COVER SHEET FOR PROPOSAL TO THE NATIONAL SCIENCE FOUNDATION

For Consideration by NSF Organization Unit(s) (Indicate the most specific unit known, i.e. program, division, etc.)	For NSF Use Only
Div. Biological Critical Systems	NSF Proposal Number
Program Announcement/Solicitation No./Closing Date: 93-17 5/17/93	

Date Received	Number of Copies	Division Assigned	Fund Code	File Location

Employer Identification Number (EIN) or Taxpayer Identification Number (TIN)	Show Previous Award No. If This is: ☐ A Renewal or ☐ An Accomplishment-Based Renewal	Is This Proposal Being Submitted to Another Federal Agency? YES___ NO X If YES, List Acronym(s)

Name of Organization to Which Award Should be Made:	Address of Organization, Including Zip Code
University of Utah Institutional Code (if known)	Office of Sponsored Projects 309 PARK Building University of Utah, S.L.C., UT 84112

Is Submitting Organization: ☐ For-Profit Organization; ☐ Small Business; ☐ Minority Business; ☐ Woman-Owned Business

Branch/Campus/Other Component (Where work is performed, if different)	Institutional Code (if known)
University of Utah, College of Engineering, S.L.C., Utah	

Title of Proposed Project:
Non-Invasive Monitoring of the Elements for Education, Compliance, Screening, and Diagnosis

Requested Amount	Proposed Duration (1-60 months)	Requested Starting Date
$ 419,834	36 months	9/15/93

Check Appropriate Box(es) If This Proposal Includes Any of the Items Listed Below:

- ☐ Vertebrate Animals
- ☑ Human Subjects, volunteers
- ☐ Historical Places
- ☐ Research Involving Genetically Engineered Organisms
- ☐ National Environmental Policy Act
- ☐ Proprietary and Privileged Information
- ☐ Disclosure of Lobbying Activities
- ☐ Facilitation for Scientists/Engineers with Disabilities
- ☐ Research Opportunity Award
- ☐ International Cooperative Activity: _____ Country/Countries

PI/PD Department	PI/PD Postal Address
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PI/PD Fax Number 801-585-5361	

Names (Typed)	Social Security No.*	High Degree, Yr	Telephone Number	Electronic Mail Address
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Co-PI/PD				
Co-PI/PD				
Co-PI/PD				
Co-PI/PD				

NOTE: The FULLY SIGNED Certification Page must be submitted immediately following this Cover Sheet.

*Submission of social security numbers is voluntary and will not affect the organization's eligibility for an award. However, they are an integral part of the NSF information system and assist in processing the proposal. SSN solicited under NSF Act of 1950, as amended.

B. Summary

The most widely used biological tissue for chemical and biochemical analysis is blood. Although blood is a convenient, minimally invasive source, there is growing interest in minimizing the need for blood samples by utilizing truly non-invasive samples. Fortunately, man has a variety of completely non-invasive windows to his inner chemistry, including hair and nails, urine, tears, saliva, sweat, and breast milk. Non-invasive gas samples include urine and expired air.

We propose to examine the feasibility and suitability of these samples as sources of chemical and biochemical information for education, compliance, screening, and diagnosis. Although these samples individually do not represent the chemical and biochemical diversity of blood, they each have their unique advantages and potential cost-effective applications. In addition, through the use of multi-variate data analysis and correlation procedures, we fully expect these methods and samples to substitute for many present day blood analyses for a wide variety of screening and diagnostic needs.

We propose to:

1. analyze hair, urine, saliva, and tears for over 40 elements;
2. perform protein separation and analyses of tears, urine, and saliva;
3. utilize existing blood-based data and diagnostic correlations, and;
4. use multivariate methods to assess the feasibility and cost effectiveness of using analysis of non-invasive samples to substitute for blood and other invasive methods.

Abbreviations

ARUP: Associated Regional University Pathologists (Salt Lake City)
CID: Charge Injection Device (a 2-D photon detector)
ESCA: Electron Spectroscopy for Chemical Analysis (same as XPS)
GI: Gastrointestinal
GPC: Gel Permeation Chromatography
HPLC: High Performance Liquid Chromatography
ICP: Inductively Coupled Plasma
LC: Liquid Chromatography
MS: Mass Spectrometry
OES: Optical Emission Spectrometry
SIMS: Secondary Ion Mass Spectrometry
PEO: Polyethylene Oxide
PPB: Parts per Billion
PPM: Parts per Million
TLC: Thin Layer Chromatography
XPS: X-ray Photoelectron Spectroscopy (same as ESCA)

CERTIFICATION PAGE

Certification for Principal Investigators and Co-Principal Investigators:

I certify to the best of my knowledge that:

(1) the statements herein (excluding scientific hypotheses and scientific opinions) are true and complete, and
(2) the text and graphics herein as well as any accompanying publications or other documents, unless otherwise indicated, are the original work of the signatories or individuals working under their supervision. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if an award is made as a result of this application.

I understand that the willful provision of false information or concealing a material fact in this proposal or any other communication submitted to NSF is a criminal offense (U.S.Code, Title 18, Section 1001).

Name (Typed)	Signature	Date
PI/PD J.D. Andrade		5/13/93.
Co-PI/PD		
Co-PI/PD		
Co-PI/PD		
Co-PI/PD		

Certification for Authorized Institutional Representative or Individual Applicant:

By signing and submitting this proposal, the individual applicant or the authorized official of the applicant institution is: (1) certifying that statements made herein are true and complete to the best of his/her knowledge; and (2) agreeing to accept the obligation to comply with NSF award terms and conditions if an award is made as a result of this application. Further, the applicant is hereby providing certifications regarding Federal debt status, debarment and suspension, drug-free workplace, and lobbying activities (see below), as set forth in Grants for Research and Education in Science and Engineering (GRESE), NSF 92-89. Willful provision of false information in this application and its supporting documents or in reports required under an ensuing award is a criminal offense (U. S. Code, Title 18, Section 1001).

