous solutions (Kur 72, Gle 71), standard additions to solutions which mimic the salt concentrations found in normal serum (Tie 70) and standard additions to serum or plasma.

Much time and effort was devoted to comparing the three methods described above for analysis of plasma copper by flameless A.A. Considerable difficulties were encountered while
using the furnace, and the results were found to be dissimilar from those obtained by XRF. Large errors and deviations were encountered which were probably due to the viscosity of the samples which affected pipetting and produced carbide and smoke in the furnace. Since these data were not used in this study, a complete description has been included in Appendix VI. After having attempted to analyze the samples via the furnace, the remaining samples amounted to approximately 2 mls each, which were diluted 1:1 with water. In order to verify the XRF results it was elected to switch to flame A.A. and analyze the remaining samples.

2.231 A.A. - Plasma Copper Measurements

One method, which was available and used for analysis of plasma samples diluted one-half with water, utilized 10% aqueous glycerol for preparation of the standard working curves (Fer 71). A stock standard solution of copper (1 mg/l) was prepared with washed copper wire dissolved in concentrated nitric acid. The concentration of this solution was verified in independent studies.

For thoroughness and to examine the hypothesis that 10% aqueous glycerol represents an average viscosity of serum and that salts affected absorbance in the flame, working curves were prepared by standard additions to serum, salt solution (B) (see Appendix VI) and aqueous 10% glycerol. The slopes of the curves generated are displayed in Table IV. Each of these data, fit by least squares, resulted in straight lines. As
### TABLE IV

Flame A.A. Comparisons of Analytical Curves Prepared With Water, Salt (B) and Serum

<table>
<thead>
<tr>
<th>Standard Solution</th>
<th>Slope of Curve $\pm 2S$ a)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>aq. gly 10%</td>
<td>0.246 ± 0.003</td>
<td>Day 1</td>
</tr>
<tr>
<td>pooled serum</td>
<td>0.233 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>aq. gly 10%</td>
<td>0.247 ± 0.002</td>
<td>Day 2</td>
</tr>
<tr>
<td>salt (B) gly 10%</td>
<td>0.251 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>serum</td>
<td>0.226 ± 0.005</td>
<td>Day 3 New Gain Setting</td>
</tr>
<tr>
<td>aq. 10% gly</td>
<td>0.266 ± 0.005</td>
<td>Day 4 New Aspiration Rate</td>
</tr>
<tr>
<td>salt (B) gly 10%</td>
<td>0.277 ± 0.004</td>
<td></td>
</tr>
</tbody>
</table>

a) S: error generated through least squares analysis.

The units for the slopes are: $\mu g/100\ mls\ Cu/0.1\%\ absorbance$
can be seen in this table, salt solution (B) apparently enhances absorbance of Cu over aqueous 10% glycerol standards, pooled serum, on the other hand, tends to decrease Cu absorbance. An intermediate and somewhat more constant slope is observed with 10% aqueous glycerol. Since the slope of the analytical curve generated with 10% aqueous glycerol is intermediate between the two others, it probably represents the average slope that would be produced by standard additions to plasma. Thus, it was this curve that was assumed most reliable and was applied to determinations of copper in unknown samples. The results of this study are presented in Section 3.

A second method, employed for the verification studies, and which has been verified itself against results obtained by neutron activation analysis (Sme 73), involved a protein denaturing step. The method, considered more reliable (Hen 74, Sme 73), is not limited by the possibility that a small variation in the plasma sample viscosity could decrease the accuracy of final measurements as could affect the other technique used (10% glyceral method).

In this procedure, a 25% trichloroacetic acid (TCA) solution is used to denature the protein which is subsequently removed by centrifugation.

To assess the impact of TCA on the working curves, and to determine if aqueous standards could be used, curves were generated by the method of standard additions to 25% TCA and aqueous solutions. Comparison of the slopes obtained while using these two standards are shown in Table V. This Table
TABLE V

Slopes of the Analytical Curves for Copper via A.A.
Obtained by Least Squares Analysis

<table>
<thead>
<tr>
<th>Standard Solution</th>
<th>Slope of Curve $^{a)} \pm s^{b)}$</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.225 ± 0.002</td>
<td>24.0 ± 0.2</td>
</tr>
<tr>
<td>II</td>
<td>0.225 ± 0.004</td>
<td>20.8 ± 0.5</td>
</tr>
<tr>
<td>III</td>
<td>0.249 ± 0.007</td>
<td>17.2 ± 0.9</td>
</tr>
<tr>
<td>Aqueous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.444 ± 0.009</td>
<td>0.25 ± 1.12</td>
</tr>
<tr>
<td>II</td>
<td>0.317 ± 0.036</td>
<td>4.75 ± 4.14</td>
</tr>
</tbody>
</table>

a) The slope represents $\frac{\mu g/100 \text{ mls}}{0.1\% \text{ absorbance}}$

b) 's' represents the error of the regression line $Y = ax + b$

is also included to show that while operating the A.A. instrument under the same conditions and analyzing identical standard solutions, distinct variations in the slopes of the standard curves result. Although A.A. has proven to be an extremely valuable analytical tool for measurement of trace elements in a wide range of matrices, the variations shown above show the possible poor reproducibility of analytical measurements via A.A. For accurate analyses therefore, the specimen must be analyzed at the same time as the standard curves are generated.
The depressant effect of TCA on the slope of the curves compared with aqueous samples can be noted in Table V. Thus, although the A.A. spectrophotometer is more sensitive to aqueous samples, TCA deproteinized plasma cannot be compared with aqueous solutions. The positive intercept for TCA indicates that the allegedly pure TCA solution (12.5%) is contaminated by approximately 0.89 ppm copper (the dry acid therefore contains 7 ppm copper). This quantity has been subtracted from all subsequent determinations. The slope generated from TCA III (see Table V) solutions was used to determine concentrations of unknown samples, because it was at this time that the samples were analyzed. Section 3 displays the results.

2.3 Anodic Stripping Voltammetry (A.S.V.)

2.31 A.S.V. - Instrumentation and Procedures

An ESA (Environmental Services Association), Model 3010, Anodic Stripping Voltammetry system was available to determine the concentration levels of lead in whole blood samples. A necessary part of such studies is a pre-digestive step which was performed using 'Metexchange' fluid provided by ESA. A digital read-out was available for observing directly the concentration of lead in a 100 µl sample of heparinized whole blood. The entire system was calibrated using blood solutions of known lead concentrations provided by ESA.

Sample handling in general was quite simple as blood samples were obtained by venipuncture into lead-free heparinized Vacutainers. One hundred microliters were then added
directly to a fixed amount of digestion fluid.

In preliminary studies the blood of 38 females was analyzed for lead levels. The average concentration of lead determined was found to be $6.8 \pm 4.7 \mu g/100 \text{ ml}$. While such measurements are of interest alone,* this low value indicated that such levels might be below the detection limits for determinations by XRF analysis. Thus, in the verification studies, known amounts of lead were added to blood samples prior to analysis. These were then available for analysis both by ASV and XRF techniques.

2.4 Specimen Collection Techniques and Problems of Contamination

It is extremely important in obtaining accurate values of trace element concentration in biological specimens that the introduction of contaminants during specimen collection and storage be kept to a minimum. Alternatively, when contaminants are introduced unavoidably, measurement of these must be performed. It was thus decided to include a detailed description of such studies here for completeness. The results of these studies were of course taken into account when necessary in the determination of the final trace element concentration profiles for copper, zinc, bromine and rubidium (Sec. 3).

In the first study completed, that of the measurement of trace elements in adult female specimens 'Vacutainers' were

* This value is much lower than the acceptable limit of 50 $\mu g/100 \text{ mls}$ for blood lead in North American subjects (Und 71), measured by other techniques.
used for blood collection. 'Vacutainers' are commercially available evacuated glass tubes with rubber stoppers. To collect blood samples, double ended needles are introduced into the tubes after having been inserted into the subject's vein. The small vacuum in the tubes is sufficient to almost fill one of these tubes.

Since it was found that the 'Vacutainers' contained high amounts of zinc, (see succeeding sections) plastic syringes and tubes were used for subsequent blood collection programs.

2.41 Contamination by Collection Devices - Study A (Females)

The 'Vacutainers' (Becton Dickinson, #4610, lot #8191254) used for the collection of blood from adult female subjects had heparin added as anticoagulant and were reported by the manufacturer, to be lead free. The rubber stoppers had been coated with a thin film of silicon grease. These "lead free" 'Vacutainers' were chosen because the blood from these tubes was to be used to measure blood levels via anodic stripping voltammetry. Although the 'Vacutainers' allegedly contained less than 0.1 µg of lead per tube, there was no guarantee that they were completely trace metal free.

Prior to determining possible contamination by the collection devices, it was found that the plastic tubes and stainless steel needles did not introduce contamination of copper, zinc, bromine or rubidium to any significant degree. For the purposes of this study therefore, the tubes were
assumed to contain no soluble metallic components.

In order to investigate contamination by the tubes used to collect the blood, outdated whole blood obtained from the Red Cross was transferred to an acid washed glass beaker and stirred continuously. Blood samples were drawn from this pool and the following samples prepared for XRF analysis:

a) Five pellets directly from the beaker.

b) Five heparinized Vacutainers were filled through doubled ended needles and tipped in the usual manner several times. Five more pellets were prepared.

Plasma samples were also prepared as follows:

c) About 9 mls of whole blood was introduced into 10 ml plastic tubes, centrifuged and transferred into 5 ml plastic tubes. From these 5 tubes, 5 pellets were prepared.

d) Five heparinized tubes were filled as in b) above, centrifuged and the supernatant plasma transferred to small plastic tubes. One pellet was prepared from each tube in the usual manner.

The results, shown in Table VI, indicate that there is virtually negligible contamination introduced by the manipulation described in step b) since the differences in the mean concentration of elements, from study a) and b), are within experimental error. A Student t Test (see Appendix II) applied to the measured means, yielded values of \( \alpha \) for copper, zinc and bromine of \( \approx 0.25 \), \( \approx 0.85 \), and \( \approx 0.55 \). The high value of \( \alpha \)
TABLE VI

Results of Contamination Study A – Blood and Plasma

\[
\left( \frac{\text{Area of Element } K_\alpha \text{ Peak}}{\text{Area of } Y \text{ } K_\alpha \text{ Peak}} \right)
\]

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
<th>Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Blood mean ± S</td>
<td>1.14±0.07</td>
<td>5.34±0.49</td>
<td>3.78±0.212</td>
<td></td>
</tr>
<tr>
<td>b) Blood mean ± S</td>
<td>1.20±0.09</td>
<td>5.28±0.33</td>
<td>3.87±0.190</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>-0.06</td>
<td>-0.07</td>
<td>+0.09</td>
<td></td>
</tr>
<tr>
<td>c) Plasma mean ± S</td>
<td>1.11±0.04</td>
<td>0.89±0.02</td>
<td>0.71±0.04</td>
<td>3.75±0.05</td>
</tr>
<tr>
<td>d) Plasma mean ± S</td>
<td>1.13±0.07</td>
<td>0.87±0.04</td>
<td>1.89±0.21</td>
<td>3.67±0.06</td>
</tr>
<tr>
<td>Difference</td>
<td>+0.02</td>
<td>-0.02</td>
<td>1.18</td>
<td>-0.08</td>
</tr>
</tbody>
</table>

a) S: standard deviation of set of measurements.
indicates that the differences in the means are probably due to random errors and are thus insignificant.

