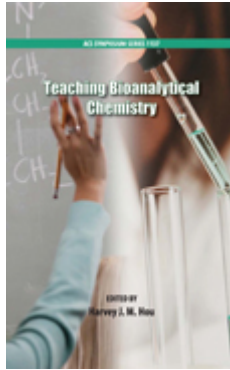
Teaching Bioanalytical Chemistry
Harvey J. M. Hou

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

ACS Books Department

Preface

It is evident that the focus of analytical chemistry in academia and industry is increasingly on the analysis of biological activity and detection of biological molecules. Bioanalytical chemistry has been becoming one of the most promising enhancements in chemical education. The purpose of this book is to present the recent progresses in terms of novel strategy and pedagogy of teaching bioanalytical chemistry in the classroom and laboratory. The effective and efficient ways are the incorporation of specific bioanalytical chemistry experiments in an existing chemistry class. In addition, brand new bioanalytical chemistry courses at undergraduate and graduate levels may be created and implemented into the chemistry curriculum. At least three layers of bioanalytical chemistry content may be developed and implemented in chemistry education: (1) introduction to bioanalytical chemistry, (2) (intermediate) bioanalytical chemistry, and (3) advanced topics in bioanalytical chemistry.

A grand challenge of bioanalytical chemistry is to build upon the explosively growing knowledge learned from quantitative and instrumental analysis, to integrate it into the extremely demanding chemistry curriculum, and to facilitate student learning by making connection between a variety of subdisciplines in science and technology. It is an honor and privilege for me to have organized four symposia on teaching bioanalytical chemistry in the biennial conference in chemical education (BCCE) in the past six years. The symposia have provided a great forum for sharing and stimulating the novel ideas of implementing the bioanalytical chemistry components into the existing chemistry classes or developing complete bioanalytical chemistry courses to enhance student learning. The authors are selected from the presenters in these symposia as well as invited experts in teaching bioanalytical chemistry at college and universities in the United States.

Because of the exceedingly rapid development and multidisciplinary nature of bioanalytical chemistry, it made educators to teach bioanalytical chemistry in classroom and laboratory tremendously challenging and demanding. The chemistry educators suffered drastically from the relatively limited textbook and confirmed teaching resources. In this ACS symposium book we present the recent advances in teaching bioanalytical chemistry, which are written in thirteen chapters by twenty-eight dedicated experts in the field of bioanalytical chemistry education in colleges and universities. These teaching innovations have been completely tested in the chemistry classroom and laboratory. The organization of the book provides a list of unconventional and effective teaching approaches to address most of the typical bioanalytical techniques. However, due to the restricted time and capability, it is impossible to present the complete advances

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in methodologies and applications of bioanalytical chemistry. It is not our intension to present a comprehensive textbook. The topics of new development in bioanalysis including, not limited to, immobilization of biomolecules, X-ray crystallography, and nuclear magnetic resonance, are beyond the scope of this book. We hope that this ACS symposium book may provide valuable information and practical innovations in teaching bioanalytical chemistry and enrich the chemistry curriculum for educators of the two-year community colleges, four-year colleges and universities at undergraduate and graduate level.

Due to the extremely limited time frame for collecting manuscripts and the strict deadline for publishing the book, the authors of this book stand for an incomplete list to represent the teaching community of bioanalytical chemistry. Some of the outstanding educators, who have presented their verified ideas and innovations in the BCCE symposia and initially decided to write book chapters for sharing their excitement and teaching experience in teaching bioanalytical chemistry in classroom and laboratory, are unfortunately unable to contribute to the book. As a result, the content of this book will be a snapshot in bioanalytical chemistry education presented in the BCCE symposia. The book introduces a head start to express and exchange new thoughts and innovations in teaching bioanalytical chemistry. I am looking forward to launching a more comprehensive book in teaching bioanalytical chemistry in the future.

I would like to take this opportunity to acknowledge the authors for writing the excellent book chapters and for being supportive and cooperative when the manuscripts were reviewed and revised. I would also like to thank the external reviewers for their timely contribution and effort in judging the value of the manuscript and delivering the constructive comments, which undoubtedly improve the quality and readability of the book. I am very grateful to Tim Marney, Arlene Furman, and Aimee Greene of the ACS Books Editorial Office for their insightful advice and meaningful assistance during the entire book project. I would like to thank the ACS Division of Chemical Education for sponsoring the BCCE symposia, which provide an exceptional venue for bringing the bioanalytical educators together and for making this book project possible. Finally, I would like to thank my wife, Du Liao for providing continuous support in all my endeavors.

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Chapter 1

Introduction: Bioanalytical Chemistry Education

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This book is written by twenty eight experienced bioanalytical educators and provides specific examples including experiments and courses in bioanalytical chemistry education. Bioanalytical chemistry is broadly defined in this book as analytical applications in the chemical and biological sciences using a variety of experimental methodologies. In this chapter, I will briefly present current statues of bioanalytical chemistry education, summarize the innovative pedagogy and approach in teaching bioanalytical chemistry, discuss the novel bioanalytical laboratory experiments, and review the new bioanalytical chemistry courses presently taught in the chemistry curriculum. I will also share my experience in teaching bioanalytical chemistry, and my views on the potential problems and possible solutions in bioanalytical chemistry education.

Bioanalytical chemistry has been becoming one of the most promising enhancements in chemical education because the focus of analytical chemistry in academia and industry is increasing on the analysis of biological activity and detection of biological molecules. During 2006-2012, teaching bioanalytical chemistry and laboratory has been one of the symposia in the biennial conference on chemical education (BCCE) sponsored by American chemical Society Division of Chemical Education. The symposia have provided an exceptional venue for bioanalytical chemistry educator to sharing and stimulating the novel ideas and pedagogies in their teaching practice.

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This ACS symposium book is written by twenty-eight authors from fourteen institutions including the liberal art colleges and the national research universities and offers specific samples including experiments and courses in bioanalytical chemical education. Bioanalytical chemistry is traditionally classified as one subdiscipline of the field of analytical chemistry, which includes the qualitative and quantitative analysis of biomolecules using chemical, biochemical, and instrumental techniques. The typical analytical methodologies are spectroscopy, electrochemistry, chromatography, and mass spectrometry. In this book, a broad definition of bioanalytical chemistry is used and includes all the analytical applications of experimental methodologies in the chemical and biological sciences. It will cover the biophysical chemistry, biochemistry, environmental chemistry, green chemistry, nanotechnology, and forensic science, in addition to the traditional bioanalytical chemistry.

It seems that much of forensic analysis and environmental science is not performed on biological samples and is based on physical methods unrelated to plants or animals. However, forensic analysis involves the analysis of illicit drugs and their metabolites (*1*). DNA profiling of biological fluids is widely used in forensic science and enriches the content of bioanalytical chemistry. In particular, microbial forensics is an emerging field of forensic science and dedicated to the detection, characterization, and interpretation of evidence from the scene of acts of bioterrorism or biocrimes (*2*, *3*). The evidences include bacteria, fungal pathogen, plant toxins, influenza viruses, and other dangerous species and belong to the context of bioanalytical chemistry. Environmental science may be associated with the detection of contamination due to the bacteria, viruses, and toxins, and can be an important component in bioanalytical chemistry.

Although bioanalytical chemistry defined in this book may overlap significantly with biochemistry laboratory, it is different from each other in two aspects. 1) Bioanalytical chemistry focuses on the sample analysis and not the purification of proteins or the molecular cloning of genes, which are the key components in biochemistry laboratory. 2) Bioanalytical chemistry includes all the applied sciences using approaches in physics and chemistry, which include biophysical chemistry, environmental science, green chemistry, material science, nanotechnology, and forensic science. These topics are not main concerns in biochemistry laboratory.

1. New Pedagogy and Approach in Bioanalytical Chemistry Education

Active learning is the key in higher education and has been applied in college classrooms and laboratories duing the past several decades. The previous ACS syposium book, "Active Learning," summarizes the innovative approaches and the active learning methods in the mode of delivery of analytical chemistry (*4*). Due to the extremely rapid growth in applcation and multidisciplinary nature of bioanalytical chemistry, it is unbelievably challenging and demending for chemical educators to teach bioanalytical chemistry in classroom and laboratory with a limited course time and vast course content. Instead of using the traditional

"expository instruction," the unconventional pedagogy and strategy must be exployed to integrate development into the curriculum, to facilate student learning by connecting the variety of subdisciplinery in science and technology.

Teaching bioanalytical chemistry may be instructor-directed (maximum guided) and student-driven (minimum guided). Based on the literature and my own experience in chemistry education, student learn the course material more by doing the activities via the hand-on experience. When it is possible, I always offer the hand-on experience or laboratory experiment in my class. Active learning is the key for students to understand the concepts and master the scientific skills. To enhancing the student learning, the active learning modules have been increasingly used in teaching bioanalytical chemistry in classroom and laboratory, which include inquiry-based approach, problem-based method, cooperative-based learning, role playing strategy, discover-based learning, research-based curriculum, context-based case studies (CBCS), small mobile instruments for laboratory enhancement (SMILE), and project-based learning. These active-learning approaches are dicussed in several chapters in this book (*5*–*9*).

Barne and Sander reviewed the nontraditional methods for teaching a specific area of analytical chemistry: spectroscopic techniques in bioanalysis (*6*). The readers may get a flavor for the different way and types of experiments that can be used to teach spectroscopic techniques in bioanalysis. The most widely used non-traditional approaches are process oriented guided inquiry learning (POGIL) and peer-led team learning (PLTL). POGIL uses strict structured student roles in group settings with well designed questions for the students to answer. In contrast, PLTL focuses on peer leaders who have been taught through separate training program for the course. These leaders work together to guide students to find solutions of the designed problems provided by faculty and become the active and coherent part of the teaching team. These innovative teaching strategies have improved student learning outcomes in the skills of higher-order thinking, problem-solving, conceptual understanding, teamwork, and communication.

2. New Bioanalytical Laboratory Experiments

Typically chemistry curriculum is incredibly difficult and demanding for undergraduates in colleges and universities. In many cases, it includes one year of general chemistry, one year of organic chemistry, one year of physical chemistry, one year of analytical chemistry (quantitative analysis and instrumental analysis), one year biochemistry, and one year inorganic chemistry (descriptive and advanced topic). Addition of a new bioanalytical chemistry might be problematic in the curriculum without deleting one existing chemistry course. The effective and efficient way is to integrate the lab component in the existing chemistry curriculum. The new experiment design should target on enhancement of the student learning, skills, competency, and enthusiasm in bioanalytical chemistry.

I have implement three experiments in the existing chemistry classes, "Procedures of Chemical Analysis" and "Chemical Biology and Technology," at UMass Dartmouth. These experiments are the determination of secondary protein

structure using Fourier transform infrared spectroscopy (FTIR), forensic analysis of illicit drugs using bioanalytical techniques, and chlorophyll fluorenscence evaluation of American cranberry plant under diverse conditions.

The objectives of the first experiment, "Protein secondary structure determined by Fourier transform infrared spectroscopy," are the understanding of the principle of infrared spectroscopy and the development of the ability to determine the secondary structures of the native and denatured proteins including myoblobin, cytochrome c, and trypsinogen (*10*). With minimized help from the instructor and extensive discussion between the peers, students are required to generate experimental procedures, prepare protein solutions, to record the infrared spectra, fit the infrared peak, calculate the secondary protein structures, and write lab report.

Forensic science is the application of scientific principles to criminal and civil laws within a criminal justice system (*1*). It involves the examination of items of evidence by analyzing alcohol, carbon monoxide, and drugs in body fluids as well as substances such as glass, soil, hair, ink, bullets, and gunpowder. To meet the increasing need of students who are interested in forensic science, another experiment "Forensic analysis of illicit drugs in uring and saliva samples" is offered. Two objectives of the experiment are 1) understand the principle of immunoassay of forensic drugs including alcohol, cocaine, and marijuana, and 2) identify the forensic drugs in urine and saliva samples using test kits including One Site Alcohol Test Kit and One Step Urine Test Cassette.

The third experiment in the chemistry course "Chemical Biology and Technology" invovles the evaluation of the photosynthetic performance in the leaves of American cranberry under diverse conditions using chlorophyll fluorescence kinetics (*11*). Three objectives of the experiment are to conprehend the principle of the chlorophyll fluorimeter, to determine the photosynthetic activity of plant tissue samples, and to examine the effects of two environmental factors including pH and temperatures. The experiment include one lecture and one lab period. The lecture provide the background of the methodogy and its application in plant biology. During the lab period, students in groups are required to devise their own experimental procedures, to conduct the experiments, and to write a lab report to summarize the experimental results.

Ho describes an outstanding lab experiment using protein conformational studies as theme to engage students in active learning (*8*). The protein conformation is probed by a variety of spectroscopic methods including UV-vis absorption, fluorescence, FTIR, and mass spectrometry. Through the well-designed course, students will learn not only the concepts and theory of the protein conformational changes, but also the principle and application of the instrumentation by collaborative learning and science writing heuristic. The emphasis is place on the critical thinking and problem solving skills. The chapter provides an effective way for instructors to turn passive cookbook lab learning into active inquiry-base activities.

Akinbo implements multiple laboratory component in analytical chemistry courses: 5 project modules in quantitative analysis and 4 project modules instrumental analysis, including one environmental and one bioanalysis project (*5*). Chohan and Sykes use SMILE initiative to transform the traditional analytical

experiment into discovery project-based labs. (*7*). It fills the need to involve students in troubleshooting instruments and to understand what goes on "inside the box." For example, students are required to design, construct, optimize and troubleshoots SMILE. Gilfilen, Lavender, Clinger, and Clinger describes a nice overiew of experiments and suggests improvement on a numbers of experiments in instrumental analysis and biochemistry to engage the pre-professional student in relevant laboratory activities (*12*). Gross, Clevenger, Neuville, and Parker develop one distinctive experiment using paper microfluidics for the undergraduate analytical chemistry laboratory (*13*). Microfluidic devices allow the analysis of tiny volume of fluids in microscale channels and wells. Research within the world of microfluidic devices, whether paper or another substrate, is currently quite common and constantly growing. Through the experiment students learn the fabrication of paper-based microfluidic devices and analysis of glucose and protein in the "unknown" synthetic using samples in the setting of clinical laboratory.

3. New Bioanalytical Chemistry Courses

A Bioanalytical chemistry course can be a laboratory-intensive or a purely lecture-based course. These courses can be offered in three levels in chemical curriculum, 1) introductory level (100 or 200 level), 2) intermediate level (300 and 400 level), and 3) advanced topics (400 and graduate-level). I have developed and implement an introductory bioanalytical chemistry and bioanalytical chemistry.

To enhance the chemical education, a new sophomore introductory bioanalytical chemistry course has been designed and implemented in chemistry curriculum at Gonzaga University in 2005. The introductory bioanalytical chemistry course is a three (3)-credit class includes three (3) hour lecture and three (3) hour laboratory component per week. The lectures covers the topics including acid-base chemistry, spectrometry, chromatography, electrochemistry, chemical kinetics, and thermodynamics.

The laboratorys focus on the applications of selected analytical methods to biological problems. Acid-base titration involves the titration of amino acid solution. Spectrometry experiment is designed to determine the chlorophyll a and chlorophyll b in spinach samples. Infrared spectroscopy is used to monitor the secondary protein conformational structures in protein samples. Inductively coupled plasma is used to quantify the content of manganese and calcium in chloroplasts. Instead of using pH electrode in traditional analytical chemistry, an oxygen electrode is utilized to obtain the oxygen evolution activity of photosynthetic electron transport in chloroplasts. An experiment using differential scanning calorimetry to assess the enthalpy change of protein denature process is developped. Additional three experiments including the separation using column chromatography, identification of proteins by electrophoresis, and chemical kinetics of enzymes are implemented.

A bioanalytical chemistry course covering the fundamental techniques in protein and DNA analysis using enquire-based approach is developped at UMass Dartmouth. The focus is placed on the methodologies to probe the structure and

function relationship of membrane proteins. The topics include spectroscopic analysis of total biomolecules, enzymes, immunoassays, electrophoresis, cnetrifugation, electrochemistry, chromatography, mass spectrometry, and other techniques in bioanalytical chemistry. The class requires two laboratory reports to assess the written skills of students, which must follow the guideline and format of a chemistry journal. There are two hourly exams to assess the student learning of principles and theories of analytical methods. Two oral presentations on the published paper with critiques in the field of bioanalytical chemistry. The same teaching strategies

Dovichi describes an excellent undergraduate course in bioinstrumental analysis which has been taught for six years between 2005 and 2010 at the University of Washington (*14*). The course covers essential bioanalytical/ bioinstrumental techniques and provides undergraduate a well-designed platform for learning bioanalysis. It is developed upon a graduate-level bioanalysis course developed by the author. The course consists of 18 lectures and eight laboratory experiments. The first half of semester focuses on the nucleic acid analysis including DNA electrophoresis, sequencing, hybridiation, PCR, and real-time PCR. The second half deals with protein analysis including protein electrophoresis, chromatography, mass spectrometry, bioinformatics, enzyme chemistry, receptors, antibody, flow cytometry, and fluorescence microscopy.

Witter and Arnold represents an unique interdisciplinary bioanalytical course for upper-level undergraduates in the field of ecological and analytical sciences (*15*). The course is designed as an advanced traning program. It is research-intensive and centers at the investigation of the ecological roles of natural products in mediating plant-insect interaction using modern analytical chemistry by team-teaching of a chemical ecologist and an analytical chemist. The lectures are designed to ensure student learning in concepts and theories in the field of ecology. The lab experiments reiterate the principles taught in classroom and allow students to perform the analyses of samples in the field and laboratory.

Ronkainen describes an exceptional advanced elective course for undergraduates, Bioanalytical Chemistry and Chemistry Sensors, and reflects in detail about the course content, learning format, and performance assessment (*9*). In particular, the course involves an emphasis on primary literature. Specifically, the focus of course is placed on problem solving, data interpretation, oral communication, and critical analysis of literature. The chapter provides detailed account of rational for the pedagogical approach, course structure, course content, learn outcomes, and course assessment instrument. It offers a well-defined idea of how to develop a similar course.

Beussman describes a lab-intensive bioanalytical chemistry course to prepare students for research in STEM field (*16*). The course have been successfully been offered several times at St. Olaf College. In the course 1) relatively common instrumentation including 1-D gel electrophoresis, UV-vis spectrometers, and HPLC systems and 2) exotic and expensive instruments including 2-D gel systems, capillary electrophoresis systems, and mass spectrometers are utilized to the topics including bimolecular structures, protein fingerprint, peptide sequencing, post-translational modification, bacteria identification, and clinical applications.

Guo, Young, and Yan describes a course design of a graduate-level bioanalytical chemistry class, "Biophysical Spectroscopy," offered at Yale University (*17*). The course is designed to teach a survey of biophysical spectroscopic methods using guided inquiry and project-based teaching strategies. Students will learn the basic principles of biophysical spectroscopy and address fundamental questions in the field of biology at the molecular level and gain the problem solving and critical thinking skills. Nguyen, Arceo, Weber, Springer and Hanrahan applied undergraduate research activities to enhance student learning in the Bioanalytical Chemsitry curriculum (*18*). The teaching activities aim to increase high-impact practices and reveal the importance of computer based projects in analytical chemistry curriculum. Specifically they describes a research-based acitivity and focused on experiential learning in classroom and laboratory.

4. Concluding Remarks

As the methodologies and applications of analytical chemistry in biological and medical sciences are increasingly developed and established, content of bioanalytical chemistry must be taught in the chemistry curriculum for the next generation of work force and world leaders. Teaching bioanalytical chemistry has been becoming one of the most important components in enhancing chemistry eduction. However, due to the fast growing knowledge and novel technology in bioanalytical chemistry, it is difficult to cover the advance and progress of bioanalytical chemistry in a single one-semester course. In addition, the arrangment of chemistry curriculum is extremely tight, it make it more difficult to have additional bioanalytical chemistry course. This is the challenging problem and dilemma we must address in current chemistry education.

As described in this ACS symposium book, the most straightforward and effective way to implement bioanalytical chemistry content is to introduce bioanalytical chemistry laboratory experiments in the existing chemistry classes. Alternatively, novel bioanalytical chemistry may replace one of the quantitative analysis or instrumental analysis class. Advanced topic classes are very feasible in graduate level chemistry classes. These novel courses include introductory bioanalytical chemistry, lab-intensed bioanalytical chemistry, biosensor, biophysical spectroscopy, and bioinstrumental analysis. Due the nature of the multidisciplinary and applied science to real world problem, the novel teaching pedagogy and strategy in the bioanalytical chemistry education must be and have been utilized, which include POGIL and PLCL as well as the inquire-based, research-based, and problem-based learning methodologies.

This ACS symposium book is to provide real stories of implementation of bioanalytical chemistry in college and university and to serve a capstone in teaching bioanalytical chemistry for chemistry educator. I intend to continuously organize the BCCE symposia on teaching bioanalytical chemistry in the years to come and encourage the participants to exchange and share new ideas and innovative experiences in teaching bioanalytical chemistry in classroom and laboratory. I believe that by working together we are able to make a difference in

bioanalytical chemistry education and enhance student learning. In such a way, we are able to prepare the students better to enter the work force and become the competitive world leader in the STEM field.

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Chapter 2

Nontraditional Instructional Approaches to Undergraduate Student Learning of Spectroscopic Techniques for Bioanalysis

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This chapter describes published instructional approaches/ materials which utilize nontraditional teaching strategies, such as problem-based methods, designed to enhance undergraduate learning, with a focus on spectroscopic techniques (UV-vis, IR, Raman, CD, NMR, and fluorescence) for bioanalysis. The chapter provides an introduction to spectroscopic bioanalysis methods, nontraditional teaching approaches such as Problem-Based Learning (PBL), Process Oriented Guided Inquiry Learning (POGIL), and Peer-Led Team Learning (PLTL), and a summarization of specific instructional resources involving spectroscopic techniques for bioanalysis. Both classroom and laboratory methods are discussed.

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Introduction

The term "bioanalysis" was derived in the 1970s to describe the process of quantifying drugs in biological fluids (*1*). Over the years, it has broadened in scope to include analytical techniques used in the quantification and characterization of biologicals, such as DNA and proteins (*1*). Currently, both definitions are used interchangeably. For the purposes of this chapter, both definitions are considered valid.

Much of bioanalysis is performed using spectroscopic techniques; common types of spectroscopic techniques include Fluorescence Spectroscopy, Nuclear Magnetic Resonance (NMR) Spectroscopy, Fourier Transform Infrared (FTIR) Spectroscopy, Raman Spectroscopy, Ultraviolet-Visible (UV-vis) Spectroscopy, and Circular Dichroism (CD) Spectropolarimetry.

Fluorescence occurs when atoms or molecules are excited by absorption of electromagnetic radiation and then give up some excess energy as they return to the ground state. It is only one pathway for molecules to relax to the ground state following excitation; most molecules do not fluoresce because their structures provide radiationless pathways by which relaxation can rapidly occur. The structures of rigid aromatic compounds cause the rate of radiationless relaxation to decrease so that fluorescence has time to occur. Fluorescence can be performed by steady state, time-resolved, quenching, polarization or energy transfer studies.

NMR spectroscopy is based on the absorption of energy in the radio-frequency region in a magnetic field by atomic nuclei that possess spin. Each nucleus in a molecule gives a signal in the NMR spectrum based on its local chemical environment. The intensity of each signal is proportional to the number of nuclei responsible for the particular transition. The number of peaks is related to the spin-spin coupling of neighboring nuclei. The ^{1}H nucleus is the most studied atom in NMR, followed by ^{13}C. NMR resonances are usually assigned from the 1-D and 2-D spectra. The major advantage of NMR is that it is a nondestructive technique, and it provides site-specific information. One major disadvantage is the need for high analyte concentrations.

Absorption of electromagnetic radiation in the infrared IR region causes vibrations in molecules to occur. The lack of site-specificity is a major drawback of vibrational spectroscopy. However, isotopically enhanced infrared spectroscopy has been useful in this regard. The absorption of water around 1600 cm^{-1} and 3400 cm^{-1} can be a problem when FTIR is used for the analysis of biological molecules in aqueous solution. Deuterated water can be use to reduce the absorbance of protonated water. In these solutions, the solvent absorption is shifted to about 1200 cm^{-1} and 2500 cm^{-1}, where it does not interfere with the absorption bands of interest.

Raman spectroscopy involves measuring the change in wavelength of light scattered by a molecule due to vibration. In addition to scattering at the same wavelength frequency (Rayleigh-scattering), the scattered light may be of lower (Stokes) energy or higher (anti-Stokes) energy than the incident light. The anti-Stokes peaks are usually weaker because they involve the excitation of molecules in a higher energy state that is less populated than the ground state. Raman spectra require higher sample concentrations than FTIR because the signal-to-noise ratio

is not as good. Raman has not been used as often as FTIR because of interference of fluorescence from the sample of from impurities. One advantage of Raman compared to FTIR is the weak water signal in Raman spectra.

UV-vis spectroscopy involves transitions of electrons from the ground state to the excited state. Molecules containing nonbonding or π electrons can absorb energy in the form of ultraviolet or visible light. The absorbance of the species in solution is most often determined using the Beer-Lambert Law: $A = 1\log_{10} (I_o/I) = \varepsilon b C$, Where A is the measured absorbance, I_o is the intensity of the incident light, I is the intensity of the transmitted light, b is the pathlength through the sample, C is the concentration of the absorbing species, and ε is the molar absorptivity or extinction coefficient.

CD spectropolarimetry is similar to UV-visible spectroscopy in that the absorbance of light as a function of wavelength is measured; however, CD measures the difference in absorbance of left and right circularly polarized (CP) light as a function of wavelength. In order to employ CD as a technique, a chiral molecule is needed. A positive or negative CD signal indicates greater absorbance of left or right CP light, respectively.

For new instructors of bioanalytical chemistry, any available teaching resource is considered a plus. This is especially appreciated when instructors consider the complexity of bioanalysis. For instance, it is very time-consuming and takes a skilled person to create illustrations of biological molecules and schematics of bioanalysis methods. Thus, materials that have been available for instructors of bioanalysis courses have been very useful. However, there is a need to alter the way in which the materials are presented to students so that there is student engagement and students see the value in learning and retaining the information for future use. A proven way to accomplish this goal is using nontraditional instructional approaches such as active learning methods.

Nontraditional Instructional Approaches

A 1996 report by the Advisory Committee to the National Science Foundation Directorate for Education and Human Resources suggested that "All students have access to supportive, excellent undergraduate education in science, mathematics, engineering, and technology, and all students learn these subjects by direct experience with the methods and process of inquiry" (*2*). Inquiry-based teaching methods involve posing a series of questions to the students so that there is an interactive discussion and exchange of dialogue between the instructor and the students. The students are allowed to engage with the instructor and the materials being presented. In a typical traditionally taught college course, undergraduate students will listen to the instructor lecture to them about each particular topic covered in the course for fifty minutes to one hour and fifteen minutes each class period. Students are expected to listen intensely and grasp the content during this period. Shortly after the lecture, students may be given some homework assignments, quizzes and exams to assess their knowledge of the course content. Successful knowledge transfer from the instructor to the students is mainly based upon the students' successful passing of a limited number of quizzes and

exams; this is difficult for students, particularly for students enrolled in classes that only meet for a few hours once a week. Although many students manage to successfully pass the course, many feel that they didn't see the value in retaining the information. Why is this such an issue? Research has shown that most students find it difficult to learn in such an environment (*3*), particularly in hard-core science courses such as chemistry. Students are frustrated by lack of involvement or not feeling valued by the instructor as a source of information (*3*). They feel bored and do not understand the usefulness of the topic to their lives; they just don't understand why they need to "learn the A" rather than "earn the A". Although it is easier for students to memorize materials for a short period so that they can pass the course, they would much rather feel that they have learned the materials; students want to understand the importance of retaining that knowledge for future courses as well as being able to identify and solve problems that they can somehow relate to (*3*). Traditionally taught laboratory courses are also difficult for students because they are usually given a "cookbook" recipe to follow with results expected and already known. With this type of approach, students spend most of their time concentrating on getting the correct answer or obtaining the correct end result rather than being involved in higher order cognitive thought processes, such as critical thinking and problem solving (*4*). Therefore, they have a false idea about how scientific research is done in the real world. Laboratory courses should present students with a problem to address first. Then, students should be allowed to perform some aspect of exploratory research, which should include searching the literature to investigate what has been done previously and how they might use the literature to assist in addressing their specific problem. Students who have been involved in the nontraditional laboratory courses feel that they have accomplished more in the laboratory course and learn by hands-on, real world experiences; they are more likely to retain what they have learned as well as use the skills they learned for other courses and other life-long learning situations (*5*).

Traditional instruction, also called Expository instruction, involves the instructor presenting information to the students. It "exposes" the learner to definitions, processes, concepts, and equations for a particular subject matter with little interaction between the instructor and the students and between the students and the material being presented (*6*, *7*). On the otherhand, nontraditional teaching methods are student-centered methods which involve active learning, anything that students do in a classroom or laboratory other than merely passively listening to an instructor's lecture or following a cookbook style experiment is considered to be active learning (*8*). Traditional methods include Discovery-based Learning, Context-based Case Studies (CBCS), Analytical Method Development, Technology-based Learning, Collaborative Learning, Cooperative Learning, Problem-Based Learning (PBL), Peer-Led Team Learning (PLTL), and Process Oriented Guided Inquiry Learning (POGIL). These methods involve student engagement. Discovery-based instruction involves students drawing on prior experiences and knowledge to interact with their environment through exploration (*9*, *10*). It occurs when students are not provided with answers to problems, but are provided with materials to find the answers themselves. Case studies allow the investigation of phenomenon within its real-life context, involving

analysis of persons, events, decisions, periods, projects, institutions, or other systems that are studied holistically (*11*, *12*). Analytical Method Development is similar to PBL, however, more information is given to the students (*13*, *14*). Technology-based instruction involves use of technologies such as the computer for simulations, demonstrations, virtual laboratories, videos, and animations, which allow the student leaner to "visualize" , and sometimes "interact with", the content of the subject matter being discussed (*15*, *16*). Collaborative learning involves groups of students working together to solve problems or complete specific tasks or activities (*17*, *18*). Learning occurs when students are able to talk things through, either face-to-face or via the internet or other means of communication. Cooperative learning is a specific kind of collaborative learning in which students work together in small groups on structured activities (*19*). Groups work face-to-face and work as a team, and are held accountable for the work both as individuals and as a group. This Chapter is mainly focused on PBL, POGIL, and PLTL and provides specific examples of these nontraditional teaching methods as they relate to spectroscopic bioanalysis.

These methods all shift focus from the instructor to the students, allowing the students to take more responsibility and be more engaged in the learning process. Research has shown that these student-centered approaches better prepare students to become life-long learners and problem-solvers; students trained in these approaches are expected to be better prepared for future job placement and acquire skills for successful work across disciplines to better address broad-based complicated problems that plague communities, states, nation, and the world.

Although there are many different names given to active learning techniques, the oldest and best known organized methods of teaching through active student participation include PBL, POGIL, and PLTL. Figure 1 shows the major feature of each of these three pedagogy (*20–22*). PBL is a long-term problem-solving approach that involves student research of a specific problem. POGIL involves structured student roles in group settings with questions for the students to answer. PLTL relies on peer leaders who have been trained for the course. These leaders work together to discover solutions to special problems created by faculty. All these methods offer benefits to students that faculty are finding successful.

Despite the benefits that nontraditional methods have been reported to provide for students, it can be difficult for instructors to make the transition to incorporate nontraditional methods in their classes. Although in most cases there is a variety of reasons, the major reasons appear to be misconceptions about nontraditional approaches, lack of training, and lack of knowledge about available resources (*23–25*). Many instructors do not understand what nontraditional methods are and how beneficial these methods really are to the students. In many cases, instructors are unaware that these methods have been used by instructors in many different settings with many different types of students, and that the students prefer these nontraditional methods over the traditional method of teaching. Some instructors feel that if they are not spending their time lecturing to the student, then they are not giving the students what they need. Others feel that nontraditional methods are only a way for the instructor to be lazy and make the students do the instructor's job.

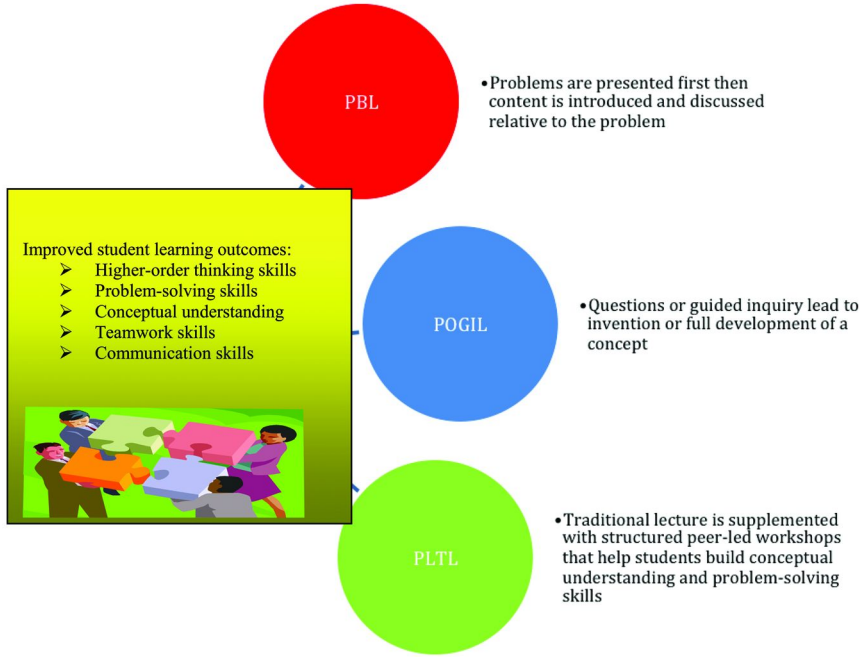

Figure 1. Features of Active Learning Methods: Problem-based learning (PBL), Process Oriented Guided Inquiry Learning (POGIL), and Peer-Led Team Learning (PLTL).

Those instructors who already realize the value in nontraditional teaching methods may be frustrated by the lack of training on how to successfully implement these approaches in their classes. Fortunately, these frustrations have been heard and training opportunities are becoming more and more broadly available (*26*). For instructors who have had training in these nontraditional approaches, having the time to develop materials may be seen as an obstacle. However, there has been an increase in the number and types of materials for nontraditional methods being made publically available. In many cases, the materials are freely accessible (*27–29*). There are even textbooks available for classroom instruction based on active learning principles (*28*).

Recent research has mostly been focused on the pros of active learning and the cons of traditional teaching approaches. However, both approaches have benefits for the students (*24*, *25*) and we suggest that instructors consider using a hybrid approach that is best-suited for their particular class or laboratory. Traditional approaches are important because the instructor is able to cover more content and fundamentals; on the otherhand, nontraditional approaches allow the students to develop higher order cognitive skills such as critical thinking and problem solving skills that they will need for successful matriculation in their field. It is important to find a balance between the two approaches that will ensure students are prepared with both the fundamental knowledge content and necessary skill set to met the global challenges of the twenty-first century.

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In order for instructors seeking to incorporate nontraditional teaching methods into their courses to make a smooth transition into teaching such methods, they must not only understand the benefits and basics of these methods, but they must also become familiar with the literature so that they can get a good start on implementing these methods and realize that they do not have to "reinvent the wheel". The literature is becoming more and more populated with examples of how instructors are using nontraditional methods in their lecture and laboratory courses (*30*, *31*). The focus of this chapter is on presenting instructors interested in nontraditional methods an overview of these methods with specific focus on spectroscopic methods for bioanalysis. The paragraphs that follow provide a discussion of available resources. The authors did not find PLTL references for bioanalysis; the references presented are for general chemistry.

Examples of Nontraditional Approaches in Spectroscopic Bioanalysis

Table I provides references that pertain to nontraditional instructional approaches/materials for bioanalysis.

Table I. Nontraditional Approaches/Methods for Bioanalysis

Instructional Approach	*Author(s)*	*Reference*
PBL	Mabrouk	(*32*)
	Jones	(*33*)
	Wilson et al.	(*34*)
	Bullen	(*35*)
	Kalivas	(*36*)
POGIL	Briese and Jakubowski	(*37*)
	MacDonald	(*38*)
	Mascotti and Waner	(*39*)
	Wilczek-Vera and Dunbar Salin	(*40*)
*PLTL	Lewis	(*41*)
	Gosser	(*42*)

* The PLTL references are for general chemistry courses.