Debt and Debarment Certifications

(If answer "yes" to either, please provide explanation.)

Is the organization delinquent on any Federal debt? Yes ___ No ___

Is the organization or its principals presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from covered transactions by any Federal department or agency? Yes ___ No ___

Certification Regarding Lobbying

This certification is required for an award of a Federal contract, grant, or cooperative agreement exceeding $100,000 and for an award of a Federal loan or a commitment providing for the United States to insure or guarantee a loan exceeding $150,000.

Certification for Contracts, Grants, Loans and Cooperative Agreements

The undersigned certifies, to the best of his or her knowledge and belief, that:

(1) No federal appropriated funds have been paid or will be paid, by or on behalf of the undersigned, to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with the awarding of any federal contract, the making of any Federal grant, the making of any Federal loan, the entering into of any cooperative agreement, and the extension, continuation, renewal, amendment, or modification of any Federal contract, grant, loan, or cooperative agreement.

(2) If any funds other than Federal appropriated funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with this Federal contract, grant, loan, or cooperative agreement, the undersigned shall complete and submit Standard Form-LLL, "Disclosure Form to Report Lobbying," in accordance with its instructions.

(3) The undersigned shall require that the language of this certification be included in the award documents for all subawards at all tiers including subcontracts, subgrants, and contracts under grants, loans, and cooperative agreements and that all subrecipients shall certify and disclose accordingly.

This certification is a material representation of fact upon which reliance was placed when this transaction was made or entered into. Submission of this certification is a prerequisite for making or entering into this transaction imposed by section 1352, title 31, U.S. Code. Any person who fails to file the required certification shall be subject to a civil penalty of not less than $10,000 and not more than $100,000 for each such failure.

AUTHORIZED INSTITUTIONAL REPRESENTATIVE	SIGNATURE	DATE
NAME/TITLE (TYPED) R. Koehn, V.P. for Research		

TELEPHONE NUMBER	ELECTRONIC MAIL ADDRESS	FAX NUMBER
801-581-7236		801-585-6212

C. Table of Contents

"I acknowledge by signing below that I understand that this is a joint venture of the National Science Foundation (NSF) and The Whitaker Foundation (WF); that submitted proposals will be reviewed by a panel jointly chosen by NSF and WF; and that funding decisions will be jointly made by NSF and WF. I authorize the disclosure of my proposal and all associated materials to WF, to panel members, and to individuals involved in the review and award process at WF.

P.I. Signature 5/14/93
Date

D. Results of Research under Prior NSF Support

Title: "Protein Interactions with Gradient Surfaces"

Award: INT-87-19079, International Cooperative Research, J.D. Andrade, PI

This international cooperative research award, which ended July 30, 1992, facilitated the collaboration between Drs. Hai Bang Lee and Jin Ho Lee at the Korean Research Institute of Chemical Technology, and J.D. Andrade, V. Hlady, and Y.S. Lin at the University of Utah. Dr. Andrade and Mr. Lin visited the Korean group in May and June, 1992 respectively, to complete the joint work. Drs. Hai Bang Lee and Jin Ho Lee will visit the University of Utah in October, 1992 to help complete the final report. Dr. Sang Il Jeon, of Kangrung University (Kangrung, S. Korea) has also been a participant in the work.

The Utah group focused on the preparation of hydrophobic/hydrophilic gradient surfaces on silica and glass, using silane chemistries in a diffusion gradient, and focused on the study of protein interactions with these gradient surfaces. The Korea group focused on the preparation of gradients by corona discharge techniques, their characterization, and their study with respect to cell adhesion and invitro cell culture.

Publications:

J.D. Andrade and H.B. Lee, "Using Gradient Surfaces for Biomaterials Education", Abst., 3rd annual fall Biomed. Engrg. Soc. Meet., 1992.

Y.S. Lin, V. Hlady, and J. Janatova, "Adsorption of Complement Proteins on Hydrophobicity Gradient Surfaces", Biomaterials (1993) in press.

J.H. Lee, J.W. Park, and H.B. Lee, "Cell Adhesion...Plasma Treatment", Biomaterials, 12 (1991) 443.

J.H. Lee, J.W. Park, and H.B. Lee, "...Hydroxyl...Gradient Surfaces...", Polymer (Korea) 14 (1990) 646.

J.H. Lee, et al., "...Gradient Surfaces...by Corona Discharge...", J. Coll. Interface Sci., 151 (1992) 563.

J.H. Lee and J.D. Andrade, "Polymer Surfaces for Cell Adhesion", J. of KOSMBE, 10 (1989) 43.

J.H. Lee, et al., "Wettability Gradient Surfaces...", Trans. Soc. for Biomaterials, 17 (1991) 133.

J.D. Andrade, et al., "Proteins at Interfaces", Clin. Materials, 11 (1992) 67.

J.D. Andrade and V. Hlady, "Vroman Effects...", J. Biomat. Sci. Polymer Ed., 2 (1991) 161

C-G. Golander, et al., "...Adsorption Studies...Hydrophobicity Gradient", Colloids and Surfaces, 49 (1990) 289.

Saliva is another easily obtained, non-invasive sample which is available in greater volumes than tears. It is rich in glycoprotein, glycosamino glycans, and enzymes, and has a very different macromolecular profile than tears or urine. Again, there are problems with variability of the sample based on differences in the nutritional and personal hygiene characteristics of the individual, and on the method of collection.

A new device, the Saliva Sack, is now available which facilitates the collection of saliva samples for chemical analysis. (BioQuant, Ann Arbor, Michigan.) The device collects a saliva filtrate with a molecular weight cut-off of about 12,000 daltons.