The Student t Test applied to the differences in the means of iron, copper, zinc and bromine in plasma, yielded values of $\alpha = 0.55, = 0.20, < 0.01$ and $\approx 0.04$ respectively. According to Eckschlager (Eck 69) the differences in the means with $\alpha \leq 0.01$ are highly significant and cannot be attributed to random errors. Thus, the increase in plasma zinc levels must be assumed to have originated from collection of the plasma. A distinct level of zinc must therefore be subtracted from all subsequent determinations while using this method of blood collection.

On the average, using the experimentally determined values, the levels of zinc contamination exists at $133 \pm \mu g/100 \text{ mls}$. In a report on zinc contamination in 'Vacutainers' (Hel 71) the highest zinc levels were found in similar 'minimal lead' 'Vacutainers' and amounted to $110 \mu g/100 \text{ mls}$. The zinc was found to originate almost entirely in the siliconized stoppers. A stopper from the 'Vacutainers' used in this study, which was analyzed via XRF, showed an extremely high zinc peak.

The level of $133 \mu g/100 \text{ mls}$ zinc represents an extremely high degree of contamination which may have originated from the stoppers, or from the lysis of the old red cells when passing through the needle into the 'Vacutainer' system. In order to assess the possibility of red cell lysis, a second contamination study (A') was set up. In the new study, fresh
blood was collected in eight 10 ml (red-topped) non-siliconized, heparinized 'Vacutainers'. This blood was transferred directly to an acid washed beaker and stirred continuously. Nine ml aliquots of this blood were transferred directly to 4 heparinized tubes, of the type used to collect blood from the females. The tubes were then tipped ten times in a manner to simulate the method of normal collection. The tubes were centrifuged and the plasma prepared into pellets in the usual manner.

Controls for Study A' were prepared by transferring 4-9 ml aliquots of the blood from the beaker into plastic tubes and centrifuging these tubes to remove the plasma. Pellets were prepared and the results of XRF analysis of these samples are shown in Table VII.

A significant difference ($\alpha < 0.01$) in plasma zinc concentrations can be noted. This difference (0.28) amounts to a mean concentration of 38 $\mu$g/100 mls zinc. When measuring zinc levels using the vacutainers it is this mean concentration of zinc which must be subtracted. When subtracting zinc contamination in this manner, the mean values of a series of analyses should not be affected.

2.42 Contamination by Collection Devices - Studies B (Males) and C (Children)

The previous study indicated that zinc, from the heparinized 'Vacutainers', contaminated the plasma samples to a large degree. Disposable plastic syringes, 5 ml polypropylene tubes, and purified heparin were then used in place of the 'Vacutainers'. These collection devices were also assessed
TABLE VII

Results of Contamination Study A' Plasma

\[
\frac{\text{Area of Element K}_\alpha \text{ Peak}}{\text{Area of Y K}_\alpha \text{ Peak}} \pm S
\]

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
<th>Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacutainers</td>
<td>$0.88 \pm 0.035$</td>
<td>$1.01 \pm 0.79$</td>
<td>$1.30 \pm 0.091$</td>
<td>$8.89 \pm 0.175$</td>
</tr>
<tr>
<td>Control</td>
<td>$0.91 \pm 0.01$</td>
<td>$1.03 \pm 0.03$</td>
<td>$1.01 \pm 0.01$</td>
<td>$9.03 \pm 0.127$</td>
</tr>
<tr>
<td>Difference</td>
<td>$-0.03 \pm 0.03$</td>
<td>$-0.02 \pm 0.08$</td>
<td>$+0.28 \pm 0.09$</td>
<td>$-0.14 \pm 0.22$</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$0.20$</td>
<td>$&gt;0.45$</td>
<td>$&lt;0.01$</td>
<td>$0.10$</td>
</tr>
</tbody>
</table>

a) As has been done in Table VI the values displayed represent the mean value b) $\pm S$

where $S$ is the mean of the set of measurements.
for their levels of contamination for the collection of blood, in a similar manner as that which was undertaken in the previous studies (Section 2.41).

Several hundred mls of freshly outdated whole blood, from the Red Cross, was placed in an acid washed beaker. While stirring, five 1 ml portions were withdrawn with one ml acid washed pipettes for the preparation of five pellets. Four 5 cc 'B.D.' plastic syringes were filled through 'Stylex' stainless steel needles (lot #C4D380D), and the blood in these syringes was expelled immediately into 5 ml polypropylene tubes containing 50 µl of U.S.P. heparin (lot #8413526). One ml of blood from each of these four tubes was used to prepare four blood pellets. The remaining blood in the tubes was centrifuged, and the plasma transferred to dry 4 ml plastic tubes. One ml of plasma for each of these tubes was used to prepare four plasma pellets in the usual manner. All samples were analyzed via XRF and the results, displayed in Table VIII, show that (since α <0.01) significant contamination is due to Fe in plasma (approximate level of 178 µg/100 mls. In verification of this finding, an XRF spectrum of the rubber component of the syringe was collected which displayed a strong iron peak.
TABLE VIII

Results of Contamination Study B - Blood and Plasma \(^a\)

\[
\frac{\text{Element} K_\alpha \text{ Peak Area}}{Y \text{ K}_\alpha \text{ Peak Area}}
\]

<table>
<thead>
<tr>
<th>Samples</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
<th>Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Blood</td>
<td>--</td>
<td>1.14±0.07</td>
<td>5.34±0.49</td>
<td>3.78±0.212</td>
</tr>
<tr>
<td>Blood; Syringe + Heparin</td>
<td>--</td>
<td>1.09±0.04</td>
<td>4.90±0.47</td>
<td>3.65±0.144</td>
</tr>
<tr>
<td>Difference</td>
<td>--</td>
<td>-0.05</td>
<td>-0.44</td>
<td>-0.13</td>
</tr>
<tr>
<td>(\alpha)</td>
<td>--</td>
<td>0.03</td>
<td>0.3</td>
<td>0.35</td>
</tr>
<tr>
<td>Plasma</td>
<td>1.11±0.04</td>
<td>0.89±0.02</td>
<td>0.71±0.04</td>
<td>3.75±0.05</td>
</tr>
<tr>
<td>Plasma; Syringe + Heparin</td>
<td>1.69±0.011</td>
<td>0.89±0.003</td>
<td>0.76±0.03</td>
<td>3.76±0.005</td>
</tr>
<tr>
<td>Difference</td>
<td>0.58</td>
<td>0.00</td>
<td>-0.05</td>
<td>+0.01</td>
</tr>
<tr>
<td>(\alpha)</td>
<td>&lt;0.01</td>
<td>0.9</td>
<td>0.70</td>
<td>0.95</td>
</tr>
</tbody>
</table>

\(^a\) See Table VII for description of this table.
3. EXPERIMENTAL RESULTS

3.1 Sensitivity Studies

As the aim of this study was to apply and assess the potential of the XRF technique for the quantitative measurement of trace elements in blood and serum/plasma samples, a series of experiments were conducted to estimate the relative analytical sensitivity of the system. Standard analytical curves (Peak area of element of interest normalized to area of $K_\alpha$ peak of Y as a function of concentration) were generated by the method of standard additions to blood and serum specimens prepared using the freeze-drying, pelletizing technique (described in Section 2).

The slopes and intercepts of these linear analytical curves were extracted using a least squares method of analysis and are presented in Table IX. These data are also displayed in Figures 9 and 10. Sensitivity is defined here as the observed area of the $K_\alpha$ peak of the element obtained for a sample containing 10 $\mu$g/100 ml of the element. The low sensitivity for elements such as calcium reflect the expected low yield of $K_\alpha$ x-rays for elements of low Z (Ber 75) and the relatively effective absorption of low energy radiation in the beryllium windows of the Si(Li) detector. The fall off in sensitivity exhibited at higher atomic numbers (>36) reflects the decrease in efficiency of the Si(Li) detector at high photon energies.
TABLE IX

Analytical Parameters Using the XRF Technique
for Blood and Serum

<table>
<thead>
<tr>
<th>Atomic Number</th>
<th>Element Line</th>
<th>Whole Blood</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope R/Ca)</td>
<td>Intercept Rb)</td>
<td>Slope R/Ca)</td>
</tr>
<tr>
<td>10</td>
<td>Ca Kα</td>
<td>1.09±0.04d)</td>
<td>0.84±0.02</td>
</tr>
<tr>
<td>24</td>
<td>Cr Kα</td>
<td>3.43±0.05</td>
<td>3.02±0.03</td>
</tr>
<tr>
<td>26</td>
<td>Fe Kα</td>
<td>3.15±0.62</td>
<td>0.62±0.05</td>
</tr>
<tr>
<td>29</td>
<td>Cu Kα</td>
<td>6.8±0.80</td>
<td>6.64±0.16</td>
</tr>
<tr>
<td>30</td>
<td>Zn Kα</td>
<td>6.82±0.25</td>
<td>12.3±0.14</td>
</tr>
<tr>
<td>35</td>
<td>Br Kα</td>
<td>17.8±0.10</td>
<td>6.6±0.06</td>
</tr>
<tr>
<td>46</td>
<td>Pb Lα</td>
<td>6.25±0.24</td>
<td>0.04±0.004</td>
</tr>
<tr>
<td>37</td>
<td>Rb Lα</td>
<td>19.3±0.40</td>
<td>5.27±0.10</td>
</tr>
</tbody>
</table>

a) \[ R/C = \frac{\text{Element (K}_\alpha \text{ or L}_\alpha \text{) area}}{\text{Yttrium K}_\alpha \text{ area}} \times \frac{\mu g}{100 \text{ ml}} \times 100 \]

b) \[ R = \frac{\text{Element (K}_\alpha \text{ or L}_\alpha \text{) area}}{\text{Yttrium K}_\alpha \text{ area}} \times 100 \]

c) As Br Kβ overlaps Rb Kα1 and Kα2 lines, the known response ratio (0.1501) of Kβ/Kα for Br (Sal 74) was taken into account in deducing the correct Rb Kα area.

d) Errors represent one standard deviation of the regression line as deduced using the least squares fitting routine.
FIGURE 9

XRF Sensitivity of the Freeze Drying Method for Whole Blood
LOG SLOPE: \[ \frac{\text{ELEMENT (K\alpha/\gamma K\alpha) \%}}{\mu g/\% \text{ ELEMENT}} \times 10^{-3} \]

ATOMIC NUMBER

\( \frac{10\pi/9}{\% \text{ AREA}} \)
FIGURE 10

XRF Sensitivity of the Freeze Drying Method for Plasma
LOG SLOPE: \[
\frac{\text{ELEMENT (K\alpha / Y\alpha)\%}}{\mu g/\% \text{ ELEMENT}} \times 10^{-3}
\]

SENSEITIVITY: \[
\frac{10^{10}/\text{ \%}}{\text{ELEMENT K\alpha PEAK AREA}}
\]
3.2 Elemental Concentrations in Blood and Plasma/Serum

To obtain preliminary information on XRF analyses of freeze dried pelletized whole blood and serum, specimens were collected from volunteers at S.F.U. and the Children's Hospital. The details of this preliminary study are presented in Appendix VII. It was found that the thickness of the samples played a more important role than hitherto assumed and that the anticoagulant EDTA contained very high concentrations of zinc.

All subsequent determinations were carried out using heparin as anticoagulant and keeping the thickness of the samples and standards constant.