Mabrouk (*32*) used a fully integrated classroom-laboratory PBL approach to Bioanalytical Chemistry course at Northeastern University. Students worked in self-directing teams of three to five members on a genuine research problem that they identified, and used research-grade analytical instrumentation. They were provided a list of past research projects and available analytical instrumentation. Learning techniques that were used included weekly group meetings, job safety analyses (JSA), a standard operating protocol (SOP), reflective journaling, and a

final technical paper. The principal role of the instructor was that of a facilitator and coach. The laboratory met 3-hours per week. One of the 65-minute class meeting times served as a weekly "group meeting" for the students in which they presented a brief progress report on their accomplishments in the laboratory and outlined their plans for the next week. The students came up with their own research projects; this promoted ownership of the whole learning experience. One disadvantage of this method was the limited course size due to available instrumentation.

Jones (*33*) used a PBL experiment to introduce students to *in vitro* protein folding of myoglobin. The extent of denaturation of horse heart myoglobin was monitored using fluorescence spectroscopy. Students prepared protein solutions with a concentration of 5 μM with final concentrations of ultrahigh-purity guanidine hydrochloride (GuHCl) between 0 and 6 M. Approximately 15 solutions were needed to adequately define the denaturation curve, with most taken between 1 and 2 M GuHCl. The solutions were allowed to equilibrate before the fluorescence measurements were obtained to ensure complete unfolding of the protein. A two-state system representing the native and unfolded state was assumed. An excitation and emission wavelengths of 285 nm and 340 nm were employed, respectively. Background fluorescence and the Raman line of water were eliminated using the fluorescence of a 0.1 M phosphate buffer solution.

Wilson (*34*) used a PBL approach with role-playing in the analytical course at the University of Kansas. Students, working in three to five member teams, were presented with a "real" problem. They were allowed to consult with their teaching assistants, faculty members, and external consultants. The first month the students were required to choose appropriate analytical methods, including sampling, sample preparation, assay validation, regulatory issues, and project cost. The students prepared a proposal which had to be approved before work in the laboratory occurred. About eight weeks were devoted to obtaining results. Students had to prepare a progress report and a final report. The latter consisted of a written report, a poster, and an oral presentation. Students complained about the heavy course work involved; however, they were intrigued by the study of "real" problems.

Bullen (*35*) used a PBL approach to integrate student-driven independent research projects in an instrumental analysis course at Northern Kentucky University. The instructor used active learning modules in the lecture portion of the course to teach different instrumental methods and concepts. Topics covered included infrared, fluorescence, and Raman spectroscopy. During the first eleven weeks of the lab, groups of four to five students rotated through instruments, which included FTIR, UV-Vis, and Flourescence instruments. They created operating manuals, produced training videos, and gave oral presentations at the end of the introductory experiments. They also had one-on-one quizzes with the instructor. The lab experiments became more and more open-ended as the semester proceeded. During the last five weeks, students worked on research projects that they designed. At the end of the semester, the students presented their research projects to the entire department. The author noted that it would have been beneficial to have a student who had taken the course before as a teaching assistant as there were multiple instruments being used simultaneously.

The biggest challenges with this approach was that not everyone could agree on the next steps and the frustration that students felt when their research project did not work. Despite those challenges, students liked the open-ended experiments and enjoyed learning.

Kalivas (*36*) incorporated PBL with cooperative learning (CL) in analytical lecture and laboratory courses at Idaho State University. The author reported that various instruments were used, although the actual instruments were not disclosed. An open-ended laboratory format was used. The instrumental analysis lab lasted sixteen weeks, with nine weeks devoted to development and evaluation of instrument standard operating procedures (SOP), followed by real-world research project which lasted seven weeks. Each group of two or three students developed a standard protocol and determined specifications for an instrument. Two other teams evaluated and edited the SOPs produced during the SOP phase. Each group wrote and presented a research proposal.

Briese and Jakubowski (*37*) introduced a project-based biochemistry laboratory to study biomolecular structure and interactions using fluorescence. Students studied phase equilibria, critical micelle concentration of single chain amphiphiles, the melting point of multilamellar vesicles, the melting points and thermodynamic constants for denatured ds-DNA and proteins, and the binding properties of proteins. They were then assigned a specific question addressing the stability/function of lipids, proteins, or nucleic acids and asked to design an experiment to answer a question using fluorescence methodologies.

MacDonald (*38*) used a student-initiated, project-oriented biochemistry laboratory course to teach protein purification and characterization techniques. Each group of students chose their own protein to study after an appropriate library search. The students then prepared a list of supplies and purification procedure. They performed relevant calculations and prepared buffers. The students all learned how to determine total protein assay using a UV-vis spectrophotometer. They were responsible for determining the protein concentration and activity of each of their purification steps in order to generate a complete protein purification table. The students then characterized the final protein fractions as a function of salt concentration or using IR or CD to determine solution dependent structures or protein folding/unfolding. The students prepared a final report written in the form of a research paper that was peer-reviewed by their classmate.

Mascotti and Waner (*39*) used a guided-inquiry laboratory project to investigate the binding of streptavidin (SA) and biotin-4-fluorescein (B4F) over two, 4-hour laboratory periods. A 1-hour discussion period followed where students critiqued the assay results from UV-vis and fluorescence techniques. Students started out with a “crude” titration of 1 mL SA into 0.6-0.8 microMolar B-4F in order to determine the breakpoint in the absorbance signal at 493nm. They were asked to estimate the SA concentration and aliquots necessary with a goal of 6-8 aliquots. Students worked in pairs during the second week. Their goal was to refine the experiment so that 10-12 points with multiple points before and after the breakpoint were achieved. Each group obtained flouorescence titration curves or UV-vis spectra. One group also investigated the titration of biotin into SA utilizing intrinsic SA tryptophan fluorescence.

Wilczek-Vera and Salin (*40*) used a method they called "spiral" to introduce fluorescence spectroscopy to students in advanced analytical laboratory at McGill University in Montreal, Quebec. The basic principles of fluorescence and the basics of instrumentation were covered in lecture. Students were expected to have read assigned material before coming to the laboratory. The experiment was designed to be completed during one 4-hour laboratory session by a group of 2-4 students. A "hands-on" approach was employed which allowed students to discover how the instrument operates. Afterwards, students studied the fluorescence of aqueous solutions of fluorescein with concentrations ranging from 0.2 ppm to 2000 ppm. Finally, they performed an analysis of an unknown sample.

Lewis (*41*) used a combination of PLTL and guided-inquiry, which they called peer-led guided inquiry (PLGI) in the first semester General Chemistry courses at South Florida. The PLGI session replaced one of three fifty-minute lectures per week. A peer-leader who had successfully completed the course was assigned to work with a group of ten students. During the sessions, students worked through activities from published texts that preceded lectures on those topics. Students who attended the sessions achieved higher exam scores than students who were not enrolled in the PLGI sessions. Eighty-five percent of the students indicated that they would take the PLGI sessions if they were offered the second semester, while fifty-eight percent would attend even if the PLGI sessions were offered on a voluntary basis.

Gosser (*42*) began using peer-led, collaborative learning at City College of New York (CCNY) of the City University of New York (CUNY) in 1991. Peer-leaders were selected from among students who were successful in a previous course. They served as facilitators for Workshop problems created by the faculty. The lecture time was reduced by one hour in order to make time for the Workshops. The peer-led Workshops resulted in an improved learning environment and an increase in student success. Students were more engaged and were not afraid to make mistakes.

Summary and Future Directions

Nontraditional active learning methods such as POGIL, PBL, and PLTL have been shown to provide students with a deeper understanding and appreciation of their subject matter and create students who are better prepared to solve problems in various disciplines. However, other fields of study, including the scientific fields of biology and physics, have progressed ahead of the area of chemistry in the number of nontraditional active learning methods that have been published. Fortunately, chemists are beginning to see the value in these techniques. Consequently, there has been a surge in the number of chemistry-related publications involving active learning methods; many of these publications are in the area of bioanalytical chemistry. Although nontraditional instructional approaches have been slow to be adopted widely in chemistry compared to other fields, the number and variety of available resources have grown and instructors are being trained for successful implementation of these techniques in their courses (*41*); it is inevitable that nontraditional instructional

approaches will begin to become more of a norm in undergraduate chemistry courses. An area of future development should include PLTL for bioanalysis.

Disclosure Statement

The authors are not aware of any biases that might be perceived as affecting the objectivity of this publication.

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Chapter 3

Teaching Bioanalytical Chemistry in an Undergraduate Curriculum: The Butler University Analytical Chemistry Curriculum as an Example Model

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Although Bioanalytical chemistry is not a common distinct undergraduate major at most institutions in the USA, the component courses, skill set, and knowledge base that are required to successfully practice the discipline are commonly taught in analytical chemistry, and biology related courses. Additionally, pedagogical approaches used in the sciences are applicable across the board and can be customized for any science course or curriculum. This chapter will therefore focus on the pedagogy, content and resources used for teaching in the analytical program at Butler University as a model that can be adapted to teach bioanalytical chemistry.

Introduction

Bioanalytical chemistry as the name implies is an interdisciplinary field of study that is right at the interface of biology (especially molecular biology) and chemistry (especially analytical chemistry and biochemistry). It is relatively new as a distinctive discipline. According to Hill (*1*), the term *Bioanalysis* was coined in the 1970s to describe the activities relating to the determination of drug molecules in biological fluids for pharmacokinetic purposes. Bioanalytical chemistry is expanding rapidly. According to Booth (*2*), some factors that will further drive the development of Bioanalytical chemistry and techniques into the future include:

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- Biomedical drug development pursuits: This requires technology that can be used for accurate quantitation of drugs and endogenous substances to provide information for pharmacokinetics, toxico-kinetics, bioequivalence and exposure-response (pharmacokinetics/ pharmacodynamics) studies.
- Development of individualized therapies: This will lead to the explorations of pharmacogenetics/pharmacogenomics, and consequently a rapid development in the use of biomarkers to identify people who are at-risk for a particular disease much earlier.
- Desire for early decision in drug development to reduce cost leads to the need for faster analysis (qualitative and quantitative), higher accuracy, sensitivity, precision, and miniaturization of technologies. For example RNA is sometimes used as biomarker. This requires sensitivities that are 2 to 3 orders of magnitude higher than a typical LCMS can handle. Also, increasing drug potency and the need for quantitation and identification of drug metabolites in complex matrices of biological fluids and tissues presents a significant analytical challenge.
- Advent of biotechnology-based drugs and the attendant challenges of quantitating large biomolecules
- The wide scope of bioanalysis including: early disease detection, disease investigations/explorations/elucidation, medicinal drug analysis, illicit drug analysis, forensic drug analysis, and environmental analysis (drugs in the environment, emerging pollutants). Analysis of drugs developed for medicinal purposes, illicit drugs, assessment of safety and efficacy by regulatory bodies (e.g. USFDA)
- Development of newer technologies will subsequently lead to other discoveries and research ideas that are not currently contemplated.

In a very recent article (2012) Horvai (*3*) recognized the rapid development in the fields of chemistry and biology and how these developments are becoming more intertwined. To this extent the author recommends that future generation of researchers should acquire sufficient background in biology and chemistry. However, the author recognized the potential challenge that this recommendation presents. Both fields are intensive due to their depth and breadth. The burgeoning prominence of bioanalytical chemistry, the recommendation and challenge noted by Horvai raises the question of how to teach the discipline in light of the expanse of information that is being generated.

Bioanalytical Chemistry: A Definition/Description

Perhaps the place to start a discussion on the teaching of Bioanalytical chemistry is to define/ describe what it is and also do a quick survey of the typical concepts that are taught in the course or curriculum. This will help to lay the appropriate foundation for the rest of the content of this chapter. Bioanalytical chemistry has been defined in a few ways. Some examples are presented below:

> [Bioanalytical chemistry is] the development and application of chemical measurement and instrumentation to problems in biology, biochemistry and medical science courses (*4*).
>
> The field of bioanalytical chemistry centers in the development of novel chemical measurements for the identification, quantification and characterization of selected molecules within biological systems (*5*).
>
> Bioanalytical Chemistry - A branch of analytical chemistry in which compounds of biological significance, such as peptides, amino acids, and carbohydrates are studied (*6*).

According to Horvai and co-workers, (*7*), the uncertainty in the definition has impacted the social aspects of its practice. It is uneven in the geographical distribution of its practice, and it is not well organized socially. There are societies and meetings devoted to subfields of bioanalysis (e.g. proteomics analysis, mass spectrometry in proteomics. Based on preponderance of publications in the area, it appears that the field is more prevalent in the US than Europe and practitioners are mostly from institutions devoted to biology and chemistry. Pharmaceutical chemists are also playing important roles. Based on their observation these authors recommend that analytical and bioanalytical chemists can benefit from each other mutually. For this to occur, analytical chemists need to be better trained in biology and biochemistry while those already in the profession should pay more attention to the rapid progress of biological sciences. Also, the education of biochemists would certainly benefit from courses given by traditional analytical chemists. Based on these definitions and viewpoint, one can potentially describe bioanalytical chemistry as an area of study in which the skills and tools of chemical analysis are applied specifically for the identification, quantitation and elucidation of the structure, behavior and properties of biomolecules (such as proteins, DNA, carbohydrates, and lipids) and/or small molecules in biological matrices (such as plants and animal tissues, blood, urine and sometimes cells). The tools that are used to accomplish this purpose include separation techniques (chromatography, electrophoresis), optical spectroscopic techniques (absorption, and luminescence mostly) and mass spectrometric techniques. Also chemistry-based (wet) techniques are used both for analysis and sample preparation (to amplify, isolate, or modify the molecule of interest to enable or enhance its detection by the instrument) as well. These techniques and the corresponding method constitute what is collectively called the bioanalytical techniques and methods.

Historical Perspective

Although Bioanalytical chemistry is relatively new as a distinctive field of study, the application of analytical chemistry in characterizing biological systems for macromolecules or small molecules is not new. For example, aspirin (introduced around 1899 (*8*) and sulfonamides (developed in the 1930s) were quantified by the use of colorimetric assays. Furthermore, the 1930s also saw the rise of pharmacokinetics, and as such the desire for more specific assays.

Toxicology, the study of adverse effects of xenobiotics on living systems (i.e. toxicology) utilizes bioanalytical techniques and methods. This field is also dated. It is perhaps as old as the history of human race (*9*). Wennig puts the origin of forensic toxicology at about 50,000 BCE (*10*). However, it should be mentioned that toxicology was recognized as a scientific discipline only in the mid-1900s. (*11*) .

Modern day toxicology has however expanded into pharmacodynamics or toxicodynamics (what the toxicant does to the body), disposition or pharmaco -/toxico-kinetics (what the body does to the toxicant) risk assessment, and safety evaluation. According to Bachmann and Bickel drug metabolism (a subspecialty of biochemistry that also utilizes bioanalytical methods and techniques) has its origins in the first half of the 20th century (*12*). It has developed rapidly since about 1950. It has also become a world-wide regulatory requirement. The reader should be reminded that chromatography (one of the commonly employed techniques in bioanalytical chemistry) was first utilized to separate plant pigments (particularly chlorophyll) by Russian botanical scientist, Mikhail Semenovich Tswett around 1900 (*13*, *14*). The field of bioanalysis has matured significantly from the early studies in drug metabolism which utilized simple colorimetry. With the proliferation of sophisticated hyphenated techniques which link advanced separation techniques (e.g. gas and liquid chromatography, electrophoresis) with mass spectrometry and other optical spectroscopic techniques (UV-Visible absorption and Luminescence instruments, FTIR and NMR spectrophotometers) as detection systems, today's bioanalyst is well equipped to deal with the modern challenges of analyzing xenobiotics in biological matrices with increased sensitivity and confidence. In addition, the incorporation of automation, robotics and computer-based instrumental control, optimization, and data processing present the advantage of higher sample throughput than previously experienced. Furthermore, bioanalysis is now utilized in the discovery, measurement and qualification of pharmacogenomic profiles, and biomarkers which is aimed at the development of diagnostic kits to individualize patient characterization and treatment.

In this chapter, we present an overview of how analytical chemistry curriculum is implemented at Butler not as an absolute readymade magical approach. Instead we present another perspective that contributes to the never ending, on-going discussion of how to teach the discipline. We offer some answer to the recurrent question of what to teach, how to teach, and what to use to teach it. In this chapter you will find a description of the rationale for our approach, a description of the approach, and the resources that we use. It is the author's hope that the reader will find sufficient information in the chapter to contribute to the discussion by adopting, adapting, or developing another approach that will address the true challenge that the analytical chemistry discipline is facing – preparing graduates that are equipped with sufficient knowledge and skill to address the scientific problems of their generation.

Bioanalytical Chemistry: A Scope of the Field

Bioanalysis was traditionally thought of in terms of measuring small drug molecules in biological fluids. However, the past twenty years has seen an increase in application of bioanalytical techniques (*15*). A few areas of these applications are shown in Figure 1.

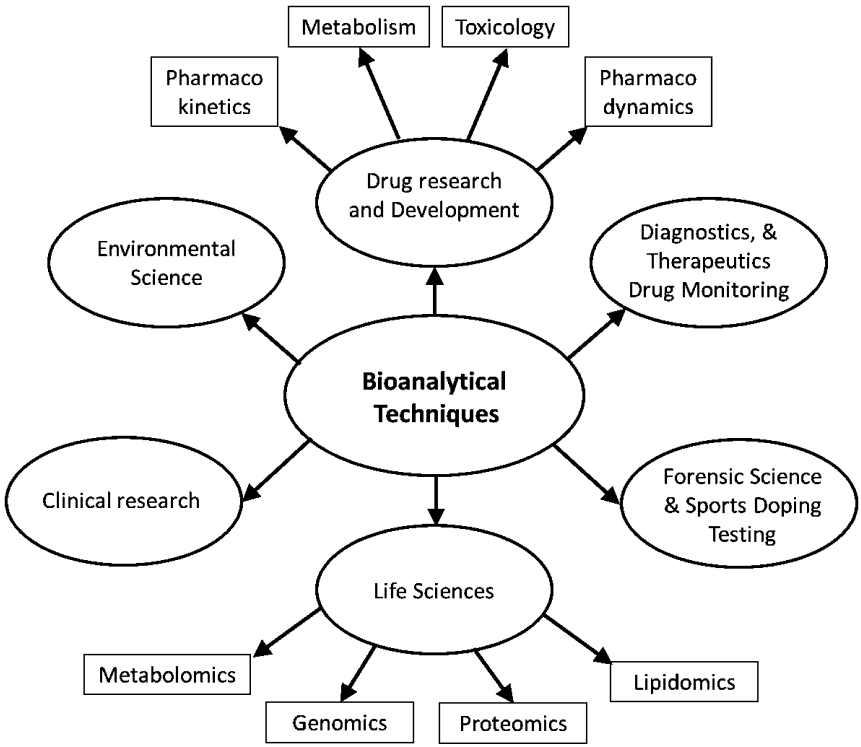

Figure 1. The scope of application of bioanalytical techniques.

Common techniques used include separation techniques (chromatography, centrifugation, and electrophoresis), mass spectrometry, and optical spectroscopic techniques (UV-Vis, Infrared, Fluorescence, & NMR). Other instrumental techniques include: radioactivity-based detection techniques, biosensors (electrochemical techniques), DNA micro arrays, surface Plasmon resonance and microscopy. Besides instrumental techniques, biochemical methods are also employed. A few examples of these include: Binding Assays, Polymerase Chain Reaction methods, Protein and Nucleic Acid Sequencing. This chapter deals with only the instrumental aspect and how to teach it. The justification for this focus resides in the fact that selecting the right analytical technique for purpose requires the analyst to have knowledge, experience and background of the techniques.

Bioanalytical Chemistry in the Undergraduate Chemistry Program: Typical Content and Resources

Bioanalytical chemistry is not a commonly taught course or area of specialty in most undergraduate chemistry programs (*3*). It is however part of the recommendation for the new European curriculum (*16*, *17*). Based on what this author gleaned from articles by Larive, and Horvai and syllabi and chemistry programs through the internet (*3*, *4*), five approaches have been used to introduce bioanalytical chemistry into the undergraduate chemistry program. In the first approach, biomedical and biochemically related topics can be used to teach some techniques and/or methods in other courses such as analytical chemistry. One such example is the utilization of enzyme-based methods to introduce kinetic methods of analysis and underscore the application of molecular UV spectrophotometry in analytical chemistry courses (*3*). A Second approach involves the introduction of experiments (or projects) of biochemical/biomedical relevance in the laboratory such as that taught by Stefan Lutz at Emory University in the USA (*18*). Others have published such experiments in literature (*19*, *20*). The third option is to teach it as a separate topic in a course such as the analytical chemistry courses. For this purpose one can use the analytical chemistry textbook by Gary D. Christian (*21*). This book has a chapter on Genomics and proteomics in which polymerase chain reaction, PCR and DNA sequencing are presented. It also has a chapter on, Clinical Chemistry in which common clinical analysis are discussed. This book also used enzymatic catalysis to present kinetic methods of analysis. Fourth option is to offer an entire course on bioanalytical chemistry such as that at St. Olaf College in the USA (*4*). In terms of resources to deliver such course, Larive highlighted three recently published textbooks: (i) Bioanalytical chemistry by Andreas et. al. (*14*). This book focuses mainly on analysis of DNA and proteins. It covers biomolecules, analytical techniques and methods such as electrophoresis, mass spectrometry, and molecular recognition techniques (including biosensors, bioasays DNA-Arrays and pyrosequencing), amplification and sequencing of nucleic acids and protein sequencing methods. (ii) Bioanalytical chemistry by Susan R. Mikkelsen and Eduardo Cortón (*22*). This textbook provides a good introduction to the subject matter and touched upon techniques that are commonly used by biochemists and molecular biologists. Some of the topics addressed in this textbook include: Spectroscopic methods for matrix characterization, Structural and functional properties of antibodies, Principles of electrophoresis, Centrifugation methods, Mass spectrometry of biomolecules, Quantitation of enzymes and their substrates, Design and implementation of enzyme assays, Isoelectric focusing, Chromatography of biomolecules. In addition to the topics listed above this textbook provides a chapter on validation of new bioanalytical methods as well. Another recent textbook is that by Gault and Neville (*15*). This book provided an introduction to biomolecules and their quantification. The book also has a chapter on the contribution by transition metals in health and diseases before diving into instrumental techniques. The chapters on instrumental techniques focused mostly on application but also gave some background information on principles. Some of the techniques discussed are biosensors (electroanalytical) mass spectrometry, separation techniques

including centrifugation, chromatography and electrophoresis. Spectroscopic techniques include the NMR, MRI, UV-Vis, IR and fluorescence. This book also addressed non-instrumental bioanalytical techniques such as Radioimmunoassay and ELISA. A distinguishing component of this textbook is its chapter on clinical genomics, proteomics, metabolomics, and applications of bioanalysis for clinical diagnostic and screening. A Fifth option is to have a whole curriculum for a major in bioanalytical chemistry. Stevens institute of Technology, New Jersey, USA has such a program (*23*). Based on the information provided above, it is clear that analytical chemistry is a major component of bioanalytical chemistry.

Bioanalytical Chemistry: How Should We Teach It?

Judging from the history and current events in most other disciplines, it is difficult to be absolute in recommending how to teach a course or develop a curriculum. But at least we can provide guidance to help beginners make choices that are appropriate for their context. For example, in the case of analytical chemistry, (one of the parent disciplines of Bioanalytical chemistry), the debate on how to teach it, the course content and resources has been on for decades. Three of the factors that led to this situation are the rapid developments in various science disciplines (leading to a rapid development in modern technologies), the continuing developments in our understanding of how people learn and increasing demands on modern technology to solve societal problems. Strobel in 1954 (*24*) and Laitinen in 1956 (*25*) reported that increased growth in analytical chemistry courses is caused by increased use of instrumental methods in industry and research environments. Torrey (*26*) in 1976 commented that the rapid developments in modern technology impacted analytical chemistry perhaps more than any other discipline. The impact resulted in an avalanche of instrumental techniques that teaching all of them presents a challenge. Kolthoff (*27*) echoed the same sentiments in 1977 and added to it that the there is an increasing demand for analytical science skills by medical, environmental and conservation related disciplines and more importantly food, forensic, bioanalytical and pharmaceutical (particularly drug development and regulation) disciplines. The need for analytical science across other scientific disciplines is not new. Consider the following statement by Lykken in 1951:

> It is generally agreed that analytical chemistry courses are an important feature of college curricula for the training of chemists and chemical engineers. However, there is not complete agreement in the role and scope of such courses because of the unpredictable placement of chemically trained college graduates. This dilemma poses a problem but it appears resolvable by compromising the needs of graduate work, industry, and teaching, the three important fields which absorb the chemically trained graduate. Actually, these three fields need about the same emphasis on basic analytical training and there is little conflict in the desired analytical coursework except in the special case where the student is being prepared for industrial analysis (*28*).

These developments have resulted in a vast content to choose from. Meanwhile, the debate has driven the development of resources for teaching the discipline including textbooks, online libraries, and electronic animations to mention a few. The desire to sustain this momentum into the future while solving the problems of our own era has forced us to keep finding ways to train scientists for the future. However, amidst this plethora of opportunities, the question remains: how do we train the new scientists?

Planning a Course: The Front-End

Planning a course starts with setting the goals and objectives of the course. In other words, questions such as: "what would the student gain from the course?", and "how will it impact their overall education, knowledge and skill set?", should precede other considerations. To further expand on this thought, one should ask the questions, "what should a graduate of the curriculum look like or be able to do at the end of the course?", or better yet "what knowledge should they acquire to be able to identify and understand current societal problems?" and "what skills should they acquire to be able to proffer solutions to these problems?" Other questions to consider are, "what preparations should they have to successfully transfer into the arena where they acquire refined capabilities (graduate programs) or the work force where they start contributing their part in the efforts to address societal problems?" Previously, the main goal of education was to get people to acquire the skills of reading and writing. However, in this current dispensation, emphasis is being placed on acquisition of technical, and social skills since the problems we face require multidisciplinary efforts to solve. As such, graduates of our programs should have the knowledge to do what they profess and the skill to solve newer problems alongside scientists from other disciplines different than theirs. Students should recognize that some problems are bigger than their individual disciplines.

Beyond these, three other questions that should follow pertains to the course content (what to teach?), the pedagogy (what instructional approach should be employed to teach the course in such a way that would engage the students and stimulate their interest in the subject matter?) and then the resources (what resource(s) should be used to facilitate students understanding?). These questions have driven many debates/discussion in course, curriculum and discipline developments over the decades. As indicated in Figure 2, these questions also drive the development of one another. In this section, we will address issues relating to instructional approaches (or pedagogies) and choice of an appropriate approach for any course.

The next section will focus on the instructional approach while issues of content and resources will be addressed in other sections appropriately.

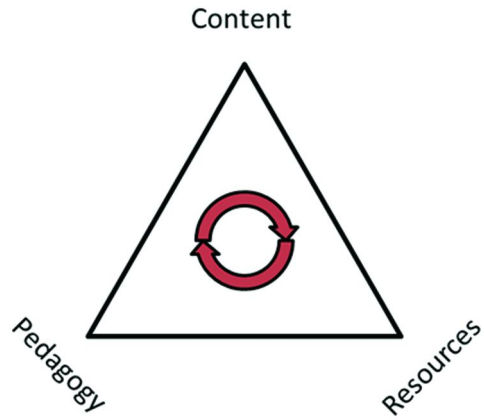

Figure 2. The three factors that drive curriculum and discipline development: What to teach (content), what to use to teach it (resources) and how to teach it (pedagogy).

Selecting an Instructional Approach

According to a document found on the Karen L. Smith Faculty Center for Teaching and Learning website at University of Central Florida (*29*) there are as many teaching models (or instructional approaches) as there are ways of learning. These models falls into a spectrum that spans the range from teacher directed (maximally guided) to the student directed (minimally guided). One broad classification bunches the instructional models into five categories. The first category is called the information processing group (of instructional models). Examples in this category are: the Inquiry-based learning, Scientific method/model, and the Creativity-based learning. The second group is referred to as the Social Learning group. This group includes: Collaborative learning models, Role playing models and the Jurisprudential style models. The third group is called the Personal Family Group. Examples in this group are the developmental models, Learner centered models (learning styles) and the Adult Learning Models. The fourth group is the Behavioral systems group. Members of this group include the mastery learning, directed learning (by expert or teacher), simulations-based learning and the feedback centered models. The fifth group is the Constructivist Models group. This group comprises of the problem-based learning, project-based learning, cooperative learning, experiential/authentic learning, situated learning, case-based learning and discovery learning.

Most of the instructional approaches that have been reported in literature for teaching analytical sciences fall into the information processing and the constructivist group. A few resources that the reader can use as starting material for planning an analytical science course that is based on active learning instructional approach are presented in Table 1.

Table 1. Resources for Active Learning Instructional Approaches

Instructional approach/method	*Resource*
Inquiry-based learning	Teaching Bioanalytical Chemistry and Forensic Chemistry Using Inquiry-based strategy by Harvey Hou. Through Online Program Guide For the 2012 Biennial Conferenceon Chemiscal Education. Through: http:// www.bcceprogram.haydenmcneil.com/conference-info/p636-teaching-bioanalytical-chemistry-forensic-chemistry-inquiry-based-strategy
Problem-based learning	Peer Reviewed: Instrumental Analysis at the University of Kansas: An Experiment in Problem-Based Learning. George S. Wilson , Marc R. Anderson , and Craig E. Lunte *Anal. Chemi.*, **1999**, *71* (19), pp 677A–681A.
Cooperative-based learning	"Cooperative Group Learning in Undergraduate Analytical Chemistry," Wenzel, T.J., Analytical Chemistry, 1998, 70, 790A-795A.
Role Playing learning	St. Olaf. Walters, J.P. "Role-Playing in Analytical Chemistry" Anal. Chem. 1991, 63, 977A, 1077A, 1179A.
Project-based learning	Developing practical chemistry skills by means of student-driven problem-based learning mini-projects. Claire McDonnell , Christine O'Connor and Michael K. Seery. Chem. Educ. Res. Pract., 2007,8, 130-139.
A collection of models and resources	Active Learning Models from the Analytical Sciences, Patricia Mabrouk (editor) American Chemical Society/Oxford University Press: Washington, D.C., ACS Symposium Series Vol. 970 (2007)

In a report on workshops organized by Ted Kuwana, which were focused on curricular developments in the Analytical Sciences (*30*, *31*), six major issues were considered as priorities: (1) course content and learning modes; (2) core technologies for undergraduate labs; (3) faculty development; (4) learning partnerships with industry; (5) the impact of technology; and (6) follow-up and dissemination. The workshop culminated in a set of recommendations. All of the recommendations are vital to the overall objective of this chapter. However, the most pertinent are that instructors should (1) develop context-based curricula that incorporate problem-based learning and (2) adapt and adopt teaching styles that accommodate students' varied learning needs. These thoughts/recommendations should guide the choice of instructional approach that is utilized in any course.

Analytical Chemistry Curriculum at Butler: Courses, Content, Instructional Approaches, and Rationale

Prior to 2008, analytical chemistry curriculum at Butler University was delivered through two courses: The Analytical Chemistry I (AC1) or quantitative analytical chemistry, and Analytical Chemistry II (or instrumental analytical chemistry). Currently, Analytical Chemistry I is still taught as a single course that comprise of lecture and lab components (see Figure 3). Analytical Chemistry II (AC2) has been split into separate courses. The lecture course is independent from the laboratory courses as outlined in Figure 4. The laboratory experience is now implemented through a series of project-based, and dynamic, theme-focused courses. More details on these courses are presented next.

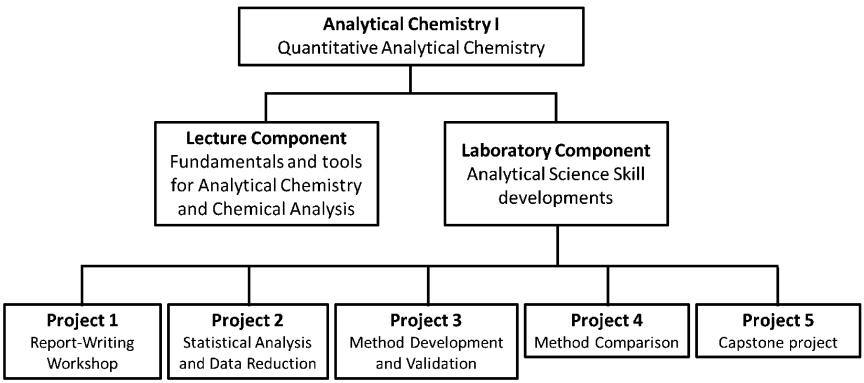

Figure 3. An overview of the Analytical Chemistry I (quantitative analytical chemistry) implementation at Butler University.

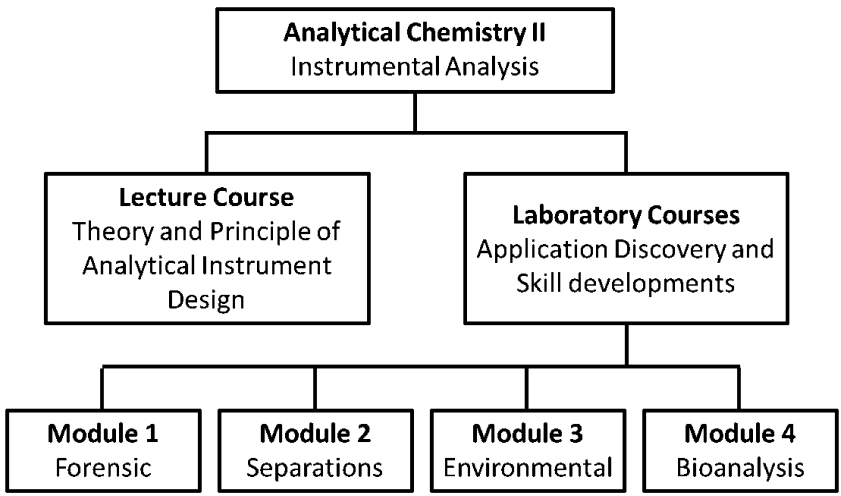

Figure 4. An overview of the structure of instrumental analysis curriculum at Butler University. Repropduced from Anal. Bioanal. Chem., 2008, 392, 1-8 with kind permission from Springer Science and Business Media. Copyright (2008).

Analytical Chemistry I: The Content

The lecture component of Analytical Chemistry I aims at presenting the principles of analytical chemistry and chemical analysis. The course is divided into two phases. The first phase focuses on the fundamentals while second focuses on the tools used in chemical analysis.

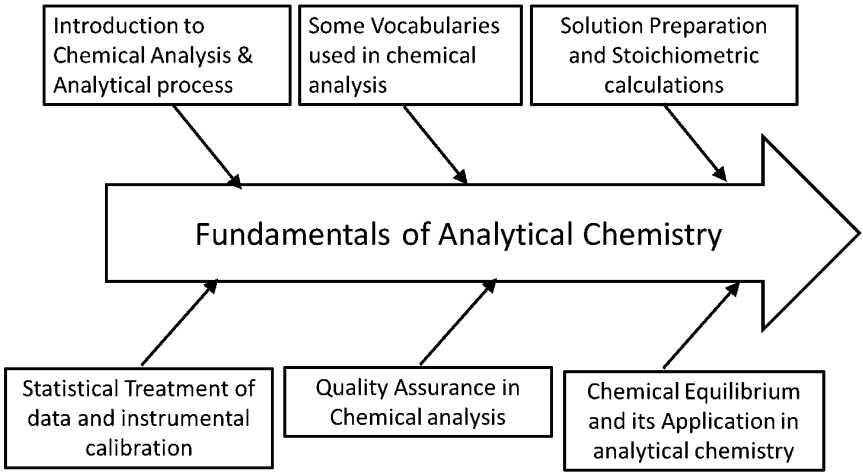

Figure 5. An overview of the fundamentals unit of Analytical Chemistry I (quantitative analytical chemistry) lecture component.