Saliva is expected to be quite sensitive to environmental and occupational inputs. It is also of interest as it appears to be the most concentrated source for the analysis of mercury and other elemental constituents of dental fillings and dental prostheses (32).

Saliva has high concentrations of lysozyme, IgA, and lactoferrin, as does tears, as well as growth factors and factors important in wound healing. Many saliva proteins are proline-rich and highly acidic, but there are also basic proteins (52, 53).

Summary: These four non-invasive, easily collected samples provide a unique opportunity with which to improve and enhance the usefulness and reliability of elemental analysis for education, screening, and diagnostic purposes. The opportunity to correlate 40 plus elements obtained in four very different samples from the same individual, to determine protein profiles of three of those tissues, and to determine the elemental component of those various protein constituents, enable the development of multi-variate correlations and possibly biochemical mechanisms for trace element deficiencies or excesses. This information, coupled with the already very extensive databases for blood and urine (93-95), will permit us to correlate and relate non-invasive sample assay to the more common and more invasive blood-based analysis and diagnosis.

b. Elemental Analysis (10, 58, 59, 61-63, 92, 93, 95)

Man's agriculture, industry, and other activities have greatly influenced the availability and potential uptake of trace metals by living organisms. So-called hard metals on the left side of the Periodic Table tend to have single oxidation states and bind weakly to ligands. Na, K, Mg, and Ca are homeostatically controlled. A number of the heavier metals can begin to substitute and compete with the more physiologic ones, such as Barium competing for Potassium and blocking Potassium channels. Biology tends to avoid the use of aluminum, yet aluminum is now widely released from soils due to acid rain, and has otherwise become widely available through industrial use and consumer products. Beryllium binds to phosphate-containing systems; living systems have virtually no protection against Beryllium. It is highly toxic. Aluminum and Vanadium also interfere with phosphate metabolism (10). Table 2 summarizes the classes and types of elements in biology and medicine.

4 Elements make up 99% of all atoms in Humans:	H, O, C, N
7 Elements make up another 0.9%:	Na, K, Ca, Mg, P, S, Cl
10 more elements are required and essential:	Mn, Fe, Co, Ni, Cu, Zn, Mo, B, Si, Se, especially Iron and Zinc.
7 more elements are probably essential:	V, Cr, F, I, As, Br, Sn
7 or more elements are generally viewed as highly toxic:	Be, Cd, Hg, Pb, Tl, As
9 or more additional elements are being studied and used for therapeutic purposes -- as drugs or components of drugs:	Li, F, Pt, Au, Bi, Sb, Ba, Se, Sn

Table 2: Chemical Elements in Biology and Medicine (10)

E. Project Description

1. Rationale

The most widely used biological tissue for chemical and biochemical analysis is blood (1). Although blood is a convenient, minimally invasive source, there is growing interest in minimizing the need for blood samples by utilizing truly non-invasive samples. Fortunately, man has a variety of completely non-invasive windows to his inner chemistry, including hair and nails, urine, tears, saliva, sweat, and breast milk. Non-invasive gas samples include urine and expired air (2) (Figure 1).

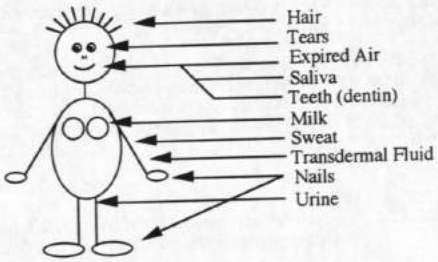

Figure 1: Non-Invasive samples useful for chemical and elemental analysis

Hair and nails have the advantage that by correlating the age of the sample with the chemistry, one has, in principle, a chemical history of the subject. The disadvantage is that hair and nails respond only slowly to significant biochemical changes in the patient (3, 4). Urine, tears, saliva and milk each have unique attributes and characteristics reflecting their particular organ and glandular sources (Table 1). Expired air reflects volatile species in blood and in respiratory tissues (2). With the exception of urine, these completely non-invasive samples are rarely used for analytical, diagnostic, or even research purposes.

Hair/Nails: (3,4,18,43, 44)	Solid Tissue: trace elements 10-100x higher concentration than in other tissues. Five days required for new hair (base of follicle) to reflect a major change in element conc.). Thirty day lag before steady state. A highly hetergenous tissue -- susceptible to environmental influences. An excretory organ? Available in large quantities.
Urine: (45-48)	Liquid Tissue -- available in large quantities; has hundreds of peptides and growth factors. Especially useful for kidney function diagnosis.
Tears: (49, 50, 51)	Liquid Tissue -- some difficulty in sampling, only small volumes available; unique protein and peptide composition, practically no elemental analysis work available
Saliva: (52, 53)	Semi-liquid sample -- large volumes available, but highly variable samples. Contains unique enzymes and mucoproteins. Only very preliminary studies will be performed
Others: (2, 47)	We do not propose to examine expired air or other volatiles, milk, sweat, semen, dentin, transdermal fluids, or other samples in this project.

Table 1: Properties of Major Non-Invasive Samples to be Used in this Project

The first row transition metals from Titanium to Zinc are utilized effectively and generally well-handled by biological systems unless they are present in significant excess. Cobalt is a component of Vitamin B_{12}. Iron is a major component of Heme and thus of many proteins. Zinc is an essential component of hundreds of enzymes.

The so-called soft metals, toward the center and right of the periodic table, include Copper, some of the noble metals, Cadmium, Mercury, Thalium, and Lead. "Biology has had little or no previous knowledge" of these metals, and they can be highly toxic (10). Copper is a constituent of many proteins and enzymes; there are over 100 copper-containing enzymes in biology. Thalium and lead are particularly toxic since they tend to bind to enzymes in the same places and sites as potassium and zinc. Cadmium tends to substitute for copper and zinc. Thalium, being very similar to potassium, blocks potassium channels. Trace elements are used in inorganic drugs. Although trace elements can bind to proteins with extremely high binding constants, that binding is in most cases still reversible and the metal is often removed, particularly in protein separation processes involving high electric fields, strong surfactants, or other denaturants.