3.3 Trace Element Profiles in Blood and Plasma

Individual studies of the concentration ranges and means of trace elements in males, females and children have been undertaken. This investigation was completed in combination with verification studies to meet the requirements of application of the described method of XRF analysis. Personal data on each individual were collected to determine if any striking conditions played a role in varying the concentrations of trace elements in human blood or plasma. Since EDXRF is a multi-elemental method, correlation statistics were also included to show the ease with which they can be generated, compared with other methods which can measure only one element per analysis. It should be pointed out that there are few
correlation studies reported in the literature and XRF could ultimately be applied to fill the void.

3.31 Profiles - Study A (Females)

Thirty-eight female nursing students between the ages of 18 and 22 donated 9 ml of venous blood during four clinics set up by Dr. Pat McLeod at the Vancouver General Hospital. Lead free heparinized 'Vacutainers' with siliconized stoppers and stainless steel needles were used to collect the venous blood. Prior to having set up the clinic, the subjects were asked to fill out a questionnaire, reproduced in Appendix VIII, listing their age, recent illnesses, any medications, vitamin preparations and drugs they were presently taking.

Immediately after collecting the blood samples they were conveyed to S.F.U. where one ml of blood from each tube was used to prepare pellets for XRF analysis. All of the remaining volume, except about one-half ml, was transferred to separate plastic tubes, centrifuged, and the supernatant plasma drawn off. One milliliter of plasma was used to again prepare pellets for analysis. Typical spectra obtained from a blood and plasma pellet are displayed in Figures 11 and 12, respectively.

The remaining volume of plasma was set aside for analysis of copper via A.A. The small quantity of blood remaining in each 'Vacutainer' was saved for analysis via anodic stripping voltammetry for lead measurements.

The results of the analysis of copper, zinc, bromine and rubidium in blood, and copper, zinc, bromine and rubidium
FIGURE 11

Typical XRF Blood Spectra
FIGURE 12

Typical XRF Plasma Spectra
in the plasma of the 38 female nursing students are shown in histogram form in Figures 13 to 16. In these figures, 'm' represents the calculated mean; 'a' the mean from Anspaugh (Ans 71); subscript S, the value for serum; *, value for adults; b, mean value calculated for subjects taking oral contraceptives. Bromine and rubidium concentrations have been reported here and in subsequent sections in both µg/100 mls and µEq/L since it is not known if these elements exist as electrolytes or free atoms.

For all analyses, the means of the distributions, displayed in Table X, are found to be in accordance with "Anspaugh's Compilations of Published Information on Elemental Concentrations in Human Organs in both Normal and Diseased States" (Ans 71). The lognormal distribution displayed for plasma copper reflects both healthy subjects, and females taking oral contraceptives. The increase observed in the copper plasma levels for these latter females is in excellent agreement with other recently reported results (Sch 72, Hor 75). Although 11 subjects indicated vitamin supplementation, no significant variation in trace element levels in these samples was observed.

3.32 Profiles - Study B (Males)

Blood samples were collected from 37 male students between the ages of 18 and 33 at S.F.U. The samples, collected with 5 ml disposable plastic syringes and 22 gauge stainless steel needles were transferred into 5 ml plastic tubes to
FIGURES 13-16

Histograms of Trace Element Levels
in Normal Healthy Females
BLOOD BROMINE

BLOOD RUBIDIUM
TABLE X

Trace Element Levels in the Blood and Plasma
of 38 Adult Females

<table>
<thead>
<tr>
<th>Element</th>
<th>Samples</th>
<th>No.</th>
<th>Whole Blood Mean±Sb) Range (µg/100 ml (µEq/L))</th>
<th>Blood Plasma Mean±Sb) Range (µg/100 ml (µEq/L))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>Normal</td>
<td>28</td>
<td>102±21 62-147</td>
<td>113±19 85-152</td>
</tr>
<tr>
<td></td>
<td>B.C.P.a)</td>
<td>10</td>
<td>149±19 126-179</td>
<td>186±34 132-246</td>
</tr>
<tr>
<td>Zn</td>
<td>Normal</td>
<td>38</td>
<td>707±73 541-840</td>
<td>142±39 85-234</td>
</tr>
<tr>
<td>Br</td>
<td>Normal</td>
<td>38</td>
<td>404±77 263-590</td>
<td>514±46 342-745</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(51±9.6) (33-74)</td>
<td>(64±5.8) (43-93)</td>
</tr>
<tr>
<td>Rb</td>
<td>Normal</td>
<td>38</td>
<td>287±45 206-386</td>
<td>36±6 7-48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(33±5.2) (24-45)</td>
<td>(4.2±0.7) (0.8-5.6)</td>
</tr>
</tbody>
</table>

a) Females taking oral contraceptives.
b) 'S' represents standard deviation of set of specimens.
which 50 μl of U.S.P. heparin had been added. One milliliter of this heparinized blood was pipetted directly into acid washed vials and was used to prepare blood pellets in the usual manner. The remaining blood was centrifuged and the plasma removed within one-half hour to form plasma pellets for analysis via XRF.

A questionnaire given to the male subjects during the blood clinic was designed to yield personal information such as age, height, weight, recent illnesses, medication and vitamin supplements which they had recently used. This questionnaire, reproduced in Appendix VIII, was used to assess the impact of recent diseases and medication on the levels of Cu, Zn, Br and Rb in the healthy male individuals.

Table XI and Figures 17 to 20 display the results of XRF analysis of blood and plasma pellets of these male subjects. As indicated in the Figures, the values found in the literature compare quite favourably with the mean values of Anspaugh (Ans 71) and Underwood (Und 71) where * = adult; a = Ans 71; u = Und 71; subscript s = serum. Levels of 608 and 774 μg/100 ml blood copper were found in two individuals, 34 and 27 years old respectively. These two data were not included in calculation of the means as they were considered statistically unsatisfactory (Q test). It is interesting to note that some subjects indicated that they used multiple vitamin-mineral supplementation in their diet, yet no significant differences in the levels of Cu, Zn, Br and Rb were found in these subjects and those not using vitamin-mineral supplementation. In addition,
### TABLE XI

Trace Element Levels in 37 Male Subjects

<table>
<thead>
<tr>
<th>Element</th>
<th>Whole Blood Mean±S*</th>
<th>Range (µg/100 mls)</th>
<th>Plasma Mean±S*</th>
<th>Range (µg/100 mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>107±25 [112±35]</td>
<td>78-189 (78-248)^a</td>
<td>120±22</td>
<td>90-158 [205]^b</td>
</tr>
<tr>
<td>Zinc</td>
<td>776±88</td>
<td>580-1023</td>
<td>104±17</td>
<td>63-140</td>
</tr>
<tr>
<td>Bromine</td>
<td>346±69 (43±8.6)</td>
<td>264-537 (33-67)</td>
<td>508±94</td>
<td>395-725 (49-91)</td>
</tr>
<tr>
<td>Rubidium</td>
<td>305±45 (36±5.2)</td>
<td>222-396 (26-46)</td>
<td>36±6</td>
<td>27-48</td>
</tr>
</tbody>
</table>

^a) One subject with 248 µg/100 mls plasma copper.

^b) One subject with 205 µg/100 mls plasma copper but not included in calculation of the means.

*S represents the standard deviation of the set of measurements.
FIGURES 17-20

Histograms of Trace Element Levels
in Normal Healthy Males
BLOOD BROMINE

\[ \mu g/100 \text{ mls} \]

BLOOD RUBIDIUM

\[ \mu g/100 \text{ mls} \]
If PLASMA RUBIDIUM.

\[
\begin{array}{c}
\text{μEq/L} \\
44 \quad 69 \\
350 \quad 450 \quad 550 \quad 650 \quad 750
\end{array}
\]

\[a^*_s \quad m\]

PLASMA BROMINE

\[
\begin{array}{c}
\text{NUMBER OF INDIVIDUALS (ADULT MALES)} \\
20 \quad 30 \quad 40 \quad 50 \quad 60
\end{array}
\]

\[m\]

PLASMA RUBIDIUM

\[
\begin{array}{c}
\text{μg/ 100 mls} \\
2.3 \quad 4.7 \quad 7.0
\end{array}
\]
no striking differences were noted for the two subjects excluded because their Cu levels were too high. Though such correlations are not the primary aim of this study, the potential usefulness of such information in other types of studies, and the availability of such data via XRF should not be overlooked.

3.33 Profiles - Study C (Children)

Whole blood was collected from 20 male children and 16 female children between the ages of 4 and 15 at the Children's Hospital in Vancouver. Since it was available, blood from 10 children known to be suffering from cystic fibrosis was also collected. The blood was obtained in the same manner as the previous study and samples were prepared in the usual manner.

Table XII displays the results of the trace element concentrations found in the 'normal' population of children and those children suffering from cystic fibrosis. Since no significant difference was noted between the concentrations of the four elements studied in males and females samples (via the Student 't' test), the results of both males and females samples were combined.

3.4 Verification Results
3.4.1 Atomic Absorption Spectrophotometry

The plasma samples, described in Section 3.31, gathered from 38 female students, were also used in the atomic
### TABLE XII

Trace Element Levels in Children's Plasma

<table>
<thead>
<tr>
<th>Variate</th>
<th>n</th>
<th>Mean ± S b)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age yrs.</td>
<td>35</td>
<td>10.0±3.4</td>
<td>4-15</td>
</tr>
<tr>
<td>Cu µg/100 mls</td>
<td>32 a)</td>
<td>129±32</td>
<td>80-217</td>
</tr>
<tr>
<td>Zn µg/100 mls</td>
<td>35</td>
<td>106±16</td>
<td>79-137</td>
</tr>
<tr>
<td>Br µg/100 mls</td>
<td>35</td>
<td>545±110</td>
<td>354-748</td>
</tr>
<tr>
<td>Rb µg/100 mls</td>
<td>35</td>
<td>33.3±7.3</td>
<td>22-56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variate</th>
<th>n</th>
<th>Mean ± S b)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age yrs.</td>
<td>10</td>
<td>11.0±4.5</td>
<td>4-15</td>
</tr>
<tr>
<td>Cu µg/100 mls</td>
<td>10</td>
<td>135±37</td>
<td>93-208</td>
</tr>
<tr>
<td>Zn µg/100 mls</td>
<td>10</td>
<td>96±11</td>
<td>79-121</td>
</tr>
<tr>
<td>Br µg/100 mls</td>
<td>10</td>
<td>487±104</td>
<td>329-623</td>
</tr>
<tr>
<td>Rb µg/100 mls</td>
<td>10</td>
<td>36±6</td>
<td>23-46</td>
</tr>
</tbody>
</table>

a) Three female children were deleted after invoking the Q test, since the levels of copper were found to be 357, 406, and 382 µg/100 mls.

b) Error determined on set of samples.
absorption verification studies. Verification was accomplished by analyzing the same aliquots of plasma, for copper, by both the XRF method and by the flame A.A. method described earlier (Section 2.21).

Comparisons of the results obtained by A.A. and XRF are displayed graphically in Figure 21. The mean value of the samples was found to be 132±40 µg/100 mls via XRF and 136 ± 40 µg/100 mls via A.A. The good agreement of these two sets of data is supported by the high value of the linear correlation coefficient; r = 0.93. (See Appendix III for discussion of 'r', the correlation coefficient.)

The second method used in the flame A.A. verification studies, discussed earlier in Section 2.21, involves denaturing the protein prior to aspirating the samples into the flame. Results of these measurements also provide data which may be used to verify the observed XRF measurements.