The *fundamentals unit* (see Figure 5) comprises of the following subunits: (1) introduction to chemical analysis and analytical process, (2) some vocabulary used in chemical analysis, (3) solution preparation and stoichiometric calculations, (4) statistical treatment of data and instrumental calibration (propagation of error and hypothesis testing (F,t, Q and G tests)), (5) quality assurance in chemical analysis (method quality criteria or figures of merit, method development and validation)and (6) chemical equilibrium, its application and relevance in chemical analysis. In this subunit (chemical equilibrium) we address topics such as the relevance of activity (relative to concentration) in thermodynamic calculations, application of chemical equilibrium for understanding solubility, complex ion stability, strengths of acids and bases, distribution of chemical species in aqueous solution and between immiscible phases, and calculation of concentration of chemical species involved in multiple equilibria. In the second unit (see Figure 6) we cover the tools for chemical analysis. The first subunit here is the wet analytical tool where we cover only the principle of titrimetry. This includes: the underlying stoichiometric calculation that is the common thread for all chemical reactions used for titrimetric analysis, the unified principle of visual indicators behavior for end point determination, principles of instrumental detection of equivalence point and the types of chemical reactions used for titrimetry. Another topic that is covered pertains to the different strategies for quantifying analytes such as back titration. Some applications of titrimetry are also presented. Note that we do not cover Gravimetry. This is because of lack of time.

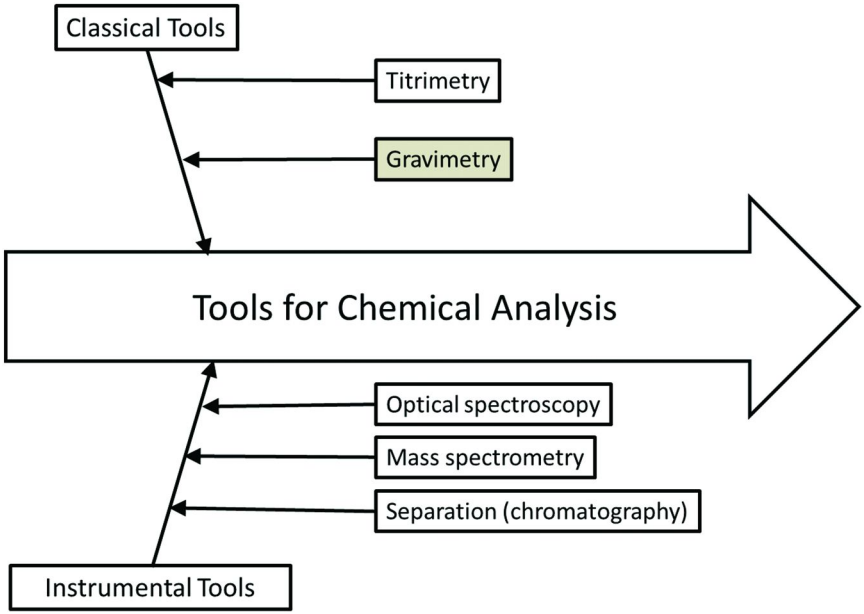

Figure 6. An overview of the chemical analysis tools unit of Analytical Chemistry I (quantitative analytical chemistry) lecture component.

In the second phase we present the *tools for chemical analysis* (see Figure 6). Topics that are covered in this unit include: Optical spectroscopy (UV-Vis molecular absorption, Atomic absorption and Atomic emission (ICP-OES)), and Mass spectrometry (basic architecture, Ion sources (EI, ESI and APCI), mass analyzers (Quadrupole, TOF and magnetic sector), and detectors). We typically cover the basic designs of Liquid and Gas chromatography. The theory (description and optimization of resolution), types of columns, pumps and detectors are also discussed.

Analytical Chemistry I: Instructional Approach

Students in the class come with varied backgrounds, learning abilities, learning styles and attitudes. Therefore a combination of expository lectures, inquiry and cooperative learning instructional approaches are used to deliver the course. The instructor presents the main principles of the topic in a short expository fashion. Student's participation and understanding is facilitated via collaborative problem solving in teams (in and out of class). Further topic exploration focuses on real world application through literature reading which in some cases students search for themselves. At other times they are guided to locate it, otherwise they are provided by the instructor. For example the article *The Role of and Place of Method Validation in the Quality Assurance and Quality Control (QA/QC) system by Konieczka* (*32*) is sometimes used as a resource to teach method validation during the quality assurance subunit coverage. Sometimes, responses have to be submitted as teams rather than individually.

Implementation of the instructional approaches is both flexible and dynamic. It varies from topic to topic and depends on the mood and attitude of the class towards the material. Sometimes the lecture may start with a question that is collectively brainstormed and worked on together. Such a question may be based on a previously presented topic and used as a Launchpad for a new topic that is to be introduced that day. Other times it may be straight expository and another time it may be a time –gated quick literature search and report by the students in teams. The main goal is to get them engaged in the learning and teaching process at each instance/meeting. Regardless of the instructional approach chosen for a particular meeting, the previous topics are always reviewed and place in context of the overall picture of the course. The topic's contribution to the goals and objective of the course is also highlighted. Fish diagrams are used to accomplish this task. An example is provided in Figure 7. The students are reminded that the entire course is one single story with many parts that link together.

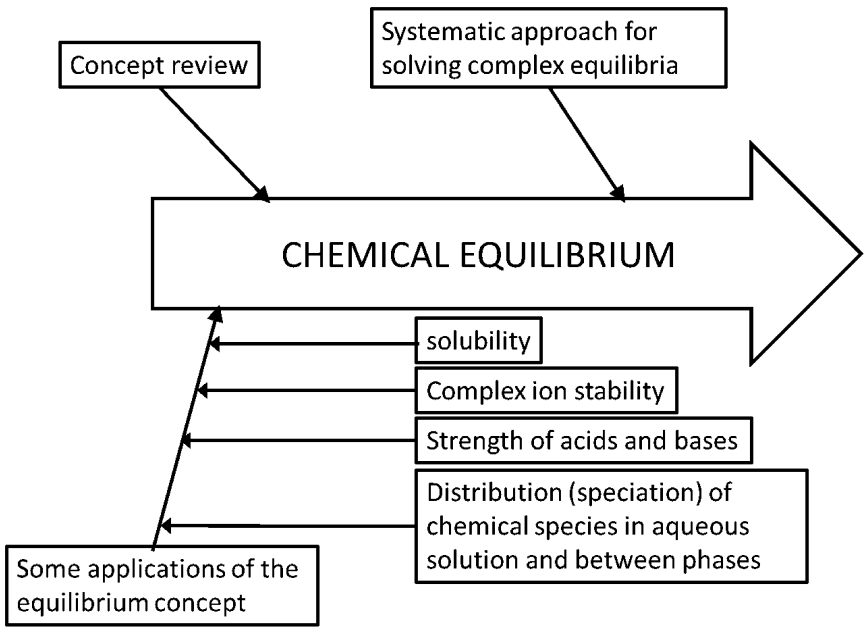

Figure 7. An overview of the topics covered in the equilibrium unit of Analytical Chemistry I.

Analytical Chemistry I: Resources

Several resources are used throughout the semester, including the course textbook and animations found on the internet particularly through such sites like Analytical Sciences Digital Library, ASDL (http://www.asdlib.org/).

Other relevant websites such as USEPA (http://www.epa.gov/QUALITY/qapps.html, http://www.epa.gov/epawaste/hazard/testmethods/index.htm), USFDA (http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/DrugChemicalResiduesMethodology/ucm113212.htm) and instrument manufacturers are also used.

Analytical Chemistry I: The Laboratory Component

The laboratory component is where the most changes have occurred in the teaching of Analytical Chemistry I at Butler University. Previously about 10 experiments were used per semester. However, since 2008, we have switched to only 5 (sometimes 4) multi-week projects. The projects are framed to address various objectives (see Figure 3a). Project 1 is the *writing workshop*. It is a one week project designed to accomplish the two goals: Firstly, it introduces students to tools and formats that are used for writing reports. Examples include word processor skills such as special characters, symbols and Greek letters, equation editors (for writing chemical and mathematical formulas), table formatting, super- and subscripts for writing simpler formulas and sometimes in-text reference numbers. Another critical example is the use of spreadsheets to carry out automated calculations on large data sets, plot and format variety of graph types, do regressions analysis and statistical test of hypothesis. Secondly it helps student to become familiar with the structure, format and language of technical articles.

Project 2 is focused on statistical analysis and reduction of data. This is a two-week project in which students generate a large set of data that they analyze by applying all the hypothesis-testing statistical tools that were covered in class. Students work in teams to implement this project. Recently we have used *Titrimetric determination of soda acidity* to generate such data. Some of the experiences that students encounter in this project include the calculation of percent purity of the sodium hydroxide used for acidity determination, statistical rejection of titration datum, application of t-test to compare results within the group, and determining similarities or differences of acidity between soda brands and types. The also, use test of variance (F-test) to compare precisions within the group. Another concept that is encountered in this experiment is stoichiometric calculations. For example, although student use approximately 0.10M NaOH solutions for titrating 25ml of each soda sample, they are asked to calculate and represent soda acidity as *the volume of a 1.0 M NaOH that is required to neutralize 50ml of the soda*. Subsequently, each group submits a report using a specified format (typically a journal format selected by the instructor).

Project three, is the method development and validation unit. The project spans three weeks. In this project, students work in groups with shared responsibilities. In the current implementations, the overarching goal is to optimize and apply a method for the determination of trace metals in spinach leaves. First step is to determine which acid cocktail (HNO_3, HCl, and HNO_3-H_2O_2) is best for digesting the spinach. The next is to determine which calibration approach (external calibration, standard addition, or internal standard) is more suited for the analysis. The optimized method is the combination of the best conditions. To accomplish this task, a standard reference material (NIST

SRM 1573a) is used. Also, the SRM is used to establish the figures of merit of the optimized method. Summaries of students result are provided in Tables 2 and 3 below.

Table 2. Summary of students method validation results. The goal was to select the best acid or acid cocktail for digesting NIST SRM 1573a (tomato leaves). Three metals (Al, Fe, and Fe) were studied. Good results were obtained for Al. External standard approach was used for instrument calibration. Students used 1:50 and 1:10 dilution of the SRM digestate.

	% Recovery for Aluminum in each acid used for wet digestion		
Student group	***HCl***	***HNO_3***	***H_2O_2- HNO_3***
1	70 ± 2	140 ± 4	85 ± 4
2	117 ± 2	111 ± 4	112 ± 6
3	113 ± 4	132 ± 4	88 ± 9
4	81 ± 2	81 ± 3	65 ± 3
5	83 ± 7	107 ± 1	114 ± 42

Table 3. Summary of students results. Three instrument calibration methods were evaluated for the determination of aluminum in tomato leaves (NIST SRM 1573a).

	% Recovery obtained for Aluminum from each Calibration Method		
Student group	***External Standard***	***Internal Standard***	***Standard Addition***
1	140 ± 4	118 ± 19	103 ± 8
2	111 ± 4	108 ± 8	128 ± 13
3	132 ± 4	83 ± 2.4	114 ± 8
4	81 ± 3	94 ± 2	92 ± 0.3
5	107 ± 1	87 ± 0.1	106 ± 9

There are many skills to learn from this project. Examples of such include: sample and solution preparation, setting quality objectives, application of performance characteristics to judge which method of sample preparation is better, statistical data analysis and quality control during chemical analysis.

Project four is geared toward method selection using self -generated empirical evidence. Criteria such as ease of implementation (e.g. ease of sample preparation, cost) and method performance characteristics (accuracy, precision, LOD etc) are used as basis for comparison. Some experiments that have been used for this project include:

- Comparison of acid-base and redox titrimetric method for the determination of ascorbic acid in *Nature Made*® vitamin C in tablets. See Tables 4 and 5 for a summary of student results.We have also analyzed fruit juices.
- Comparison of ion chromatography and ion selective electrode for determination of fluoride ion in tooth paste and mouthwash.
- Comparison of atomic absorption (AAS) spectrophotometry and complexometric titration for the determination of Ca and Mg in tap water.

Table 4. Results of method validation (accuracy and precision) for titrimetric determination of ascorbic acid in *Nature Made*® Vitamin C tablets. Accuracy was calculated as percent recovery while precision was calculated as standard deviation. Each method was validated using pure ascorbic acid. Each group consists of a pair of students.

Student Group	*Acid-Base titration method validation: accuracy and precision*	*Redox titration method validation: accuracy and precision*
1	92.2 ± 1.2	113.7 ± 4.0
2	122.3 ± 10.2	98.6 ± 4.3
3	193.4 ± 6.3	98.9 ± 1.3
4	95.5 ± 2.3	95.4 ± 0.41
5	97.0 ± 2.6	98.8 ± 0.8
6	105.3 ± 3.9	99.2 ± 1.3
7	90.7 ± 4.9	104.3 ± 1.6
8	95.8 ± 1.0	100.6 ± 2.9

Each of these experiments presented a number of practical experiences for the students. For example the need for significant dilution of tap water during application of AAS for determination of Ca and Mg, was 'unusual' to their experience. Why would you dilute water for analysis when water is the solvent that we typically use to dilute other samples? Another revealing experience is the impact of the chemical form of ascorbic acid on the method of analysis. Also the impact of the chemical form of fluoride (sodium fluoride, NaF versus sodium monofluorophosphate, Na_2PO_3F) on their detectability facilitated teachable moments in the lab.

Table 5. Results of titrimetric determination of ascorbic acid concentration in *Nature Made*® vitamin C tablets. The acid-base titrimetric method utilized 0.1M NaOH as titrant. The redox method utilized 0.03M KIO_3 for the titration. Accepted concentration (based on information on vitamin C bottle label) is 500mg ascorbic acid/vitamin C tablet. Each group consists of a pair of students.

Student Group	*Concentration of ascorbic acid (mg/g of Vitamin C tablet) found by using by Acid-Base titration method*	*Concentration of ascorbic acid (mg/g of Vitamin C tablet) found by using by Redox titration method*
1	402 ± 75	533 ± 15
2	703 ± 46	541 ± 2
3	968 ± 14	508 ± 8
4	487 ± 12	491 ± 19
5	481 ± 7	498 ± 11
6	516 ± 39	465 ± 62
7	500 ± 51	516 ± 19
8	509 ± 12	492 ± 4

Project five is a capstone experience which is focused on getting the students to utilize all the skills and knowledge that they have gained in the course. Once a question for the project focus has been identified, the class is divided into groups to address various parts of the question. At this point the students use literature to identify the methods needed to fulfill their obligation. The report for this project is in poster form (one poster per group). They present the poster at the end-of–semester departmental poster session which is a celebration of the project works that have been done in the department in every course. High ranking university administrators and local alums are invited. Posters ranked as the best are given awards. One such project has been based on safety issue. The group that chose this project researched the safety of urban soils for gardening. They collected and analyze sample from faculty and staff in the department who live in the rural area, in the city and in the sub-urban areas. They also collected samples from the university garden. Students used ICPMS and atomic absorption to profile about 20 trace elements (toxic and essential) the student. Students also used ion chromatography to determine common inorganic ions in the soil.

Although the individual project foci are well defined and fixed, the experiments used to implement each of them can vary from semester to semester. For example project four could be implemented by comparing gas chromatographic and liquid chromatographic methods, or ICP-OES versus atomic absorption, or ICP-OES versus ICPMS. It is important to note that the overarching goal of this course is to facilitate learning and acquiring of fundamental knowledge and skill typically used in chemical analysis. Also,

because each project is reported in a technical article format, the writing skill is refined. Alongside these, social skills are further developed through the group-work. We stimulate students' interest in the project by using real world samples and focusing on answering real questions.

Summary

As presented above the Analytical Chemistry I course is presented with multiple instructional approaches in a flexible manner that adapts to the mood and attitude of the students dynamically. It provides guidance and sets the students free to explore and discover for themselves. Rosenshine (*33*), in an article titled "Principles of instruction: Research Based Strategies That All Teachers Should Know", noted that (1) the most effective teachers ensured that the students efficiently acquired, rehearsed and connected knowledge. (2) "Many [teachers] went on to hands-on activities but always after, not before, the basic material was learned" and (3) "The most successful teachers spent more time in guided practice, more time in asking questions, more time checking for understanding and more time correcting errors". The author also provided a list of 17 principles of effective instruction. These are at the center of what we hope to accomplish with these re-tooling of the course.

Analytical Chemistry II: Instrumental Analysis at Butler University

The most significant change made to analytical chemistry curriculum at Butler University was in Analytical Chemistry II. As previously mentioned, the lecture and laboratory experiences are now separate courses (see Figure 4). The lecture course (CH422) is designed to highlight the recurrent and unifying principles of chemical instrumentation architecture across all techniques. Multiple laboratory courses are now used to help student gain hands-on experience on instruments, practice the full analytical process and develop critical thinking, technical and social skills. This section is dedicated to discussing the rationale, its origin, details of content, resources and instructional approaches that are used to accomplish these tasks.

Analytical Chemistry II:
Rationale for The Approach - An Echo from the Past

What should be the driving philosophy of the instrumental analytical chemistry course? What criteria should be used to set the goals and objectives for the course? To address these questions comments and opinions from the past and present were considered. In an introductory remark to the Division of Chemical Education at the 128th meeting of the American Chemical Society meeting in 1955, Laitinen (*25*) distinguished between instrumental analysis and instrumentation. Instrumental analysis is the application of an instrument to accomplish a task while instrumentation is focused on the design and function of the instrument. At the same meeting, Kolthoff (*34*) recommended that academia

should focus on the theoretical fundamentals, principles and limitations of the measurement systems and leave the training of technique specialists to industrial laboratories. This view was implicitly re-echoed in 1977 when Torrey (*26*) commented that analytical chemistry was then being taught in dispensation, as if it were an abbreviated orientation course for technicians. According to the author, students were instructed to flip switches and be blackbox operators instead of being students of chemistry. Strobel (*24*), suggested that the course should focus on the characteristics sought in the students completing the course and the relevance of the subject matter to their overall chemical training. Furthermore the instructor should ask the following pertinent questions: what particular instruments and experiments will best demonstrate general principles without losing students in in operational details? Will the students' imaginations be stimulated so that they will learn to ask relevant questions as well as perform the assigned project? To what extent should the emphasis in lectures and discussions be on the fundamentals of theory rather than application? Strobel's picture of an instrumental analytical course is better captured in the following statement:

> ...an instrumental course may be described as intending to produce chemists with insight into and some experience with instrumentation. Broadly it should turn out students who not only look beyond the particular to recognize general characteristics of the different methods with which they work, but also can draw on their theoretical background with reasonable success for intelligent operation, adaptation, and if necessary extension of present physical methods.

Clearly, the author envisages a student centered course that is founded upon the fundamental theories and principles of instrument, their limitations and their design. On the question of how we should teach the course, Torrey (*26*) reiterated the need to teach the fundamentals instead of instruments. The author wrote:

> One may ask: How is it possible to equip a student with all the many necessary procedures and techniques required to cope with the outside technical world by exposure to one or two courses in analytical chemistry in college? The answer, of course, is very clear−Teach the Basic Fundamentals.

Additionally, Smith (*35*) added that employers expect that students would be provided with a background in the analytical process alongside their chemistry training. In 2001, Enke (*36*) identified the "*the lack of a unifying theme with which to bind the various techniques*" as the major problem of the discipline. To summarize the views of these authors, instrumental analytical chemistry should be taught based on fundamental theories and principles of the techniques in the context of the analytical process. A graduate of the course should possess sufficient knowledge and skill to address current issues and solve newer problems. Also the instructor should highlight the unifying theme of the techniques. All of these are directly or indirectly embedded in the recommendations of the Ted Kuwana led workshops on curricular development in the analytical Sciences (*30*).

The next challenge is centered on course content. Given the vast array of possible topics and limited time, which topics should be included and which should be excluded from the lecture course? Ewing and van Swaay (*37*) addressed the issue of content. They acknowledged the vastness of the content and noted that a compromise between the breadth and depth is difficult. The authors deferred to the instructor to make the call as to where to draw the line in terms of content. Pemberton (*38*) noted the disagreement in the analytical community concerning what should be taught in a modern chemical analysis curriculum. The author disagreed with the chapter-by-chapter teaching of topics and recommended that analytical chemistry should be taught in the context of the analytical process. In the same year, Girard and Diamant (*39*) published the result of their survey that was conducted in 1998. The goal of their study was to find out what topics are being taught in instrumental lecture courses and which instrumental techniques were being used for experiments in the labs. They found that about 37 topics covering spectroscopy, separation, electroanalytical techniques were common. No school indicated that all 37 topics were taught in one term. Topics most often included were: UV–Vis spectrophotometry, infrared spectrophotometry, molecular fluorescence spectroscopy, atomic absorption spectroscopy, NMR, mass spectrometry, electrochemical cells, cyclic voltammetry, HPLC, and GC, GC–MS. Others include ICPAES, Capillary Electrophoresis and supercritical fluids. They compared their results with an earlier survey conducted in 1981 (*40*) and found a reduction in the frequency of teaching certain topics such as optical rotary dispersion, circular dichroism and polarimetry. The authors (Girard and Diamant) concluded by noting that the content of the analytical curriculum and the instrumental analysis course is constantly evolving and that the curriculum reflects the sophistication of the society. They also made the recommendation that the instructor of the instrumental analysis course will always have to make the call on what topic to include or exclude.

With regards to instructional approach, there are as many approaches as there are learners. So what approach should we use? Pemberton (*36*) commented that, not all approaches are equally effective at teaching the basic principles of modern chemical analysis. The author rejected what was described as the topic-by-topic (or chapter-by-chapter) approach and favors the case study and problem-based learning. Savery (*41*) defined PBL as "an instructional (and curricular) learner-centered approach that empowers learners to conduct research, integrate theory and practice, and apply knowledge and skills to develop a viable solution to a defined problem."

All of the above factored in to the rationale for the approach that we use to teach instrumental analytical chemistry. As previously noted in earlier section, the lecture course (CH422) is designed to highlight the recurrent and unifying principles of chemical instrumentation architecture across all techniques. The instructional approach used is a combination of expository, collaborative and inquiry-based approaches depending on the topic, the student attitude and many other factors. The overall goal is to get the students involved in the process of learning and to lay the foundation that wets their appetite to become lifelong learners of the discipline. Also, the same resources that were mentioned under the Analytical Chemistry I section are also utilized for the instrumental analytical

course. However, this author will like to draw attention to a new repository of resources on the ASDLIB website (http://www.asdlib.org/ActiveLearning.php). This is being developed by a consortium of educators and the effort is led by Thomas Wenzel and Cynthia Larive.

Analytical Chemistry II at Butler University: A Description of the Lecture Course

The focus of this course is to introduce the architecture, theory, principle of operation, analytical capabilities, and application of instruments used for chemical analysis. The goal is such that at the end of the course the student should be able to:

- Identify the major building blocks of an analytical instrument
- Explain/discuss the theory and operational principles of analytical instruments and the component modules
- Evaluate the performance of a chemical instrument
- Compare and systematically select methods for atomic and molecular analyses
- Optimize, calibrate, validate and apply chemical instruments for analytical problem solving
- Identify sources of error and interferences in instrumental analysis
- Make appropriate choices and efficient use of analytical instrumentation

In terms of logistics, the breakdown is presented in Table 6.

It should be noted that due to time limitation only the optical spectroscopy, mass spectrometry, and separation (electrophoresis, liquid chromatography and Gas chromatography) techniques are covered. The instructor introduces the major unifying principles of the techniques in an expository format. The students are charged with the discovery of different types of instruments under each technique with their unique features.

Table 6. Agenda of Analytical Chemistry II

Time frame	*Focus*
Week 1-2	The instructor uses expository approach to familiarize students with the unifying principles of instruments. This discussion focuses on • Function of analytical instrument • Fundamental principle of operation of analytical instruments • Basic building blocks of analytical instruments (the unifying theme) • Parameters to characterize the abilities of an instrument (figures of merit)
Week 3-14	Each technique is studied along the following pattern: • The instructor presents the unifying principles of the technique • Students are then guided through an inquiry based, collaborative

Continued on next page.

In Teaching Bioanalytical Chemistry; Hou, H.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Table 6. (Continued). Agenda of Analytical Chemistry II

Time frame	*Focus*
	approach to discover the instruments under each technique based on the following pattern − Instrumental design/Architecture − Highlight the component modules of each instrument along with • Their uniqueness • Alternatives (e.g. different sample introduction devices) • Principle and/or theory of operation − Figures of merit − Application

The Unifying Principles of Instrument Architecture

Nature is constantly sending signals/information that man cannot process naturally. The first unifying principle of an instrument resides in their function. They are designed to resolve the communication impasse between man and nature (see Figure 8).

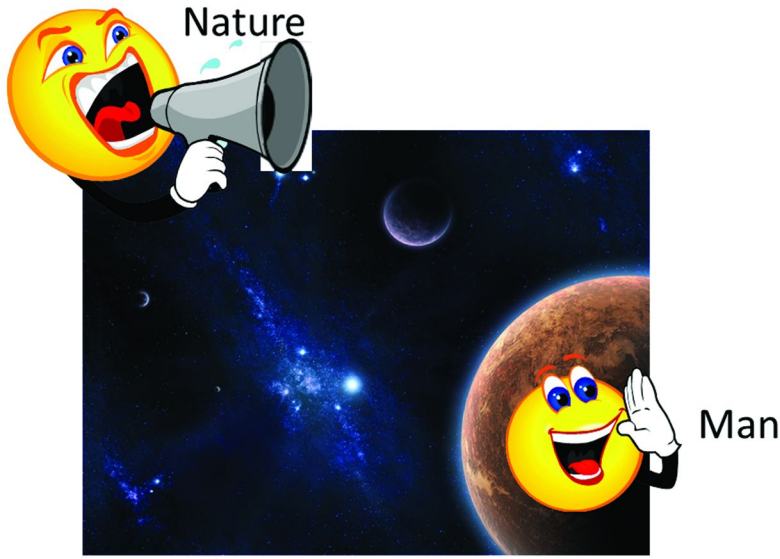

Figure 8. A depiction of the communication impasse between nature and man.

This impasse could be caused by a lack of sensitivity on the part of man or by a weak inaudible signal that is imperceptible to man sent by nature. The imperceptibility could also be due to the presence of signals from other component parts of nature, the toxicity of the source or the signal, the distance between the source and man or a complete lack of ability to understand (or decode) the information naturally.

The second unifying principle of instruments is observed in the overall design/ architecture of the instruments (see Figure 9). The instrument sends a stimulus to

nature (a chemical system) to probe it (i.e. elicit a response from nature), next it monitors the response from nature, sifts it from other signals and translates it into information that humans can comprehend. These two principles set the tone for the rest of the semester.

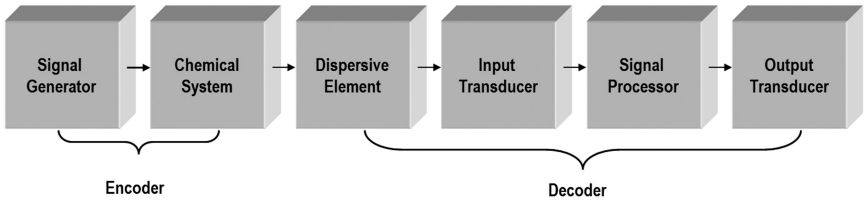

Figure 9. The unifying structure of analytical instruments.

Optical Spectroscopy Module as an Example of the Principle-Based Approach Used in Lecture Course of Instrumental Analytical Chemistry at Butler University

In the introductory portion of the module, the instructor presents information on the common building block of optical spectroscopic instruments (see Figure 10) and fundamental theory of optical spectroscopic techniques. First, the nature of the stimulus (i.e. electromagnetic radiation, emr) that is used for probing the chemical system is presented. This includes emr characteristics such as the speed, frequency, wavelength, and energy and their association. Next, the events that happen when the emr and the chemical system interact are also discussed. First we look at what happens to the chemical system as a result of the interaction (i.e. electronic, vibrational and rotational excitations), then we look at what happens to the emr itself (absorption, transmission, emission, reflection, refraction, interference and diffraction). Such events provide us with information that can be used to identify (in part) or quantify the chemical system. These events/phenomena present concepts instruments could be based on.

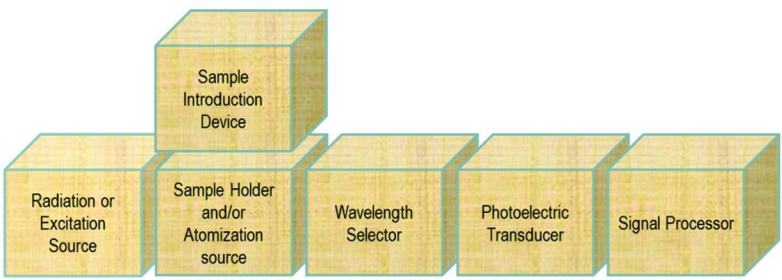

Figure 10. General building block of optical spectroscopic techniques.

At this point the approach is switched to inquiry based where the students are guided to discover the different types of material used in optical spectroscopic instruments and criteria for selection for purpose. Next the students look at

types of radiation sources and regions of usefulness; types of wavelength selector and the theory of operation; and types of photoelectric transducers and a brief view of analogue-to-digital converters. In the next phase students study general configurations of optical spectroscopic instrument based on the phenomenon of absorption, emission, and luminescence. The next phase is to guide students into the discovery of the various instruments under the optical spectroscopic technique (see Figure 11).

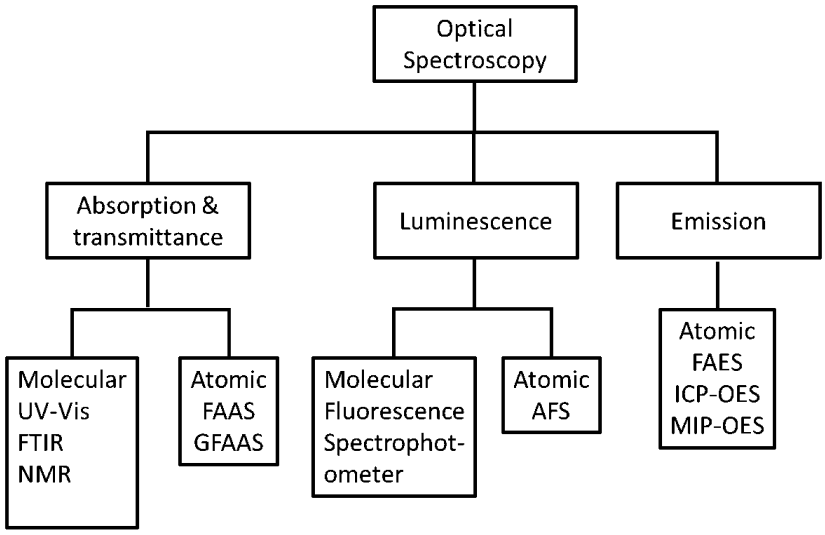

Figure 11. Relatedness of instruments used to deliver the optical spectroscopic module.

The template used to search pertinent information is presented in Table 6. This will typically culminate in a problem set. With regards to resources used for delivering the lecture course, a combination of textbook, primary literature, manufacturer's technical documents and most recently the ASDL website are utilized.

Instrumental Analytical Chemistry Laboratory Courses: A Description of the Theme-Based Approach

The main objectives of the theme-based laboratory courses are: to introduce multiple advanced instrumental techniques, promote their application in inquiry-based problem solving quests, challenge students to think critically, and improve their communication skills. The expected outcomes (or learning goals) are that each student will improve in their:

(1) Ability to identify scientific problems that are relevant to societal concerns
(2) Ability to break problems down into focal questions that guide experimentation
(3) Ability to conduct literature search to gather relevant information

    a. To underscore the significance of the project
    b. To provide sufficient background information that is necessary for designing the experiment and for appropriate data interpretation to answer the questions set forth

(4) Ability to adapt, adopt, validate and implement methods (or protocols) for chemical analysis
(5) Ability to integrate knowledge to address complex issues/questions
(6) Social skills. Particularly ability to work within a team and provide peer review
(7) Ability to present scientific information in written and oral forms

The framework for implementing the modules has already been reported elsewhere (*42*). Each module must comprise of multiple projects where at least two techniques (instruments) must be used. Results from each project must contribute information to the central goal/objective/question of the module. Elements of the objectives and learning goals enumerated above must be present in the course. In terms of logistics, the semester can be divided into three time frames and the general activities that occur in each time frame depending on the module and instructor. One such time-frame-activity plan that was used for the environmental analysis module is presented in Table 7.

Table 7. Logistics of Implementation for the Environmental Analysis Focused Laboratory Course

Time Frame	***General Activities***
Front-end (1- 2 weeks)	• Planning • Selection of project • Development of question for experiment focus • Distribution of responsibilities • Development of quality control strategies
Mid-point (9-10 weeks)	Method identification • Experiment implementation • Weekly research updates, criticism and suggestion for improvement/direction • Scaling back because of resources and time • Optimization/validation of methods • Development of quality criteria for acceptance/rejection of results

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In Teaching Bioanalytical Chemistry; Hou, H.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Table 7. (Continued). Logistics of Implementation for the Environmental Analysis Focused Laboratory Course

Time Frame	*General Activities*
	• Gathering and formulating the background aspects of the final report/presentation
Back-end (2-3 weeks)	• Writing of final reports and creation of posters

Bioanalytical, forensic, environmental, and food analysis focused courses have already been implemented.

Environmental Module as an Example of Implementation of the Theme-Based Approach

The environmental module has been implemented a few times. Its main objectives are embedded in the framework described above. However, in addition to that, we intend to use this to expose students to regulatory methods, improve their application for open-ended projects, and to further engrain the concept of generating reliable and defensible data (via quality control, quality assurance, and method validation) into the curriculum. Generally, the module is divided into four related projects. At any given time during the semester, the class is divided into four groups with each working on a project. Group members are rotated throughout the semester on member at a time, so that every student contributes to each project.

During the first meeting, students were provided with a list of 'seed' ideas from which they could choose. Alternatively, they can come up with their own idea for the semester long project. One list that was used recently contained the following ideas: *is the cost of bottled drinking water worth it?, investigating of the impact of human activities (industrial and domestic) on the quality of surface water, aquatic animals and soil of a watershed, sediments and their impact on source water used for potable water, impact of traffic on quality of soil and dust is the urban environment actually worse than rural areas? What is the impact of agricultural practices (e.g. pesticide residues, and other chemicals) on agricultural products?* More than often the students will choose from the list and over assume the time and resources available. However, after choosing the focus, they are sent to the library to read technical articles that have information that are related to the topic. This will help identify the sub projects, and generate questions and identify instrument in brainstorming session that follows.

One cohort rejected the seed ideas and came up with their own. Some members of the group noted that there was a difference in taste of water obtained from faucets and fountains in various building on campus. With guidance they decided that the overarching objective for their work was to evaluate the quality of drinking water on campus. As a result, the following four potential questions were generated: (1) what is the impact of the activities (industrial, commercial and agricultural) in our watershed on the quality of the source water used for our water supply?, (2) what is the impact on our water supply of the city distribution

system (water pipes), campus water distribution system and building plumbing distribution systems? Based on limitation of time and resources the last question (impact of building plumbing and distribution system on our water supply) was adopted as the focus. The students were then mandated to use literature and the EPA website to identify relevant contaminants and potential analytical methods to accomplish the task. Subsequently, the work was broken down into four projects as shown in Figure 12.

The first project focused on determining the common inorganic anions using USEPA method 300.1. The second project utilized USEPA method 200.7 and 200.8 to determine trace metals with ICP-OES and ICPMS respectively. For the third project, USEPA method 8015C and 5030 were attempted to determine petroleum related organic compound using GC-FID and GC-MS. The fourth project focused on utilizing USEPA method 532 and 632 with LC-UV to determine pesticide residues. The four projects were implemented concurrently in two phases (see Figure 12). In the first phase the methods were validated by establishing their figures of merit. Each analyte was deliberately spiked into drinking water and canal water samples. Additionally, NIST SRM 1640 (trace element in natural water) was used to validate the ICP methods. In phase two, samples were collected from seven buildings on campus. Composite samples from each building were used for analysis. This is based on a decision to reduce the number of samples due to time limitations.