A one part per billion concentration of the metal is consistent with an association constant of 10^{-12} molar. This is well within the binding constant range for many if not most metalloproteins (23-26). "Most trace elements exert their effects within the physiological system via their incorporation in enzymes or transport proteins (78)." In the majority of cases, the interaction is via binding to enzymes. Because enzymes can turn over large quantities of substrate to product, a particular trace element can have a very disproportionate effect on enzyme biochemistry. "The trace element is the ultimate regulatory factor for the system's activity level. The absence of a trace element in an enzyme can result in the total failure of a vital physiological system (78)." Specific trace elements can serve as co-factors for various enzymes. A toxic trace metal may substitute for an essential element in an enzyme, protein, or DNA; although it can substitute and bind strongly, the fact that its orbitals and chemical environment are different generally lead to a change in the protein which results in inactivation and therefore a biochemical defect or abnormality.

Trace metal absorption and regulation is generally in the GI tract, and excess trace metal concentrations are usually eliminated in the feces; there is some excretion through the urine. Trace metals introduced by non-GI routes, such as via aerosols or lung particulates, via the slow corrosion or degradation of metal implants, via direct transdermal absorption such as from jewelry or other direct skin contact (29), or by other routes can lead to an increase in circulating trace metal concentrations which bypass the normal GI homeostatic regulation processes.

The analytical methods and correlations which we develop must cover the generally low range required for an essential nutrient to the higher concentration needed for that nutrient to become a toxin or poison (10, 27, 31). Fortunately, modern methods of elemental analysis permit the detection and measurement of about 4/5 of the periodic table at parts per billion concentration, thereby easily covering the needed range for practically all of the elements of interest (28, 37, 38, 54-57). Figure 3 (courtesy of Fisions Inst.) shows the detection limits for one of the most inexpensive ICP-MS systems currently available.

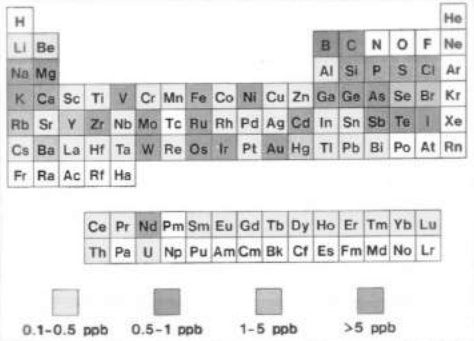

Figure 3: Detection limits for a common ICP-MS analysis system, which includes a full 7 orders of magnitude linear dynamic range, handled by the instrument without operator intervention. (Courtesy of Fisions Inst.)

The selection of an optimum and most cost-effective technique for the measurement of trace elements in biological tissues is complex (37, 38, 41). We have carefully considered the methods available, the costs of the equipment, the time and expense involved with sample preparation and insertion, the through-put of the instrument, the ability to analyze 40 or more samples simultaneously and quickly, the ability to use very small volumes, such as tear samples, the costs and difficulty associated with operation and maintenance of the instrument, and a variety of other factors.

Instruments and methods are now readily available with which to detect ppb concentrations of most of the elements of interest. We have focused on two major techniques, both utilizing an inductively coupled plasma (ICP) as the means to break the sample down into its atomic and ionic constituents (54-56). The only real problem with an ICP approach is that it generally requires a liquid sample with a relatively low solids content. ICP methods have been used with all of the biological samples we propose to study, and are the methods of choice for most blood, serum, and tissue elemental analysis as well.

There are 2 basic methods of detection of the elemental constituents of the sample: optical emission spectroscopy (OES) and mass spectroscopy (MS). In optical emission, the excited state atom and ion atomic orbital optical emission lines are monitored in the range of roughly 200 to 800 nanometers. There are perhaps 50,000 major lines in this range representing some 80% or more of the periodic table. There are some interferences and problems, although most of the elements of interest can be detected in the part per million to part per billion range with this technique. Our samples will of course have high sodium, chlorine, potassium, calcium, and the other major biological elements which provide some interferences and background levels which can be a problem in the analysis of specific elements.

The second major detection method is mass spectrometry (19, 28). Here a slightly different region of the plasma torch is directly input into the mass spectrometer via a complex but highly reliable differentially pumped vacuum system. Through a set of extraction optics, electrostatic lenses, and final quadropole charge to mass ratio separation and analysis, mass spectrometer/ICP systems are generally capable of near part per trillion analysis and even below, although the costs such of such systems tends to be above $250,000.

Modern optical emission systems are equipped with a wide array of photo multiplier tubes to detect 20 to 40 or more specific optical emission lines simultaneously, or are equipped with a CID chip allowing detection of a much larger number of lines and therefore larger number of elements,

The highest resolution, highest sensitivity protein separation technique for the analysis of highly complex mixtures which is in wide use today is 2-dimensional gel electrophoresis (75). This technique normally utilizes a long, thin rod isoelectric focusing gel in one dimension. The protein bands in that gel then serve as samples for a second dimension gel slab utilizing poly-acrylamide gradient gels, resulting in separation in the second dimension on the basis of size. So-called Isodalt and related 2-dimensional gel electrophoresis mechanisms generally lead to many hundreds of spots or bands on these 2-dimensional gels (45, 47, 75). These are normally developed or detected with a sensitive silver stain. This is the method of choice for the highly sensitive, high resolution analysis of complex protein mixtures today. This technique is routine in our laboratories. We developed the method for the analysis of the competitive adsorption characteristics of plasma proteins on high surface area particles where the 2-D gel electrophoresis is used as an analytical method for the study of complex solute depletion. We routinely monitor and analyze over 40 different plasma proteins and their competitive adsorption processes by this method (11, 79, 80). That's the good news. The bad news is that this technique, in order to get the high separation efficiency, breaks the protein up into its constituent poly-amino acid chains, disrupts all disulfide bonding, and, because of the high and low pH characteristics of the isoelectric focusing gel, the high field strengths involved in the separations, and the fact that SDS surfactant is used, elements which are not covalently attached are stripped from the proteins during the separation processes.