Four different specimens were analyzed by XRF and A.A. The results of analyses of three to five aliquots from these four specimens are shown in Table XIII. Analysis of the differences in the means, by the Student 't' test (see Appendix II), yield probability or α values which have also been included in this table. Since α is greater than 0.05, the differences in the means probably originate randomly and the two techniques may be assumed to produce indistinguishable plasma copper measurements.
FIGURE 21

Comparison Between Plasma Copper Determinations via Flame A.A. and XRF
Y = 6.1 + 0.93x
r = 0.92
### TABLE XIII

Results of XRF and AA Analysis on the Same Samples

<table>
<thead>
<tr>
<th>Specimen (Lot #, origin)</th>
<th>A.A. Mean ±2S&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>XRF Mean ±2S</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>3659K00Al Hyland</td>
<td>131±7</td>
<td>132±6</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>3659T00Al Hyland</td>
<td>124±16</td>
<td>132±5</td>
<td>0.55</td>
</tr>
<tr>
<td>3659V00Al Hyland</td>
<td>109±7</td>
<td>116±9</td>
<td>0.35</td>
</tr>
<tr>
<td>Pooled Serum</td>
<td>148±10</td>
<td>146±7</td>
<td>0.60</td>
</tr>
</tbody>
</table>

<sup>a</sup> S: standard deviation of set of measurements.
3.42 Anodic Stripping Voltammetry (A.S.V.)

As described in Section 2.31, samples of blood with added amounts of lead were analyzed both by the XRF technique and by an A.S.V. method. Results of this comparison study, with estimated errors, are presented graphically in Figure 22. Statistical correlation functions were used and the linear correlation coefficient, r, was calculated to be 0.99. This high value of r is an indication of excellent agreement between the measurement of blood lead by the two methods described.

3.43 Analysis of IAEA Reference Blood

In order to further verify the XRF method, a reference sample of whole blood was analyzed. The sample, consisting of 80 grams of homogenized, spray dried animal blood from cattle and pigs, was obtained from the International Atomic Energy Agency (IAEA). Since this dried organic powder would not easily suspend in water, samples were prepared by pelletizing the solid material directly. It was necessary to convert the response curves obtained earlier to ppm instead of μg/100 mls by determining the density of the whole blood used to generate the initial analytical curves. The slopes of the analytical curves used to determine the levels of trace elements in the dried whole blood were taken from Figure 9.

The results obtained using XRF analysis compared against the IAEA accepted values, are shown in Table XIV. The IAEA values represent the mean values of measurements
FIGURE 22

Comparison Between Blood Lead Determinations via XRF and A.S.V.
Pb IN BLOOD

\[
\frac{\text{AREA } \text{Pb} \text{ L}\alpha}{\text{AREA } \gamma \text{ K}\alpha} (\text{XRF})
\]

ANODIC STRIPPING ANALYSIS (\(\mu g / 100ml\))
TABLE XIV

Results of Analysis of IAEA Reference Blood

<table>
<thead>
<tr>
<th>Element</th>
<th>XRF</th>
<th>IAEA Accepted Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>1029 ppm</td>
<td>1038* ppm</td>
</tr>
<tr>
<td>Mn</td>
<td>102 ppm</td>
<td>123±18% ppm</td>
</tr>
<tr>
<td>Fe</td>
<td>3829 ppm</td>
<td>3410±7.6% ppm</td>
</tr>
<tr>
<td>Cu</td>
<td>51 ppm</td>
<td>45±6.6% ppm</td>
</tr>
<tr>
<td>Zn</td>
<td>145 ppm</td>
<td>89±14% ppm</td>
</tr>
<tr>
<td>Br</td>
<td>12.3 ppm</td>
<td>14* ppm</td>
</tr>
</tbody>
</table>

* Data available from one lab only.

Reasonably good agreement is observed between these two sets of data. The zinc level appears somewhat higher via XRF than that quoted by IAEA. The source of this discrepancy is not known, but the sample may have been contaminated with zinc en route, or during preparation.
This discrepancy noted here does not affect the validity of the previous results particularly when considering that our values were in good agreement with published values (Sec. 3.31, 3.32, 3.33). It is probable that the sample was either contaminated, or the IAEA values are suspect, because the zinc photopeak was about four times the intensity of the copper photopeak. (The EDXRF technique used here is more sensitive to zinc than copper.)

3.5 Analysis of Precision of the XRF Method

There are several steps involved in the preparation and analysis of samples via XRF, each of which can contribute distinctly to the error associated with a single measurement (see Section 1.514). In the analysis of error for the A.A. method used herein, aliquots of the same specimen were analyzed repeatedly and a standard deviation calculated from these results. On the other hand, in the analysis of error of the XRF method, each contribution was determined separately and combined in quadrature to produce the relative magnitude of the overall error. Each error was determined individually in order to assess the size of the contributions of each component so that improvements in either the preparation method or analytical device could be made or suggested. As has been mentioned (Section 1.514) the overall error has been broken down into 4 separate areas; counting, placement, drift and preparation. The counting error is determined by applying formula 9 (Section 1.514) to the areas of the element and standard photopeaks. The placement error is determined by
analyzing the same pellet several times after re-orienting it. The error associated with electronic drift and changes in some components of the instrument, called the drift error, has been calculated from results of analysis of one pellet over several months. After preparing the same aliquots from a single large specimen, and analyzing the resulting pellet, a preparation error could be determined. The net relative magnitude of these errors ($\varepsilon\%$) is displayed in Table XV.

As can be seen in the above table, for Cu, the largest error is associated with the preparation. The preparation is largely a result of inhomogeneity in the pellets. It is the processes of freeze-drying and grinding which affects the integrity of the prepared samples to the largest extent, and must be particularly regulated during sample preparation.

For Zn, Br and Rb, the counting statistics play a large role. In order to diminish the counting error ($\varepsilon$(count)) by a factor of 2 (for example) a counting time of one hour would be necessary. If we wished to apply this method to a clinical setting rather than count for extended periods an increase in the exciting beam intensity may be more practical to reduce the counting error.

The errors displayed in the above Table may be combined in quadrature, assuming they are random, as follows:

$$\varepsilon_{\text{total}} = [\varepsilon_{\text{count}}^2 + \varepsilon_{\text{place}}^2 + \varepsilon_{\text{drift}}^2 + \varepsilon_{\text{prep}}^2]^{1/2}$$

The resulting total error is displayed in Table XVI.

It should be emphasized that this is the total error and includes such variables as drift and counting statistics. In order to compare the usual error quoted with other analytical
measurements, only the observed error associated with $\epsilon_{\text{prep}}$ should be reported. This error is not reported here but it is slightly greater than the net preparation error shown in Table XV (see Section 4.1, p. 122).
TABLE XV

Net Relative Errors Associated with the XRF Method (1 Standard Deviation)

<table>
<thead>
<tr>
<th>Element</th>
<th>Peak</th>
<th>( \varepsilon(\text{count}) )%</th>
<th>( \varepsilon(\text{place}) )%</th>
<th>( \varepsilon(\text{drift}) )%</th>
<th>( \varepsilon(\text{prep}) )%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>( K_\alpha )</td>
<td>3.8</td>
<td>3.4</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Cu</td>
<td>( K_\alpha )</td>
<td>2.2</td>
<td>0</td>
<td>3.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Zn</td>
<td>( K_\alpha )</td>
<td>2.6</td>
<td>0</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Br</td>
<td>( K_\alpha )</td>
<td>1.3</td>
<td>0.64</td>
<td>0</td>
<td>0.84</td>
</tr>
<tr>
<td>Rb(^a)</td>
<td>( K_\alpha )</td>
<td>1.8</td>
<td>2.6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\( a\) Rb \( K_\alpha \) error determined from Br \( K_\beta \) + Rb \( K_\alpha \) single peak plus the error of the Br \( K_\alpha \) peak.
TABLE XVI

Total Relative Error of the XRF Method

<table>
<thead>
<tr>
<th>Element</th>
<th>1ε%</th>
<th>2ε%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>15.9</td>
<td>31.8</td>
</tr>
<tr>
<td>Cu</td>
<td>6.6</td>
<td>13.2</td>
</tr>
<tr>
<td>Zn</td>
<td>4.27</td>
<td>8.54</td>
</tr>
<tr>
<td>Br</td>
<td>1.69</td>
<td>3.38</td>
</tr>
<tr>
<td>Rb</td>
<td>3.13</td>
<td>6.26</td>
</tr>
</tbody>
</table>
4. DISCUSSION OF RESULTS

4.1 Application

Not all of the essential elements present in blood can be observed using the XRF technique described herein. Of the nine elements that could be easily measured (S, Cl, K, Ca, Fe, Cu, Zn, Br and Rb) only four (Cu, Zn, Br and Rb) were used to show the potential capabilities of the method. Copper and zinc were chosen because of their importance in human health, nutrition, and disease. Bromine and rubidium, which probably act as electrolytes, were chosen not only because they were relatively easy to measure but also because of their possible clinical importance and potential correlations in health and disease.

A major aspect of assessment of the XRF procedure required application of the method. For this, the trace element concentrations in the blood and plasma of adult males, adult females and children were measured. This does not represent a comprehensive study of trace element levels in the blood and plasma of healthy individuals. Rather, since successful, the results are valuable in indicating the strength of this technique in its capacity for characterizing trace element levels in human blood or serum/plasma.

Because XRF is sufficiently sensitive to the four elements studied (Figures 9 and 10), differences may be noted in the concentration profiles produced. For example, besides the distinct difference in copper concentrations between
'normal' females and those on oral contraceptive therapy is the difference noted between females', males' and children's plasma copper; 113±19, 120±22, and 129±32 μg/100 mls respectively.

The error associated with normal values above, is the distribution about the mean value assuming that the deviations are distributed randomly. To quote trace element 'profiles' both the mean and this deviation must be reported. In the case of male and female samples, although the means may be different, their profiles (width and height of the distribution) are essentially the same.

There is also an error associated with a single measurement which is primarily a result of variations in sample preparation. The magnitude of the observed error (±2S) has been determined, for copper, as 10.7%; zinc 7.1%; bromine 3.4% and rubidium 5.4%. The discrepancy between these values and those in Table XVI reflect the difference in the total error of the method which includes instrumental drift and counting fluctuations and the total error of sample preparation.

The errors just quoted are also not the net preparation error (Table XV) but rather, the observed preparation error determined by analyzing several pellets prepared from the same aliquot. By an assessment of the error associated with a single A.A. plasma copper measurement, in a similar manner, a 6.1% error was determined. Although the precision of XRF is almost twice as high as that of A.A., a maximum of an 11% error is not excessive, and reliable measurements may safely
be reported. Since this error is primarily a result of drying, grinding and pelletizing, a strict regulation of these steps would probably diminish the deviation. Note also that the errors for the other elements are considerably less. This is a result of the increased sensitivity (high relative counting rate) for elements of higher atomic number.

A thorough assessment of the XRF method required verification of some of the results with other established analytical techniques. Consequently, A.A. was successfully utilized to establish the accuracy of plasma copper XRF analyses. Excellent agreement was observed between XRF and flame A.A., since the correlation coefficient calculated (0.93) indicates that there is less than a 0.1% probability that a random distribution would yield similar results.

A.A. was chosen because of its reliability in determination of trace elements in some biological material. It should be emphasized that due to the organic nature of whole blood, few A.A. methods are available for determination of elemental concentrations in this matrix. Because x-rays are scattered and absorbed in a predictable fashion in particular matrices, whole blood does not affect XRF measurements to the extent that it affects A.A. measurements. One of the more difficult biological materials to analyze may therefore be easily assessed for Cu, Zn, Br and Rb using the method described herein.