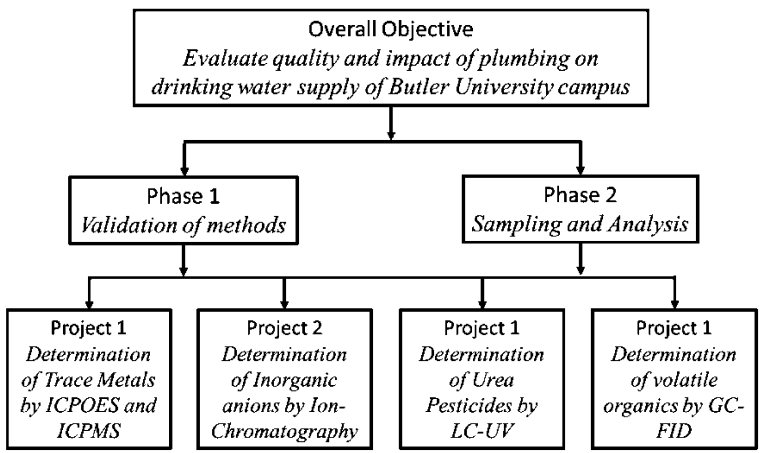
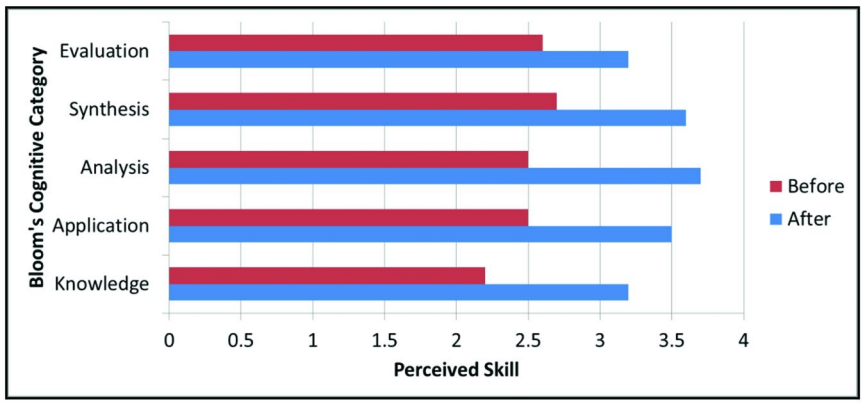


Figure 12. An overview of the projects used in a recent implementation of the environmental module. Repropduced from Anal. Bioanal. Chem., 2008, 392, 1-8 with kind permission from Springer Science and Business Media. Copyright (2008).

At the end of the semester, all targeted goals for projects 1 and 2 were accomplished. The methods were validated and the samples were analyzed. Concentrations of the anions were below the maximum contaminant levels set by EPA. Similar results were found for the trace metals except in two buildings where the concentrations of lead were near or over the action level. This information was

brought to the attention of the administration and action was initiated to remedy the situation. With regards to the third project, only the validation phase for six urea-based pesticides was accomplished. The students were able to compare two extraction methods using spiked water samples. Project four required a purge-and-trap system but the students investigated the use of Tenax GR as an alternative. The validation was incomplete. The students wrote reports for each project using the directions provided by authors' guide of *analytical chemistry* (an American Chemical Society Publication). They also presented their work at the departmental end-of-semester poster session. The impact of the approach on student learning was assessed using SALG and the data was analyzed using both the Blooms and Krathwahls taxonomies. The results are presented in, Figures 13a and b. As seen in these figures, students made gains both in the cognitive and affective areas.



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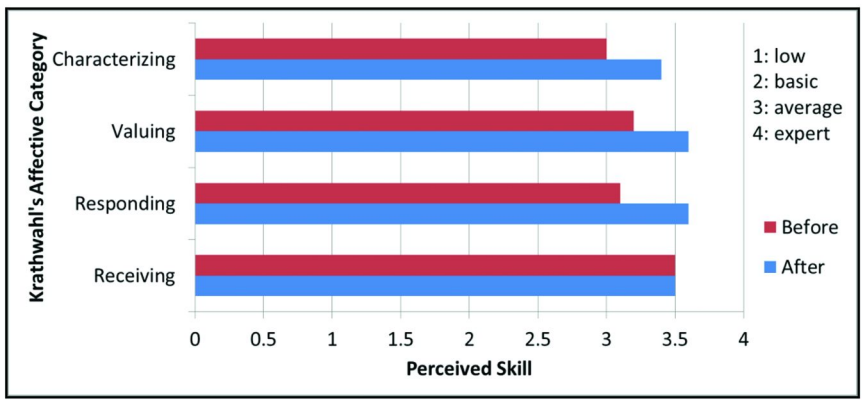

b

Figure 13. a- Cognitive achievements and b- Affective gains of students in the 2009 implementation of the environmental analysis laboratory course using the Blooms and Krathwahl's taxonomies respectively.

In describing their experience, some students wrote the following:

> I learned many various methodologies, how to read a professional procedure and make sense of it, how to tackle a project with little guidance, to multitask and delegate responsibilities when overwhelmed.

> I [learned] that there is no simple answer. This is because chemistry does not work this way: if there is a simple answer to a question, then you probably did something wrong in the method. In order to make the issue at hand less stressful, it is necessary to use teamwork because no sole individual has all the right answers. In other words, not only did I gain the actual information that we found from all of the analyses, but I also learned how to work in a team to solve the big picture. I feel as though this will help me for graduate school as I will be working in a group on a large scale project.

> It is good to have this experience of independent thinking and working in a group. I may not have directly similar experiences in my career, but I am gaining skills that I will be able to use.

> I find this style of lab experience very helpful because in graduate school and industry there isn't a cookiecutter procedure for everything. It allows us to see what other scientists are doing and allows us to make our own decisions.

From the assessments and comments we can tell that the theme-based modular approach to laboratory experiences is beginning to make positive impact on the students. Also, the approach has basically been adopted to the extent that our entire advanced level courses have either adapted or adopted the model. A similar approach has been used to teach entomology (*43*) and project-based laboratory in instrumental analytical chemistry. The authors also provided how to adapt it to large courses (*44*).This further contributes to the strength of this instructional approach.

Conclusion: A Reflection on Choosing the Content, Approach/Method/Pedagogy, Resource and the Overall Purpose of Teaching Analytical Science

Bioanalytical chemistry is an interdisciplinary field at the intersection of biology and analytical chemistry. Although it is new as a distinct discipline, its progenitors are not. Moreover, the application of analytical techniques and methods for determination of small molecules in biological matrices or the characterization of macromolecules is very dated. Bioanalytical chemistry is rapidly evolving as it is being used in many life and health related sciences. Bioanalytical chemistry can be taught by adapting or adopting one or more of the instructional approaches and principles which are used for teaching its

progenitor disciplines. An example model used at Butler University for the implementation of the analytical chemistry curriculum was presented along with the rationale undergirding it. The principles that should guide choice of content, instructional approach and resources used in teaching must evolve alongside our context. We must help students to acquire the skill set and knowledge that are necessary to tackle the issues of their time. Additionally, the students should be equipped to draw on their knowledge and experience to tackle newer problems. To accomplish this, instructors must be flexible and adapt/adopt the appropriate instructional approach that matches the students' learning styles. This will get their attention and motivate them to participate actively in the teaching-learning process. These recommendations have been addressed by many authors in the past. A few include:

> Creative individuals with original ideas must be nurtured, for they enable us to adapt intelligently to changing conditions. Furthermore, individuals must be encouraged to act on their ideas, or else they will be ineffectual, governed by entrenched traditions instead of their own experience (*45*).

> I wanted to learn the names of the birds, so I bought a book and learned their names. I wanted to learn to swim. So I bought a book on how to swim and I drowned. Clearly, match-making is important. Those of us who teach introductory college science courses have great opportunities to match what we teach both to the current interests of our students and to their long-term concerns as citizens, parents, voters, professionals and inhabitants of our planet. Much is at stake. If we continue match our student with a science curriculum that is largely devoid of real-world context that can motivate and inspire them, we risk far more than drowning. There are options for match-making. It is urgent that we make good use of them (*46*).

> Our ability to respond to some of the most critical challenges of the near future – global health, climate change, energy – depends on our ability to fully tap the intellect, passion and creativity of the next generation of scientists and engineers (*47*).

With regards to choosing instructional approach or pedagogy, instructors should realize that

- There are as many instructional approaches out there as there are learners; different people learn in different ways and there is no one-cap fits all solution in this matter, and
- No one instructional approach is wrong or right. The choice of approach used for teaching must be guided by the learning goals and the learner's learning style.

According to Walker and Solstice, we need to realize that the quality of a curriculum or course depends on many factors; it is complex and multifaceted.

The fitness of a course or curriculum depends on its context, philosophical and educational viewpoints (*45*). As such, a course or curriculum design must be based on a philosophy that takes into account what the student completing it should be able to do. Such an endeavor must consider the societal issues of their time and how the students can be equipped to contribute towards solving them. Platz (*48*), the director of the Division of Chemistry at NSF, echoed these same sentiments in the following quote:

> In the 21st century, meeting humanity's increasing demand for food, water and energy will be one of the greatest challenges facing civilization and one that will surely occupy the thoughts of world leaders. Defining the constraints associated with the food/water/energy nexus, and optimizing this complex function of interdependent variables will require the discovery of new chemistry. I sincerely hope that chemists vigorously accept this challenge and that chemistry becomes the "go-to" science for solving this and other pressing problems. The younger generation is full of creative individuals who want to build a sustainable world. It is up to us to convince these idealistic students, by word and example, that research in chemistry, around great global problems, is the way to realize their personal and professional dreams. Chemistry and chemists have simply never been more important to humanity. Spread the word.

Finally, a well-designed course/curriculum should be learner, knowledge, community and assessment centered (*49*), and there are several ways this can be achieved.

Acknowledgments

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Chapter 4

Protein Conformation: Engaging Students in Active Learning

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This chapter describes using protein conformational studies as a theme to engage students in active learning of the physical and analytical methods of biochemistry. The conformational studies can be applied to a variety of spectroscopic methods, from UV-Vis absorption, fluorescence, FTIR, to Mass spectrometry. Students not only can learn the concepts involving protein conformation, but also the methodology related to the instrumentation. Through collaborative learning and Science Writing Heuristic, students can practice crtical thinking and problem solving skills. This chapter provides practical suggestions for instructors to find strategies that turn passive, cook-book lab learning into active, inquiry-based activities.

Introduction

The traditional undergraduate analytical or physical chemistry labs (*1*) are taught by focusing on laboratory techniques and operation of instruments. In this expository, cook-book approach students are not effectively engaged in higher-level scientific reasoning and critical thinking skills. As the result, students do not have a deep understanding of the subjects and the ability to carry out scientific investigation and problem solving as a real chemist does. In this chapter, we will demonstrate a different learning strategy for students to actively engage (*2*) in the studies of protein conformations. Our approach is a problem-based learning (*3*), with collaborative learning strategies (*4*) and Science Writing Heuristic (*5*). The goal is to provide students with a complete experience in how a scientific problem,

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in this case, the study of protein conformations, should be investigated as a real chemist.

The study of protein conformation change is chosen as the topic because it plays an important role in many aspects of our lives, thus making this problem more relevant (*6*). It is a interdisciplinary study that is related to many important applications in medicine and the food industry. For example, the misfolding and aggregation of proteins was found to be related to cystic fibrosis, early-onset emphysema, and Alzheimer's disease, which are also called the protein conformational diseases. Protein conformational stability is also important in pharmaceutical protein formulation. The effect of lyophilization on protein conformation, for example, can induce unwanted denaturation of protein formulation and may inactivate or reduce the activity of the biomolecules. In food industry, protein stability studies also provide valuable information about how to maximize, or even increase, the stability of proteins thus extend the storage time for food products. Providing exposure to students in the experimental techniques of monitoring protein conformation can be a useful experience for students pursuing biological related fields.

The conformation of proteins can be followed by several spectroscopic techniques (*7*): UV-VIS absorption, fluorescence maximum and intensity, amide vibrational IR bands, mass spectrometry, hydrogen exchange, NMR, EPR, and circular dichroism. There are also non-spectroscopic methods, such as calorimetric measurements, polarization interferometry, viscosity measurements, and x-ray crystallography. In this introduction, we will focus on the use of most basic instruments that are already available in undergraduate teaching lab, such as UV-VIS spectrophotometer, Spectrofluorometer (or fluorimeter), FTIR spectrometer, and Mass spectrometer (*7*).

These experiments can be best implemented as problem-based leaning. One possible scenario is where students work in small groups and each group is assigned to different techniques for the study of the same protein or different proteins of the same kind. They are provided with minimal information necessary to start the discussion for the development of experiments. The students collaboratively develop and complete the experiments, process data cooperatively through group discussions, and write conclusions by practicing scientific reasoning skills. The results of each group's study are then compared by discussion and students' reflective report.

Prerequisite Knowledge

We expect students to have the basic concepts in the following three areas before they start their investigations: polarity and charge of amino acids, intermolecular forces, and thermodynamics of equilibrium. Students also need to be equipped with the basic knowledge of the instruments they use and the molecular basis of the spectroscopic measurements. For example, absorption in the UV-visible region is the result of electronic transition of chormophores, fluorescence is an emission process after molecules are excited by light, infrared spectrum comes from the energy transitions of vibrations involving a specific

bond or a group of bonds, and mass spectrometry provides mass-to-charge ratio of molecules or of fragments of a molecule. The techniques they learn from the conformational studies will reinforce and expand their knowledge to the applications of analytical instrumentation.

These concepts can be required through pre-lab reading assignments or discussions. Intermolecular forces and equilibrium are usually covered in the general chemistry sequence for science majors and the structures of amino acids are typically the topics of organic chemistry, biochemistry, biology, or genetic courses. A good review can be done by referring students to the textbook from these courses.

Intermolecular Forces Responsible for Protein Conformation

In order for students to discuss the intermolecular forces that hold a conformation, they need to learn or review different levels of protein structures that constitute conformation. This can be done through a lecture, literature reading, small group discussion, or any combination of the above. Through the selected learning activities, we expect them to now have a good understanding of the following questions:

- What are the primary, secondary, tertiary, and quaternary structures of proteins, and the forces determining these structures?
- What is the conformation of a protein, and how are the conformational changes related to structural changes and intermolecular forces?
- What are the thermodynamic contributions of the conformational changes of proteins? Which contributions favor folding and which favor unfolding?

The following is a summary of these questions.

All protein-building amino acids contain two functional groups: amine ($-NH_2$), carboxyl (-COOH) and a side-chain. Based on the structure of side-chains, they are either polar or nonpolar, positively, negatively charged, or neutral. Amino acids are combined through amide bonds, called peptide bonds, to form peptides. A protein is a polypeptide. The particular order of amino acids in the peptide sequence is the primary structure of protein. The intermolecular forces between side-chains, particularly H-bonding, cause the primary structure to fold. As a result, there are repeated structures of the amino acid sequence that we call the secondary structures, such as α-helix, β-sheet, and turns. Because of the intermolecular forces from the side-chains in the secondary structure, the repeated structures (α-helix and β−sheets) are further packed into a more compact structure called the tertiary structure. A tertiary structure can repeat itself in different areas of a large protein to form the quaternary structure. The intermolecular forces are clearly the root of all levels of proteins structure, except the primary structure which is formed by peptide bonds.

Protein conformation is referred to the three-dimensional arrangement of the secondary, tertiary, and quaternary structures. When proteins show all levels of structures, it is considered folded. When proteins lose these structures, it is unfolded (or called denatured). An unfolded protein is a random coil which also loses its biological function. What are the factors holding proteins in the folded (native) form? Research has provided the following known factors:

Intermolecular forces (*8–10*) – this factor favors protein folding. All types of intermolecular forces contribute to the folding. These include hydrogen-bonding (*11*, *12*) between amine and carboxylic groups, and between particular side-chains, electron static forces between charges within protein, and the weak and short ranged van der Waals interactions.

Hydrophobic interaction (*13*) – this factor also favors protein folding. The nonpolar side-chains are buried inside the core of protein structure, leaving the polar side–chains to be exposed to the exterior of protein in the aqueous environment. This arrangement is seen in the folded proteins.

Conformational Entropy (*8*) – this factor favors protein unfolding. There is only one or a few folded states of proteins but many unfolded conformations. Therefore, the entropy change from random coils to folded state is negative, making the folding less favorable. This is the only negative contributor to the energy of protein folding.

With all but one factor favorable for folding, we expect the overall Gibbs free energy of protein folding to be negative. Among these factors, hydrophobic interaction and H-bonding are the two most significant contributors of ΔG (*9*).

In a typical study of protein conformation, proteins are purposely unfolded (denatured) to observe the change of spectroscopic observable. Commonly seen denaturants include urea, guanidine hydrochloride, temperature, pH, or alcohols.

Experimental Consideration of Denaturants

Although there are many denaturants that can be used, most studies of protein conformation were done using urea and Guanidine Hydrochloride (*14*). These are the preferred denaturants because both provide consistent, highly reproducible spectroscopic measurements and the unfolding, using these reagents, is completely reversible. High purity forms of these reagents are available from several commercial resources. The molarity of the stock solutions can be accurately calculated with refractive index (*15*, *16*). A variety of impurities were reported present in Guanidine hydrochloride and urea that could affect the conformation of a protein. The most concerned impurity comes from the decomposition of urea to form cyanate and ammonium ions, particularly at high pH. The cyanate ions can modify amino groups of proteins (*17*). Therefore, urea should not be used after 24 hours of the stock solution being prepared. However, Guanidine hydrochloride remains stable after months. The highest

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molarities that can be prepared for urea and Guanidine hydrochloride are 10 M and 8 M, respectively. Students usually found it easier to dissolve urea in water than Guanidine hydrochloride. Alternatively, the commercial 8 M Guanidine hydrochloride stock solution is available for purchase.

It is also crucial to use a high purity buffer and water in the preparation of the stock solution of denaturant to reduce interference. Watch also for any organic content in water. It was reported that bacterial growth from buffers can interfere with fluorescence measurements (*18*). A pre-scan is necessary for background fluorescence from the buffer and denaturant solutions prior to the measurements of protein assays.

Basic Two-State Model of Unfolding

The simplest model of conformational change is the two-state equilibrium model. In this model only two forms of proteins exist, folded or unfolded, and a dynamic equilibrium between them

$$\text{Folded (F)} \leftrightarrows \text{unfolded (U)}. \qquad \text{Eqn 1}$$

The thermodynamic parameters, from the following two-state model, are expected to be a straightforward derivation by the students through discussion. The two parameters are the expression of the equilibrium constant in terms of concentrations of folded ([F]) and unfolded ([U]) proteins:

$$K = [U]/[F], \qquad \text{Eqn 2}$$

and the Gibbs free energy from the equilibrium constant:

$$\Delta G = - RT \ln K. \qquad \text{Eqn 3}$$

Further discussion about how these parameters, K and ΔG, can be determined experimentally should be pursued by the students.

Beer-Lambert's Law and UV-Vis Spectrophotometry

Although the most direct experimental approach to equation 2 is to measure the concentrations of folded and unfolded proteins in the solution, this approach is not always the best choice for multi-component systems. This is potentially the best time to review Beer-Lambert's law for the relationship between absorbance and concentration:

$$A = \varepsilon l C \qquad \text{Eqn 4}$$

where $A = -\log (I/I_o)$, I and I_o are the intensities of incident and emerging light, respectively, Eqn 5

ε: molar absorptivity or molar absorption coefficient, and

l: path length of sample.

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At this point, students should be given information from the chromophers responsible for the absorption of proteins in the UV-Visible range. The molar absorptivity of the proteins is resultant of the electronic absorption of light by three aromatic amino acid residues, tryptophan, tyrosine, and phenylalanine in the near UV range (240-300 nm) by the S-S bonds (as in cystine) as well as by the π to π* transition of peptide backbone in the far UV range (180-220 nm). At 280 nm, tryptophan has the strongest absorption ($\varepsilon = 1490\ M^{-1}cm^{-1}$) followed by tyrosine ($\varepsilon = 5500\ M^{-1}cm^{-1}$) and cystine ($\varepsilon = 125\ M^{-1}cm^{-1}$) (*19*). The absorption of phenylalanine is negligible. Therefore, the total concentration of proteins can be accurately estimated at this wavelength from the total molar absorptivities of tryptophan, tyrosine, and cystine in the proteins.

The determination of native and denatured protein concentrations in a solution by UV absorbance measurements is well documented (*19*). The molar absorptivities of the native and denatured proteins are first determined either empirically or by calculation. The concentrations of native or unfolded proteins can then be calculated by using equation 4. This approach is most reliable for proteins with tryptophan and Tyrosine residues.

The absorption of proteins is also sensitive to local environment where the amino acid chromophores are located. For example, a small blue-shift (2-3 nm) is observed when the tryptophan or tyrosine residues are moved into a more polar environment (*7*, *19*, *20*). Consequently, when proteins unfold, tryptophan or tyrosine will be moved from hydrophobic core into more polar aqueous region, expecting a blue-shift in the absorption spectrum. This shift of wavelength is useful for monitoring the change of protein conformation. The wavelength which is definitive of the maximum difference can be used to follow the unfolding. However this wavelength shift, due to conformational change, is small in comparison to the UV absorption, but is more sensitive to the CD.

Another range of wavelengths for the detection of conformational change is the Soret band (400-410 nm) from Heme-containing proteins (*21–23*), such as myoglobin and cytochrome. The molar absorptivity of the Soret peak decreases when proteins are unfolded. Figure-6 shows a Soret band of myoglobin when the proteins are unfolded by pH changes. If time allows, an effective approach is to ask students to run a quick UV-visible spectrum of native myoglobin solution at pH 7 and use the spectrum to discuss different regions of absorption.

Unfolding Curves

The process of unfolding (or denaturation) can be conveniently followed by an unfolding curve (*14–16*). Unfolding curves are analogous to titration curves where the change of pH is monitored at different reaction stoichiometries between the acid and base; the unfolding curve also shows how the measured spectroscopic variable is changed at different degree of denaturation of proteins. In a typical unfolding curve, there is a region where all proteins are in the folded state, a region where all proteins are in the unfolded state, and a transitional region where different fractions of unfolded proteins are shown. An unfolding curve can be prepared by using a chosen spectroscopic variable (such as absorbance,

fluorescence intensities, etc). The spectroscopic variable is plotted as the y-axis and the degree of denaturation represented by the concentration of denaturant as the x-axis. If the temperature is used to denature the protein, this is called the thermal unfolding curve. Figure 1 shows a hypothetical unfolding curve of myoglobin using fluorescence intensity measurements.

Although the unfolding curve can be generated by different spectroscopic measurements, it would be useful to convert different measurement variables into a unified measure. This measure is the *fraction of unfolding*. The fraction of unfolding is simply the fraction of unfolded protein in the entire sample of protein

$$f = [U]/([U]+[F]). \qquad \text{Eqn 6}$$

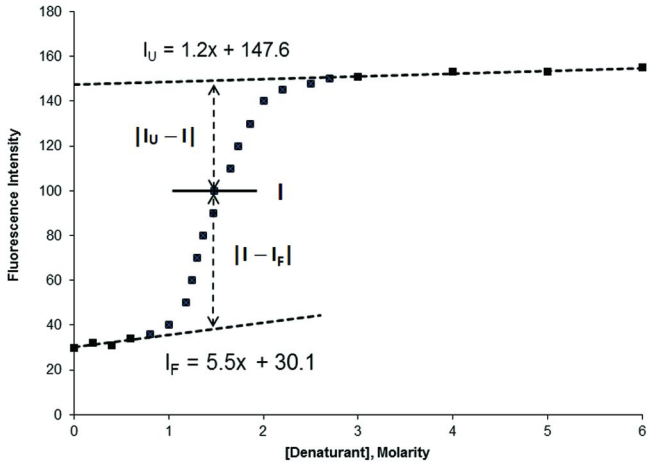

Figure 1. Illustrated unfolding curve of myoglobin using fluorescence intensity. The tryptophan fluorescence intensity is low when myoglobin is folded due to energy transfer from tryptophan to the heme group, and is high when myoglobin is unfolded because tryptophan is moved away from the heme group.

Alternatively, the fraction of unfolding can be determined directly from spectroscopic measurements. For example, when proteins are monitored by UV absorbance, the fraction of unfolding can be calculated as

$$f = (A_C - A_F) / (A_U - A_F) \qquad \text{Eqn 7}$$

where A_U, A_F, and A_C are, respectively, the absorbencies of protein solutions that contain all unfolded protein, all folded protein, and protein with denaturant concentration C presented. The fraction of unfolding can also be obtained from the unfolding curve as

$$f = \frac{|I - I_F|}{|I_U - I_F|} \qquad \text{Eqn 8}$$

The unfolding curve plotted, using *fraction of unfolding*, is demonstrated in Figure 2.

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The Equilibrium Constant

The equilibrium constants of protein unfolding can be calculated from the unfolding curve instead of using the concentrations. The formula of this calculation is another useful exercise for student to derive. With the help of quantities labeled in Figure 1, and the expression of K in equation 2, we expect students to derive the following equation:

$$K = \frac{|I - I_F|}{|I_U - I|}$$

Eqn 9

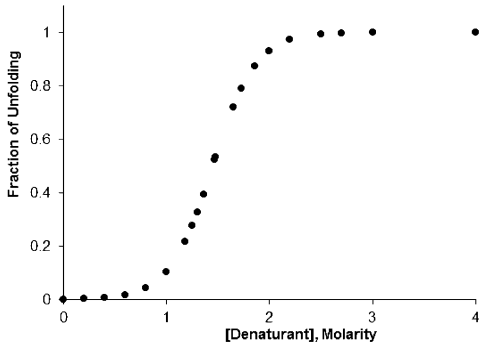

Figure 2. Unfolding curve using fraction of unfolding converted from Figure 1.

Gibbs Free Energy of Unfolding

The free energies of unfolding for each protein conformation, in the transitional region of the unfolding curve, can be calculated from K as shown in equation 3. However, these energies are associated with protein conformations in the presence of different concentrations of denaturant. *How should the Gibbs free energy without the presence of denaturant (denoted as ΔG_w) be determined?* This is an important post-lab question for students to discuss. Students can be hinted by plotting the relationship between Gibbs free energy and the concentration of denaturant, such as one demonstrated by Figure 3. We ask students to discover what kind of relationship from their experimental data is shown and how to use the relationship to determine ΔG_w.

The following discussion questions are recommended:

- How are Gibbs free energy, equilibrium constant, and the concentration of denaturant correlated?
- How do you determine the trend of Gibbs free energies with different concentrations of denaturant with a mathematical equation?
- What are the contributing elements of ΔG_w discussed in the factors responsible for protein folding? What is the sign of the each contributing energy and the overall ΔG_w you predict, and why?

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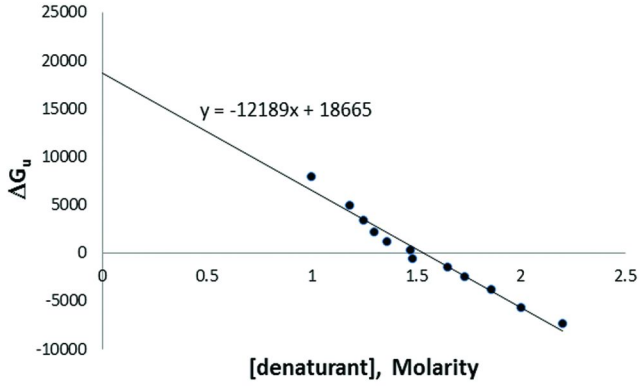

Figure 3. Gibbs free energies of protein conformation in the transition region of the unfolding curve plotted against the concentration of denaturant. Students will discover the relationship between the two variables is linear and ΔG_w can be obtained as the intercept at zero concentration.

The Linear Energy Model

From this energy model (*24*), the Gibbs free energy (ΔG_u) is linearly related to the concentration of denaturant (C) such that

$$\Delta G_u = \Delta G_W - mC, \qquad \text{Eqn 10}$$

as seen in Figure 3. The trendline intercepts the concentration axis ($\Delta G_u = 0$) where

$$\Delta G_w = mC_m, \qquad \text{Eqn 11}$$

Equation 11 provides an alternative way of calculating ΔG_w. The unfolding curve can be modeled as a sigmoid function of m (an empirical constant represents how ΔG is varied with denaturant during the transition region) and C_m (the middle point of the unfolding curve), shown in the following equation:

$$f = \frac{\exp(-m\frac{C_m - C}{RT})}{1+\exp(-m\frac{C_m - C}{RT})} \qquad \text{Eqn 12}$$

The values of m and C_m can be found by fitting the unfolding curve to equation 12. Once m and C_m are found, ΔG_w can be calculated using equation 11. To fit experimental unfolding curve to the above equation, a non-linear curve fitting program is required. We have used the EXCEL solver plug-in with a satisfactory result. Figure 4 and 5 show ΔG_w determined by both methods (extrapolation and equation 12) from the same experimental data. The two methods should lead to similar values of ΔG_w.

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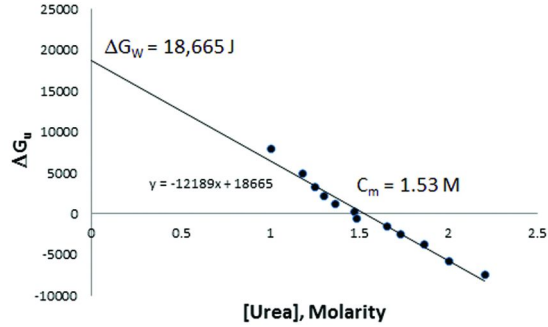

Figure 4. ΔG_w was determined by extrapolation to the y axis.

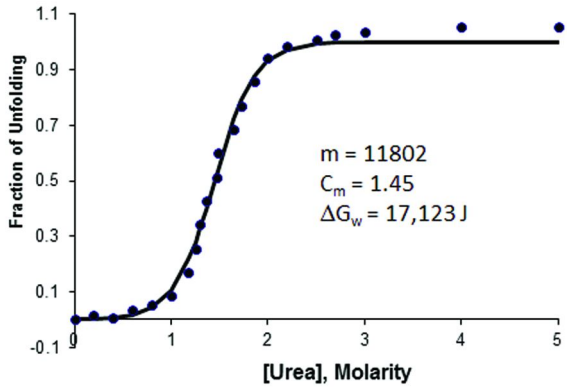

Figure 5. ΔG_w was determined by nonlinear curve fitting to equation 12 (solid line) and calculating using equation 11.

In the following sections, the instrumental methods of absorption using UV-VIS spectrophotometer, fluorescence intensity using Resonance Energy Transfer (RET), vibrational transition using FTIR, and Mass spectrometry using ESI-MS, are introduced. All experiments demonstrated were developed for myoglobin.

The Soret Band

As mentioned previously, three characteristic absorption regions of proteins are 180-220 nm (peptide bonds), 240-300 nm (Try and Tyr), and 400-410 nm (Soret band for porphyrin). The Soret band is unique for heme proteins and is absent from apomyoglobin (myoglobin without the heme).

Measurements of the Soret band of myoglobin are best done with a dual-beam spectrophotometer and a temperature controlled sample compartment to keep a constant temperature of the protein assays. A pair of matched cuvette should be used. For single-beam or diode array spectrophotometers, see reference (*7*). In a typical experiment, the myoglobin solutions are prepared in phosphate buffer at pH 7, and denatured by 8 M guanidinium chloride or 10 M urea. The amount

of protein to be used can be determined by a preliminary run of folded protein. Proteins can also be denatured by pH, temperature, or alcohol. A series of myoglobin solutions with different concentrations of the denaturant are prepared and incubated at a constant temperature bath until equilibrium is accomplished. The time required to reach equilibrium varies with temperature, pH, and proteins and should be determined by monitoring the absorbance. The protein assays are then scanned in the Soret range (300-500 nm) at the same temperature protein assays were incubated. The absorbencies at the Soret peak are recorded for generating the unfolding curve. More description of the experiment can be found in references (*7*, *21*, *22*, *25*). With 10-15 assays, students can finish the experiment within a 3 to 4 hours lab period, not including the incubation time. Guide the students through the design of experiment with guiding questions and a detailed discussion. Figure 6 shows the Soret bands of myoglobin in different pH's.

Tryptophan Fluorescence and Resonance Energy Transfer

In proteins, the sources of fluorescence (fluorophores) are three aromatic amino acid residues: phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) (*18*). Proteins without these amino acid residues generally do not fluoresce.

Tryptophan gives a stronger fluorescence than tyrosine and phenylalanine. It absorbs in the range of 200-305 nm and fluoresces from 300 to 440 nm. The intensity and wavelength of maximum fluorescence of Trp are solvent dependent. The maximum fluorescence shifts to shorter wavelength and the intensity increases as the polarity of the solvent decreases. In the native state of protein, Trp residues are buried inside the hydrophobic core. Upon unfolding, the Trp residues are exposed to more polar water molecules, thus, the intensity of fluorescence decreases and the maximum fluorescence shifts to a longer wavelength (*26*).

Tyrosine absorbs from 240 to 290 nm (maximum at 280 nm) and fluoresces from 270 to 360 nm. Although Tyr has a smaller emission than Trp, it usually presents in a larger number than Trp and can give significant fluorescence. However, many factors can reduce (quench) the tyrosine fluorescence. The energy transfer to nearby Trp residues is the most commonly seen quenching of tyrosine fluorescence. The energy transfer is due to the overlap of Trp absorption (200-305 nm) and Tyr fluorescence (270-360 nm). In many cases, energy transfer to Trp completely diminishes Tyr fluorescence.

Phenylalanine absorbs from 200 to 270 nm and fluoresces weakly from 240 to 320 nm. Therefore, the Phe fluorescence is only observed when both Trp and Tyr are absent.

The fluorescence of these amino acids decreases when they are incorporated into a polypeptide chain. Therefore the fluorescence intensity of a protein containing any or a combination of these amino acid residues will be much lower than those from free amino acids. The intensity change between the folded and unfolded protein is sensitive to environmental change of these amino acids, particularly Trp since it constitutes a major portion of the total fluorescence intensity.

The intensity change when proteins are unfolded varies from protein to protein. For some proteins, the intensity of fluorescence increases when unfolding, whereas for others intensity decreases. However, the shift in maximum fluorescence is more consistent based on the environmental change upon unfolding. The exposure of Trp to a polar solvent, when unfolding, causes the shift in maximum fluorescence. Both fluorescence intensity and fluorescence maximum can be used to monitor the protein conformation change. Figure 7 shows how wavelength maximum shifted when the myoglobin unfolded.

Energy transfer is another important factor in intrinsic fluorescence. When energy of a donor group is transferred to an acceptor group, the fluorescence of the acceptor group decreases (*27*). This is the reason Tyr fluorescence is usually not observed due to the energy transfer to Trp as mentioned before. Another example is when Trp residues are nearby heme prosthetic group in the folded state, hemoglobin shows almost no Trp fluorescence. The energy is transferred from Trp to heme. When hemoglobin is unfolded, Trp residues start to move away from the heme group leading to an increase in Trp fluorescence. By observing the increase of Trp fluorescence, we can monitor the unfolding status of heme-containing proteins.

Binding a quenching agent to proteins is another example of using fluorescence techniques in probing unfolding state of a protein. ANS exhibits high fluorescence quenching in aqueous solutions, however fluorescence increases when ANS binds to hydrophobic folding packets. Therefore, in the presence of ANS, folded protein solutions show greater fluorescence than unfolded protein solutions. This technique is called extrinsic fluorescence.

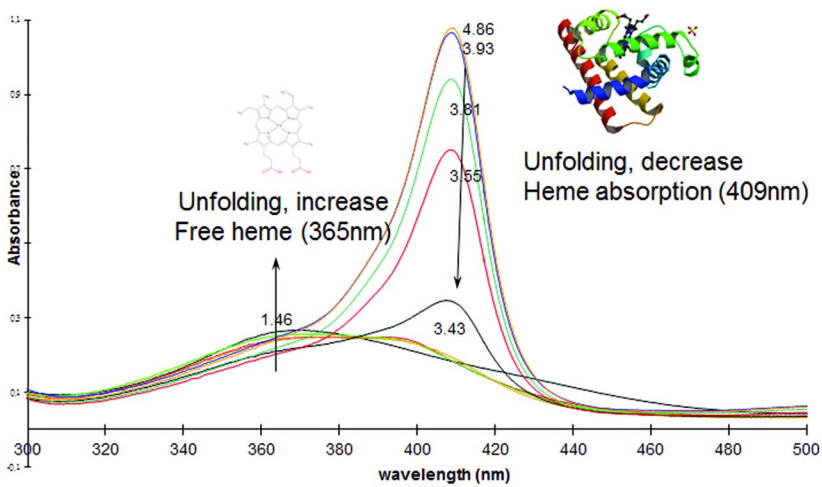

Figure 6. The Soret band of myoglobin decreases its intensity as pH decreases (indicated as 4,86, 3.93, 3.81, 3.55, 3.43, and 1.46). In the meantime, absorption of free heme group is increased as the pH is low and the heme group departs from myoglobin.