There are much less traumatic and "softer" protein separations, such as via column liquid chromatography (71, 73). However, such processes are generally tuned or designed to separate only a very small number of specific proteins from a mixture. Although chromatography has been scaled up for preparative purposes for the separation of specific proteins from complex mixtures such as plasma, the systems utilized are relatively complicated in that they involve sophisticated buffer or other solvent elution profiles and often require several or even many different chromatographic separation procedures or processes.

Our group has studied protein adsorption and interactions at interfaces for many years (81, 82). We have correlated the adsorptive characteristics of proteins, their 3-dimensional structure (83), their amino acid composition, and, in the case of complex multi-domain proteins, the particular characteristics of their major structural domains (84). We really do understand how proteins bind and interact with solid supports (85). We also know how to prevent protein interactions by the use of optimized surfaces which tend to exclude proteins from the surface region by non-specific steric or entropic exclusion processes (86, 87).

Our work on protein adsorption from complex mixtures, coupled with that of many of our colleagues studying competitive protein adsorption (the so-called Vroman Effect), have given us an appreciation and a partial understanding of the complex protein adsorption hierarchy and exchange/desorption processes which occur (81-91). We can now prepare surfaces which can tune for the adsorption of certain proteins semi-specifically and quantitatively using so-called non-specific interactions (88). We have developed a wide range of surfaces with a 1-dimensional gradient in surface properties which are very powerful research tools with which to understand and to control protein surface interactions (88, 89, 91)). That basic science surface modification technology is now ready for application in this project.

We are also well aware of the developments and advances in the protein separation and purification community using capillary, column, gel, and planar chromatographic and electrophoretic approaches (64-74). One of our on-campus advisors, Dr. Karin Caldwell, who also directs the University Center for Biopolymers at Interfaces, has worked extensively in the areas of protein chromatography, field flow fractionation, and protein separation. We have also worked extensively on several biosensor and immunodiagnostic projects using capillary, planar, and radial geometries in which high resolution protein separations were required for site specific immunosensing and immuno diagnostic analysis (90).

simultaneously. These tend to cost about $150,000. There is a series of less expensive mass spectrometry ICP systems which has now become available, selling in the range of about $150,000 which allow part per billion to part per trillion sensitivity (see Figure 3). Although mass spectrometry tends to be more sensitive, largely because of the essential zero background between lines and a very high sensitivity and specificity of the quadropole mass analyzer and ion detector, a number of interferences are common, largely due to multiply charged ions, of which a major culprit is sulphur. Given the high sulphur content in proteins and particularly in hair proteins (77), this is a major problem for certain elements.

Optical emission and mass spectrometry used together permit the resolution of practically all of these interferences. Dual detection systems have recently become available and provide a very cost-effective solution to the problem of analyzing 40 or more elements at very high sensitivity and throughput with minimal sample volumes on a regular basis, 24 hours a day, 7 days a week. We have budgeted such a system in this proposal. Details are given in the Budget Justification section. Briefly, the ICP/OES system is budgeted in year 1 at $140,000, 50% of the cost attributed to this grant and another 50% attributed to a parallel grant to the Whitaker Foundation (8). The University of Utah has agreed to provide 50% of the NSF share of the instrument. We have budgeted the mass spectrometry attachment for year 2 at a total cost of about $100,000 with the same funding formula.

Although one could argue that spending the $240,000 on a much more sensitive mass spectrometry system would be a better use of the funds, such a system would not have the mass resolution to distinguish the multiply charged ion and other interferences. Although such a system would provide sub parts per trillion sensitivity, our analysis indicates that such sensitivity is not really required for the elements of interest. As we have indicated in the earlier discussion, virtually all of the elements we wish to detect are already present under normal conditions in the sub parts per billion range. Going to parts per trillion, although interesting, is probably of little diagnostic significance for all but a very few elements.

Although we are already very familiar with the major ICP/OES and ICP/MS instruments and companies, we will of course conduct an extremely thorough instrument evaluation process prior to committing to a specific model and manufacturer. This process will include direct analysis of the four different sample types we will use. This would all be done in the first month of the grant. Normal delivery of these instruments is 30 to 60 days. Space for the instrument is already available in our laboratory and an operator skilled in the operation of such instruments is already in place and budgeted in this grant (Mr. Paul Dryden), so we would expect to be up and operational within the first 3 months of the grant. We anticipate little or no problem with sample preparation techniques. We will utilize standard preparation techniques for these samples. We have considerable experience in surface analysis by X-ray photo electron spectroscopy. Our lab is used to working with highly purified chemicals, solvents, and solutions, and we anticipate little or no problem in dealing with sample preparation methods which require sub-part per billion levels of trace contaminants.

c. Protein Separation and Detection

This is really the most difficult part of the project. Basically the problem is that we wish to separate the peptides and proteins present in urine, tears and saliva using methods which allow for very high resolution separation and detection but which do not result in removal of bound trace elements from the particular protein band or fraction.

Many of the trace elements of interest bind to proteins by various ionic, orbital complexation, and chelate-like mechanisms. Although these binding interactions can be very strong and the resulting binding constants very high, they are nevertheless reversible, and under appropriate conditions the element is removed from the protein. That is probably why the study we have proposed has not already been done.