Another method that has been applied primarily to whole blood analysis is A.S.V. Verification studies completed,
by addition of lead to whole blood, showed excellent agreement between the measurement by XRF and A.S.V. \( (r = 0.99) \). It was necessary to add lead to XRF because no photo peaks were observable in normal samples.

In summary, EDXRF analysis using a freeze drying-pelletizing method of sample preparation has been successfully verified by flame atomic absorption spectrophotometry and anodic stripping voltammetry.

4.2 Detection Limit of the XRF Method

To determine and compare the effectiveness of one analytical technique with another, the limits of detection must be determined. Using formula (6) (Section 1.513) the lower limit of detectability (LLOD) for 8 elements was calculated and is shown in Table XVII. It must be emphasized that a very generous deviation \( (\pm 3\sigma) \) of the background is taken into account in the above calculation. It has been stated elsewhere that only \( \pm 2\sigma \) is a sufficiently high deviation to include all fluctuations in background and in this case a slightly lower LLOD, than that reported here, would result.

For the measurement of the elements used to assess the technique, the LLOD calculated is sufficiently low for determination of Ca, Fe, Cu, Zn, Br, Rb and in some cases Pb. As Table I shows, the concentrations of Cu, Zn, Br and Rb are lower than those associated with any of the disease states listed and therefore this method may be used to help assess specific disease conditions. Although the LLOD of Fe is low
### TABLE XVII

Lower Limit of Detectability of the XRF Freeze-Drying Technique

<table>
<thead>
<tr>
<th>Element</th>
<th>Whole Blood</th>
<th>Plasma</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/100 ml</td>
<td>µEq/L</td>
<td>µg/100 ml</td>
<td>µEq/L</td>
</tr>
<tr>
<td>Ca</td>
<td>100</td>
<td>49.8</td>
<td>31</td>
<td>15.4</td>
</tr>
<tr>
<td>Cr</td>
<td>34</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>--</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>26</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>22</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br</td>
<td>10</td>
<td>1.3</td>
<td>8</td>
<td>1.2</td>
</tr>
<tr>
<td>Rb</td>
<td>26</td>
<td>3.0</td>
<td>15</td>
<td>1.8</td>
</tr>
<tr>
<td>Pb</td>
<td>22</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
enough, inconsistent photopeak areas in the background spectra compounded with the contamination by iron in the syringes made it difficult to measure iron reliably, and was therefore not included in this study.

The upper acceptable limit for blood lead levels is 50 µg/100 mls (Tie 70) and the average concentration is probably less than half of this value. Indeed, an average level of 6.8 µg/100 mls was found in the blood of 33 adult females. Since the LLOD for blood lead concentrations is 22 µg/100 mls, the XRF freeze drying pelletizing technique could not be used for blood lead measurements. Instead, XRF could easily be used as a screening procedure for subjects with suspected high blood lead levels.

The concentration of calcium in serum ranges between 8.7-10.7 mg/100 mls or 4.4-5.4 mEq/L (Tie 70). Thus the LLOD calculated of 31 µg/100 mls or 15.4 µEq/L is several orders of magnitude smaller than that necessary to measure this element. In addition to calcium, the other low atomic number element may also be measured via XRF easily.

The normal concentration levels of chromium in human tissues is considerably less than that shown in Table XVII. This element may therefore not be measured via the XRF technique assessed herein. In fact, the features of this table which parallels, to some extent, the sensitivity of XRF to these elements (Figures 9 and 10) (high detection limit for low atomic number and low detection limit for high atomic number), reflects a limiting feature of all EDXRF analysis
while using an essentially monochromatic exciting source. The further advantages and disadvantages of this feature are presented in succeeding sections.

The further advantages and disadvantages of this feature are presented in succeeding sections.

The variation in LLOD's do not follow smooth functions and some LLOD's are higher than would be expected from the sensitivity plots (Figures 9 and 10). For example, the detection limits of Fe, Cu and Zn are relatively high because distinct background peaks, which must be taken into account, exist for these elements. Also, although the yield for RbKα radiation is high under MoKα exciting radiation, the LLOD does not reflect this. The higher LLOD for this element compared with the Br LLOD is due to an overlap by the BrKβ on the RbKα. An error is associated with subtracting this overlap which tends to increase the LLOD.

Table XVIII has been compiled from the literature for comparison of LLOD and methods of sample preparation using x-ray induced XRF. A striking and common feature of all these reports is the lack of thoroughness in characterizing EDXRF for trace element analysis in blood and plasma. The only comparable report (Flit 75) to the present study fails to report LLOD, analyzed only serum samples and did not verify the results.

The table shows LLOD in ppm for comparison purposes only. To equate ppm to ug/100 mls requires a simple multiplication by 100. Different sources of exciting radiation are used along with variations in counting times. The most direct comparisons with the system and method employed at S.F.U. are those
TABLE XVIII

Sample Preparation and LLOD of Elements
Analyzed Using X-ray Induced XRF

<table>
<thead>
<tr>
<th>Exciting Radiation</th>
<th>Sample Preparation</th>
<th>Time mins</th>
<th>Elements Analyzed</th>
<th>LLOD ppm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo-1</td>
<td>1 gr As doped freeze dried serum</td>
<td>10</td>
<td>As</td>
<td>1.65</td>
<td>Lub 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sr</td>
<td>4.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>W</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hg</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pb</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>n/s*</td>
<td>100 mg/cm² freeze dried, pelletized blood</td>
<td>16.7</td>
<td>Pb</td>
<td>0.1</td>
<td>Wol 73</td>
</tr>
<tr>
<td>Se-2</td>
<td>dried and ashed Ni doped serum</td>
<td>n/s</td>
<td>Fe</td>
<td>est.</td>
<td>Ong 71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cu</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mo-2</td>
<td>freeze dried, pelletized serum</td>
<td>30</td>
<td>Cr</td>
<td>1</td>
<td>Gia 73</td>
</tr>
<tr>
<td></td>
<td>30 mg/cm²</td>
<td></td>
<td>Fe</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cu</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zn</td>
<td>n/s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Br</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rb</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Mo-2</td>
<td>freeze dried blood pelletized; 0.5 mm thick</td>
<td>n/s</td>
<td>Pb</td>
<td>0.5</td>
<td>Gou 74</td>
</tr>
<tr>
<td>Mo-2</td>
<td>0.5 ml V doped freeze dried and ashed serum</td>
<td>160</td>
<td>K</td>
<td>n/s</td>
<td>Fli 75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>n/s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fe</td>
<td>n/s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cu</td>
<td>n/s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zn</td>
<td>n/s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Br</td>
<td>n/s</td>
<td></td>
</tr>
</tbody>
</table>

* n/s = not stated
1 = primary target
2 = secondary target
using the secondary targets and analyzing freeze-dried pelletized serum (Gia 73, Gou 74). All of the LLOD's reported in these works are higher than those reported here. For example, Giaque (Gia 73) reports an LLOD for serum copper of 0.3 ppm (30 µg/100 mls). The level reported here is about half of this value, 0.17 ppm (17 µg/100 mls).

4.3 Correlation Analysis

Although not the primary aim of this study, this section is presented to show the potential of x-ray fluorescence for the determination of correlations or interrelationships between trace elements in health and disease. Generally, the other analytical devices which have been applied to analysis of trace elements in biological material, are not multielemental as is XRF. Therefore, correlation statistics are not readily obtainable. This may be particularly important in a clinical setting where the time available for analysis is limited.

Correlation in trace element studies have generally been investigated between the concentration of a specific metal and the state of a disease or condition. For example, definite relationships have been discovered between the concentration of copper in serum, age, ceruloplasmin levels, and oral contraceptives (Yum 74, Sch 72). Similar relationships have been found between copper levels and diabetes mellitus (Kan 67). Other relationships have been sought between elements. For example, since imbalances of copper and zinc have
been implicated as a major factor in the etiology of cardiovascular diseases (Kle 75) the copper/zinc ratio could be used to assess the clinical condition of patients suffering from these diseases. Another example of two element correlations are changes in relationships between K and Pb in patients with and without excessive x-ray exposure (Sco 73).

Further studies on the interrelationships between many elements (14) have been investigated in control children and children with Pica (Del 73) (ingestion of lead with an excess lead in the blood stream). Spark source mass spectrometry was used since it permitted multielemental determinations of all the elements of interest. Many of the changes in correlations observed between controls and patients were assumed to be a result of the ingestion of toxic metals such as lead.

The mathematical definition of the correlation coefficient (r) is shown in Appendix III. To reiterate, 'r' is a measure of the dependence of one variable on another. This dependence may be positive or negative. In a positive correlation, as one metal increases, so does the other. It follows that in a negative correlation, as one metal increases the other decreases. If a correlation coefficient attains a low value it may not necessarily mean that a poor correlation exists. For this reason probability values, 'α', are reported based on the degrees of freedom and magnitude of 'r'. Definite correlations occur and are generally reported when the probability value is equal to or less than 5%, (α ≤ 0.05).

In order to determine quantitatively if any correlation
existed between the levels of trace elements for a group of individuals, coefficients of correlation (r) were calculated for healthy females and females taking oral contraceptives. The results of the calculations, with the APL programs "Multiple Regression" and "Simple Correlation" (S.F.U. Computing Centre), performed on the IBM 370 computer, are shown in Table XIX.

Generally, the low values of the correlation coefficients indicates a lack of definite interrelationship between any set of two variables. An exception to this is the slightly higher 'r' values and lower levels of significance (α or probability values) for copper-zinc levels in healthy females and the bromine-rubidium levels in B.C.P. subjects. Between these two sets of subjects therefore, one correlation is lost, and another becomes apparent. The significance of these changes is not known.

As was undertaken previously, coefficients of correlation were calculated for the levels of trace elements in the plasma of male subjects. These values, shown in Table XX, indicate the poor correlation between the levels of one element and another. Interestingly however, the sign of the correlation coefficient for copper-zinc and bromine-rubidium is reversed from that in the female subjects. A high correlation for Br-Rb levels is noted. The male population therefore shows changes in correlations compared with the normal females. The significance of these changes is also not known.

Prior to investigating the interrelationships between
TABLE XIX

Correlation Coefficients Between Plasma Trace Element Concentrations in Female Subjects

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Normal Females n=28</th>
<th>B.C.P. a) n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>α</td>
</tr>
<tr>
<td>Cu-Zn</td>
<td>-0.366</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Cu-Br</td>
<td>0.163</td>
<td>≥0.10</td>
</tr>
<tr>
<td>Cu-Rb</td>
<td>-0.140</td>
<td>≥0.10</td>
</tr>
<tr>
<td>Zn-Br</td>
<td>-0.055</td>
<td>≥0.10</td>
</tr>
<tr>
<td>Zn-Rb</td>
<td>0.140</td>
<td>≥0.10</td>
</tr>
<tr>
<td>Br-Rb</td>
<td>0.263</td>
<td>≥0.10</td>
</tr>
</tbody>
</table>

a) B.C.P. - subjects on oral contraceptive therapy.
### TABLE XX

Coefficients of Correlation Between the Male Plasma Trace Element Levels

<table>
<thead>
<tr>
<th>Elements</th>
<th>$r$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-Zn</td>
<td>+0.304</td>
<td>$\geq$0.05</td>
</tr>
<tr>
<td>Cu-Br</td>
<td>-0.108</td>
<td>$\geq$0.10</td>
</tr>
<tr>
<td>Cu-Rb</td>
<td>-0.086</td>
<td>$\geq$0.10</td>
</tr>
<tr>
<td>Zn-Br</td>
<td>+0.094</td>
<td>$\geq$0.10</td>
</tr>
<tr>
<td>Zn-Rb</td>
<td>-0.114</td>
<td>$\geq$0.10</td>
</tr>
<tr>
<td>Br-Rb</td>
<td>+0.417</td>
<td>-0.01</td>
</tr>
</tbody>
</table>
any two sets of metals in the normal children's samples and sick children, and between normal children and adult males, the significance of the difference in the mean concentrations between the groups was investigated. Results of a Student 't' test (a values only) are shown in Table XXI. A difference in zinc concentration can be noted, between cystic fibrosis children and normal children, however, since a is not less than 0.05 the difference is not highly significant. No other significant differences are noted.