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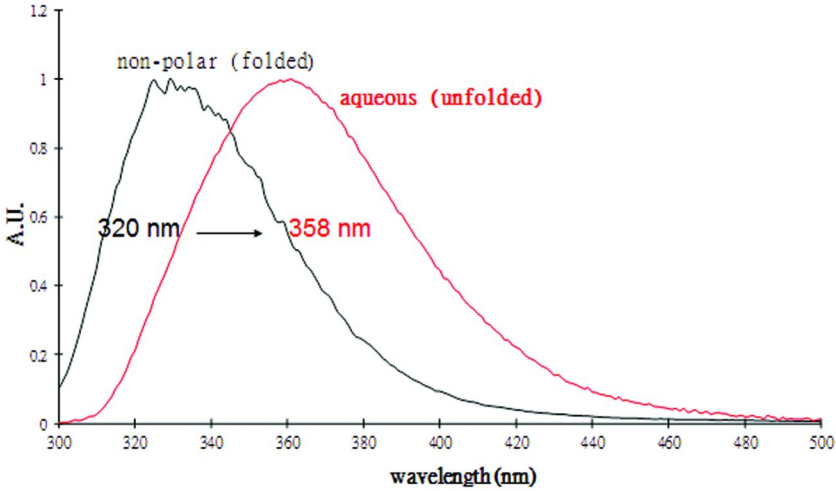

Figure 7. Tryptophan fluorescence of myoglobin from folded and unfolded forms. The unfolded myoglobin has the wavelength maximum shifted from 330 nm to 360 nm.

In a typical undergraduate experiment (*28*), a series of protein assays are made consisting of the same concentration of proteins, buffer solution to maintain the pH, and different concentrations of denaturant. The assays should contain solutions of a few folded states (absence of denaturant), unfolded states (high concentration of denaturant), and many transitional states (different concentrations of denaturant). A quick run of a few assays of the transitional states can help students find the best distribution of concentration of denaturant for the assays. The assays should be prepared and incubated at a constant temperature bath until all assays reach equilibrium. An experiment for 10 to 15 assays should be sufficient.

The required instrument for fluorescence measurements is either a spectrofluorometer or a fluorometer with a temperature-controlled sample compartment. The temperature control is crucial for obtaining stable measurements since folding-unfolding equilibrium is sensitive to temperature. The choice of excitation and emission wavelength, and other instrumental parameters (slit width, etc) could be determined by students. In a typical setting, the excitation wavelength should be chosen from the range of 200 to 205nm, and the emission in the range of 270 – 360nm. A preliminary run of the emission spectra of folded and unfolded proteins will be helpful for determining the excitation, emission wavelengths, slit widths, and other instrumental parameters. The students can also save the entire emission spectra and decide which wavelength to be used to plot the unfolding curve later. The Raman scattering of water can interfere with the protein emission. See reference (*7*) for how to identify and subtract them. Figure 8 shows a typical student's fluorescence spectra from myoglobin protein assays with methanol as the denaturant.

IR Amide Bands for Secondary Structures

As early as 1950's, Elliot and Ambrose (*29*, *30*) have demonstrated an empirical correlation between the Amide I & II absorption and the secondary structures of proteins determined by x-ray diffraction. It was not until 1986, when Byle & Susi published their work (*31*), that the determination of secondary structures of proteins from FTIR spectra began. The amide I absorption (1600 ~ 1700 cm^{-1}, Figure-1) is a broad band containing many unresolved components consisting of 80% C=O stretching and 20% C-N stretching of the amino acids that constitute the protein. These components are caused by the influences of H-bonds on C=O or C-N stretchings. We know the secondary structures (mainly α-helix and β-sheet) are determined by H-bonds. It is therefore possible to find the correlation between these components and secondary structures.

Similarly, the amide II absorption contains in-plan N-H bending components (~60%) and C-N and C-C stretching (~40%) that can be correlated to secondary structures. This band is found in the 1510 and 1580 cm^{-1} region. However, the influences of H-bonds on N-H stretching are more complicated than those in amide I region. The correlation is thus less reliable in this region. We will focus on amide I in the study. Figure 9 demonstrated how amide I and II band changed when protein is unfolded.

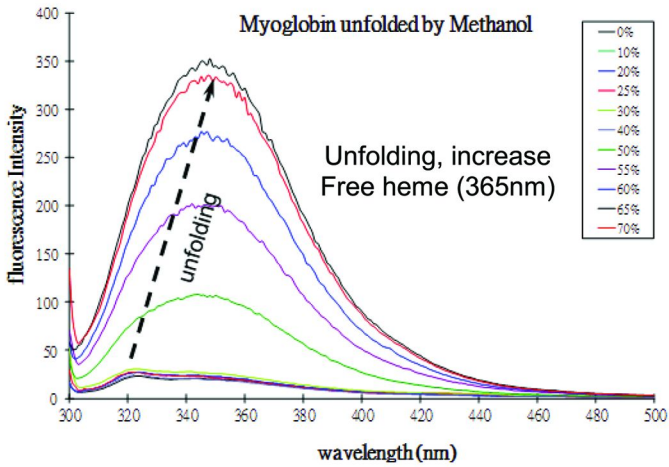

Figure 8. Myoglobin denaturation monitored by fluorescence spectra. 60 mg of myoglobin were dissolved in 15 mL buffer to make the stock solution. 1 mL of protein stock was mixed with 9 mL of methanol-water solvent with different percentages listed in the figure. Assays were kept at 0°C for 8 hours for equilibrium. The excitation wavelength was 296 nm.

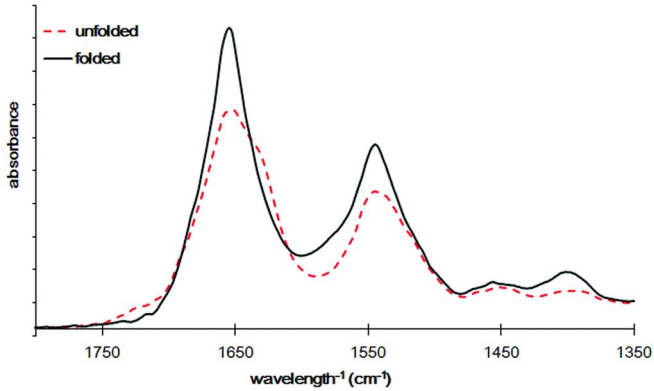

Figure 9. Amide I and II bands of a folded and unfolded protein.

The Secondary Structures of Proteins

There are three basic secondary structures of proteins: beta sheet, alpha helix, and beta turn structure. The studies of infrared spectra of synthetic polypeptides have been very helpful in characterizing the content of secondary structures of proteins. Some good and comprehensive references are given by Barth & Zschep (*32*), Krimm & Bandekar (*33*) and Barth (*34*)

In aqueous solution, there are two main frequencies of beta sheet. The first frequency ranges from 1612 to 1641 cm^{-1} with the average value of 1633 cm^{-1}. The second frequency is ranged from 1687 to 1695 cm^{-1}, averaged at 1984 cm^{-1}.

The bands of alpha helix will be shifted to lesser energy in deuterated hydroxide (^2H_2O). The two frequencies are 1615 ~ 1638 cm^{-1} (averaged at 1630 cm^{-1}) and 1672~1694 cm^{-1} (averaged at 1679 cm^{-1}). For alpha helix in aqueous solution, the main frequency is averaged at 1654 cm^{-1}(1649~1657cm^{-1}) for amide I band with a half-width of 15 cm^{-1}. In ^2H_2O, the band is averaged at 1652cm^{-1} (1642~1660cm^{-1}).

The beta turn frequencies are overlapping with the other two secondary structures, with an exception of 1672 cm^{-1} (1662~1686cm^{-1}). The random structure also gives amide I band centered at 1654cm^{-1} (1642~1648cm^{-1}) for aqueous solution and at 1645cm^{-1} (1639~1654cm^{-1}) for ^2H_2O.

Figure 10 summarizes the contributions of secondary structures in amide I band of proteins.

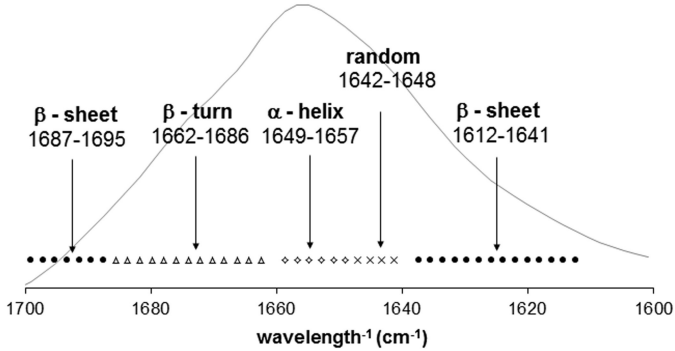

Figure 10. The frequency ranges of different secondary structures of amide I band.

The Data Analysis of Amide I Band

The resolution of the amide I band can be improved to reveal more underlined peaks. This can be done with a Fourier self-deconvolution and second-derivative of the band (*35*). Figure 11 shows the second-derivative of the amide I band of myoglobin using Grams AI. The two methods may yield different identified peaks, depending on the parameter setting of the computer program. These components will be compared to the secondary structures of proteins (eg, alpha-helix, beta-sheet, etc) obtained from the X-rays crystallography (*36*)

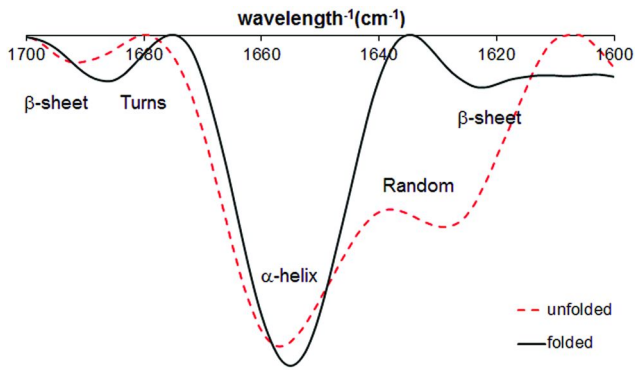

Figure 11. The second derivative of an amide I band of folded and unfolded myoglobin. The unfolded band reveals more random structure.

After the components are identified, we will run an iterative curve fitting procedure to enhanced amide I regions. When a proper curve-fit is found, the area of each component will be recorded and can be used to calculate the % of each structure. Figure 12 shows the change of secondary structure of myoglobin revealed by comparing the % of secondary structures obtained from curve-fitting of amide band I.

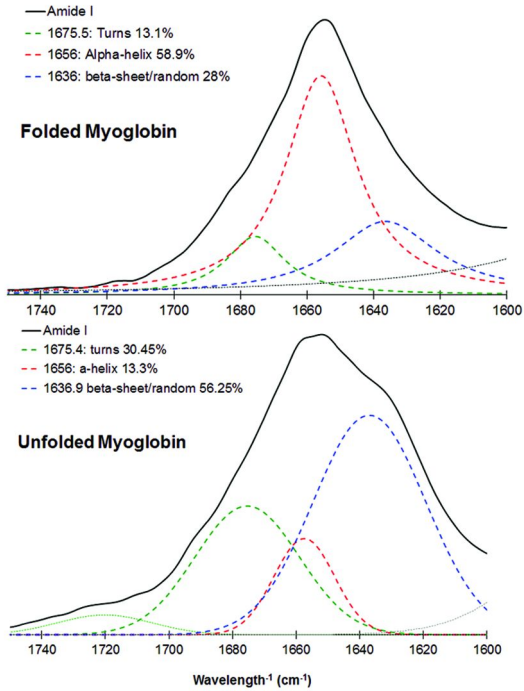

Figure 12. The secondary structures revealed by curve-fitting of the amide I band of folded and unfolded myoglobin. The myoglobin was unfolded by pH. Both spectra were taken using ATR-FTIR with 2 cm^{-1} resolution, 128 scans.

Experimental

The protein samples can be prepared using a sealed liquid cell or through Attenuated Total Reflectance (ATR) (*37*, *38*). A typical sealed liquid cell uses two salt windows and a spacer to form the sealed liquid compartment. When in use, the liquid sample is injected into the sealed compartment with a syringe. By using a liquid cell, the IR light will interact with the sample via transmission. ATR, however, uses a different approach for sending IR light through a sample. Instead of using transmission, the IR light enters the ATR crystal at a specific angle producing internal reflectance. This internal reflectance generates an evanescent wave that extends beyond the surface of the crystal and interacts with the sample. The use of ATR simplifies sample preparation and is an alternative method for the study of protein conformation.

There have been suggestions that the use of D_2O instead of H_2O is preferred in the preparation, but the resulting amide bands were too difficult to be interpreted by students. High protein concentration (> 30 mg/ml) is required when H_2O is used to overcome the high intensity water peak. The advantages of using ATR method are due to the lower concentration requirement and less interference from high concentration denaturant. It is also possible to analyze protein structures from

a thin film using ATR. To make the thin film, a small amount of protein assay is applied to the ATR crystal and the assay is allowed to evaporate. The background spectrum should be taken with the buffer and denaturant. With the liquid cell, the sample compartment of the spectrometer should also be purged continuously with nitrogen to eliminate water vapor. Both the background and the protein sample should be taken with 125 scans or more at the resolution of 2 or 4 cm^{-1}.

To ensure the signal of water is subtracted in the sample spectrum, the proper subtraction is judged to yield a flat baseline (approximately) from 1900 to 1720 cm^{-1}, avoiding negative side lobes, and the removal of the water band near 2130 cm^{-1}. The amide I band is from 1600 to 1700 cm^{-1}.

We have run the FTIR experiments of myoglobin by using pH change as the means of denaturing the protein to avoid problems caused by a high concentration of denaturant.

Mass Spectrometry

Mass Spectrometry is a technique for studying the masses of atoms, molecules, and fragments of a molecule. In mass spectrometry, a sample is ionized and accelerated by an electric field and the charged particles are separated by magnetic or electric fields and analyzed according to their mass-to-charge ratio (m/z). The result is presented as the detector's response versus m/z, called mass spectrum.

There are many ways to carry out ionization. For example, chemical ionization, fast atom bombardment (FAB) and liquid secondary ionization MS (LSIMS), matrix-assisted laser desorption ionization (MALDI) developed by Hillenkamp and Karas, Tanaka, and electrospray ionization (ESI) by John Fenn. We will focus on the ESI method.

Electrospray Ionization (ESI)

The concept of electrification of liquid droplets produced by spraying has been known for centuries. The development of ESI was started in 1960's and had a breakthrough by John Fenn, who later received the Noble Price in 2002, demonstrating the ESI mass spectra of proteins and synthetic polymers. Currently, applications of ESI-MS for bipolymers analysis constitute a large and still rapidly expanding field.

Electrospray ionization is a process that involves many steps. First, a dilute solution of biopolymer is allowed to flow (sprayed) out of a tip of a hypodermic needle. The fine droplets are electrically charged and due to their instability they are prone to be broken into finer particles. The process of breaking the droplets into finer particles continues until there is only one macromolecule remains in each particle.

A very distinct feature of ESI is the accumulation of multiple charges on a single analyte molecule during ionization. Consequentally, the multiple charging in ESI gives the appearance of multiple peaks (as m/z values) in the mass spectrum. Depending on the solvent and buffer solution used in the preparation of the analyte

solution, there are many different charge carriers that are commonly associated with an ESI process (H^+, Na^+, K^+ etc). Figure 13 shows a mass spectrum of myoglobin.

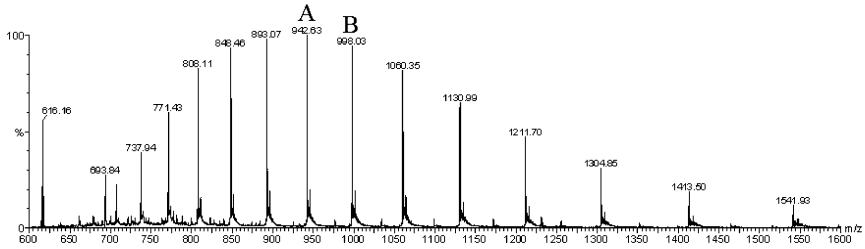

Figure 13. ESI Mass spectrum of myoglobin demonstrating multiple peaks with a distribution of charges.

Through discussions, students will learn how charges can be determined. Assuming H^+ is the only source of charge, the following basic equation is provided to students:

$$m/z = (M + n(1.008)) / n \qquad \text{Eqn 13}$$

where M is the molar mass of the protein and n is the charge. Can both M and n be calculated from equation 13? Help the students to realize that two equations are needed.

Assigning Charges to Mass Spectrum

Students are now discussing how to use equation 13 on two adjacent peaks (A and B) with charge n and n+1,

$$m/z_n = (M + n(1.008))/n \qquad \text{peak A}$$
$$m/z_{n+1} = (M + (n+1)(1.008))/(n+1) \qquad \text{peak B}$$

By substituting $M = n\,(m/z_n) - 1.008n$ from peak A into the equation for peak B, we can get

$$n = (m/z_n - 1.008)/(m/z_n - m/z_{n+1}) \qquad \text{Eqn 14}$$

Notice that the molar mass M is not required using equation 14.

As an example, by using Figure 13 the two peaks labeled as A and B have m/z = 942.63 and 998.03, respectively. We can calculate the charge for B as

$$n = (998.03 - 1.008) / (998.03 - 942.63) \approx 17$$

The charge for A is then n + 1 = 18. The two adjacent peaks are different by one charge. Students can assign the charges for the rest of the peaks in the spectrum by adding to or subtracting from the charge of the adjacent peak by one.

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Protein molecules demonstrate an isotope distribution based on the natural abundances of the constituent atoms. High resolution mass spectrum can easily distinguish peaks due to the mass difference of isotopes. This is the most accurate calculation of the charge from a single-charge-state peak. This method can also be used on a lower resolution mass spectrum through a mathematical resolution enhancement process (such as deconvolution, maximum entropy, etc). You can find these references (*39–44*) for more details.

Calculating Mass

Although the mas can be calculated from equation 13 after n is known, this is not the most accurate way. Mass accuracy often is improved considerably by averaging the charge envelope over the N charge states (*45*):

$$M_{avg} = \frac{\sum_i M_i}{N} \qquad \text{Eqn 15}$$

The Charge Distribution and Protein Conformation

The multiple charged peaks of a protein form a charge distribution that is sensitive to the folding state of a protein. An unfolded protein in solution leads to the formation of higher charge states than the same protein in its native, folded conformation. Although the physical basis of such change in ESI-MS spectrum has not been fully known, it has been found that the charge state distribution is more sensitive to the tertiary structure than to the secondary structures (*46*). Evidences also show that the change of charge distribution can be linked to the steric accessibility of possible protonation sites and alterations of their specific pKa values, and to the increased surface area of the peptide chain. The change of the charge distribution is the basis of this experiment to obtain an unfolding curve of myoglobin.

The average charge of each mass spectrum is calculated as

$$\overline{n} = \frac{\sum_i n_i \, I_i}{\sum_i I_i} \qquad \text{Eqn 16}$$

where I_i is the intensity and ni the charge of peak i. Figure 14 shows the average charges of the folded and unfolded myoglobin. The average charge is decreased from 20.66 to 11.73 when myglobin was denatured by a pH change. It will be a good exercise for students to calculate the average charge of the two mass spectra and determine the trend by themselves.

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The Positive mode vs Negative Ion Mode

ESI can be operated in a positive or a negative ion mode. The positive mode adds proton to the analyte whereas the negative mode abstracts proton from the analyte. Studies (*47*) have shown that the positive ion mode demonstrates more pronounced shifts to the higher charge states when protein is unfolded than the negative ion mode can do.

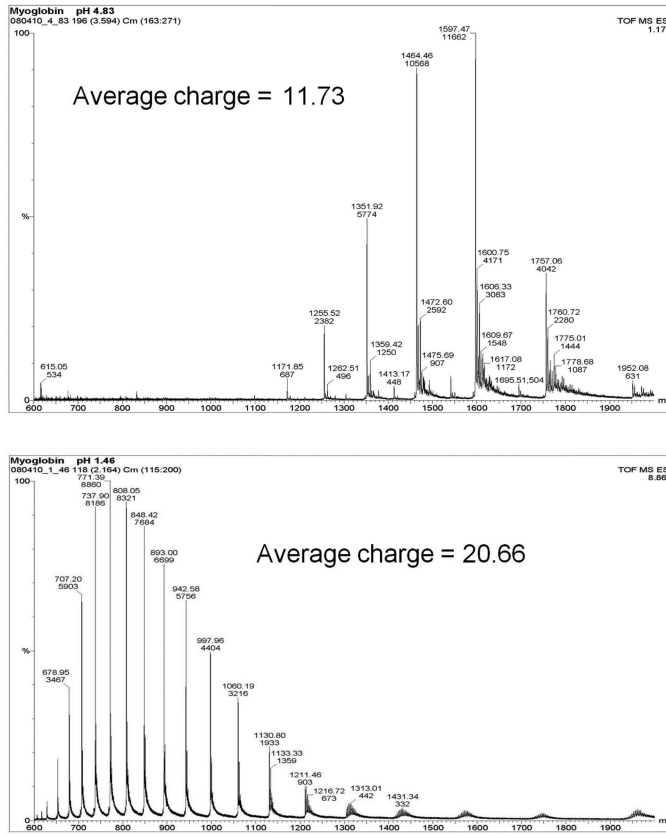

Figure 14. Top: the mass spectrum of the folded myoglobin shows the average charge 11.73. Bottom: The mass spectrum of the unfolded myoglobin shows averaged charge of 20.66. It demonstrates when protein is denatured, the average charge from MS-ESI increases.

The Calculation of Fraction of Unfolding

Provided with the average charges from each mass spectrum of protein assays, the unfolding curve can be plotted by using the average charges or fractions of unfolding converting from the average charges. We expect students to be able to derive the equation for the conversion of average charge to fraction of unfolding:

$$f_k = \frac{\overline{n}_k - \overline{n}_{Folded}}{\overline{n}_{unfolded} - \overline{n}_{folded}} \qquad \text{Eqn 17}$$

Experimental Consideration

To avoid the interference, Guanidine hydrochloride or urea is not used for ESI-MS method. Instead, myoglobin is unfolded by using either methanol-water (*48*) or pH (*49*, *50*). Students will be given the references to read before discussing how the experiment should proceed. Since this is a specialized experiment, more guidance needs to be provided to students.

Methanol-Induced Unfolding

Myoglobin stock solution is prepared at pH 4, adjusted by HCl. The methanol-water solvents with different percent (0 to 90%) are also prepared at pH 4. The same amount of protein stock solution is added to each methanol-water solvent. The assays are stored at 0°C for four hours until running through an ESI-MS.

Acid-Induced Unfolding

Myoglobin is dissolved in 3% methanol water solvent. Adjust the pH of the protein solvent mixture by using HCl or KOH to give a range of different pH's (2 to 7) verified by a micro pH electrode. Let the samples be kept at 0°C for 2 hours and run them through an ESI-MS.

Data Processing

Each MS spectrum has to be processed to determine the charge distribution and average charge, as explained in equations 14 and 16. The average changes are then converted to fractions of unfolding for the generation of the unfolding curve (equation 17). The unfolding curve is then fitted to equation 12 for the determination of C_m and m. Gibbs free energy of unfolding can then be calculated as the product of m and C_m as shown in equation 11. Figure 15 and 16 exhibit an example of the unfolding curve of myglobin determined by MS-ESI spectra.

An Example Result

Figure 15 shows a series of mass spectra obtained from the methanol-induced unfolding of myglobin at pH 4. By examing the mass spectra in Figure 15, students were asked to analyze the relationship between charge distribution and the percentage of methanol. It was not difficult for them to discover the association of high charge with a high methanol ratio and the unfolded state, and conversely, low charge with a low methanol ratio and the resulting folded state. Students then calculated the average charges of these spectra and made an unfolding curve with the average charge and its associated percentage of methanol. They then used the average charge from 0% methanol for the folded state ($\overline{n}_{folded}$) and the average charge from 100% methanol for the unfolded state ($\overline{n}_{unfolded}$) to calculate the fractional unfolding for each percentage of methanol :

$$f_{exp,\,k} = \frac{\overline{n}_k - \overline{n}_{Folded}}{\overline{n}_{unfolded} - \overline{n}_{folded}} \qquad \text{Eqn 17}$$

To fit their data of the experimental fractional unfoldings to equation 12,

$$f_k = \frac{\exp(-m\frac{C_m - C}{RT})}{1 + \exp(-m\frac{C_m - C}{RT})} \qquad \text{Eqn 12}$$

students used the EXCEL program to find the best values for m and C_m by applying the principle of least squares. EXCEL Solver plug-in can be used to derive a non-linear least squares fit. To use Solver, initial values for m and C_m were chosen and the fitted fractional unfolding was calculated using equation 12 for every percentage of methanol. The sum of squared differences between the experimental fractional unfolding ($f_{exp,\,k}$) and the fitted fractional unfolding (f_k) for each percentage of methanol in the transitional region were calculated. These values were derived from the pre-defined m and C_m. Students used Solver to determine the values of m and C_m that gave the minimal sum of squared differences.

Both the experimental fractional unfolding and the final fitted fractional unfolding were plotted against % methanol as shown in Figure 16. From the curve fitting, the two parameters, m and C_m, were determined as 890 and 38.934, respectively. The Gibbs free energy of the unfolding was then calculated as 890 x 38.934 = 34651 J/mol. This value was in fair agreement with 33 kJ/mol obtained from fluorescence intensity measurements.

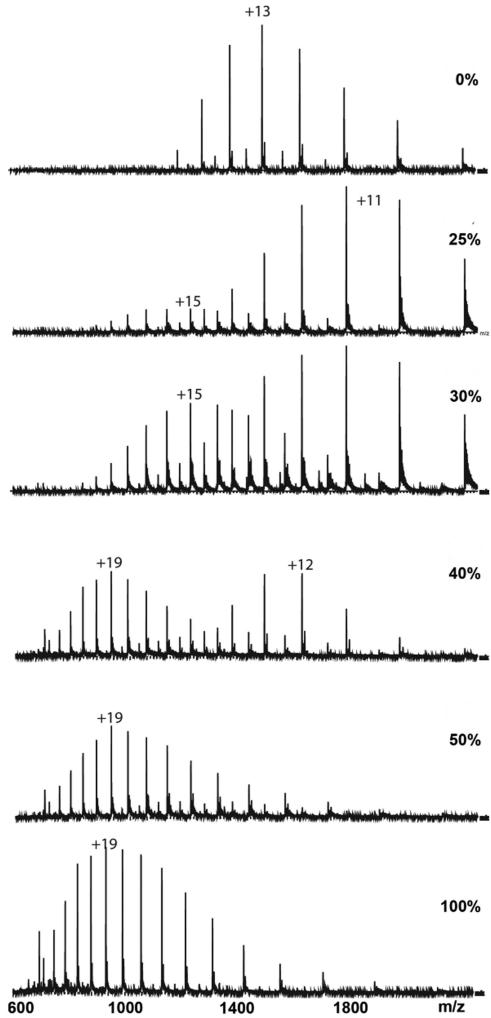

Figure 15. Mass spectra of myoglobin with different percentages of methanol, as indicated in the figure. All protein samples were kept at pH 4. This figure shows the charge distributions are shifted from low charge value to high charge value from top (0%) to bottom (100%). During the transition region (25%-40%), each spectrum has two distinct charge distributions indicating both folded and unfolded conformation coexisted. The numbers (+13, +11, etc) are the maximum charges of each peak of the charge distributions.

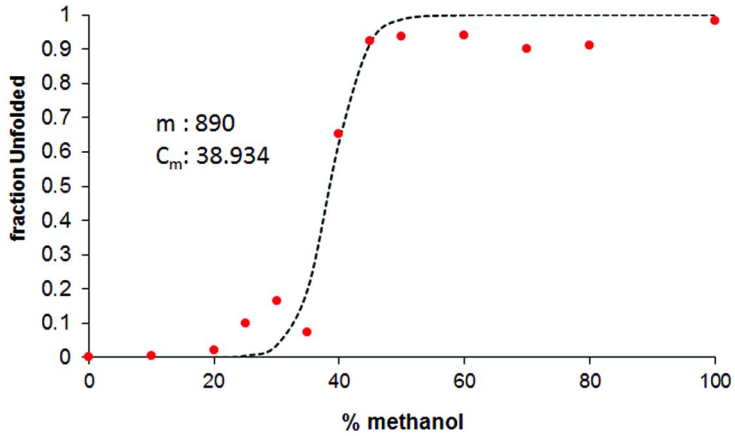

Figure 16. The unfolding curve from the methnaol induced denaturation of myoglobin followed by ESI-MS. Plots were generated by student data and a non-linear curve fit (dashed line) with Microsoft EXCELSolver plug-in.

Conclusions

Protein conformation and conformational change can be studied with basic instrumentation available to undergraduate students. By actively participating in the design and interpretation of the experiment, students can gain deeper understanding of not only the chemistry problem they have investigated, but also the instruments they used for generating the data. Not all instruments they used can provide the same quality of data. Each method has its advantages and limitations. Through the small group discussions and reflective writing, and working collaboratively during this effort, they have more chance to practice critical thinking and problem solving skills.

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Chapter 5

A Bioanalytical Microfluidics Experiment for Undergraduate Students

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A paper microfluidics experiment has been developed for the undergraduate analytical chemistry laboratory. In this laboratory experiment, students fabricated paper-based microfluidic devices and prepared the device for simultaneous colorimetric glucose and protein measurement. Students were given an "unknown" synthetic urine sample and a set of diagnosis protocols. The students prepared glucose and protein standards and analyzed the urine sample for glucose and protein content. The reagents changed color in the presence of glucose and protein in the urine sample. Students visually compared the extent of the color changes in the unknown to color changes from standard solutions. From these colorimetric results, students recommended whether the patient should undergo further testing.

Introduction

Microfluidic devices allow the control of tiny (*i.e.* nL) volumes of fluids in microscale channels and wells (*1*). Wired magazine has called them "computer chips with plumbing" (*2*). A citation report on the Web of Science® database for the term "microfluidics" resulted in over 1100 published items per year in 2011 versus less than 100 in 2001. The "holy grail" of microfluidic devices has been the development of a "lab-on-a-chip" (*3*). Analytical chemists call

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this a micro-total-analysis system (μ-TAS). The small sample sizes required by μ-TAS systems make them ideal for bioanalytical assays. For these reasons, it is beneficial to expose undergraduate students to bioanalytical assays on microfluidic devices. Traditionally, the fabrication and utilization of microfluidic devices have been confined to research laboratories with microfabrication facilities. The high cost associated with the equipment and materials has precluded general hands-on exposure of microfluidics to undergraduate students. Recently, different groups have reported on inexpensive fabrication processes for microfluidic devices including paper-based (*4–9*), shrinky dink-based (*10*), and thin plastic film-based (*11*). Nevertheless, there is a dearth of reports in the literature regarding microfluidics and the undergraduate laboratory curriculum.

Paper-based microfluidic devices (μ-PADs) have been utilized for glucose and protein assays in urine. These devices were intended for telemedicine applications in developing nations (*6*). This particular report fabricated devices using a photoresist in a "FLASH" rapid-prototyping method which created hydrophobic barriers to form channels (*5*). Subsequently, wax printing was demonstrated as an economical, efficient and reproducible method for patterning devices (*12*, *13*). We have developed an undergraduate laboratory experiment combining the colorimetric assays used for telemedicine (*6*) and the wax printing method for device fabrication (*12*, *13*). This experiment exposes students to clinical chemistry and bioanalysis, along with the emerging field of microfluidics. It also gives students an opportunity to discuss societal issues such as health care costs and accessibility.

A laboratory experiment titled "Simultaneous Determination of Glucose and Protein on a Paper-Based Microfluidic Device" was developed for the Quantitative Analysis Laboratory at Creighton University. Undergraduate research students helped develop the procedure, and the experiment was tested on students in Quantitative Analysis Laboratory for two semesters. Students were given a pre-lab assignment to aid in preparation for the experiment. When they arrived in lab, they were given a sheet of paper-based devices, an artificial urine sample for diagnosis, and a diagnosis protocol. Students worked alone or in pairs, depending on class size. Students made standard solutions and prepared the microfluidic devices for analysis. The preparation included spotting the devices with the correct colorimetric reagents for glucose and protein determination. Each set of devices contained the following: blank, unknown, glucose standards, and protein standards (see Figure 1 for sample student testing devices; diagnosis protocols are shown later). The students performed the analysis and estimated the glucose and protein levels in the urine sample by comparing to the color changes to those of the standards. From these semi-quantitative estimations, they diagnosed the patient. After completion of the experiment, students filled out a survey regarding the experience and completed a post-laboratory assignment. Survey results were positive, and students enjoyed the "real-world" application of the experiment. In the most recent test of the experiment, students diagnosed the glucose level correctly 80% of the time, while they were correct in the protein determination 50% of the time. Undergraduate research students, who scanned in all the images and analyzed with an imaging program, performed quantitative analysis. A 100% correlation was determined for the digital results and visual

results. A scanned image of a student's testing devices is shown in Figure 1. This student prepared the set of six devices (blank, unknown, protein standards [30 mg/dL and 80 mg/dL] and glucose standards [0.17 mM and 1.40 mM]) in duplicate. The experimental procedure given to students and instructor notes are subsequently reported in the remainder of this chapter.

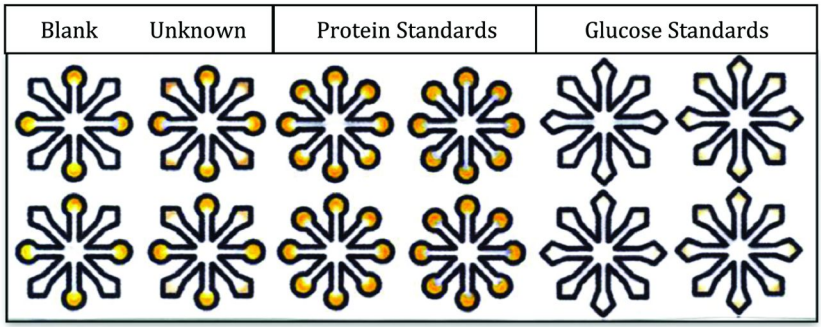

Figure 1. A scanned image of paper-based microfluidic devices used by a student. Each column represents duplicate devices (left to right): blank; unknown; protein standards (30 and 80 mg/dL) and glucose standards (0.17 and 1.40 mM). Note: the yellow reagent turns blue in the presence of protein and the colorless reagent turns reddish-brown in the presence of glucose. (see color insert)

Experimental Procedure Given to Students: Simultaneous Determination of Glucose and Protein on a Paper-Based Microfluidic Device

Pre-Lab

For some background on this experiment watch the following ~15 minute video, "George Whitesides: A lab the size of a postage stamp" url: http://www.ted.com/talks/lang/eng/george_whitesides_a_lab_the_size_of_a_postage_stamp.html Take some notes over the movie in your laboratory notebook.

If you are interested in this topic, another interesting video to watch is "George Whitesides: Toward a science of simplicity" *http://www.ted.com/talks/lang/eng/george_whitesides_toward_a_science_of_simplicity.html*

Read through this entire procedure and perform the calculations for the standard preparation procedure. You will be preparing *"working standard"* solutions from *"stock standard"* solutions. Obtain the exact concentration of the glucose and protein stock solutions from your instructor and calculate the volume of each stock standard required to prepare each dilute, or "working", standard solution. Prepare a table of the volumes of protein stock, glucose stock and artificial urine that you will be using.

Purpose

The purpose of this procedure is to simultaneously perform two bioassays, one for glucose and one for protein, on an (artificial) urine sample. Your assay will be performed on a microfluidic device that you fabricate in the laboratory. Given a diagnosis protocol, you will use the results of the bioassay to diagnose or make recommendations for further testing to the "patient".

Background

In our everyday lives we have seen many of the technologies we use such as computers, phones, and stereos become miniature versions of their predecessors. The field of microfluidics has allowed for the miniaturization of the scientific laboratory. Microfluidics involves the manipulation of fluids in micron-sized channels and wells. Various laboratory tasks such as pumping, mixing, filtering, and analysis have been performed on microfluidic devices (*1*). Microfluidic devices can also serve as microreactors to study hundreds of reactions in just a few days.

Many of the initial applications of microfluidic devices are in the area of analytical chemistry. Their small size has many advantages such as small sample and reagent consumption, low cost, short analysis time, portability, and a small "footprint". Microfluidic devices have the potential to ultimately be incorporated into lab-on-a-chip devices, also known as micro-total analysis systems (μ-TAS).