We wish to assess the feasibility of developing a planar multi-modal, multi-dimensional "soft" protein separation process which will result in highly separated protein bands or zones with the tertiary and quaternary structure largely intact, and with tightly bound metals and other elements remaining in place. Our hypothesis is that many of the trace elements which may have medical and diagnostic significance are important because they bind to and activate or inactivate specific proteins and peptides (10, 61, 78). That concentration range requires requires fairly high binding constants. The binding can be maintained during the "soft" separation process, particularly if aqueous buffers and media are utilized which, if necessary, are spiked with the key elements of interest.

We propose to use gel permeation chromatography as the first separation dimension. This technique separates proteins on the basis of general molecular size using minimally interactive gel matrices with different cross-link densities and porosities. By the appropriate selection of gel porosity and a variety of other variables, molecules can be separated ranging in molecular weight from several 100 to several million. We propose to do this using a hollow tube geometry and to examine the effectiveness of gel particles as well as a continuous gel with a linear gradient in porosity. Although this does not lead to sharp or high resolution separations, it will be useful as a preliminary separation dimension. This gel will then serve as the feeder gel for the next dimensional separations.

Proteins collide with a particular surface in many different orientations, some of those orientations being more favorable to adsorptive interactions than others. In addition, proteins in mixtures competitively collide with the surface, and eventually those proteins with the strongest interactions will tend to dominate the interfacial composition. This leads to a hierarchy and change in the surface population with time, which is known in the plasma protein adsorption community as the Vroman effect (91). The simple rule is what you see on the surface depends on when you look.

Since most complex proteins, including most tear and plasma proteins, have multiple domains each with different surface activities, one of the domains or regions of the protein may tend to drive or dominate the adsorption process (84, 85). On a hydrophobic surface for example, the most hydrophobic or most readily denaturable domain may dominate. On a positive recharged surface the highly negatively charged domain or region may dominate, etc. We are now in the process of mapping the domain surface activity for human albumin and human fibrinogen. A very preliminary analysis of tear lactoferrin, plasma hemopexin, and plasma vitronectin have also been performed (79, 80).

The anionic and cationic electrostatic interactions and the hydrophobic interactions provides three modes for potential separation (64, 69, 70). The plan is to use the size exclusion gel, containing the proteins separated by molecular size, to feed planar gels containing gel networks or positive charge, negative charge and hydrophobicity. By forcing the separation through the use of an applied buffer flow field (in the inside of a hollow semi-permeable tube), one basically has a planar kinetic chromatography separation which, at the prescribed time, can be stopped and fixed in position. This is very similar to the planar thin layer chromatography which has been extensively used for the separation of low molecular weight compounds but commonly for peptide and protein separation (65-68). The difference is that in our case the matrices will be prepared so as to minimize or eliminate all but the desired interactions with proteins. Using our experience with polyethylene oxide surface modification to provide a steric exclusion/steric repulsion layer, which minimizes the adsorption of proteins (85-87), coupled with specific derivitization of the gel with positively charged, negatively charged, or apolar residues, will allow a much cleaner separation with far less interferences than is normally the case with thin layer chromatographic media.

We will approach this problem similarly to the way we approached 2-dimensional gel electrophoresis many years ago as a quantitative, multi-protein analysis technique, by preparing and

evaluating a number of media using a range of model proteins whose molecular weight, adsorptive properties, domain structure and electrophoresis/chromatographic separations characteristics are well-known (12). Using this small array of proteins, we will optimize the separation gel characteristics and parameters. The separated protein bands and patterns will be detected by standard fluorometric and silver staining procedures and semi-quantitated using 2-dimensional optical detection with our existing CCD camera facility (12). Once the protein coordinates, i.e. the map, is known, then in future runs that portion of the gel can be removed, solubilized and analyzed by the ICP/OES/MS technique for overall elemental composition.

Multi-dimensional thin layer chromatography is not a new technique (65), but it is a set of techniques which has not been widely applied to protein separation. We feel we are in a good position to take what is already known about multi-dimensional protein separation in the liquid chromatography mode (64, 70), coupled with our knowledge of protein adsorption, protein structure, minimization of non-specific interactions, and surface derivitization and gradient technologies, and develop a means to perform multi-dimensional thin layer chromatography of complex protein mixtures with high resolution and high sensitivity.

Our approach utilizes so-called soft interactions (71, 73), utilizing phases and separation modes which completely minimize the denaturation or other structural and chemical alteration of the proteins involved.

The pioneering paper by Gercken and Barnes used size exclusion chromatography to separate blood proteins, followed by ICP mass spectrometry, to measure lead, copper, zinc, and iron levels in the various fractions (76). Theirs was a relatively low resolution size exclusion separation but it shows the feasibility of determining ppb metal levels in separated plasma protein fractions. We plan to do similar experiments in the early stages of our study, verifying the size exclusion separation methodology and the ion exchange, and hydrophobic interaction separation through the detection and measurement of model metalloproteins (23-26, 39). These studies would begin as soon as the instrument is up and operational using the existing chromatographic and separation facilities in the Center for Biopolymers at Interfaces. We will also perform a variety of conventional TLC and planar chromatography separations on model protein systems and utilize those separations to develop the methodology for the high sensitivity elemental analysis of various chromatographic media. By the middle of year 2, we expect to have the multi dimensional planar separation approach developed to the point where we can begin to establish the feasibility of metalloprotein detection and quantitation.

The goal is a multi-dimensional map representing the separation of the proteins as a function of size, cationic character, anionic character, and hydrophobic interaction potential.

Most proteins are expected to have only one size or size range, except for those which might undergo monomer, dimer, or oligomer equilibria within the concentration range studied. Our initial consideration of multi-modal protein separation will result in cationic retention, anionic retention, and hydrophobic retention parameters for each protein. These parameters would then be plotted or mapped as a function of molecular weight as determined by the size exclusion separation. A protein can be retained by all three of the interaction mechanisms, either due to the interactions of different regions or faces of varying characteristics of a single domain or through the interactions of separate structural domains with different characteristics.