Although no real differences were observed (via the t test, Table XXI), correlation coefficients were calculated to compare the relationships of trace element concentrations between normal children and children afflicted with a disease. The results are displayed in Table XXII.

In normal children definite and previously unobserved correlations occur between Cu-Br and Br-Rb concentrations. The former correlation is different as compared with normal males (Table XIX) but the latter parallels that of normal males. The positive correlation between Cu and Br is somewhat surprising since one is a metal involved in enzyme function, the other, an electrolyte.

The strong age-Cu correlation, calculated only for children (shown in Table XXII), has been observed elsewhere (Tes 73). This agreement does increase the credibility of our studies and aids in exhibiting the analytical sensitivity of the method.

All correlations in trace metal concentration, and
with age, are apparently lost in the C/F patients. Thus, although there are no significant differences between the mean concentrations of the elements studied, definite differences in the correlation statistics do occur. These changes may therefore be used to characterize not only C/F patients but also other subjects suffering from other maladies where even stronger interrelationships might exist.
TABLE XXI

Results of Student t Test Between Normal Children and Cystic Fibrosis Patients
and Between Normal Children and Adult Males

<table>
<thead>
<tr>
<th>Variate</th>
<th>C/F Children $\alpha$</th>
<th>Males $\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.42</td>
<td>--</td>
</tr>
<tr>
<td>Cu</td>
<td>0.59</td>
<td>0.20</td>
</tr>
<tr>
<td>Zn</td>
<td>0.08</td>
<td>0.6</td>
</tr>
<tr>
<td>Br</td>
<td>0.11</td>
<td>0.15</td>
</tr>
<tr>
<td>Rb</td>
<td>0.70</td>
<td>0.10</td>
</tr>
</tbody>
</table>
TABLE XXII

Correlation Coefficients Between Trace Element Levels in Children's Plasma

<table>
<thead>
<tr>
<th>Variate</th>
<th>Normal Children</th>
<th>C/F Children</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>r</td>
</tr>
<tr>
<td>Age-Cu</td>
<td>32</td>
<td>-0.542</td>
</tr>
<tr>
<td>Age-Zn</td>
<td>35</td>
<td>-0.145</td>
</tr>
<tr>
<td>Age-Br</td>
<td>35</td>
<td>-0.216</td>
</tr>
<tr>
<td>Age-Rb</td>
<td>35</td>
<td>-0.089</td>
</tr>
<tr>
<td>Cu-Zn</td>
<td>32</td>
<td>-0.034</td>
</tr>
<tr>
<td>Cu-Br</td>
<td>32</td>
<td>0.372</td>
</tr>
<tr>
<td>Cu-Rb</td>
<td>32</td>
<td>-0.101</td>
</tr>
<tr>
<td>Zn-Br</td>
<td>35</td>
<td>-0.126</td>
</tr>
<tr>
<td>Zn-Rb</td>
<td>35</td>
<td>0.020</td>
</tr>
<tr>
<td>Br-Rb</td>
<td>35</td>
<td>0.460</td>
</tr>
</tbody>
</table>
5. DISCUSSION

5.1 Methods of Sample Preparation

Of the four methods investigated for the preparation of blood and plasma specimens, the freeze-drying technique and subsequent formation into self-supporting pellets of consis-tant mass has been found to represent a reliable method for the determination of copper, zinc, bromine and rubidium. It has been proven to be with reasonable accuracy compared with the A.A. and A.S.V. methods and the method is sensitive enough to assure the capability of determining differences between samples from subjects suffering different diseases. The precision is also within acceptable experimental error. This error, which is maximum for copper, may be considered somewhat high, (overall error ~13%, sample prep ~11%) but by controlling the preparation of samples to a higher degree it is conceivable that it could be decreased to the error associated with a single A.A. measurement.

Preparing samples by ashing also represents a potentially valuable technique, however, it has numerous disadvantages. Although the analysis of raw ash may yield higher sensitivities, and subsequently better LLOD's, the potential sources of error are high; this method is prone to contamination; it is much more time consuming, and leads to an inconsistent loss of some elements. Further, the samples are difficult to handle and a much larger original volume is required to form an ash of reasonable mass for analysis in
the XRF spectrophotometer. Finally, to form self-supporting samples, a binder is necessary in some cases which would dilute the ash and lower the observed sensitivity. An element such as chromium, which occurs in serum at about 10 µg/100 mls (Ans 71) may be measured if the ashing process is capable of concentrating this element by tenfold from that in the freeze dried material. Other elements that occur at approximately equivalent concentration as Cr, which could be measured if the samples were concentrated by about tenfold, are V, Ge, Se, and Cd (Ans 71). (Concentrations up to 100x would be necessary to measure elements such as Co and Mn.)

The advantages of the freeze drying process, although not as sensitive as the ashing methods are many. The time required is considerably less, there is no contact with toxic or corrosive chemicals, and the freeze dried product is relatively easy to handle and may be re-analyzed easily, if necessary.

While involved in trace element research, we must be very aware of the role of contamination during contaminant, storage, preparation and analysis of the samples. The greater the number of steps involved in the preparation of the final product, the greater is the possibility of effects of extraneous metals. Since there are fewer manipulations associated with the process of freeze drying, the probability of contamination is less than that of the ashing processes.

Contamination was the major determinant in the decision to abandon iron analysis in blood plasma. It was found that
an inconsistent level of iron occurred in the samples while using the disposable plastic syringes.

The size of the sample required also plays an important role in deciding upon a sample preparation procedure. To form freeze dried pellets, only one milliliter of sample is necessary, for ashed samples, from 10-20 mls of sample is required. Compared with flame A.A. which is suitable for Cu+Zn analyses, the sample sizes required are about equivalent with that needed for the freeze drying process. For elements in low concentration, such as Cr, the more sensitive electrically heated graphite furnace is generally employed. The quantity of sample usually required for these measurements in the furnace (flameless A.A.) is between 5 and 25 μl. Thus, for a single analysis, flame A.A. may be preferred. However, if XRF could be made more sensitive by using a large volume of initial sample (viz. the ashing process) the multielemental nature of XRF may outweigh any of the advantages of the flameless A.A. method.

A.S.V. is particularly applicable to blood lead determinations. At present, because XRF is not sufficiently sensitive to this element, as mentioned, XRF may only be used as a screen for high lead concentrations. A 2-3 fold increase in concentration would be sufficient for lead measurements. Thus, if the ashing was relatively reliable, it may be applied to investigation of this element also.
5.2  EDXRF System

5.2.1  Electronics and Data Handling

As was shown in the previous section (5.1), some changes in the sample preparation procedure might increase sensitivity and precision with a subsequent much desired decrease in the LLOD. Here is presented in a general sense some of the changes or improvements that may be made to the method of data handling and electronics which could ultimately reach the same goals: a decrease in the LLOD and capacity to characterize more of the essential and toxic trace elements.

One of the simplest procedures to increase the sensitivity of XRF is to count the samples for longer periods of time. Although the sensitivity may be increased, the precision will increase by only a $\sqrt{N}$ where $N$ is the number of counts in the photopeak. Thus, a one hour counting time would be required for each sample to increase the sensitivity by fourfold and the precision by twofold.

Longer counting times would decrease the LLOD also, but since the LLOD $\alpha 3\sqrt{N_b/N_c}$ ($N_b$ background count, $N_c$ characteristic photopeak count of a pellet with constant concentration of trace element) a counting time of one hour would decrease the LLOD by a factor of only $1/2$. In the case of an element such as Cr a $1/10$ decrease in the LLOD is necessary for reliable measurements.

Sample preparation, which was covered in the previous section, plays an important role in the capacities of the nuclear electronics used for trace element work via XRF.
Previously it was shown that a constant optimum thickness was necessary. On increasing this thickness, a relative decrease in the photopeak/backscatter results. This process is due to the incapacity of the amplifier and pulse shaping electronics to handle the high count rates originating from this increase in backscattered radiation (primarily MoKα coherent and incoherent scatter). More sophisticated electronic apparatus, capable of handling count rates up to 100 times that of the present instrument would in effect allow an increase in the sensitivity and precision with an ultimate decrease in the LLOD.

Let us consider a one-hundred fold increase in count rate (with electronic apparatus capable of handling this count rate). This count rate may be accomplished relatively easily by the combination of two methods; a large increase in x-ray tube current, and an increase in sample thickness. It must be borne in mind, however, that the thickness is limited by the 'infinite' thickness (see Figure 1, Section 1.51) and the quantity of sample available. One method of increasing the thickness without the need for increased sample size is to utilize a smaller diameter pellet press. This option should not be overlooked because the collimator diameter is much smaller than the present samples. A one-hundredfold increase in flux reaching the detector could decrease the LLOD by the desired factor of 10 and make it possible to analyze many of the elements in very low concentration in human blood and serum/plasma.
In addition to the aforementioned improvements to produce higher sensitivity and lower LLOD are changes that could be made to geometry and orientation of the x-ray radiation and variations in target and filter assemblies. For example, if the exciting beam was closer to the sample, a higher flux of radiation might reach it. If attempting to measure a specific group of elements (e.g., V, Cr) a secondary target of iron or cobalt could be utilized since the highest fluorescent yield is observed with exciting radiation just greater than the absorption edge energy of the element of interest.

Both the contributions of the background continuum and the background photopeaks play a large role in affecting the LLOD. By re-designing the beam chamber and sample chamber (with Al for example) the background photopeaks could be completely eliminated.

With the use of a different or thicker filter assembly the background would not be decreased to any appreciable extent, however. The background would not be affected because it results primarily from the production of bremsstrahlung radiation from photoelectrons in the sample itself.

Improvements in data handling, reduction, and acquisition might also afford a more accurate method of quantitative analysis. The method presently utilized for XRF analyses at S.F.U. relies primarily on a single
computer code, SAMPO (Rou 69) which was developed for gamma-ray spectra. No options for corrections of interelement effects, matrix corrections, absorption effects or background calculations are possible. In addition, once the spectra are stripped, no opportunity exists for ready manipulation of the data such as statistical analysis and relating the intensities directly to concentrations. There have been some other methods available for calculating concentration directly from stripped data (Gia 73) but these methods rely on both accurate experimentation and theory. Even with this, a computer code capable of integrating the data is necessary.

A study by Statham (Sta 76) compares various methods of background subtraction and calculation, filtering, integrations of peaks with overlap, (interelement effects), least squares fitting, computed peak profiles, and iterative spectrum stripping. Incorporations of many of these improvements into XRF analysis would require interfacing a laboratory minicomputer for data reduction, retrieval, storage, presentation and statistics (Gun 76). An ultimate improvement in reliability and accuracy would result.

These improvements shown above, could be made to the present apparatus, samples and method of data reduction, but the cost would be quite enormous. A more practical and less time consuming approach would be to rely on commercially available instruments (Kevex Corporation, Ortec, Siemens, Philips) which have corrected and supply many of the features of the instrument we have available at S.F.U. The potential
of one of these instruments is vast and could be used in many other areas of trace element research such as nutrition and toxicity studies where the elements in low concentrations play a dynamic role in health and disease.