The primary procedures used to fabricate microfluidic devices are based on those already in place in the microelectronics industry to develop silicon-based computer chips. Coincidentally, microfluidic devices have been called "computer chips with plumbing" (*2*). These processes involve working in a cleanroom environment in which the number of dust and contaminate particles are controlled. These types of environments are typically only found at large research universities or in industrial or government laboratories. Recently, more economical methods for fabricating paper-based microfluidic devices have been reported in the literature (*4–6*). These methods used photolithography, but a wax-based printer can also be used (*12*, *13*). One intended use of paper-based microfluidic devices is for in-field medical diagnostics (telemedicine) in developing nations, where access to sophisticated instrumentation is limited (*6*).

In this experiment, you will use a wax-printer to fabricate a microfluidic device for chemical analysis. This process does not require a cleanroom, specialized equipment, or costly chemicals. You will prepare paper-based microfluidic devices and perform two bioassays ***simultaneously*** on your device. The sample flow in the device is controlled so that two assays are performed concurrently on a single sample. The assays are colorimetric tests for glucose and protein. The anticipated biological sample is urine, as urine tests are generally a noninvasive first screen for markers of disease. Although these tests are colorimetric, they can provide more quantitative results than just a "yes" or "no" for the presence of a compound. Using a camera or scanner as a "detector", a quantitative value can be determined. You will make a preliminary diagnosis

based on the color change and then submit your device to the instructor/TA's for digitization by a scanner and subsequent quantitative analysis.

Materials and Equipment

Materials for paper device preparation and for solution preparation will be provided.

Safety and Special Handling Procedures

Protective eyewear must be worn at all times. Tetrabromophenol blue is a dye that will stain skin and clothing.

Experimental Procedure

Fabrication of Paper Devices

If necessary, turn on the oven. Set to 150°C. Print or obtain from your instructor a sheet of 12 testing devices (two rows of six). A sample set of six is shown in Figure 2. Note that the glucose test zones are **diamond** shaped and the protein test zones are **circular**. A strip of six devices contains: two sets of combined tests, two sets of protein-only tests, and two rows of glucose-only tests. Note that you will introduce sample in the center. The hydrophobic channels (printed with a wax printer) will guide the solution through the channel to the test zone on the periphery. Note that each device is approximately one inch in diameter.

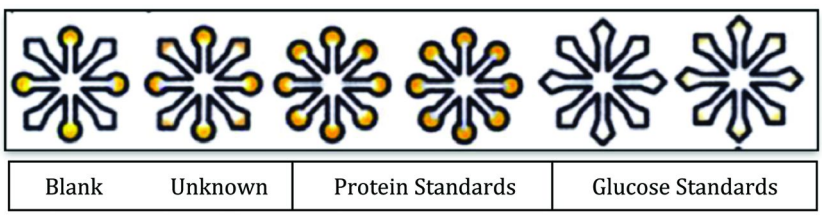

Figure 2. A scanned image of microfluidic devices used by students. You will need six devices for your assays. After appropriate spotting (protein reagents in circles and glucose reagents in diamonds), you will deliver the sample as follows: blank (artificial urine), unknown, protein and glucose standards. (see color insert)

The minimum number you need is six, but you can keep some extra as "back-ups". Note that the wax ink (black) creates a hydrophobic barrier on the hydrophilic paper.

Number six devices as shown in Figure 2 and as listed below. You may write on the paper to label the devices:

#1 = blank (a combination of protein and glucose test zones)
#2 = for your unknown (a combination protein and glucose test zones)
#3-4 = for protein standards only (circular test zone)
#5-6 = glucose standards (diamond test zones).

Ensure that the oven is at **150°C**. Place your sheet of paper on the oven rack. Close the oven door and start timer. Allow the sheet to remain in the oven for **90 seconds**. The heat melts the wax, allowing the wax to spread through the chromatography paper to make a hydrophobic barrier that will contain your solutions. The heat also spreads the wax laterally so your channels will looks smaller than when you printed them. Turn the paper over and check that the ink has spread through as shown in Figure 3.

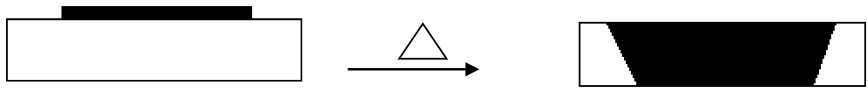

Figure 3. Cross-sectional representation of wax spreading laterally and vertically through the chromatography paper when heat is applied. The white area represents a cross-section of the paper; the black represents the wax ink.

Preparation of Devices for Assays

You will be using micropipettes for your analyses. You should verify the accuracy of your micropipetting. Your instructor will demonstrate proper pipetting technique. Instructions are in Appendix A at the end of this procedure. Before using the micropipettes (100 µL and 1000 µL) to prepare your standards, verify their precision and accuracy by measuring the amount of water delivered at a set volume on the analytical balance. Document the verification in your notebook. Your goal should be to achieve 3 trials with acceptable precision and accuracy (less than 1% error). Table 1 shows a sample table for your notebook.

Table 1. Sample Table for Micropipette Verification

Set volume (µL)	*Delivered mass (mg)*	*Delivered volume (µL)**	*Absolute Error (µL)* $\mu L_{delivered} - \mu L_{set}$	*% Relative Error* $\frac{\textbf{Abs. Error}}{\textbf{set volume}} \times \mathbf{100}$

* For example, the density of water is **0.9982 mg/µL** at 20°C.

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From your instructor, obtain the following solutions (*with the indicated labels*):

- **100 µL glucose reagent solution** (*GOx*)
- **100 µL priming solution** (*Pr*, pH 1.8 citrate buffer with 2% (w/v) Tween-20)
- **100 µL protein reagent solution** (*TB*)
- **~5 mL artificial urine** (*AU*) **solution, pH 6.0**
- **200 µL glucose stock standard** (*GS*, 25.0 mM)
- **400 µL protein stock standard** (*PS*, bovine serum albumin, 2.00 mg/mL)
- **100 µL unknown urine sample** (*record #*)

Also obtain a sheet of Al foil, the required micropipettes, and four centrifuge tubes in which to prepare your solutions. Most of the solutions you obtain will have been stored in a refrigerator or freezer. Before using them, 1) allow them to warm up to room temperature then 2) mix on the vortex mixer.

In your notebook, record the actual concentration of the stock standard solutions and your unknown number. Determine the volume of each standard required to prepare the following working standard solutions. Show your calculations and volumes to be delivered in your notebook.

1000 µL of 0.17 and 1.40 mM glucose
500 µL of 30.0 mg/dL and 80.0 mg/dL BSA

Prepare each solution in the given 1.5 mL centrifuge tubes. Dilute all solutions to the final volume with AU solution, and mix on the vortex mixer. *It may be helpful to make a table indicating the volume of standard and volume of artificial urine used to prepare each solution. Remember, the two volumes should equal the total desired volume.*

Analysis of Unknowns

Before beginning, completely read through this section and devise a plan for performing the assays. You will be performing an assay on a urine sample for glucose and protein simultaneously on the same device. The reagents will undergo a color-forming reaction. The intensity of the color in your unknown samples will be compared to that of samples containing standard solutions. Two standards will be analyzed for each assay. If you do not have a timer on your watch or phone, obtain a timer from your lab instructor. As shown in Figure 2, you will need a total of 6 devices: 2 for glucose standards; 2 for protein standards; 1 for your unknown; and 1 for your blank. You can cut the devices from your sheet in whatever manner you prefer, individually or in groups. Leave some room around the sides for labeling. It is important to label each device and even the specific test zones for the unknown and blank devices. Clearly write your name and date on the device.

Protein Assay Preparation

Use the 2.5 μL micropipette to spot 0.40 μL of priming solution into each protein test zone (circle-shaped) only. This solution keeps the pH sufficiently low so that the protein reagent, tetrabromophenol blue (TBPB), remains yellow (Figure 4). As indicated in Figure 2, the small circles along the periphery are the protein test zones. *An effective way to spot small amounts onto paper is to touch the pipette tip to the paper (which will begin to wick the fluid) then deliver the solution by pushing the pipette to its first stop. Do not push the second stop.* ***Your instructor will demonstrate this to you.*** Spot all eight test zones of the two devices that will analyze standard solutions. Also spot four circular test zones on the device for the unknown and four circular test zones on the blank device. Allow the devices to dry for five minutes at room temperature. *Do not allow the reagents to enter into the channel. This would make analysis of the test zones difficult.*

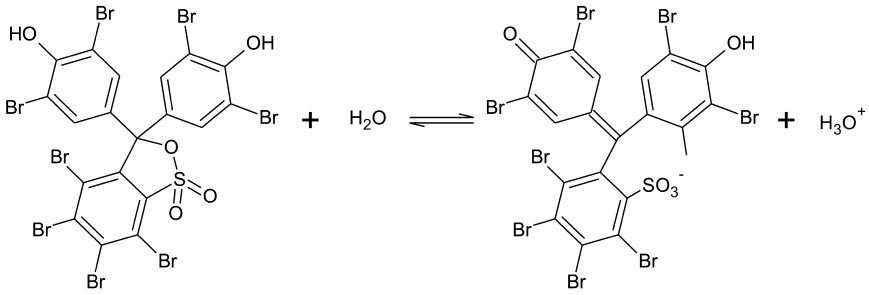

Figure 4. Acid-base equilibrium of tetrabromophenol blue. It is an acid-base indicator in the pH range 3.0-4.6. The acidic form appears yellow and the basic form appears blue. TBPB also appears blue when bound to protein.

Next spot each "primed" protein test zone with 0.30 μL of protein reagent solution (TBPB) using the same micropipette (new tip). Your test zones will now appear yellow as in Figure 5. Note that zone #5 has been perfectly spotted. Allow the devices to dry for 10 minutes in the dark (in a drawer or under a piece of Al foil) at room temperature. TBPB is a dye and will turn your skin blue. Be careful not to get TBPB on your hands!

Note: *Do not deliver TBPB in the channel (as in zone #8 in Figure 5). If the priming solution, which acts as a buffer, is not in the channel, a color change will occur even with pure AU solution since TBPB is an acid-base indicator. The buffer lowers the pH and prevents any color change occurring from pH. If this happens in one of your trials, do not include that test in your results. If necessary, adjust the volume of TBPB used to spot each zone. Perform the spotting until you have at least 3 properly spotted circles.*

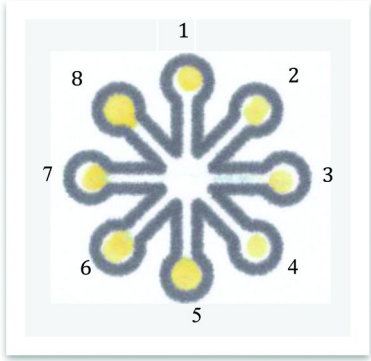

Figure 5. Proper and improper spotting. Ensure that the whole test zone is filled but that TBPB is not in the channel. Zones 1, 2, 3, 4, 6, 7 do not contain sufficient TBPB. Zone 8 has too much TBPB, with TBPB in the channel. Zone 5 is properly spotted. (see color insert)

Glucose Assay

Spot 0.50 µL of glucose reagent solution into each glucose test zone (diamond shaped). The number of glucose test zones includes eight for each standard; four on the unknown; and four on the blank. Refer to Figure 2. Allow the devices to dry for 10 minutes in the dark at room temperature.

Use a micropipette to deliver 14 µL ***each*** of the appropriate blank, unknown, and standard solutions to the center of each device. Use the same delivery method as before (touching the tip of the pipette to the paper and allowing the paper to begin wicking the fluid, then slowly expel the fluid). Your blank is the artificial urine solution. Perform this step efficiently and start the timer. See the note below before beginning.

Note: To ensure that the solution remains on each paper device and does not go through onto the benchtop, you can set the devices on a test tube rack or weighing boat. Another method is to prepare the back of the device with packing tape. You can remove the devices from the weighing boat once they are dry. Also, when you deliver the 14 µL with the micropipette, do not push the button to the second stop. The second stop delivers a burst of air that can cause splashing. The important factor is to treat all of your standards, samples and blanks the same. The best method is to hold the drop to the device and allow fluid to wick up the paper.

You should observe the fluid travel through the channels and notice color changes occurring in the test zones after a few minutes. The glucose zones will turn brown/orange as triiodide (I_3^-) is formed in a redox reaction. The edge of the protein test zones will turn from yellow to blue. **Obtain a scan or photograph of each device. Section 6 describes the scanning process.** While you are waiting,

note any other relevant observations. Also read through the next step and begin to make observations on the color changes of your unknowns relative to your standards. *The protein color will begin to fade, so be sure to capture its picture and make observations in a timely manner.*

Looking at the relative colors of the test zones on your devices, estimate the glucose and protein concentration ranges of your unknown sample. For example, is the glucose concentration lower than your lowest standard, higher than your highest standard, or in between your standards? When looking at the protein test zones, ignore any blue color in the channel; the reagent will turn blue when interacting with "un-primed" paper. Document your observations in your notebook. Make a table such as Table 2 to quickly record your observations.

Table 2. Sample Table for Color Change Observations

Unk #_____	*Below low standard*	*Between standards*	*Above high standard*	*Diagnosis*
Glucose level				
Protein level				

Given the information on Tables 3 and 4, determine your recommendation or diagnosis for the patient. Evaluate your standard concentrations/relative colors. Then, based on the relative color intensity of your standards and unknown, mark in what range your unknown glucose and protein samples are likely to lie. Check the appropriate boxes and write down your diagnosis or recommendation based on Tables 1 and 2. Note if your results are inconclusive.

Table 3. Random Urine Glucose Screen Protocol*

Urine glucose levels	*Diagnosis*
0.17 mM – 1.4 mM (3 – 25 mg/dL)	No diabetes
1.40 mM (> 25 mg/dL)	Patient should undergo further blood testing

* Adapted from reference (*14*).

Table 4. Protein in Urine as a Disease Indicator*

Urine protein levels	*Diagnosis*
< 30 mg/dL	Trace levels, normal
30 mg/dL – 80 mg/dL	Further testing, possible proteinuria
> 80 mg/dL	Further testing, possible subnephrotic range proteinuria or nephrotic syndrome

* Adapted from reference (*6*).

Reporting Requirements

Use the results of your visual and/or quantitative analysis to diagnose the patient or determine if the patient should undergo further testing. Explain your reasoning. Make a table in you notebook similar to Table 2 to indicate where your glucose and protein unknown concentrations lie relative to the standard concentration ranges. Table 5 shows the grading rubric for this experiment.

Table 5. Grading Rubric

Work	*Possible points*
Pre-lab work documented and correct	10
Micropipette verification	20
Glucose assay and diagnosis	20
Protein assay and diagnosis	20
Correct procedure followed	10
Post-lab questions	20
TOTAL	100

Waste Disposal

Your used paper devices can be thrown in the trash. Turn in any unused devices to your instructor. Also return the Al foil pieces to your instructor, as these can be reused. All aqueous solutions can be poured down the drain. Return your unknown solution, glucose reagent, priming solution, and TBPB to your instructor for disposal.

Appendix A: Operation of Eppendorf Adjustable Pipettes

Volume Setting

The volume is adjusted by pressing down the lateral catch and turning the control button at the same time.

It is advisable to carry out volume setting from the higher down to the lower value (*i.e.* first go above the desired volume and then return to the lower value).

Pipette Tips

Typically the color of the control button will correspond to the color of the Eppendorf tip or tip rack. ***For the best precision and accuracy, pre-wet all new tips by aspirating and dispensing liquid 2-3 times before pipetting.***

Aspirating Liquid

Attach suitable pipette tip to the pipette firmly. Press down the control button to the first stop (measuring stroke). Immerse the pipette tip vertically ~3 mm into the liquid. Allow the control button to slide back *slowly*. Pull the tip out of the liquid *slowly*. To remove any remaining droplets, dab with non-fibrous cellulose material, ensuring that liquid does not come out of the tip. You can also dab on the side of the beaker containing the liquid you are pipetting.

Dispensing Liquid

Hold the tip at an angle against the inside wall of the tube/flask. Press down the control button slowly to the first stop (measuring stroke) and wait until the liquid stops flowing. Press down the control button to the second stop (blow-out) until the tip is completely empty. Hold down the control button and pull the tip out of the inner wall of the tube/flask. Allow the control button to slide back slowly. Pressing the control button to the final stop ejects tip.

Do not lay down the pipette when a filled pipette tip is attached as this may result in liquid entering the pipette.

Verification of Pipette

You can verify that the pipette is performing accurately by dispensing nanopure water from a pre-wetted tip into a tared flask or tube onto an analytical balance. Typically, one might test full volume and ½ volume, e.g. 500 and 1000 µL for a 1000 µL pipette.

Convert the mass to volume by dividing by the density at room temperature. For example, the density of water is **0.9982 mg/µL** at 20°C. This number is the

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volume actually delivered by the pipette. Determine the error relative to the set value. Repeat a few times to verify that the pipette is accurately delivering water. If not, consult your instructor. Refer to Table 1 for a sample data table.

Instructor Notes

Instructor Notes for Simultaneous Determination of Glucose and Protein on a Paper-Based Microfluidic Device

Pre-Lab

Less than one week before lab (the closer to lab the better) the instructor or TA must prepare the solutions outlined in Tables 6 and 7. Table 6 describes the solution identity and volume needed per assay and the storage conditions for each solution. Table 7 describes the preparation of each solution and the CAS number of each component. Bulk solutions can be prepared and aliquotted into appropriate centrifuge tubes. A set of seven tubes containing solutions along with four empty tubes will be given to each student or pair of students. The instructor must also draw and print out the device designs as shown in the experimental procedure. For this work, we used AutoCad to draw the devices, but other drawing programs may be used.

Table 6. Solutions Needed To Perform Glucose and Protein Assays

Tube #	*Solution identity and concentration*	*Volume needed per assay*	*Storage conditions*
1	Artificial urine (AU) solution, pH 6.0	5 mL	Refrigerated Freezer (long term)
2	Glucose standard solution (~25 mM, prepared in AU)	200 μL	Refrigerated
3	Glucose reagent solution (5:1 solution of glucose oxidase-horseradish peroxidase, 0.6 M KI, and 0.3 M trehalose in a pH 6.0 phosphate buffer)	200 μL	Freezer
4	Protein standard solution (2.0 mg/mL, bovine serum albumin (BSA) obtained from Thermo)	500 μL	Refrigerated Freezer (long term)
5	Priming solution (92% water, 8% ethanol v/v and 750 mM citrate buffer (pH 1.8) with 2% w/v Tween-20)	100 μL	Room temperature

Continued on next page.

Table 6. (Continued). Solutions Needed To Perform Glucose and Protein Assays

Tube #	*Solution identity and concentration*	*Volume needed per assay*	*Storage conditions*
6	Protein reagent solution (95% ethanol, 5% water v/v and 9 mM tetrabromophenol blue)	100 µL	Room temperature and in the dark
7	Unknown (glucose and protein mixture)*	100 µL	Refrigerated Freezer (long term)

* Use the diagnosis protocols (Tables 3 and 4) to determine appropriate unknown concentrations. Table 8 shows two suggested concentrations for unknowns that students analyzed.

Table 7. Solution Preparation and CAS Numbers

Chemical	*CAS*
Artificial urine (AU) solution, pH 6.0*	
1.1 mM lactic acid	50-21-5
2.0 mM citric acid *or sodium citrate, dihydrate*	77-92-9 *6132-04-3*
25 mM sodium bicarbonate	144-55-8
170 mM urea	57-13-6
2.5 mM calcium chloride *(dihydrate)*	10035-04-8
90 mM sodium chloride	7647-14-5
2.0 mM magnesium sulfate *(heptahydrate)*	10034-99-8
10 mM sodium sulfate	7757-82-6
7.0 mM potassium dihydrogen phosphate *aka potassium phosphate, monobasic (H_2KPO_4)*	7778-77-0
7.0 mM dipotassium hydrogen phosphate *aka potassium phosphate, dibasic (HK_2PO_4)*	7758-11-4
25 mM ammonium chloride	12125-02-9
adjust pH with hydrochloric acid solution	7647-01-0
D-(+)-Glucose	50-99-7
Glucose Reagent Solution (in a pH 6.0 phosphate buffer, 0.25 M) 5:1 solution of glucose oxidase-horseradish peroxidase (120 units of glucose oxidase enzyme activity and 30 units of horseradish peroxidase enzyme activity per mL of solution)	
glucose oxidase, Type X-S: from aspergillus niger (freezer) *(100,000 – 250,000 units per gram of solid)*	9001-37-0

Continued on next page.

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Table 7. (Continued). Solution Preparation and CAS Numbers

Chemical	*CAS*
peroxidase, type I, from horseradish (lab fridge) *(50 – 150 units/mg of solid)*	9003-99-0
potassium iodide (0.6 M solution)	7681-11-0
D(+)-Trehalose dehydrate from corn starch (0.3 M solution)	6138-23-4
Protein Standard Solution (2 mg/mL, bovine serum albumin)	9048-46-8
Priming Solution (protein assay)	
92% nanopure water (v/v)	
8% ethanol (v/v)	64-17-5
600 mM citrate buffer (pH 1.8)	6132-04-3
Tween-20 (2% w/v)	9002-89-5
Reagent Solution (protein assay)	
95% ethanol (v/v)	64-17-5
5% nanopure water (v/v)	
tetrabromophenol blue (9 mM solution)	4430-25-5

* Prepared per reference (*15*).

Table 8. Suggested Unknown Solution Preparation*

solution	*Glucose conc. (mM)*	*Protein conc. (mg/dL)*	*Glucose stock vol.*	*Protein stock vol.*	*AU vol.*	*Total vol.*
Unknown #1	0.70	100				
Unknown #2	2.50	20				

* The unknowns will be prepared from the stock standard solutions: 25 mM glucose and 200 mg/dL protein. You can fill in the remainder of the table, depending on the total volume of unknown required.

The following **materials and equipment** are required to perform the experiment (semi-quantitative analysis):

- Whatman #1 chromatography paper (20 x 20 cm, cat. # 3001-861)
- Wax printer (e.g. Xerox Phaser 8560)
- Oven or hot plate set at 150°C
- Assorted micropipettes (e.g. 2.5, 100 and 1000 μL micropipettes)
- Centrifuge tubes (1.5 mL and 5 mL)
- Weigh boats
- Test tube racks

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If a quantitative analysis is performed, image analysis software is needed (e.g. Adobe Photoshop or ImageJ (http://rsbweb.nih.gov/ij/), or equivalent).

Protein Assay Chemistry

Tetrabromophenol blue (Figure 4) is an acid-base indicator in the pH range 3.0-4.6. The acidic form appears yellow and the basic form appears blue. TBPB also appears blue when bound to protein. This can be explained to students in the text or could be the subject of questions on a pre or post-lab assignment.

Glucose Assay Chemistry

Glucose reactions:

Glucose $\rightarrow$ $\mathbf{H_2O_2}$ + gluconic acid (catalyzed by GOx)

$\mathbf{H_2O_2} + 3I^- + 2H^+ \rightarrow I_3^-$ *(brown)* $+ 2H_2O$ (catalyzed by horseradish peroxidase)

These reactions could be given to students or the subject of questions on a post or pre-lab assignment.

Safety/Special Handling Procedures

Protective eyewear must be worn at all times. Tetrabromophenol blue is a dye that will stain skin and clothing. For the instructors or TAs preparing buffers: hydrochloric acid is corrosive and must be handled carefully.

Experimental Procedure Tips for Instructors

Working Standard Preparation. Table 9 shows the calculated solution volumes for working standards the students will prepare from 25 mM glucose stock and 2 mg/mL (200 mg/dL) protein stock. Before preparing these solutions, students will calibrate or verify two micropipettes: a 10-100 µL and a 100-1000 µL. The instructor should demonstrate proper use and handling of micropipettes.

Table 9. Example Table for Standard Solution Preparation

Working standard Conc.	*Stock standard vol. (μL)*	*AU vol. (μL)*	*Total vol. (μL)*
0.17 mM glucose	6.8	993.2	1000
1.40 mM glucose	56	944	1000
30 mg/dL protein	75	425	500
80 mg/dL protein	200	300	500

Chemical Reactions

Students can be given the chemical reactions in the procedure or may answer questions (pre-lab or post-lab) where they determine or investigate the various reactions that result in the observed color changes. The reactions include acid-base, biochemical and redox.

Device Spotting

The instructor should demonstrate proper handling and spotting with the 0.1 – 2.5 μL pipette. A GC syringe can also be used for the spotting. Particular care should be used for spotting the protein reagents as they do not elute *with* the sample solution. Thus the blue color forms at the front edge of the test zone. The instructor should also reiterate which test zones (diamond vs. circular) correspond with which reagent (glucose and protein, respectively). Figure 2 from the procedure can be referred to.

The minimum number of devices required per analysis is six, but students can keep some extra as "back-ups" or perform the analysis in duplicate. Student skills improve with repetition of micropipetting.

The students should number six devices as shown in Figure 2 and as listed below. They should write on the paper to label the devices:

#1 = blank (a combination of protein and glucose test zones)
#2 = for your unknown (a combination protein and glucose test zones);
#3-4 = for protein standards only (circular test zone);
#5-6 = glucose standards (diamond test zones).

Assay Procedure

It is important to emphasize that students read through the analysis procedure in its entirety beforehand. Once the unknown or standard solution is introduced into the center of the device, the students must quickly make and document their

observations about the relative color changes of the unknown versus the standards. Students should have their notebooks set up to record these observations.

Other Suggestions

When spotting the test zones, students can adjust the micropipettes to optimize the reagent spotting (i.e. priming solution range 0.300 – 0.350 μL and TBPB solution range 0.375 – 0.425 μL)

When making the diagnosis, students can cross out or disregard the test zones that were improperly spotted)

Ensure that the TBPB is stored in the dark. Devices spotted with TBPB can be covered in Al foil while drying.

Grading

Grading will vary depending on how instructors modifiy the laboratory experiment. For example, some instructors may want to have students evaluate different device designs. The grading rubric shown in Table 8 was used in our test of the experiment.

Example Post-Lab Questions

For a post-lab assignment, students could be asked to read through some of the literature references (e.g. reference (*6*) was given to students) or look up some of the colorimetric chemical reactions. Below are some sample post-laboratory questions that were given to students.

1. Which part of your device is hydrophilic? Which part is hydrophobic? Use this information to explain why the aqueous solution in contained in the channels and is able to flow through the channels on the device.
2. The glucose assay contains the following reagents: glucose oxidase (GOx), horseradish peroxidase (HPOx), potassium iodide, and trehalose. a) What is the role of each reagent? b) The color change results from a redox reaction between hydrogen peroxide and iodide (products are water and the brown-colored tri-iodide I_3^-). Write the half reactions below. From the half reactions, write the balanced overall reaction. The half reactions can be found in the appendix of your analytical chemistry textbook, or you can balance them yourself.

3a. Tetrabromophenol blue (TBPB) is not only used as a dye that binds to proteins, but is an acid-base indicator. Its chemical structure is in the experimental procedure. a) What is the color (yellow or blue) of:

   i. The acidic form of TBPB
   ii. The basic form of TBPB

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iii. The protein-bound form of TBPB
iv. The unbound form of TBPB

3b. What is the approximate pH of the priming solution? What is the pH of the AU solution? What do you suppose is the purpose of the priming solution?
4. During the development of this procedure, the volume capacity of a device had to be determined. Suggest an experiment to determine the volume of water that a device can hold.

Micropipette Operating Instructions

A demonstration may be required to demonstrate to students the proper usage and handling of micropipettes. Instructions for the Eppendorf brand micropipettes were added to the experimental procedure (Appendix A). A verification protocol has also been added to the procedure.

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Chapter 6

Teaching Bioanalytical Chemistry: Application of the SMILE initiative to Bioanalytical Chemistry Instruction

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Analytical chemistry labs have traditionally focused on students doing just enough in the scheduled time to obtain the correct answer, with a very limited focus on developing the essential critical-thinking skills, the ability to question the significance of the numbers that they have experimentally obtained, and the limitations of the instrument used to find the magic number. In an effort to enhance the student's skills in these areas, to boost enthusiasm, and increase competency, we have instituted a major pedagogical change in the instrumental and quantitative analysis courses. The transforming discovery project-based labs that we have incorporated are intended to inspire students to design, construct, optimize, and perhaps even troubleshoot small mobile instruments for laboratory enhancement (**SMILE**). To engage students further, the SMILE initiative requires students to develop a comprehensive suite of bioanalytical experiments to go with the instruments that are directed towards lower-level college, high school, and middle school science students. The highly multidisciplinary nature of SMILE allows for co-curricular experiences, and provides an ideal platform for lively discussions in analytical and bioanalytical chemistry.

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Analytical chemistry has developed into an interdisciplinary science with rather close relations to mathematics, physics, biology, computer science, and instrumentation science. At Penn State University, science and engineering majors are required to take a set sequence of freshman-level General Chemistry courses. Chemistry and chemistry-related majors then progress to sophomore-level Organic Chemistry courses. How they choose to proceed beyond this level depends on the specific degree program and students' interest. The Analytical Division is involved in a sophomore-level Analytical Chemistry and senior-level Environmental and Forensic Chemistry courses, as well as a variety of specialized Instrumental Analysis courses focusing on Chemical Spectroscopy, and Chromatography and Electrochemistry. Graduate chemistry students are offered several analytical courses, including Electroanalytical Chemistry, Analytical Separations, and advanced Chemical Spectroscopy. The overarching goal of these analytical chemistry courses is not just to train our students to make careful and reliable measurements, but to have them understand chemistry concepts at a level that they can assimilate, generalize and then apply this understanding to a new situation, instrument or analytical methodology. At the same time, we wish to capture student interest, increase student engagement, focus on topics that concern the practice of modern analytical chemistry, and perhaps develop a desire in our students to pursue the subject matter upon graduation.

Many of the undergraduate students making their way through a chemistry curriculum have a strong interest in the biological, pharmaceutical and medical sciences, where the importance of precise and consistently accurate bioanalytical measurements is paramount. For these reasons our analytical chemistry framework, particularly the Quantitative Analytical Chemistry, Instrumental Analysis and Forensic Chemistry courses have been revised over the years to include a significant biological content, to the point where bioanalytical chemistry is now an integral part of our undergraduate chemistry education.

The combined courses make use of an experiential initiative that builds understanding through a process that designs, develops, and then makes use of small mobile instruments (SMILE). The lab component is the centerpiece of the students' learning experience. This approach reverses the traditional format where early mastery of factual material is strictly required before the students are allowed to explore the really interesting hands-on problems. The lecture component of our analytical chemistry courses interweaves a traditional lecture format with online and 'on-demand' laboratory instruction. The online format requires students to review lecture material at their leisure, prior to attending class (flipped classroom, flipped learning, reverse instruction method), and then attend separate lab and discussion sections where they work in small groups on project related issues. The problems directly apply to concepts and principles that the students need to successfully complete the project-based labs. We believe that students respond and perform better with this format simply because they have more exposure to the material, using both on-line preparation and regular problem-solving sessions. As the projects proceed, students can also identify supplementary lecture topics based on their needs, for instance if they come across some stumbling block with which they need assistance and a more in-depth explanation. By allowing students to dictate certain aspects of the course, they

take responsibility for what happens in the laboratory, an approach that we have found strongly facilitates student learning. This tried and tested initiative, deliberately entwined into our analytical chemistry offerings, successfully allows students to not only gain an understanding of how instrumentation works, but also provides the students with a hands-on experience of how the instruments are used to measure biological substances.

The SMILE Initiative

Students that plan on pursuing STEM related careers must be technologically literate, be competent in operating various types of instrumentation, and have some knowledge of the components and limitations of the instrument and the technique employed. Although students encounter instrumental techniques in courses other than analytical chemistry, instrumental analysis courses are the primary mechanism for providing technology education in the chemistry curriculum. It is within the instrumental analysis course where students get hands-on operating experience or detailed explanations of how instruments work. Many instrumental analysis courses survey 10 to 15 instrumental techniques within a typical one-semester (15-week) course. The laboratory component offers hands-on experience with a subset of these methods. Clearly, the existing structure compromises the ability of even the best students to have a meaningful experience, i.e. to be able to obtain deep conceptual understanding of any one technique, simply due to information overload. Unfortunately, this "National Model" for instrumental analysis renders a senior-level capstone course to nothing more than a general survey course. Our own informal survey of 50 chemical and pharmaceutical companies (28 respondents) indicates that an ability to modify, troubleshoot and maintain existing instrumentation is highly valued but remains a skill that is lacking in almost all chemistry undergraduates.

In an effort to help students develop critical-thinking skills – both conceptual and practical, we have instituted the SMILE initiative. This approach represents a much needed pedagogical transformation of undergraduate STEM education, at the heart of which is a strong desire to increase the engagement of students and teachers alike through a successful synergy between faculty research and all levels of course curricula. Although the breadth of material is reduced (number of instruments discussed in the course), the initiative does develop a more rigorous in-depth exposure to a select set of instruments, thus making it easier for the student to grasp the more difficult concepts.

Our upper-level students are required to build a functional instrument as part of a semester-long class research project. The students build, from scratch, calibrated and quantitative instruments, using their rudimentary electronic and soldering skills. Students spend a significant amount of time tweaking, adjusting and repairing their instrument in order to pass validation tests. An important part of the project requires that they must develop and/or adapt an experimental lab, making full use of their instrument, and geared towards the lower-level chemistry student. The guided-inquiry learning experience requires students to be the principal investigators; to first submit a literature-based research proposal,

and then work as a team to complete the tasks, present their research at a chemistry-wide poster symposium, and write a final technical project report.

The basic design and methods for probing chemical systems between commercial instruments and the student-built instruments are identical. The most substantial differences are the lack of computer control, software ("bells and whistles"), and a variety of the high-tech electronic devices that give superior sensitivity. To-date, the SMILE initiative has created a handful of low-maintenance, low-operating cost, mobile instruments (*1–5*), including a bar-code scanner, an electrostatic lifter, a static NMR probe that is compatible with an existing liquid-state NMR spectrometer, a colorimeter, two types of fluorimeters, a Karl-Fisher apparatus, a dissolved oxygen probe, a conductivity meter (currently being used as part of a water quality educational initiative in Ghana), high-pressure liquid chromatography columns, and a cyclic voltammeter, all of which cost between $50 and $180 in parts to construct. A number of instruments continue to be designed and developed, such as a GC-FID, a temperature-gradient gel electrophoresis unit, a portable nitroaromatic explosives detector, a photoacoustic/piezoelectric cell, and a magnetic susceptibility balance. The course instructor is no longer an unapproachable authority figure, acting as the primary source of information; the SMILE initiative demands that the instructor is a facilitator, one who makes sure that students understand the tasks on-hand, assists students to discover the analytical concepts for themselves, and helps students stay within the allotted time.

The senior-level student's focus is primarily on the instrument and its capabilities. Whereas the lower-level student's focus is on the experiment, taking full account of the performance characteristics of the analytical instrument and significance of the quantitative results. As the younger peers advance through the curriculum, their knowledge of instrumentation and their ability to use instruments in more sophisticated ways increases until they are involved in designing a new instrument, or problem-solving and modifying existing instruments, that are then used by their younger peers to achieve valid analytical results. The additional benefit of such instruments is that the low-price, low-maintenance and low-operating costs allows for deployment of multiple units in the lower level courses. Some of the new instruments are donated to high schools and middle schools, thus allowing Penn State to build vital connections with pre-college teachers.

It is important to stress that these instruments involve relatively simple electronic components. Although the science or chemistry major may never become an instrumentation expert, he/she should have a reasonable understanding of the basic principles and applications of the more common electronics devices. The scope intended for the course at the undergraduate level is one of familiarization. So, the inclusion in the Instrumental Analysis course of a few experiments that involves the use of modular electronic components (snap-circuits) helps to give valuable insight into their role and function in all analytical instruments. We make it very clear to our students that we do not seek to turn them into electronic engineers in the space of a few weeks. The lecture material is restricted to topics relevant to understanding the workings of analytical instruments and modules that are in general use in chemical/biochemical analytical

laboratories, such as electromagnetic sources and detectors, optics, mirrors, filters, signal amplifiers, recorder and integrator assemblies, analog/digital converters, and how to computer interface. One aim of the course is to enable students to develop an understanding of how to make their analytical instruments more versatile or more appropriate to their needs by way of optimization towards a particular experiment. Our hope is that students will develop a very hands-on and practical approach to their measurements and be able to read a circuit diagram, fault-trace and pinpoint problems with the aid of a tester screwdriver and a multimeter, and perhaps even rectify problematic issues with the aid of relevant instrument manuals.