Superimposed on the separation axes, we will have a set of elemental composition axes involving over 40 components, which is where the multi-variate analysis comes in.

e. Summary and Limitations:

This is an enormous project. It is a feasibility study. We expect that as various phases of the project begin to prove feasible, grants will be written and funding hopefully obtained to expand that particular phase of the project beyond the feasibility stage. Reviewers can argue that we should take all of this one step at a time. If we do that and focus virtually all of our efforts on one part of the project, then we essentially lose the vision of the integrated whole. We know we cannot accomplish the integrated whole in depth in a three year period with these limited resources, but we also feel it is very important to maintain that broad multi-disciplinary approach and vision as we move forward. We fully expect that the resources available to us for this project will exceed the budget requested in this proposal. We expect several students supported by fellowships to work on this project.

In summary, we propose to:

- develop the methodologies and protocols with which to analyze four readily obtained, non-invasive tissues for at least 40 trace elements,
- develop means to separate the peptide and protein components of three of those tissues using soft, relatively non-perturbing protein separation methods of very high resolution and sensitivity,
- determine the elemental content of those various protein bands for the same 40 or so elements,
- assess the diagnostic potential and significance of multi-element, multi-protein data, and finally
- assess the feasibility and cost effectiveness of this approach and make specific recommendations for its continuance or elimination.

d. Multi-variate Analysis:

There are six components to the multi-variate analysis part of the project. Given that we will have data on over 40 different elements from four different tissues from a variety of subjects and sources, we will begin with a correlation of elemental concentrations across the four tissue types. We expect some crude correlation between urine, tears, and saliva and only a weak correlation if any between hair and the other three tissues, in part because of the long time delay expressed by hair samples. We also propose to perform cross-element correlations among the 40 or so elements. Based on the brief earlier discussion, some elements displace others in particular binding sites, some elements may occupy unfilled sites (such as the binding of aluminum to the transferrin fraction which does not contain bound iron) and certain groups of elements may reflect common modes of input (such as iron, nickel, and chromium in an individual with a stainless steel prostheses).

As the protein separation part of the project develops in year 2, we will then be in a position to do cross tissue protein correlations. This has been done in a limited way using high resolution 2-D gel electrophoresis and the 2-D maps available for plasma, urine, saliva, and semen, but there has been little such effort for more soft separation processes such as we propose to utilize and develop.

With the development of the soft high resolution protein separation, followed by the elemental analysis of the various protein bands or patterns, we will then have the 40 plus elements measured for each of the 100 or so protein bands in the various tissue samples. We will therefore be able to do multi-element, multi-protein correlation which can then be extended to a multi-element, multi-protein, multi- tissue correlation.

Finally, with most of that methodology in place and having given a sufficient time to begin to become thoroughly familiar with the extensive computer databases now in place in clinical chemistry, clinical laboratory, and diagnostic medicine (16, 17, 93, 94), we will be in a position to at least preliminarily initiate a multi-variate analysis of trace elements, metalloproteins, and clinical states.

Fortunately, multi-variate analysis has developed very rapidly in the last decade, and there are now a variety of relatively standard and easy to use software packages. Our local consultant is Dr. Bonnie Tyler from Montana State University, a recent graduate from the University of Washington who worked extensively with the Chemometrics group there, and utilized modern multi-variate analysis tools extensively in her thesis and subsequent work. Dr. Tyler will work with us during the entire three years of the project so that we have the multi-variate analysis methodologies up and going by the end of the first year, so that there will be little or no delay in beginning to develop the correlations and analyses as the data are generated. As more and more data are generated, the analyses will be regularly updated.

We will experiment with means of presenting the element/protein/tissue and disease/diagnostic correlations using what Vogl, et al have called their Vector Diagrams and Cluster Models (17). We have used a similar approach in utilizing a multi-variate radial plotting process, which we have called the "tatraplot," to cross correlate structural, chemical, and stability properties of model proteins with their surface activity at air/water and solid/liquid interfaces (80, 85). This "poor man's multi-variate analysis" is an extremely effective way to show patterns and correlations particularly to those with no appreciation of or background in the complexities of multi-variate processes. We fully expect that at the conclusion of the study we will have an array of such patterns which tie to certain disease states.

4. Time Plan

Table 3 is an approximate time plan for the various parts of the study over its 3-year duration.