5.3 Trace Element Concentration Profiles

5.3.1 Present and Future Areas to Search for Possible Clinical Correlations

One of the striking observations while applying XRF to measurement of trace elements in normal samples was the difference in concentration of copper in the blood of females with and without oral contraceptive therapy. This is verified elsewhere (Sch 72). The higher copper levels in some females, which is correlated with high ceruloplasmic levels (Hor 75), may be due to increased stress placed on the liver due to a high and constant level of estrogens which the liver is forced to metabolize.

Analysis of serum or plasma copper may therefore be helpful in the evaluation of the health of the liver and the overall felicity of the body. Multielemental XRF analysis, if pursued, could ultimately show up even more similar correlations between other elements and other conditions.

Since the population of individuals utilized in this study was small, a definite correlation between age and copper concentrations, which are apparently pronounced for males (Yum 74, Tes 73) was not observed. This increase of copper concentration with age remains undefined, but it might
"conceivably play a role in the aging process" since increased serum copper has been associated with a number of chronic illnesses such as coronary atherosclerosis, myocardial infarction, diabetes mellitus and various hematologic disorders (Sin 70, Sch 74). When present in increased amounts with increased age, it was thought that copper might tend to increase the rate of tissue peroxidation and hence accelerate atherosclerosis (Ham 65).

There is one other hypothesis with regard to high copper levels correlated with increased age. Klevay (Kle 75) suggests that a metabolic imbalance with regard to zinc and copper is a major factor in the etiology of coronary heart disease. Rather than a high copper level reported hitherto, she feels that it is actually a low copper level (high zinc to copper ratio) which may be either causative, or a manifestation of the disease. The increase of copper observed with age, she states, is due to a selective mortality caused from coronary heart disease because of a low copper serum concentration. Since the XRF method assessed in this study is capable of analysis of both copper and zinc reliably and accurately, this technique could be used to investigate the copper-atherosclerosis or copper-zinc-atherosclerosis relationship in a large group of individuals.

No clinical correlations for bromine blood and plasma levels have been observed. However, since the XRF technique

developed here is capable of quick and reliable bromine determinations, it may be employed, if necessary, to ascertain the connection with clinical symptoms, if any exist. Sometimes bromine containing drugs, used for sedation, are taken in overdosage. Unfortunately, the method generally employed in detecting plasma bromine, clinically, determines only the bromide ion so that the organic bromides go undetected (Tie 70). XRF bromine analysis would circumvent this problem.

The possibly essential element rubidium, too, can be easily determined. Apparently, there is recent interest in the application of Rb in the research and therapy of the affective disorders or other psychiatric illnesses since the behavioural, biogenic amine, and EEG changes produced by Rb can be described as "opposite" to those produced by lithium (Fie 73). Rubidium appears to affect intracellular potassium metabolism, while lithium has its major effect on sodium metabolism. In this area therefore, XRF could yield information related to studies in psychiatric health. Also, high levels of erythrocyte Rb may be associated with occupational x-ray exposure (Sco 73). Here, too, XRF could have some application.
5.4 Originality of the Work

The aim of the present project was to assess by verification and application EDXRF spectrophotometry for quantitative trace element analysis in whole blood and plasma. Although other studies have applied EDXRF to trace element analysis to blood (see Table XVII, p. 128) none of the results of these studies had been thoroughly verified by other techniques. The only comparable report to the work presented here is that by Flint (Fl1 75). Counting times were over ten times longer than that used here and the report includes neither error analysis, verification, nor calculation of L.L.O.D. Since we optimized samples and counting rates, the L.L.O.D.'s reported in this work are generally slightly lower than the L.L.O.D.'s reported prior to Flint's work.

A single publication (Whe 74) has compared the precision of serum copper measurements by three different techniques. The relative precisions (± 2S) followed, from greatest to least: flame A.A. (2.8%); photon induced EDXRF (8.8%); proton induced EDXRF (16.2%). Here we report for the EDXRF method a relative standard deviation of 10.7%. The two reports are not directly comparable however because two different secondary targets were used: Se and Mo. In addition it was found here that the precisions decreased with increasing atomic number as expected and the greatest contribution to this error was sample preparation and counting statistics.
6. CONCLUSION

A secondary target energy dispersive x-ray fluorescence spectrophotometer has been successfully applied to the quantitative measurement of four elements in blood and serum/plasma.

By using a freeze-drying pelletizing method of preparing samples, the lower limits of detectability of the four elements were found to be sufficiently low to conclude that these elements could be detected in blood and plasma under almost all conditions of health and disease. In addition, the sensitivity of the method used is high enough to assure reflection of changes in the levels of these elements, at low concentrations. The average relative standard deviation (±2S) of about 10% compares favourably with other analytical techniques (Whe 74) and XRF can, with proper preparation of samples, be utilized in the analysis of other biological material such as bone, hair, tissue, and developed further for the analysis of urine samples (Aga 74).

Since XRF is a multielemental method, correlation analyses can be performed relatively easily. Since many of the interrelationships between trace elements have yet to be understood (Del 73) these types of correlation statistics may be valuable. Such studies may characterize diseases to a greater extent and lead to our knowledge of interactions between various trace elements.

With the employment of the newer more sophisticated commercially available XRF apparatus, other elements such as
(V, Cr, Mn, Co, Se, Ge, Pb and Cd) may easily be measured. An increasing awareness of the importance of these elements in low concentration in biological material is beginning (Rec 74) and recent concern over their roles in the body and in intravenous formulations have resulted (Hul 74, Ham 74). There are many other areas in human and animal health, nutrition and disease which would benefit from application of a more sensitive and rapid multielemental method. The use of EDXRF now, and in the future, in these areas does therefore now not seem unrealistic.
APPENDIX I

Relationship between sample thickness and intensity of emitted characteristic line. (Lie 72)

**FIGURE IA** - Arrangement for X-ray absorption

1. Initial intensity (exciting radiation) = \( I_0 \)

2. Effective intensity \( \bar{I}_o = \alpha_1 \bar{I}_0 \)

(\( \alpha_1 \); fraction effective in exciting characteristic lines)
3. Effective intensity at \( d \):

\[ I_1 = I_0 e^{-\lambda_1 d} \]

\( \lambda_1 = \mu_1/\rho \) mass absorption coefficient for incoming radiation

\( d \) = thickness of sample where elements are excited

4. Decrease in effective intensity

\[ \frac{dI}{dd} = d(I_0 e^{-\lambda_1 d}) \]

\[ dI = [I_0 e^{-\lambda_1 d}] dd \]

5. Quanta generated in small unit of volume

\[ d\bar{I} = |\alpha_2 dI| \]

\( \alpha_2 \); fraction generated from those excited

6. Fraction of these quanta detected

\[ \bar{I}_d = \alpha_3 e^{-\lambda_2 d} ddI \]

\( \lambda_2 = \mu_2/\rho \) for characteristic radiation

\( \alpha_3 \); fraction of those x-rays detected
7. Total quanta/sec detected

\[ I_d = \frac{\csc \theta}{0_3 e^{-\lambda x} d} \]

\[ = \frac{\csc \theta}{0_3 e^{-\lambda_2^d a_2 a_1 I_0 e^{-\lambda_1^x} d} d} \]

\[ a = a_1 a_2 a_3 \]

\[ \lambda = \lambda_1 + \lambda_2 \]

\[ \theta = \text{angle from plane of exciting and emitted radiation} \]

\[ I_d = \frac{\csc \theta}{0_3 a_1 I_0 e^{-\lambda x} d} \]

\[ = \frac{a_1 I_0 (1 - e^{-\lambda \csc \theta})}{\lambda} \]
Student t test

The Student t test is used to evaluate a small number of results. In this case, the normal distribution is replaced by the STUDENT $t$ distribution. The distinguishing feature of the $t$ distribution over that of the normal distribution is the greater frequency of larger errors and smaller frequency of smaller errors for the $t$ distribution. When the number of observations reaches infinity the Student $t$ distribution is equivalent to the normal distribution.

The Student $t$ test permits the calculation of the confidence interval for the unknown parameter $\mu$ (the true value), and therefore permits the accuracy of the mean to be tested if the true value is known or the difference in the means to be tested if the two means from two different methods are known. In the latter case the $t$ distribution may be expressed as follows (Eck 69):

$$
t = \frac{\bar{X}_A - \bar{X}_B}{\sqrt{\frac{(n_A + n_B)(n_A S_A^2 + n_B S_B^2)}{n_A n_B (n_A + n_B - 2)}}}
$$

where $X_A$ and $X_B$ are the arithmetic means of the results of the two methods, $S_A$ and $S_B$ are estimates of the standard deviations and $n_A$ and $n_B$ are the numbers of parallel determinations carried out by the methods A and B respectively. The degrees of freedom $= (n_A + n_B - 2)$. 
 Calculation of the t value permits the determination of the significance level \( \alpha \), of the difference between the two means. If \( \alpha \) is determined to be greater than 0.1 there can be assumed to be an insignificant difference in the means or that there is a 0.1 probability that the difference is caused by random errors. As \( \alpha \) approaches 0 there is a smaller probability that the difference in the means are caused by random errors so that therefore the differences become highly significant.
APPENDIX III

The correlation coefficient (Men 71)

The linear correlation coefficient 'r' can be used to characterize the degree of mutual dependence between two variables when the two variables X and Y are expressed through the relationship

\[ Y = a + bX \]

where the slope is given by

\[ b = \frac{n \sum_{i=1}^{n} X_i Y_i - \sum_{i=1}^{n} X_i \sum_{i=1}^{n} Y_i}{n \sum_{i=1}^{n} X_i^2 - (\sum_{i=1}^{n} X_i)^2} \]

and N is the number of pairs of measurements.

In trying to determine the dependency of Y on X, X can be considered a function of Y of the form

\[ X = a' + b'Y \]

in which case b' is similar to b and can be described as:

\[ b' = \frac{n \sum_{i=1}^{n} X_i Y_i - \sum_{i=1}^{n} X_i \sum_{i=1}^{n} Y_i}{n \sum_{i=1}^{n} Y_i^2 - (\sum_{i=1}^{n} Y_i)^2} \]

In complete correlation, a definite relationship exists between the variates X and Y and the coefficients a,b and a',b':

\[ Y = -a'/b' + X/b' = a + bX \]

=> \[ a = -a'/b' \]

=> \[ bb' = 1 \]

If no correlation exists, both b and b' are null.
As a measure of this correlation, the coefficient of correlation is defined as \( r = \frac{\sum X_i Y_i - \sum X_i \sum Y_i}{\sqrt{[\sum X_i^2 - (\sum X_i)^2][\sum Y_i^2 - (\sum Y_i)^2]}} \), thus:

The correlation coefficient may achieve values from -1 to +1; the positive values corresponding to relationships where both variates increase or decrease and negative relationships where one value increases while the other decreases.

The value of the correlation coefficient is dependent on the degrees of freedom and therefore in order to determine the significance of the coefficient, critical levels are chosen at the 5% (\( \alpha = 0.05 \)) or 1% (\( \alpha = 0.01 \)) levels of significance.
Standard Counting Error

Correction for background.