The SMILE projects are woven into the existing instrumental analysis curriculum. For example, in the laboratory component of the chemical spectroscopy course, students build simple R-C-L-diode-transistor-op amp breadboard circuits during the first two weeks of the course then build an AM radio in the third week. The students thoroughly enjoy building the radio (which they get to keep) as it incorporates each of the above components into a single elegant, and yet simple device. The radio also fosters a more seamless introduction to NMR spectroscopy later in the semester. In the fourth week, students focus their attention to a breadboard-based mini-spectrophotometer, for which they design a power supply to drive a diode laser and a photodiode transimpedance circuit with variable gain coupled to an analog-to-digital converter, which they configure. Students then make use of their breadboard spectrophotometer to measure the absorption of several standard dye solutions and compare their results to that of a commercial instrument. In the fifth week, students begin work on their SMILE instrument. No additional formal lab time is allotted for work on their SMILE instrument after the fifth week; students are simply required to work on their project in the lab on their own time. For the remainder of the semester, students must also work on three laboratory instructional modules. The modules vary each year, for example, one module incorporates fluorescence, UV-VIS absorption and IR/Raman spectroscopy using research-grade instrumentation (Fluorolog 3, Cary 4000, Nicolet 6700 models), where students investigate excimer and non-excimer formation in pyrene and anthracene solutions. Students collect the absorption spectra, obtain the appropriate excitation wavelengths then obtain the fluorescence emission profiles for pyrene and anthracene as a function of concentration. They then investigate the inner filter effect using synchronous scanning fluorescence (SFS) on a mixture of four polynuclear aromatic hydrocarbons (fluorene, anthracene, perylene, benzofluorene). SFS is a useful technique for isolating the fluorescent signals of each component in a simple mixture for quantitation purposes without requiring a prior chromatographic separation step. Finally, students collect the emission spectra of water at five different excitation wavelengths, calculate the corresponding IR/Raman vibrational modes and plot the calculated frequencies on an IR spectrum of water.

The instrumental analysis courses are writing-intensive courses which satisfy university-wide general education requirements. Graded elements include lecture exams, lecture activities (in-class group work on problems), homework problems, laboratory notebooks, written reports and a poster presentation. The SMILE

projects constitute 75% of the course grade. Faculty guidance does not ensure a successful end to any SMILE project. The outcome is very much based on the students' initiative, motivation, time-management and organization skills, and work ethic. SMILE project grades are based on the student's laboratory notebook, the literature-based research proposal (submitted and accepted prior to initiating any work on the project), a journal-style manuscript submitted in successive iterations (introduction, experimental methods, results and discussion; each section with several review and revision cycles), a laboratory guide for each of the experiments developed by the students geared towards lower-level chemistry students, and an end-of-semester poster symposium.

Lab Adoption

We are not advocating radical change in the manner in which instrumental analysis courses are taught at college. However, we do believe that the college curriculum can be more responsive to industry needs as well as graduate programs in the chemical sciences. The SMILE approach, whether instituted as a single modular component or as a unifying theme to achieve integration of course topics, is tailor made for adoptive use. Faculty, students, and school teachers have carefully compiled a set of instrument manuals and appropriate lab protocols. The manuals are clearly written, packed with practical information, and sprinkled with illustrations throughout. The manuals also include an introduction to laboratory safety, and a detailed tutorial on Excel for data and statistical analysis.

The confidence we place in the SMILE initiative as a tool to enhance understanding of how measurements are made, to boost student enthusiasm, and increase student engagement in analytical chemistry, has lead to its inclusion in a DoE funded Upward Bound-TRIO summer workshop. This novel program at Penn State is designed for professional (grade 7-12) school teachers, and high-risk inner city students to experience the joys and opportunities that are available to them *at* and *through college*. In this combined outreach-program the teachers learn new topics and lab techniques in chemistry, and the residential school students assist in the construction of small analytical instruments and test out the newly developed lab experiments. The students serve as a 'living laboratory' for the teachers to try out newly acquired skills from the SMILE workshop. The suitability of the analytical chemistry module for high-school adoption is discussed at length during the summer workshop. Newly constructed instruments, well-tested lab applications, manuals for both students and instructors, and some essential supplies and lab materials are made freely available to the school teachers. All of the lab procedures are ready for classroom use, and unfamiliar techniques are illustrated in detail in the instructor's manual as a ready reference. The overall feedback from this workshop over the years from both school teachers and Upward Bound-TRIO students has been overwhelmingly positive and enthusiastic.

A local middle school has recently adopted the SMILE initiative by introducing the colorimeter project as a cross-curricular vehicle to connect the science, technology, engineering, and math (STEM) curricula. Five teams of 8th

grade technology students each built a colorimeter over a period of several weeks. Once completed, the students used the devices in their science class to quantify food colorings in a variety of products. In their algebra class, the students utilized Beer's Law to convert the percent transmittance data to absorbance, and then graphed the results in Excel. Upon returning to their science class, these 8th grade students then analyzed, discussed, and incorporated the results into their lab reports. Such inspiring and supportive middle school experiences can help change students' trajectories for the better, preparing them well for success in high school, college and the workforce.

SMILE Initiative and Bioanalytical Labs

The Instrumental Analysis course at Penn State is an introduction to the measurement of chemical and biochemical systems using analytical instruments. Each type of instrument has a unique set of strengths and weaknesses that makes it suitable for some measurements, but not for others. Throughout the course, the fundamental principles underlying common instrumental methods are discussed. Our goal for the course is to enable students to class the instruments based on a similar measurement of a chemical or physical property, and be able to operate any analytical instrument based on similar principles. Students should ultimately be able to identify with confidence suitable instrumental methods for particular analytical problems.

The use of electronic instruments over classical methods of chemical analysis allows for greater sensitivity, selectivity, accuracy, precision, speed, lower limits of detection and automation. The aim of our analytical chemistry courses is to provide a thorough education in the theoretical fundamentals of methods of analysis, with emphasis on the possibilities and limitations of the method chosen and the instrument being used. We are also mindful that our courses must stay current, and that our undergraduate courses should appeal to a wide audience; from the student with an interest in meteorology to the pre-medical student, from the student interested in polymeric materials to the geochemist. The greater majority of our upper-level chemistry students have already declared that they wish to pursue the biological, biotechnological, and medical sciences, and because of this a number of our chemistry (organic, physical, inorganic, and analytical) labs include a significant biological aspect. Bioanalytical science is the development of new measurement approaches and their application to solve problems in biological systems. Several lab experiments with a biological incline have been developed in our analytical chemistry courses, thus equipping future biologists and medical professionals with a solid intellectual foundation that will better enable them to understand the science involved in making and interpreting chemical measurements. Each of the experiments we describe here has been carefully selected by faculty, fully developed and tested by senior-level students, and refined over a number of years according to the feedback from our lower-level students. Each of the experiments has been fully incorporated into our syllabus, and with the appropriate preparation can be completed in a three-hour laboratory class.

A brief introduction to four of our analytical instruments is given below, together with a discussion of how they have been utilized in our chemistry courses. Unless otherwise mentioned, each set of experimental data shown below was collected either by a small group of students or the entire class. Each data point is the mean average of at least three readings. The calibration curves give the students a measure of the sensitivity and reproducibility of the measurement. We have found the between-group variability for the calibration curves to be less than 10%, thus highlighting the robustness of the technique and method utilized for teaching purposes. When the same students repeated the analyses relative standard deviations were found to be less than 4%. *Detailed experimental procedures are available on request along with instructor notes.*

1. Fluorimeters (360-nm and 460-nm LED Excitation)

Molecular fluorescence occurs when a compound absorbs light at a particular wavelength then reemits that light at a longer and less energetic wavelength. A fluorimeter measures the concentration of fluorescent compounds by first illuminating the solution containing the compound of interest, and then measuring the amount of light emitted at wavelengths at or near the emission maximum.

Two types of portable hand-held fluorimeters were constructed (Figure 1), one makes use of a 360-nm LED and the other a 460-nm LED for excitation. A silicon photodiode is used for detection. The output of the photodiode is converted to a voltage using a transimpedance amplifier, and the analog signal is then converted to a digital output and displayed on an LCD. Material costs to manufacture each instrument are less than $80.

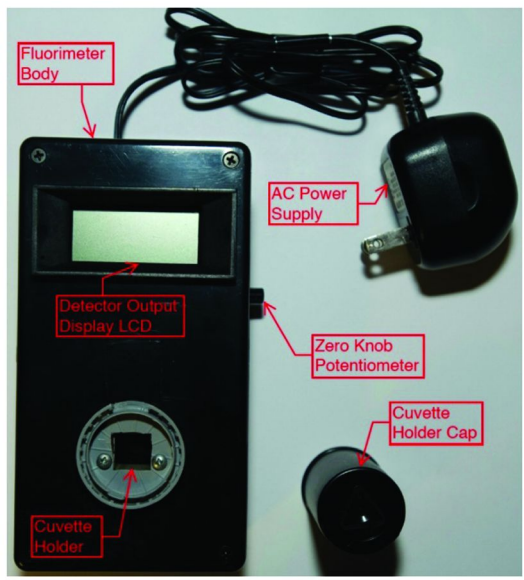

Figure 1. External configurations of the student-built fluorimeters. (see color insert)

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Fluorimeter Labs

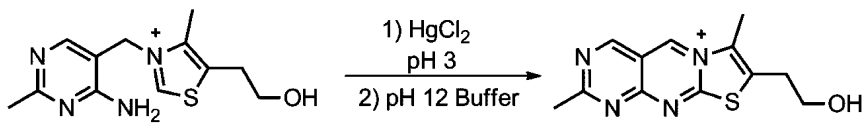
During the development phase, fluorescence data were first collected on the student-built instruments and then collected on a Perkin-Elmer LS-50 luminescence spectrometer. The results obtained from the student-built instruments are of the same quality as the results obtained from the commercial instrument.

(a). Determining the Concentration of Thiamine in Vitamin Supplements Using Kinetic Fluorescence

The 360-nm LED instrument was used to quantify the amount of thiamine (vitamin B1) in one or more brands of multivitamin supplements. The experiment strongly emphasized techniques in fluorimetry, quantitative dilution, collection and graphical treatment of kinetic data, the importance of rate equations and rate constants, and the generation and use of calibration curves.

Thiamine ($C_{12}H_{17}N_4OS$) is a water soluble vitamin involved in the metabolism of carbohydrates and fats, and is found abundantly in cereal grains, peanuts, meat, potatoes, egg-yolk, bananas, lentils, and tuna fish. Thiamine deficiency can lead to edema, muscular atrophy, and Beriberi, a disease that impairs the nervous and cardiovascular system.

Thiamine does not fluoresce, however, its concentration can be determined indirectly by oxidizing thiamine to thiochrome in the presence of a catalyst, in this case Hg^{2+} (Scheme 1). Thiamine will undergo competing reactions during this oxidation, so to avoid this problem the thiamine solution is kept acidic (pH of 3 to 4). The reaction proceeds rather slowly at this pH, so a phosphate buffer of pH12 is added. The rate of the reaction and production of the fluorescent signal is proportional to the thiamine concentration (*6*, *7*).



Scheme 1. Oxidation of thiamine to thiochrome for analysis of vitamin-B1.

Commercially available thiamine hydrochloride was used as the calibrant in these experiments. The kinetics of the oxidation of thiamine to thiochrome was carefully monitored using the 360 nm LED fluorimeter. Fluorescence increased as thiochrome is produced and the rate of increase in fluorescence is based on the amount of thiamine that was originally present, and corresponds to first order kinetics. The initial rate of the reaction is a good indicator of the overall rate, and so to save time, the reaction was monitored over a period of four minutes, and a calibration curve was generated, as illustrated in Figure 2. To determine the concentration of the thiamine in the B_1 tablet, the rate constant of the unknown

multivitamin supplement sample was compared to the known concentrations on the calibration curve. Student experimental data corresponded remarkably well to the manufacturers claim.

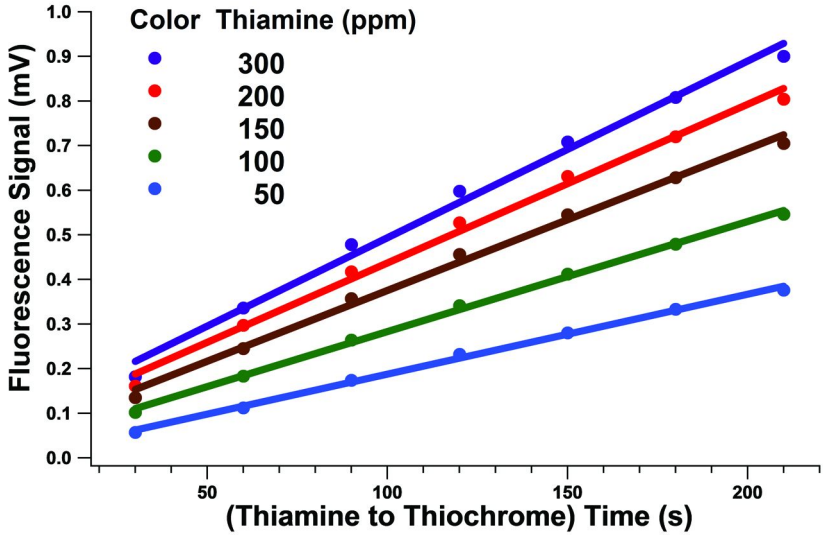


Figure 2. Fluorescence vs time plots for the various thiamine (300, 200, 150, 100, 50 ppm) to thiochrome oxidations, obtained from the 360 nm fluorimeter. (Correlation coefficients, r^2 = 0.996, 0.997, 0.997, 0.999, and 0.999, respectively). Reproduced with permission from reference (2). Copyright 2011, American Chemical Society. (see color insert)

(b). Extraction and Quantification of Chlorophyll-a from Fresh Spinach Leaves

The 460-nm LED instrument was successfully used to quantify chlorophyll-*a* pigment in fresh spinach leaves. This experimental lab exposed students to common extraction and purification techniques, and provided a thorough exercise in spectrometry and Beer's Law. Students generated a calibration curve using commercial chlorophyll-*a*, and then isolated and quantitatively measured chlorophyll-*a* from fresh spinach leaves. A simple thin layer chromatography (TLC) procedure provided an opportunity for students to distinguish and discuss the many components in their leaf extract, and develop an appreciation for the methods involved in purifying natural products.

Chlorophyll is the most important pigment involved in photosynthesis, the process by which light energy is converted to chemical energy by green plants and algae. Chlorophyll occurs in several distinct forms, and chlorophyll-*a* and -*b* are the major types found in the chloroplasts of green plants. Chlorophyll-*a* fluoresces around 660 nm, giving it a red "glow". In this experiment, the concentration of chlorophyll-*a* (Figure 3) was determined in an extract of fresh spinach.

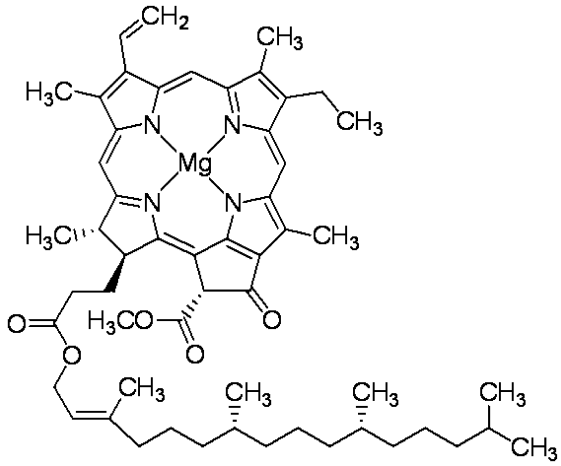


Figure 3. Molecular structure of chlorophyll-a, ($C_{55}H_{72}O_5N_4Mg$).

A number of reviews and procedures have appeared on the laboratory methods for preparation of chlorophyll from plant materials (*8–15*). The method that we chose to adapt involved the least number of steps and can be conveniently and cost-effectively done within a period of 40 minutes, in an introductory chemistry lab setting.

A number of standard solutions were first prepared using commercially available chlorophyll-*a* in hexane solvent. To stay within budget, the method of serial dilution of the stock solution was adopted. A calibration curve was constructed using the 460 nm LED fluorimeter. A plot of the relative fluorescence Intensity (mV) *versus* concentration (ppm) is shown in Figure 4.

For preparation of the leaf extract, fresh spinach leaves were ground in acetone, and the green paste diluted with a hexane/water mixture and then gravity-filtered. The hexane layer was carefully separated from the green colored filtrate, dried with anhydrous $MgSO_4$, filtered, and then analyzed with the 460 nm fluorimeter. Using the calibration curve, the chlorophyll-*a* concentration was readily calculated. All the values obtained by students correspond to chlorophyll-*a* concentration that is on the lower end of those reported for fresh spinach leaves in literature (*16–20*). The value obtained by our students is predominantly due to the short period of time that was available for the extraction process. The chlorophyll-*a* content for spinach is highly dependent on factors such as freshness, brand, storage temperature, extraction solvent, exposure to oxygen, and time required to conduct the extraction (*21–23*). Considerable analyte losses can occur during sample preparation, and it is important to carefully maintain low temperature and light conditions. However this can be a tall order in an introductory lab setting with limited time.

As a short exercise in TLC, students spotted the spinach extract onto a silica TLC plate, which was then placed in a beaker containing a hexane/acetone mobile phase. The students worked with the instructor to identify the pigment components (carotenes, pheophytin-*a* and -*b*, chlorophyll-*a* and -*b*, and xanthophylls) using

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reference colors and R_f values from the literature (*8*). The chlorophyll content was found to be highly dependent on temperature and light, and students were able to see this as the chlorophyll degraded on the TLC plates. Chlorophyll degradation has a number of pathways, leading to many products, not all of which are detectable by TLC analysis. Fresh spinach tends to have the most intact chlorophyll-*a*, and the least pheophytin-*a*. The results of TLC analysis allowed students to qualitatively assess the freshness of the spinach samples and the various factors one needs to consider during the extraction and preparation of naturally occurring chemicals.

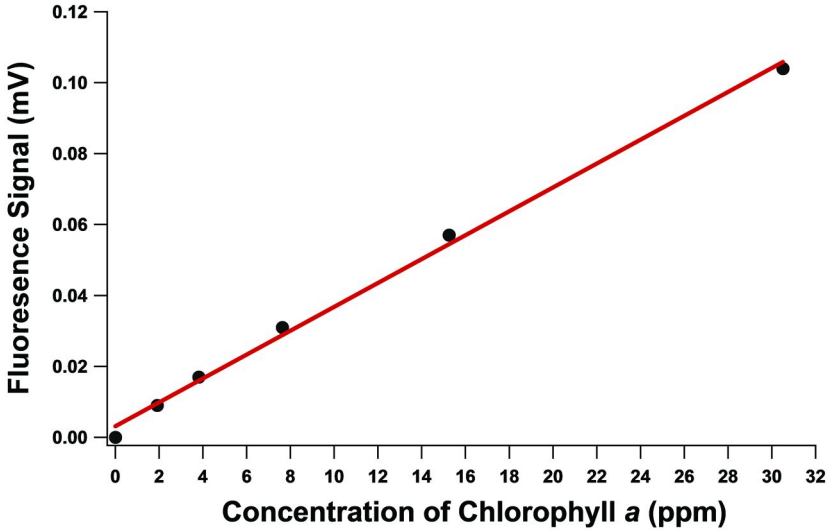

Figure 4. Calibration curve for chlorophyll-a using the 460 nm fluorimeter. Correlation coefficient, $r^2 = 0.999$. Reproduced with permission from reference (3). Copyright 2011, American Chemical Society.

This lab can be easily expanded into a two lab period experiment with an increased focus on chromatography separations and its importance in the isolation of natural plant substances: For instance, a chemistry-focused lab could feature a silica column chromatography to extract and identify the chlorophyll-*a* and -*b* from each other. A more biology-focused lab could feature isolation of the chloroplasts, and a determination of how much (% w/w) chlorophyll-*a* there is per chloroplast.

2. Colorimeter

The colorimeter is able to quantitatively measure the absorption of visible radiation by a sample in terms of the red (640 nm), green (524 nm), and blue (470 nm) components of light. The device utilizes a low-voltage LED as the light source. A tricolor 8x8 silicon photodiode sensor is employed to measure the transmitted light. A color light current-to-frequency converter generates a signal

that is displayed as percent transmittance on an LCD. The instrument is powered using four 1.5 V AA batteries making it field portable, and is manufactured for less than $50 (Figure 5).

Figure 5. Student-made colorimeter was designed and built for <$50. (see color insert)

Colorimeter Labs

The colorimeter has been used in several experiments in the Analytical and General Chemistry courses. During the testing and development phase, colorimetry data were first collected on the student-built instrument and then collected on an Ocean Optics USB 4000 spectrophotometer. The final results show that data from the student-built colorimeter are quite comparable in terms of reproducibility and accuracy to the commercially available Ocean Optics instrument.

(a). Spectrophotometric Determination of a Mixture of Tartrazine and Amaranth Using the Method of Simultaneous Equations

The student made colorimeter was used to quantitatively determine a mixture of tartrazine and amaranth (Figure 6) using the method of simultaneous (Beer's Law) equations.

Synthetic food colorings such as amaranth (FD&C Red #2, E123, CI 16185) and tartrazine (FD&C Yellow #5, E102, CI 19140) were once extensively used in the pharmaceutical, cosmetic, personal care products, and food industry. This pair of organic azo dyes absorb visible light intensely, amaranth displays three intense absorption bands at 219, 322, and 521 nm, and tartrazine displays two

intense absorption bands at 257 and 428 nm (*24*, *25*). Amaranth is a suspected carcinogen, and has been banned in the US since 1976 (*26*). Tartrazine appears to cause the most allergic and intolerance reactions of all the azo dyes, a variety of immunologic responses have been attributed to tartrazine ingestion, including anxiety, clinical depression, blurred vision, itching, and sleep disturbance (*27–29*). The existence of a sensitivity reaction is well-known, but the existence of more extreme effects and the specific link to tartrazine remains controversial. The US only requires manufacturers to declare tartrazine usage on food and drug products.

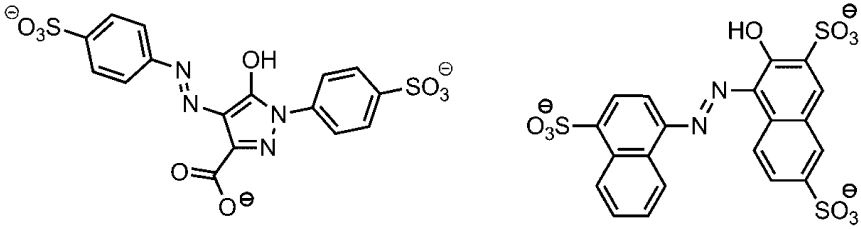

Figure 6. Molecular structures of tartrazine (left) and amaranth (right).

On occasions, the desired analyte is not the only compound in the sample that absorbs at a particular wavelength. Sometimes a separation procedure can remove the interfering compound from the solution; however, when the interfering compound is chemically similar to the analyte, it can be difficult to remove the interfering compound using a quick and simple separation step. One technique that can be employed to quantitatively determine the analyte is to select a new wavelength for the analysis, one that is absorbed by the analyte of interest exclusively. However, failure to find an appropriate wavelength requires a method that makes use of simultaneous spectrophotometric analysis, where the absorbing solutes absorb independently of each other.

The sum of the absorbances of an analyte and an interfering compound is equal to the absorbance of the mixture of the two if the concentration of each is held constant. For a two-component mixture, the following forms of the Beer-Lambert expression can be applied:

$$A_{\lambda 1} = (\varepsilon_A)_{\lambda 1}\, b\, C_A + (\varepsilon B)_{\lambda 1}\, b\, C_B \qquad (1)$$

$$A_{\lambda 2} = (\varepsilon_A)_{\lambda 2}\, b\, C_A + (\varepsilon_B)_{\lambda 2}\, b\, C_B \qquad (2)$$

where b = path length, C_A and C_B are the analyte concentrations, (ε_A) and (ε_B) are the molar absorptivities at wavelengths λ_1 and λ_2. In order to find C_A and C_B, the absorptivities need to be determined by measuring the absorbance at λ_1 and λ_2 of solutions of known concentration of the two components in their pure form.

In this experiment students prepared a series of standard solutions of tartrazine and of amaranth. They then made use of a student-built colorimeter to obtain a series of absorption spectra for these dyes at the different concentrations (with and without optical filters), and used this data to generate a pair of calibration curves (Figure 7 and Figure 8).

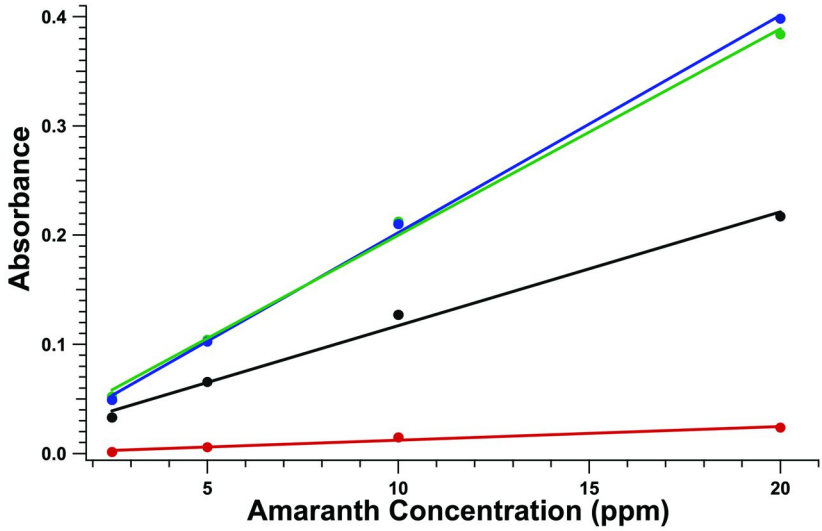

Figure 7. Calibration curve expressing the relationship between the concentration of the amaranth solution and the clear (no optical filter, black line), red (red line), blue (blue line), and green (green line) absorbance. Correlation coefficient, r^2 values are 0.996, 0.984, 0.999, and 0.998, respectively. (see color insert)

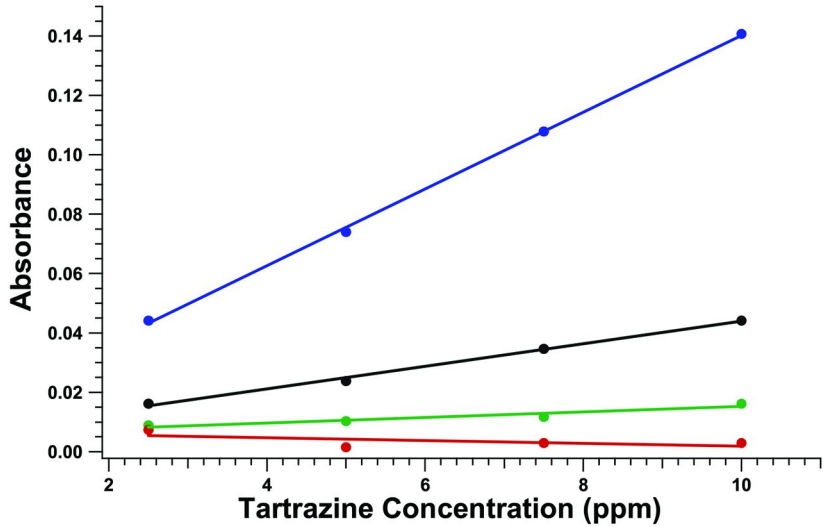

Figure 8. Calibration curve expressing the relationship between the concentration of the tartrazine solution and the clear (no optical filter, black line), red (red line), blue (blue line), and green (green line) absorbance. Correlation coefficient, r^2 values are 0.998, 0.604, 1.000, and 0.954, respectively. (see color insert)

This calibration data allowed for calculation of the effective molar absorptivity coefficients for pure amaranth and pure tartrazine at three different wavelengths (470, 524, 640 nm). The absorbance at 640 nm was performed

with (red) and without (clear) an optical filter. The concentration of an unknown tartrazine-amaranth solution mixture was then determined *via* the method of simultaneous equations (Equation 1 and Equation 2 above), by making use of the measured absorbance at the blue and green wavelength, and the appropriate effective molar absorptivity values for that particular wavelength from the calibration curves.

(b). Spectrophotometric Determination of Total Protein in Whey Protein and Bovine Serum Albumin (BSA) Using Copper Binding Methods

Total Protein assays are used on a regular basis to analyze industrial, pharmaceutical, agricultural, and biotechnology products. They are also utilized in the research lab for determining the specific activity (Total Activity/Total Protein) of enzymes and antibodies.

The presence of protein in a sample can be rapidly determined by measuring the amount of light absorbed at 280 nm. Proteins, such as tryptophan, tyrosine and phenylalanine have aromatic side chains that absorb light at 280 nm. The A_{280} is routinely used in conjunction with chromatography to purify and determine which eluted fractions contain protein. Because proteins have different numbers of aromatic residues, many of which are sensitive to pH and ionic strength, the A_{280} methodology can only be used qualitatively. Many cellular components, including nucleic acids, absorb UV light (A_{260}). The ratio of A_{280}/A_{260} is often used as a non-invasive and quick way of gauging the purity of protein or nucleic acid samples during the separation and purification stage. For increased sensitivity, the absorbance wavelength can be lowered to between 210 and 225 nm. This measures the amide bond in proteins, but is subject to interference from other cellular components and buffer solutions. Much more sensitive methods involve binding the protein to transition metals or dye molecules, such as the Biuret, Lowry, BCA (bicinchoninic acid) and Bradford assays.

This particular experiment was a two-week exercise. Students created a series of standard BSA solutions which were then used to generate calibration curves for each of the four protein assays (Biuret, Lowry, BCA and Bradford) using the student-made colorimeter instrument. The calibration curves were used to determine the Total Protein content in several unknown samples of BSA and samples of dry whey protein powder, using each of the protein assay methods for comparison (*30*, *31*). Each of the basic protocols should require no more than 75 minutes for preparation, measuring sample, mixing, incubation if required, and reading the absorbance on the colorimeter. The experiment provides an excellent introduction to the application of colorimetry to biological problems. Students not only developed their quantitative technical skills, but also learned about the advantages, the disadvantages, the meaning of limits of detection and useful working ranges, and also about the interference issues concerning each of the biochemical techniques employed to quantify Total Protein in biological samples.

(i). Biuret Assay

The Biuret method depends on the presence of peptide bonds in proteins. When a solution of proteins is treated with cupric ions in an alkaline medium (1% NaOH), a purple colored Cu^{2+}-peptide tetradentate complex is formed (Figure 9), which can be measured quantitatively with a colorimeter.

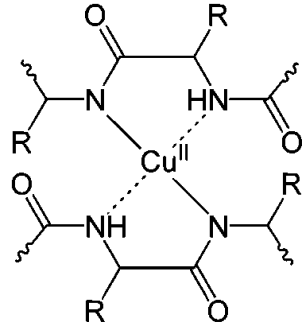

Figure 9. Molecular structure of copper-Biuret complex.

The intensity of color produced is proportional to the number of peptide bonds that are reacting, and therefore to the number of protein molecules present in the reaction system. The reaction cannot occur with single amino acids because of the absence of peptide bonds, or with dipeptides because of the presence of only one peptide bond. The purple colored complex can be measured at 550 nm. The color formed is stable for about two hours and consequently all spectrophotometer readings must be made as soon as possible after the incubation step. The Biuret assays lack sensitivity, requiring relatively high concentrations of sample (1 mg/ml to 10 mg/ml, which is diluted 5-fold by the added reagent to give a concentration of 0.2 mg/ml to 2 mg/ml final assay volume) (*32*).

A calibration curve, which plots the concentration of the BSA standard solution versus absorbance, prepared using the Biuret method is shown in Figure 10.

(ii). Lowry Assay

The Lowry assay is similar in many ways to the Biuret assay except that the Folin reagent increases sensitivity (*33*) (5 µg/ml to 0.10 mg/mL). The Lowry method relies on two different reactions; the first reduces copper in alkaline solutions and forms a copper complex (Biuret chromophore). The second step involves the reduction of Folin-Ciocalteu reagent (a mixture of phosphomolybdate and phosphotungstate) by tyrosine and tryptophan residues. The reduced Folin-Ciocalteu reagent is blue and thus detectable with a spectrophotometer in the range of 500 nm to 750 nm. The major disadvantage of the Lowry method is the very narrow pH range within which it is accurate (pH 10 to 10.5). A number of compounds interfere with the method and thus limit the Lowry assay. Interferences include some amino acid derivatives, ammonium ions, zwitterionic

buffers, nonionic buffers, lipids, sugars, nucleic acids, sulfhydryl reagents, magnesium and calcium. These substances need to be removed or diluted before running the assay, which is not always possible because many of these are constituents commonly used in buffers for preparing proteins.

A calibration curve, which plots the concentration of the BSA standard solution versus absorbance, prepared using the Lowry method is shown in Figure 11.

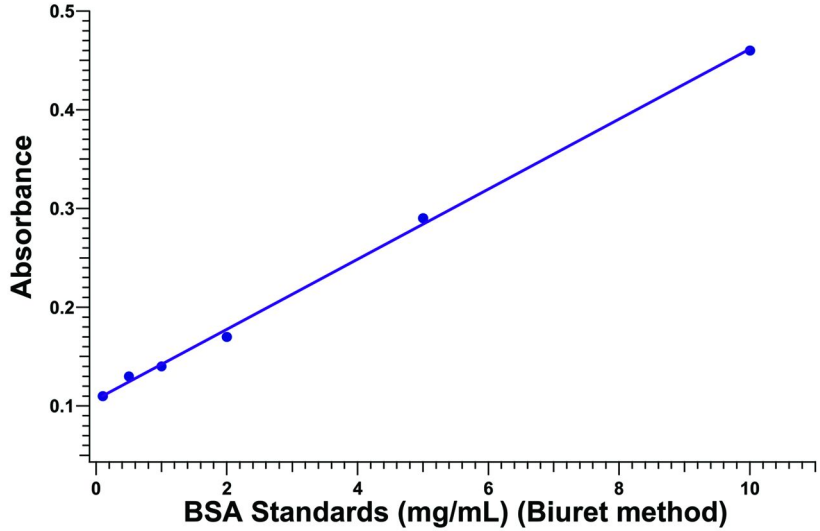

Figure 10. Biuret protein assay calibration plot for BSA. Absorbance measured at 552 nm. Correlation coefficient, $r^2 = 0.999$.

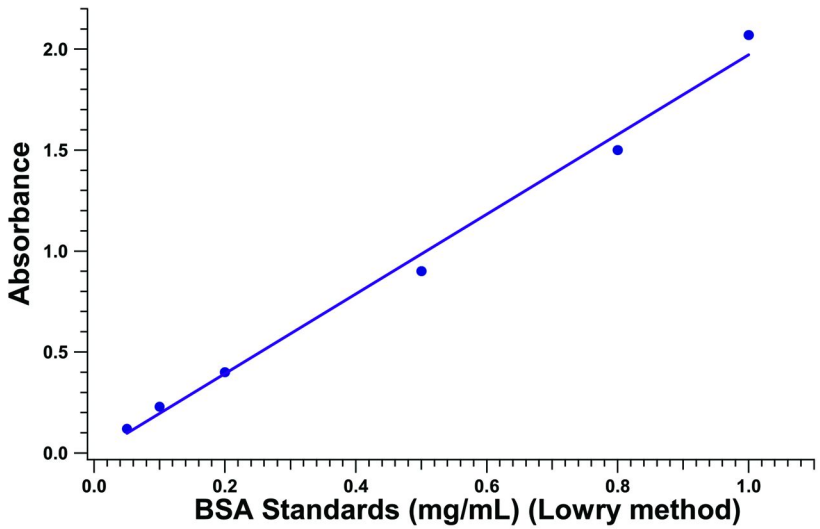

Figure 11. Lowry protein assay calibration plot for BSA. Absorbance measured at 650 nm. Correlation coefficient, $r^2 = 0.996$.

(iii). BCA Assay

The BCA reagent replaces the Folin-Ciocalteu reagent used in the Lowry assay with bicinchoninic acid (BCA). The BCA assay is a two-step process, the first occurs at low temperatures and involves the familiar Biuret reaction - reduction of Cu^{2+} to Cu^{+} by a protein (or peptides containing three or more amino acid residues) in an alkaline medium to form a light blue complex. In the second step two molecules of BCA (Figure 12) selectively react with one Cu^{+} ion to form an intense purple-colored reaction product, which has a strong absorbance at 562 nm (*34*).