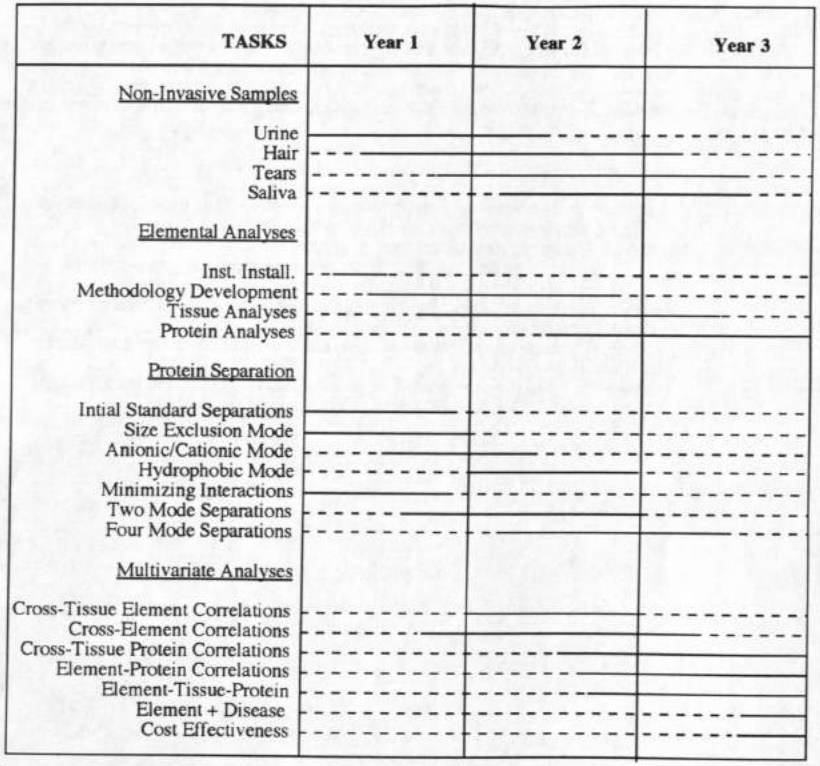

Table 3: Approximate Time Plan for the various parts of the study. Dotted lines indicate preliminary or ongoing routine activities; solid lines indicate major effort and focus.

5. Significance and Cost Effectiveness

The cost effectiveness or cost benefit-risk ratios for public health and medical procedures analysis is in a state of flux and disarray. There is certainly a growing tendency in Washington to look to prevention and screening as a means to decrease the overall costs of health care. The recent federal approval of the Oregon plan will lead to increasing emphasis on the allocation of limited

medical and public health resources in a manner so as to optimize the health of the state population. This plan places an emphasis and a premium on means to improve the overall health and well-being of the entire state population through appropriate education, prevention and screening.

We anticipate a growing national interest in education, prevention, screening, and early diagnosis. By the time this project is concluded,the actual cost of obtaining quantitative data on 40 or 50 elements will be greatly decreased, possibly to only several dollars for a complete profile. ICP mass spectrometers are already being mounted in vans and used for environmental analysis purposes by the EPA and the Department of Defense. Such analyses are already available by a variety of commercial laboratories. The problem is that not much is known about what to do with that data, largely because the correlations with known medical problems are not fully established and the correlations with other measures used for medical diagnosis do not exist.

This project will help determine the feasibility and cost effectiveness of such efforts by taking what is known from the public health and environmental community with respect to trace elements, what is known from the protein biochemical and organic biochemistry community about metalloproteins, and what is known in the medical informatics, clinical laboratory, and diagnostics community about lab tests and diagnosis.

By the time we are ready to analyze the cost effectiveness of these approaches, we hope and expect that the state of Oregon, the state initiatives dealing with prevention and early diagnosis, and hopefully a new Federal initiative to help resolve or at least greatly simplify the present health care mess, will have developed methodologies and guidelines for cost effectiveness which will be appropriate to this project.

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G. Biographical Sketches

Biosketches for J.D. Andrade, P.I., for Dr. Owen Ash, key advisor, and Dr. Bonnie Tyler, consultant, are included in this section, together with letters of collaboration. On-Campus advisors include:

Dr. Owen Ash, Professor of Pathology, Chief Executive Officer of ARUP (Associated Regional University Pathologists), a major clinical chemistry laboratory adjacent to the University of Utah. Dr. Ash has had a personal interest and extensive experience in trace element analysis for the past 20+ years. His letter of collaboration and biographical sketch are included. He will meet with the project staff on a regular basis to give them the benefit of his experience and advice regarding the design and conduct of the study and the correlations and the conclusions generated. Through Dr. Ash we will have access to major parts of the ARUP database, access to their technical staff, particularly their ICP mass spectrometry facility, to help develop the methodologies and sample preparation techniques for the elemental analysis studies. Their staff in the area of protein separation, characterization, and analysis will also be available to provide advice and input. ARUP is the major clinical chemistry laboratory in the Intermountain United States, with branch offices in Chicago and Cleveland. It conducts over 1000 tests and assays (14).

Dr. Douglas E. Rollins is Professor of Pharmacology and Toxicology and Director of the Center for Human Toxicology at the University of Utah. Dr. Rollins' particular expertise is toxicology and poisoning. His facility has experience in certain aspects of this project and his input and advice will be very valuable. He currently has a large project on detection and measurement of drugs in hair.

Dr. James McClowsky, Professor of Medicinal Chemistry, is an expert in the area of biological mass spectrometry. Dr. McClowsky recently edited, with A.L. Burlingam, the volume Biological Mass Spectrometry, published by Elsevier, 1990. He is expert in all areas of mass spectrometry as applied to biological samples and systems.

Dr. Karin Caldwell directs the Center for Biopolymers at Interfaces at the University of Utah and is Associate Professor of Bioengineering. Dr. Caldwell and Dr. Andrade, the P.I. on this program, have shared laboratories over the years and have shared technical staff, post-docs, and students. They have worked closely in the area of tear protein interactions with contact lenses. Dr. Caldwell will advise in the general area of tear collection, tear analysis, and tear protein studies. Dr. Caldwell is an expert in the area of separations of particles and macromolecules, and has a great deal of experience in the area of protein chromatography and protein electrophoresis.

Dr. J. Janatova, Research Associate Professor of Bioengineering, is a protein chemist with particular experience in the complement system and on the plasma protein albumin. Dr. Janatova has worked closely with Drs. Caldwell and Andrade over the years, and will provide advice and input in the general area of protein separation and analysis.

Dr. Robert Huefner, Professor of Political Science and holder of the FHP Chair in Health Policy, is an expert in the general area of health economics and health policy. He was a participant at the AIMBE meeting in March, 1993 on The Future of Health, was a key participant in the April 1992 NSF workshop and has chaired the Governor's Task Force on health policy matters for the state of Utah. Dr. Huefner and Dr. Andrade have been working closely together for the past three years on areas related to the costs of health care and the cost-effectiveness of medical technologies. Dr. Huefner will meet with the group on a regular basis, specifically to address the cost-effectiveness and the potential health policy aspects of the work.