Let $N_u$ be the total count and $N_b$ the proper background count over the counting interval. The counting error for the difference $N_u - N_b$ is

$$s_{\text{count}} = \sqrt{(S_u)^2 + (S_b)^2} = \sqrt{N_u + N_b} \quad (i)$$

The relative counting error is simply:

$$\epsilon_{\text{count}} = \sqrt{\frac{N_u + N_b}{(N_u - N_b)}} \quad (ii)$$

When a standard is used and the results reported as a ratio of this standard and the unknown the relative standard counting error is calculated as follows:

$$\epsilon_{\text{count}} = \left[ (\epsilon_{ST})^2 + (\epsilon_u)^2 \right]^{\frac{1}{2}} = \sqrt{\frac{\left(\sqrt{N_{ST}}\right)^2}{\left[\frac{(N_u)^2}{N_{ST}}\right]}} + \frac{\left(\sqrt{N_u}\right)^2}{\left[\frac{(N_u)^2}{N_{ST}}\right]} \quad (iii)$$

$$= \sqrt{\frac{1 + 1}{N_{ST} N_u}} \quad (iv)$$
In the case where the ratio must be corrected for background (ii) is substituted for $\varepsilon_u$ in (iii):

$$
\varepsilon_{\text{count}} = \left[ \left( \varepsilon_{\text{ST}} \right)^2 + \frac{[\sqrt{N_u + N_b}/(N_u - N_b)]^2}{(N_u - N_b)} \right]^{1/2}
$$  \hspace{1cm} (v)

$$
= \sqrt{\frac{[\sqrt{N_u + N_b}/N_{\text{ST}}]^2}{(N_u - N_b)^2}}
$$  \hspace{1cm} (vi)

$$
= \left[ (N_{\text{ST}})^{-1} + (N_u + N_b)(N_u - N_b)^{-2} \right]^{1/2}
$$  \hspace{1cm} (vii)
Verification Studies with A.A.
(Measurements Performed Externally)

A comparison study between the Essondale Clinical Lab and SFU was set up to assist quality control at their lab and help verify our preliminary XRF results. Twenty-three 3 ml serum samples were forwarded to SFU in polyethylene tubes. These samples had been previously analyzed for copper by flame A.A. using the method outlined in 'Clinical Methods for Atomic Absorption Spectroscopy' (Han 73)

A plot of the values obtained via XRF against those from A.A. is shown in Figure 2A. Background was subtracted by determination of the expected copper background peak area based on the nickel area from each sample pellet. Although the results of the two methods are well correlated \( r = 0.86 \), the means differ significantly, 194±42 µg/100mls for XRF and 155±45 µg/100mls for A.A. Part of this discrepancy may be due to the fact that the samples were prepared into 37.3 mg pellets and the standard curves were prepared using 30.4 mg pellets. By analyzing 30 and 50 mg samples prepared from the same material it was found that the normalized results between the two samples were significantly different. This difference amounted to a depression
of the results from the thicker pellets compared to the thinner pellets by 23 \mu g/100 ml. The large difference noted between A.A. and XRF could not be accounted for completely by variations in thickness of the pellets however. It was thus assumed that the differences in the means could also be due to the method used to subtract background. A larger nickel peak in the blank spectra than that in the samples would tend to increase systematically the apparent copper levels found in the samples analyzed. It was decided, at this point, to use the method which measured changes in background more directly (see section 2.143).

In order to investigate the possibility of contamination by the apparatus and collection devices used in collecting and preparing the samples, XRF spectra of the metal spatula, steel pellet press, needle and Vacutainer top were collected (see Figure 3A). Although the spectra do not display concentrations of element directly, they do indicate that there is essentially no contribution from copper in these devices. The discrepancy noted between XRF and A.A. was therefore assumed to originate from the development of the standard curves for (either analytical apparatus) and that noted previously, the method used to subtract background.

Since the A.A. data presented in Figure 2A had been performed outside of S.F.U. on an older model spectrophotometer the reliability of some of the data may be suspect. Rather than rely on external labs over which we had no control, it was decided to pursue A.A. studies at S.F.U. on the Perkin Elmer Model 305 spectrophotometer.
Comparison Between Serum Copper Determinations via XRF & A.A. (Essondale)
FIGURE 3A: XRF Spectra of Metal Devices used in Preliminary Sample Preparation

A - Metal Spatula
B - Pellet Press
C - Stainless Steel Needle
APPENDIX VI

Assessment of Flameless A.A. for Analysis of Plasma

In order to compare the three methods of sample preparation, (originally discussed in section 2.22), standards were prepared with water, pooled serum, and salt solution 'A'. The salt solution (A), which is shown in Table IA, parallels the normal levels found in serum (Tie 70).

TABLE IA

| Original Salt Solutions Used to Develop Working Curves |
|-------------|-------------|
| **Solution A** | **Solution B** |
| gr/l | gr/l |
| NaCl | 0.52 | NaCl | 12.04 |
| CaCl₂ | 1.11 | Ca(NO₃)₂ | 1.18 |
| KHCO₃ | 1.0 | KAc | 0.79 |
| MgSO₄·7H₂O | 0.74 | MgSO₄ | 0.49 |
| NaHCO₃ | 2.52 | NaAc | 6.40 |
| H₃PO₄ | 0.166 | NH₄PO₄ | 0.23 |
| Na₂EDTA | 5.58 | LiNO₃ | 0.14 |

Stock copper standards were prepared with washed copper wire dissolved in concentrated nitric acid and diluted with d.d. water to a concentration of 1 mg/l. This solution was tested against a second solution made up independently in the same manner. The absorbance of the solutions of identical concentrations were found to be the same to within measurement error.
Six standard solutions (41.5 to 311.4 μg/100mls) were prepared by serial dilutions of the original stock solution. Working standards were prepared by diluting these solutions 1:1 with water, salt solutions or control serum. Ten microliter aliquots of these solutions were pipetted directly into the furnace. The three cycles of drying, ashing and atomization using the graphite furnace for salt and aqueous standards was set at 10, 10, and 4 seconds respectively. For serum, a 45 second ash was found necessary. All analyses were carried out at a chart expansion of 8.

It was found that salt, but not organic constituents tended to depress the signal of copper absorbance. This verified the results of Dawson (Daw 68) and substantiated a recent report by Smeyers-Verbeke (Sme 76) which indicated that all chlorides depressed significantly, copper absorbance in the graphite furnace.

The results of standard additions to serum showed poor reproducibility where, on one day the signals approximately paralleled salt solutions, and at another time, were depressed. Also, due to the increased viscosity and decreased surface tension of the serum solutions, a large pipetting error resulted.
Because the EDTA salt solution (solution A) tended to precipitate with time, copper-salt solution B (see Table IA) was prepared and compared with the aqueous and serum standards. Once again, both the salt and serum standards were depressed over the aqueous standards and considerable variation was still observed while pipetting serum. The resultant curves are shown in Figure 4A which display the deviations as ±2σ. For aqueous and salt standards the relative standard deviation was found to be 3.1%, whereas that for serum standard additions was calculated as 14%. The error was determined by measuring the absorbance of a single sample many times.

Although a greater error resulted while analyzing serum samples this analytical curve was used to generate values of unknown samples. The serum curve was used because the aqueous and salt samples produced curves too dissimilar to the curves generated by using serum. The results of 37 female plasma samples using this working curve yielded a mean of 187±52 μg/100mls compared with 132±40 μg/100mls for XRF (mean ±3). The error represents the standard deviation of the set of samples.

Obviously the means via XRF and A.A. do not compare and are probably a result of the pipetting problem and processes which occur in the furnace while analyzing biological material. These processes, which decrease the reliability of the measurements, include carbide and smoke formation in the furnace.
FIGURE 4A: Flameless A.A. Standard Curves

- Aqueous
- Salt (B)
- Serum

ABSORBANCE vs. ADD. COPPER

0.8  0.7  0.6  0.5  0.4  0.3  0.2  0.1

µg/100 ml. serum

0  100  200  300
Preliminary Application of the PFD Technique

Experiments designed to measure quantitatively only the elemental concentrations of Cu and Zn in available blood and plasma/serum specimens were conducted with little effort to assemble data on both the method employed and samples analyzed.

The pelletizing freeze drying (PFD) method was applied to the analysis of the levels of copper and zinc in healthy volunteers at SFU and the Childrens Hospital (CH) in Vancouver. Vacutainers containing EDTA as anticoagulant were used to collect the blood. Since this was a preliminary study, and an assessment of the technique was the primary aim, we used any readily available collection device. The samples were prepared in the usual manner. The mass of the pellets prepared for blood and serum was 46.6±6.8 mg and 40±6.4 mg respectively.

The results are shown in Table IIA. While the majority of the results are in good agreement with published values (Table 1, section 1.3), blood zinc levels are about twice the value expected. Such discrepancies may have been caused by the high level of zinc found in the anticoagulant used since it was found, by analyzing this anticoagulant (see Figure 5A), spotted onto
FIGURE 5A: XRF Spectra of EDTA Solution from a Vacutainer

EDTA spot from Vacutainer on Whatman #42

Whatman #42

CHANNEL
Whatman #42 filter paper, that it contained a high concentration of zinc. A spectrum of a clean filter paper is shown for comparison. Plasma copper levels in females from the Childrens Hospital (CH) also seem somewhat high, but this may be a result of the majority of these adults ingesting oral contraceptives (Sch 72). Other discrepancies noted may be caused by the variations in the thickness of the samples.

TABLE IIA

Results of Preliminary Application of the Freeze Drying Pelletizing Method

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Specimen</th>
<th>Origin</th>
<th>Cu μg/100ml mean ±s</th>
<th>Zn μg/100ml mean ±s</th>
<th>Cu μg/100ml mean ±s</th>
<th>Zn μg/100ml mean ±s</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Males</td>
<td>CH</td>
<td>116±36</td>
<td>1527±280</td>
<td>160±43</td>
<td>108±18</td>
</tr>
<tr>
<td>8</td>
<td>Females</td>
<td>CH</td>
<td>136±31</td>
<td>1612±218</td>
<td>221±85</td>
<td>121±32</td>
</tr>
<tr>
<td>7</td>
<td>Males</td>
<td>SFU</td>
<td>89±14</td>
<td>1222±362</td>
<td>110±30</td>
<td>137±30</td>
</tr>
<tr>
<td>2</td>
<td>Females*</td>
<td>SFU</td>
<td>126</td>
<td>1620</td>
<td>156</td>
<td>178</td>
</tr>
</tbody>
</table>

* mean level reported only
s: standard deviation calculated on set of measurements.
APPENDIX VIII

Questionnaire (Females)

Surname  Initials  Birthdate  Age

Address

Private Physician's Name  To-day's date

Have you used any oral contraceptives during the past three months?
Yes  No

If 'Yes' Brand

Date of first day of last menstrual period

Have you used any vitamin preparations during the past month?
Yes  No  Brand

Obstetrical History: Are you pregnant now?  Yes  No

Total number of pregnancies  miscarriages  stillbirths  living children

Date of termination of last pregnancy
Appendix VIII cont'd. . . .

BLOOD CLINIC QUESTIONNAIRE
(Males)
Sept. 15-18, 1975

<table>
<thead>
<tr>
<th>Name</th>
<th>Tube Number</th>
<th>Age</th>
<th>Ht.</th>
<th>Wt.</th>
<th>Sex</th>
</tr>
</thead>
</table>

Time of last meal
List any recent illnesses or any past serious illnesses or diseases which you have had:

<table>
<thead>
<tr>
<th>approx. date</th>
<th>approx. date</th>
<th>approx. date</th>
</tr>
</thead>
</table>

List any medications or drugs such as antibiotics, vitamins, mineral supplements, cod liver oil, or kelp etc. which you are now taking:

This information will be kept strictly confidential.
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