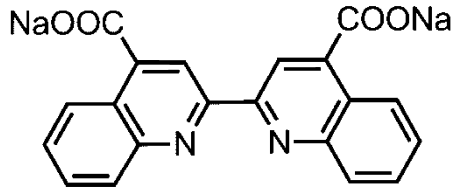

Figure 12. Molecular structure of bicinchoninic acid (BCA) reagent.

The reaction that leads to BCA color formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in proteins. In addition, and particularly at elevated temperatures, the peptide backbone also contributes to color formation. Thus, performing the assay at 37 to 60°C increases the sensitivity (0.5 µg/mL to 1.5 mg/mL) and reduces the variation in the response caused by protein amino acid composition. The BCA method is advantageous in that it does not interact with as many substances as the Folin-Ciocalteu reagent, especially detergents and buffers. The BCA assay is limited in that it interacts with most reducing agents and copper chelators (*35*). In general, these are not critical components of buffers and can be easily eliminated prior to assay.

A calibration curve, which plots the concentration of the BSA standard solution versus absorbance, prepared using the BCA method, is shown in Figure 13.

(iv). Bradford Assay

The Bradford assay is a popular and widely used method because it is simple, rapid, does not require heating, inexpensive, sensitive (1 µg/mL to 1.4 mg/mL), and gives a more stable colorimetric response than the assays described above (*36*). The method is based on the proportional binding of the dye Coomassie brilliant blue G-250 dye (CBBG, Figure 14) to proteins; as the protein concentration increases, the color of the test sample becomes darker. The assay is monitored at 595 nm.

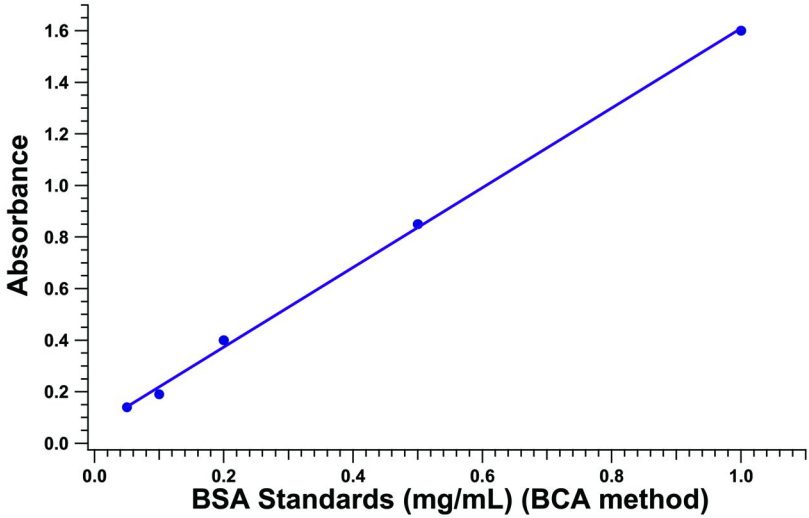

Figure 13. BCA protein assay calibration plot for BSA. Absorbance measured at 562 nm. Correlation coefficient, $r^2 = 0.999$.

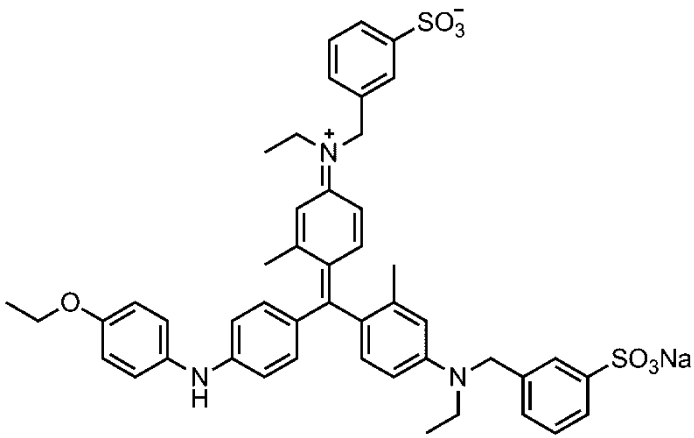

Figure 14. Molecular structure of Coomassie brilliant blue G-250 (CBBG) reagent.

The CBBG dye specifically binds to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues. The assay responds to arginine residues almost eight times as much as any of the other residues. The free dye in solution is in the cationic form, with an absorbance maximum at 470 nm (red), this shifts to 595 nm (blue) upon binding to these residues in the anionic form. It should be emphasized that the absorption spectra of the two ionic forms of the dye overlap, which causes the assay to respond non-linearly in the standard curve (*37*). Commercial kit providers of the Bradford assay, and many lab researchers insist

that the assay performs linearly. However, when a standard curve is performed it is noted that a second order curve (second degree polynomial, $y = ax^2 + bx + c$) will fit much better than a linear curve (first degree polynomial, $y = ax = b$). The assay does perform linearly over short concentration ranges, and this has most likely resulted in the overall conclusion that the assay is linear. The nonlinearity compromises the sensitivity and accuracy of this method over a wide-range of protein concentrations. There are actually two forms of the assay commonly used, one for low amounts (1 to 25 µg/ml) known as the microassay, and one for higher amounts (20 to 150 µg/ml) known as the standard assay.

In this lab protocol, our students were required to use the more convenient microassay and dilute the protein down to that concentration range. The microassay also generates less chemical waste. The issues behind the non-linearity for the data obtained with our student-made colorimeter are currently being investigated. However, reasonable values were obtained when the unknown protein samples fell within the range of the calibration curve.

Other experimental issues worth highlighting include: (1) The CBBG dye used in the assay binds to quartz cuvettes quite strongly. Therefore, glass or plastic cuvettes should be utilized. (2) The use of sodium hydroxide in the assay helps to fully solublize the protein. Some proteins, especially hydrophobic, membrane or "sticky" proteins tend to precipitate in the presence of Coomassie dyes. The response therefore is protein dependent, and varies considerably with the composition of the protein. Such limitations do make protein standard solutions all the more necessary.

A calibration curve, which plots the concentration of the BSA standard solution versus absorbance, prepared using the Bradford method, is shown in Figure 15.

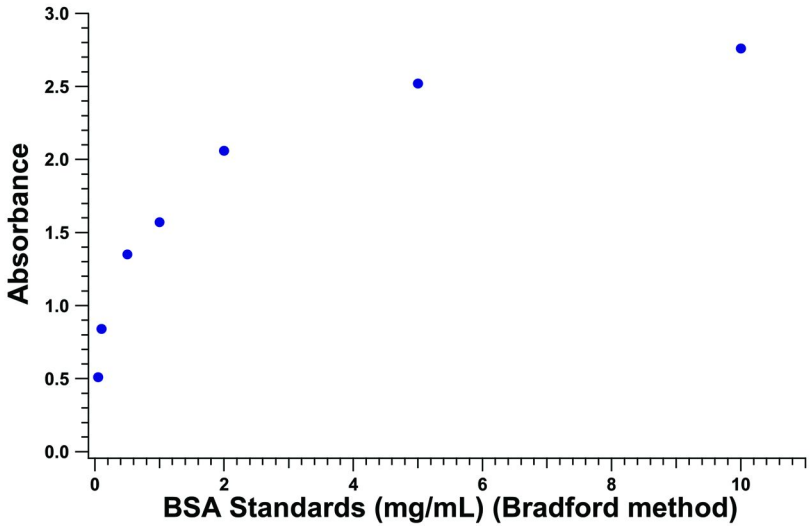

Figure 15. Bradford protein assay calibration plot for BSA. Absorbance measured at 595 nm.

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3. Karl-Fisher Apparatus

The Karl Fischer (KF) coulometric titration is a widely used analytical technique for the quantification of moisture (water) in various substances including laboratory solvents, pharmaceuticals, cosmetics, and food products.

Although most analytical chemistry curricula at the undergraduate level teach students the principles of KF titration, few courses are able to incorporate it into a laboratory setting. We have developed a low-cost, custom-built KF titration apparatus that facilitates the practical application of KF analysis within standard undergraduate analytical chemistry laboratory courses. The water content of a sample is quantified based on the number of electrons transferred during titration, which in turn is determined by the time necessary to reach the endpoint of an electrolytic titration of water with iodine under constant current conditions.

The KF technique is based on the Bunsen reaction, and occurs within a liquid medium containing an alcohol (ROH), a base (B), SO_2, and iodide. At the generator, iodine titrant is anodically produced from iodide with a corresponding reduction of H^+ to H_2 gas (**Reaction 3** and **Reaction 4**)

$$I^- \longrightarrow I_2 + 2e^- \quad (3)$$

$$2H^+ + 2e^- \longrightarrow H_{2\,(g)} \quad (4)$$

The alcohol and SO_2 initially form an alkylsulfite. When H_2O is present in the system, the alkylsulfite is further oxidized by the iodine to an alkylsulfate (**Reaction 5** and **Reaction 6**). Under these conditions, two moles of electrons correspond to one mole of H_2O, when H_2O and I_2 are consumed in a 1:1 ratio:

$$ROH + SO_2 + B \longrightarrow BH^+ + ROSO_2^- \quad (5)$$

$$H_2O + I_2 + ROSO_2^- + 2B \longrightarrow ROSO_3^- + 2BH^+I^- \quad (6)$$

The end-point of the titration occurs when all of the water present is consumed by I_2, the presence of excess I_2 is then detected voltammetrically by the platinum (Pt) detector electrode. The reagent solution will also appear yellow in color as the titration end point is reached.

The KF titration apparatus that our students have built costs $185. The apparatus utilizes a galvanically isolated bipotentiometric diaphragm-less cell, consisting of a pair of generator and detector platinum electrodes that are both immersed in a single commercially available KF reagent that serves as both anolyte and catholyte (Figure 16). The electrodes and KF reagent are housed in a custom-designed round bottomed 50 ml glass tube that provides the required stirring rate and wide-mouth with a good seal for a rubber stopper. The electronics

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portion of the KF device is comprised of two main components: The first is the constant current control, timing, and human interface, that is built around a PIC (peripheral interface controller) microprocessor and a standard liquid crystal display (LCD). The second is the detection electronics which determine the reaction endpoint. At the equivalence point of the titration, excess iodine is present in the system and is reduced back to iodide at the cathode of the detector, which results in a rapid voltage drop between the two electrodes.

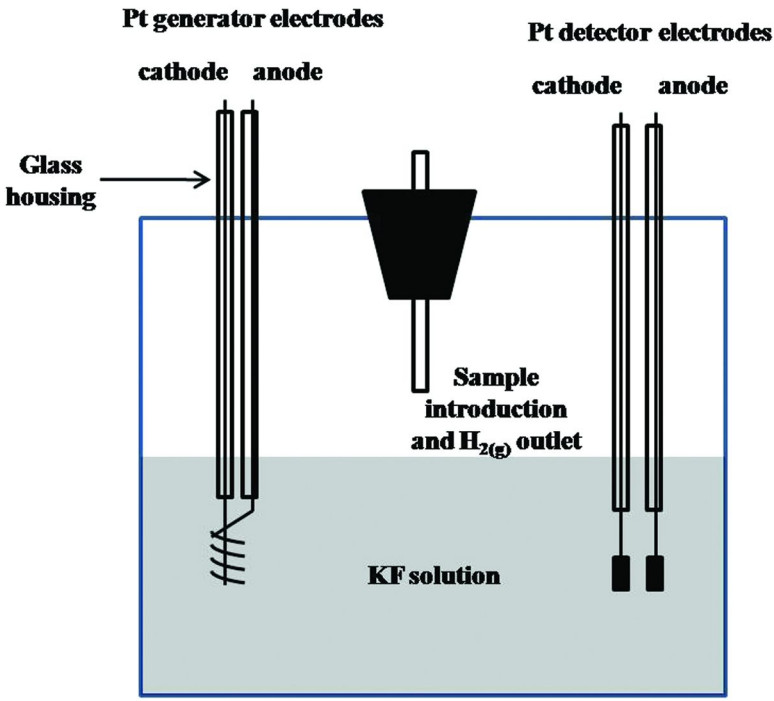


Figure 16. Schematic of a diaphragm-less KF cell. Reproduced with permission from reference (1). Copyright 2010, American Chemical Society.

KF Labs

The KF instrument is used to quantify moisture levels in (**a**) various brands of hand lotion and (**b**) licorice. The experiment emphasizes a widely used coulometric technique. It exposes students to some very common extraction and sample preparation techniques, and highlights the importance of various factors that go into generating good calibration curves.

The Hydranal Coulomat-AG KF reagent is hygroscopic and so the residual water within the solvent was first removed. Blank runs were repeated several times in order to condition the KF cell. A calibration curve, plotting the volume of nanopure water versus experimental run times was then constructed, as shown in

Figure 17. The magnitudes of the error bars reflect the uncertainty in the volume of injection using the micropipettor (x-axis) and the standard deviation of the four replicate runs relative to the average run time (y-axis).

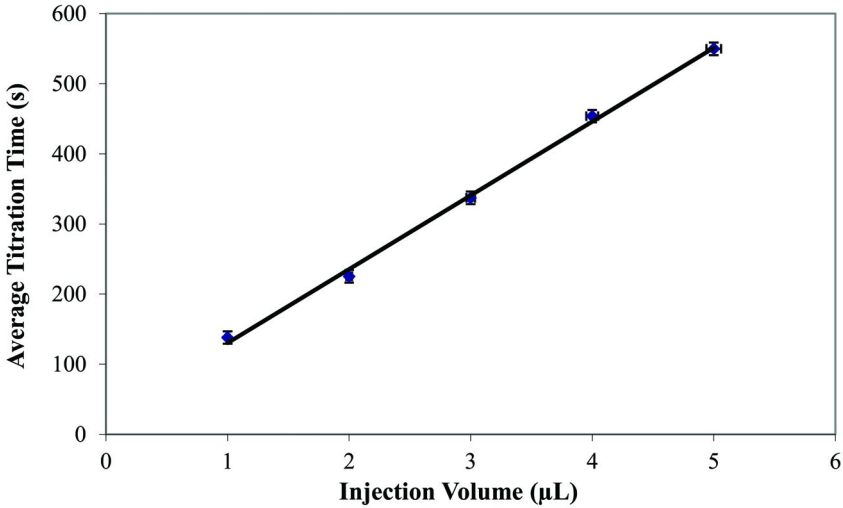


Figure 17. A calibration curve expressing the relationship between the volume of nanopure water and the coulometric titration time. Mean average values are shown with standard error bars. Correlation coefficient, $r^2 = 0.997$. Reproduced with permission from reference (1). Copyright 2010, American Chemical Society.

(a). Determining Moisture Content in Hand Lotion (Aveeno Active Naturals Positively Smooth and Rusk Sensories Calm)

Two different brands of hand lotion, Aveeno and Rusk, were prepared using a procedure similar to that reported in literature (*38*), by extracting the moisture content from the lotion with methanol. The sample was filtered using a 0.22 µm pore size syringe filter and known amounts of the filtrate were then carefully injected into the titration cell. Experimental run times were adjusted by subtracting the average run time of several methanol blanks in order to exclude the presence of trace amounts of water in the extracting solvent. Using the calibration curve in Figure 17, the moisture contents in Aveeno and Rusk hand lotions were calculated. Water is listed as the largest ingredient in both hand lotion samples but the manufacturers do not list concentrations; therefore, it is not possible to evaluate the accuracy of the results obtained by our students. However, our calculated moisture contents compare favorably with those reported (*38*) for other hand lotions.

(b). Determining Moisture Content in Red-Colored Licorice (Hershey's Twizzlers)

The licorice samples were prepared by shaving the strips into thin slivers using a paring knife, dissolving them in a 1:1 mixture of formamide and methanol, and then filtering the solution extract through a 0.22 μm pore size syringe filter. The addition of formamide improves the solubility of polar substances. Although not necessary for this variety of red licorice, solubility problems for such food samples can often be overcome by adding co-solvents such as chloroform or 1-octanol. Known amounts of the filtrate were then carefully injected into the titration cell. Experimental run times were adjusted by subtracting the average run time of several solvent (1:1 mix of formamide and methanol) blanks in order to exclude the presence of trace amounts of water in the extracting solvent. Using the calibration curve in Figure 17, the moisture content of the licorice samples was calculated. The moisture content calculated using the student-built KF apparatus was very similar to the manufacturer's stated value. The standard deviation in student data is most likely a function of incomplete sample dissolution and the problems faced by those new to micropipetting. The pipetting aspect was highlighted as a major source of frustration, particularly by our lower-level students.

4. Cyclic Voltammeter

Cyclic voltammetry (CV) is a versatile electroanalytical technique used to monitor the redox behavior of chemical and biochemical species within a potential range. The CV instrument that we have designed, constructed, and characterized, facilitates the practical application of CV analysis within standard undergraduate laboratory courses. The entire miniaturized and portable instrument was designed for less than $50, thus allowing for deployment of multiple apparatus in a lab with a modest budget. All of our instruments remain under constant development by our students, and the latest CV model has allowed us to obtain some remarkable cyclic voltammograms on a number of chemical compounds from the biological world.

The student-built CV instrument consists of two parts (Figure 18): A commercial miniLAB-1008 unit, and a student-built CV potentiostat. The miniLAB is a data acquisition (DAQ) device (Measurement Computing Corporation, Norton, MA) that provides an interface for Analog to Digital conversion, and allows for computer analysis and manipulation of the acquired data. Data processing and interpretation was done with MathWorks Matlab (version 7.14).

The CV experiment is carried out in an electrolytic cell with three electrodes: The reference electrode keeps the potential between itself and the working electrode constant, and the current is measured between the working and counter electrodes. The student-built potentiostat employs a platinum 2 mm wire or a commercially available glassy carbon working electrode (BASi inc), a platinum auxiliary electrode (2 mm wire), and a simplified student-built Ag/AgCl reference electrode. The electrodes were housed in a 50-mL beaker or a shot-glass cell with a customized rubber or drilled teflon stoppers.

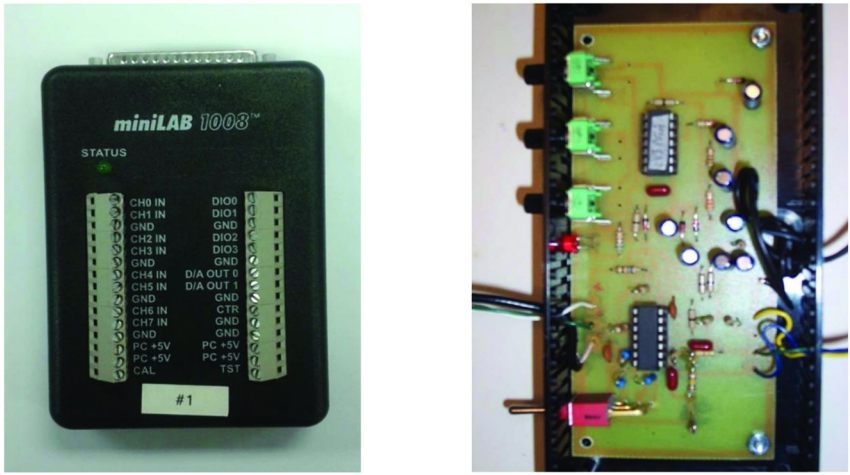


Figure 18. (a) MiniLAB 1008 for data acquisition from the potentiostat to a computer. (b) View of the circuit board found inside the student-made potentiostat. (see color insert)

In a typical CV experiment, the potential of the working electrode is varied from an initial value to a predetermined limit where the direction of the scan is reversed. Often an initial reduction is undertaken to form a product, and then the voltage scan is reversed to oxidize the product of the reduction back to the starting material. A voltammogram is obtained by measuring the current at the working electrode during the potential scan. Depending on the information sought, single or multiple cycles, of varying scan rate and potential limits can be used.

CV Labs

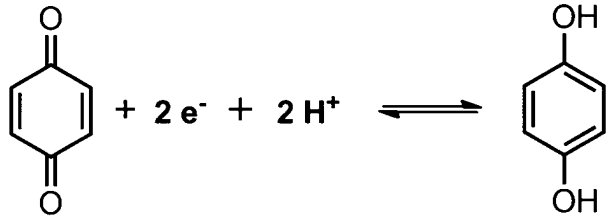
During the testing phase, CV data were first collected on the student-made instrument and then collected on a commercial BASi Epsilon CV50W electrochemical analyzer for comparison. In a number of cases the results obtained from the student-built instrument were found to be surprisingly superior to those obtained from the commercial instrument.

(a). Determining the pH of an Unknown Solution Sample of Quinone/Hydroquinone (Q/H_2Q)

The CV was used to create a calibration curve of pH *versus* E° (mV) for the p-benzoquinone/hydroquinone (Q/H_2Q) chemical system, from which the pH of an unknown hydroquinone sample was calculated.

Quinones represent a class of compounds that are widely distributed in nature, as the key electron carriers in photosynthesis to the class of molecules involved

in blood coagulation. The biological action of quinones is intimately linked to their electron transfer rates and redox potentials. The Q/H_2Q experiment focused on a well behaved and reversible redox couple (Scheme 2), and is an excellent system for illustrating the Nernst equation to students (*39*), and the relationship this equation has to the pH, and the features that make this compound of such importance to the biological world.



Scheme 2. The two-electron/two-proton coupled reaction for benzoquinone/hydroquinone (Q/H_2Q).

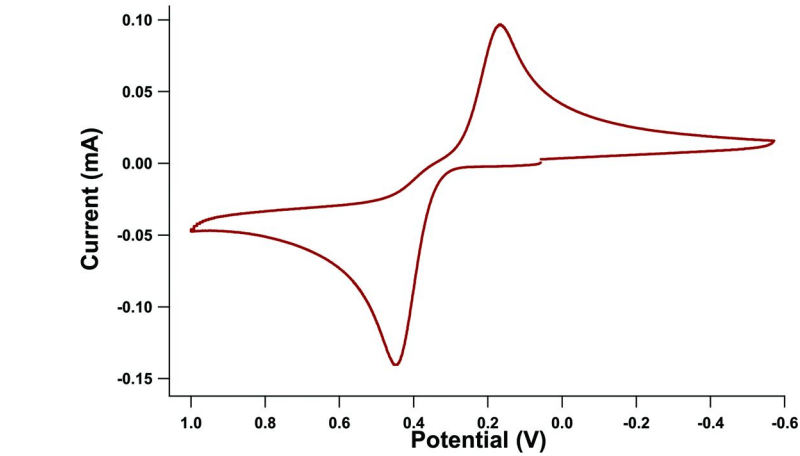
A second aspect of this experiment (not discussed here) required students to make use of the Nernst equation and the Randles-Sevcik equation to determine the number of electrons transferred in the redox process, and calculate the diffusion coefficient of the electrochemical system.

In this experiment students identified various features of a cyclic voltammogram, determined the anodic and cathodic peak currents, the formal reduction and oxidation potentials (E°), and commented on the influence that pH has on the voltammogram of Q/H_2Q. In addition, students prepared several hydroquinone solutions in phosphate buffer, with a pH between 2 and 6, and then obtain CV data for each solution. The Henderson-Hasselbalch equation was employed to help with the calculations, and the exact pH of each solution was checked just prior to use in the CV experiments.

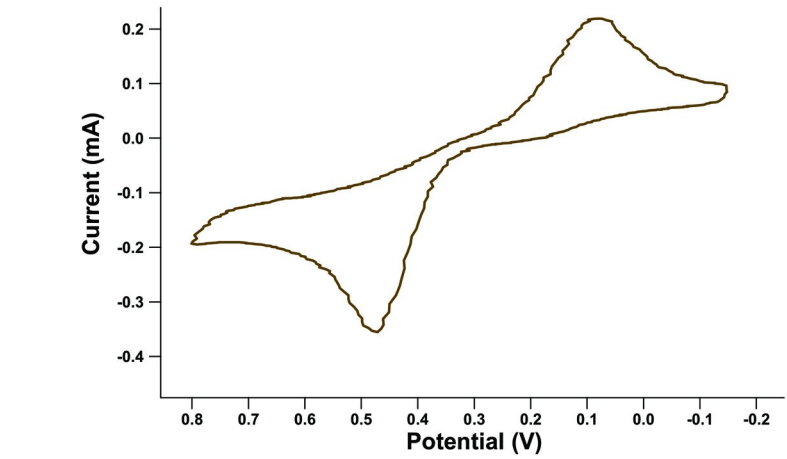
A typical cyclic voltammogram at pH3 is illustrated in Figure 19, it shows one cathodic peak in the forward (negative-going) scan due to the reduction of Q to H_2Q, and one coupled anodic peak in the reverse (positive-going) scan that corresponds to the oxidation of H_2Q to Q. The voltammogram from our student-built instrument and the commercial instrument are both remarkably similar. Although the data from the commercial instrument has much better signal-to-noise, the student-built apparatus is clearly able to provide very good data at a fraction of the cost.

Student data shows that as the pH of the buffer solution increases, both the anodic and cathodic peak potentials shift to the negative, indicating a decrease in the redox potential for the couple as is predicted by the Nernst equation. The observed cathodic shift illustrates that the redox potential is dependent on the equilibrium concentration of the redox species involved. Experimental data were used to plot a calibration curve of pH *versus* E° (mV) as shown in Figure 20, from which the pH of an unknown hydroquinone-buffer solution (as prepared by the lab instructor) was determined. Students were required to adjust the concentration if necessary, and to keep track of any dilutions, so that the anodic and cathodic peak

current values (Ip_a and Ip_c) fall within the calibration curve. The calibration curve was found to be linear, with a slope of 43 ± 4 mV/pH. Although this value isn't quite the ideal 59 mV/pH unit that is expected (*40*) for a 2-electron, 2-proton redox system, it does clearly show the pH dependence of the Q/H_2Q redox potential, and that the reaction involves about two electrons.



(a)



(b)

Figure 19. (a) Voltammogram from the BASi Epsilon instrument for 2.0 mM hydroquinone at pH 3.0. Scanned from 0 mV to 1000 mV, then to -600 mV, and back to 0 mV, at a scan rate of 100 mV/s. (b) Voltammogram from the student-built CV for 5.0 mM hydroquinone at pH 3.0. Scanned from about 800 mV to -200 mV, and back to 800 mV, at a scan rate of 74 mV/s.

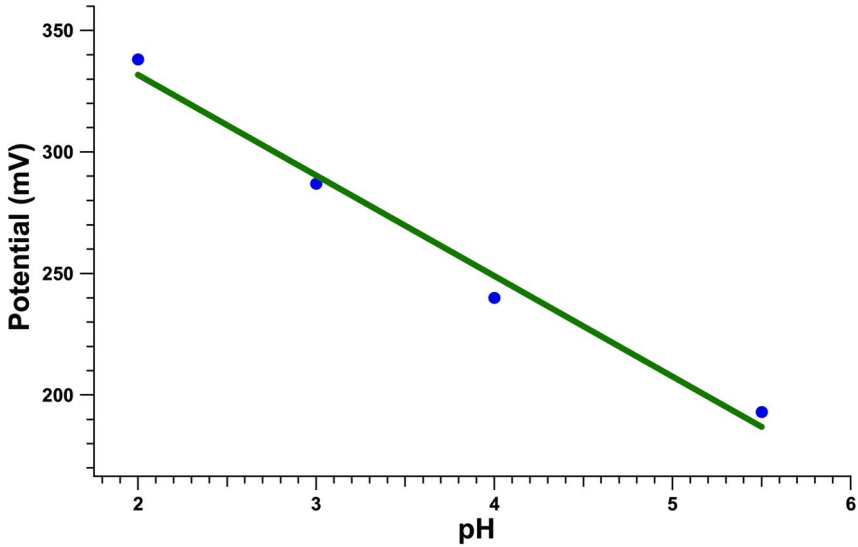


Figure 20. E° (mV) vs pH calibration curve for a 5mM hydroquinone-buffered solution concentration. Correlation coefficient, r^2 = 0.993, with a slope of 43 ± 4 mV/pH. Potentials (mV) are given vs Ag/AgCl.

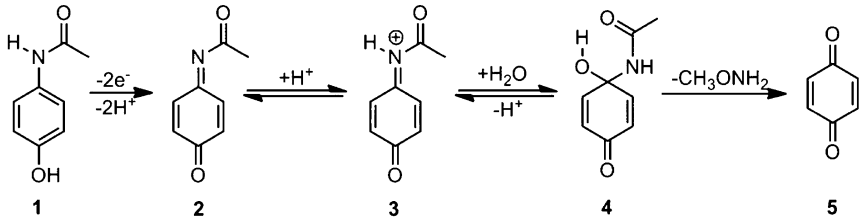
(b). Determination of 4-Acetaminophenol (APAP) in a Sample of Tylenol Cold Medicine

APAP (paracetamol, acetaminophen) is an analgesic and antipyretic. Its analgesic properties are comparable to those of aspirin, while its anti-inflammatory effects are significantly weaker. APAP is non-carcinogenic and an effective substitute of aspirin for those folks unable to tolerate aspirin. It is commonly used for minor aches and pains, and is a major ingredient in numerous cold and flu remedies (such as Tylenol, Anacin, Acamol, Panadol). Since the molecule is readily oxidized at low potentials, electroanalytical methods (*41*, *42*) are very well suited for the quantitative determination of APAP in over-the-counter cold remedies.

One aspect of this CV experiment (not presented here) dealt with the chemical and electrochemical mechanism that is observed when 4-acetaminophenol (APAP) is oxidized to benzoquinone (Scheme 3) as the solution pH is varied. This second aspect of this experiment made use of the student-built CV apparatus to determine the amount of APAP active ingredient in Tylenol Extra Strength Rapid Release capsules. The experiment described herein is a modified version of that given in literature (*42–44*).

The CV experiment involved use of the Randles-Sevcik equation to construct a calibration curve of Ip_a (mV) *versus* APAP concentration (mM), at a constant buffer pH of 1.7 (Figure 21). Because of its dependence upon pH, the cathodic current (Ip_c) was found not to be suitable for quantitative analysis. The anodic

peak current (Ip_a), however, was found to be proportional to the concentration of the benzoquinone species and therefore the APAP species. Students were allowed to make use of the Henderson-Hasselbalch equation to help with the buffer calculations, and the exact pH of each buffered solution was checked before running the CV experiment.



Scheme 3. Proposed mechanism for the oxidation of APAP.

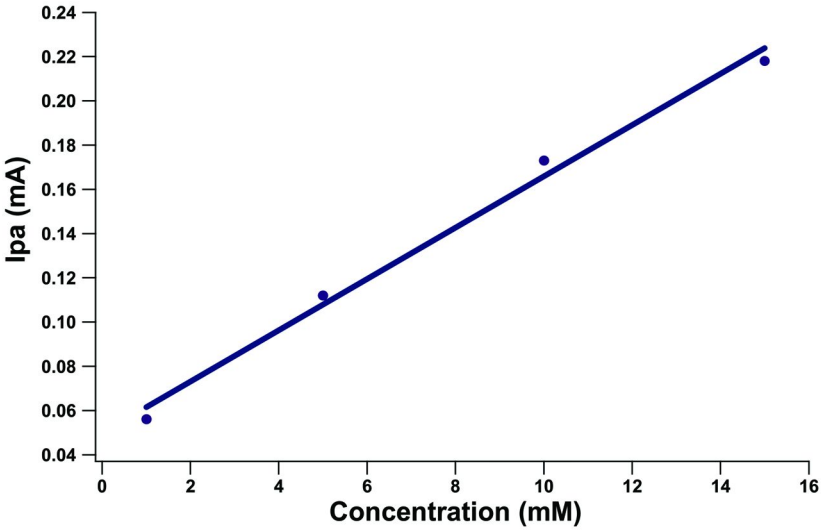


Figure 21. A calibration curve for APAP-buffered solution concentration, Ip_a vs concentration (mM). Solutions were buffered at pH1.7. Correlation coefficient r^2 = 0.996.

The linear calibration curve was then used to calculate the concentration of an unknown solution sample of APAP made from Tylenol capsules, at this same pH value. The unknown sample was prepared by taking the contents of a weighed "Gelcap of Tylenol Extra Strength Rapid Release", and dissolving in pH1.7 buffered solution, in a volumetric flask. Students were advised to make adjustments to the concentration if the voltammogram Ip_a values fell outside

the calibration curve, and to make note of any dilutions performed in order to calculate the concentration of the original Tylenol solution. The concentration of the Tylenol capsule was calculated to be precisely the same as what the manufacturers claim on the label. This is an excellent and pleasing testament to the accuracy, reproducibility, and reliability of the student-built CV instrument and the methodology employed.

Summary and Conclusions

Analytical chemistry assignments should involve as wide a range of instruments as possible. However, training in the mere use of analytical instruments, or simply learning how to navigate commercial software, is not enough. Some appreciation of the fundamental working principles must be imparted in the academic course of study. The course that we have developed for our upper-level students, teaches principles rather than detailing cookbook step-by-step procedures to be followed. Lecture material provides the theory; problem sets and lecture activities reinforce those concepts; and the extensive laboratory experiences provide the practical application of the theory. The inclusion of the SMILE initiative ties together the physics, electronics, engineering, and the mathematics associated with the design, construction and use of analytical instruments. The low-maintenance, mobility, and low-operating cost of the student-built analytical instruments are capable of providing accurate quantitative information for a fraction of the cost of commercial instrumentation. SMILE enhances student understanding of how analytical measurements are made. We have found that the benefits of implementing SMILE has lead to a significant increase in student enthusiasm, class-participation, and has fostered cooperation, group learning, and scientific communication amongst the students. Furthermore, we find that the research nature of the SMILE initiative has helped students develop their critical-thinking and deductive reasoning skills. In these respects SMILE has created a unique and successful learning environment.

Analytical chemistry labs have traditionally focused on students doing just enough in the scheduled time to obtain the correct answer, with a very limited focus on developing the essential critical-thinking skills and the ability to question the significance of the numbers that they have experimentally obtained. The biological applications we have chosen are of particular interest to our undergraduate population and have provided an ideal platform for lively discussions in quantitative and instrumental analytical chemistry. The experiments presented here provide for highly interdisciplinary training for undergraduate students, and also represents an opportunity to introduce students to *the joys* of undergraduate research.

It is clear to us that students' perceptions and behaviors in the science laboratory are greatly influenced by the instructors' expectations and by the quality and orientation of the associated laboratory guides. Assessment data shows that building small analytical instruments and then making use of these student-built instruments clearly promotes student competency in the STEM subjects. Our experience has shown that students can construct the relationship

between the data collected in an experiment and the conclusions we want them to reach when they have a say in the design of the instrument and the procedures used to collect data, when they are encouraged to work together, and when the class as a whole discusses the instrumental issues and the experimental results. On the whole, our analytical chemistry courses have been transformed over the past few years, and our students have responded positively, they appreciate group-work and knowing that the lab experiment does not come to an abrupt end when they have finished collecting data. We have found that those involved in building and testing the instruments spend a significant amount of time and effort outside of the regular lab schedule in support of their instrument project and data collection activities. The high-quality oral presentation and technical papers written in the format of a peer-reviewed journal paper at the end of the course have greatly exceeded our original expectations.

On the basis of survey responses, students find the SMILE lab experience far more enjoyable than any of the other science lab courses they have taken to date. Over the past five years, 80% of the students that design, build, and calibrate the analytical instruments have consistently indicated that these SMILE projects are by far their favorite activities in the course. Student feedback highlights that the best features of the SMILE labs were the independence afforded, thus improving research abilities, and the development of real-life and practical applications particularly those related to biological systems. Students were also enthusiastic about the value of their lab work and its importance to the scientific research team, where the results could end up being presented and published. Feedback also included specific suggestions and ideas for future improvement of both the instrument and course. Informal assessment of the SMILE program clearly indicated positive learning outcomes: Analysis of our examination data shows that students in the SMILE program scored a mean average of 90% on the relevant lecture material, compared to a score of 78% in control groups.

Negative comments included the amount of outside class time required to successfully complete a SMILE project, finding time to meet as a team outside of class, not being allowed to take the instruments home to work on them, and some complaints about members of the group not pulling their weight and not doing their fair-share of the work. Each year, one to several students negatively associate SMILE as a physics-based curriculum rather than chemistry and register their displeasure for electronics in course evaluations.

Overall, we have found that our SMILE initiative is a natural conduit for co-curricular experiences. The greater degree of active participation by students in the science laboratory, coupled with the close student-faculty interactions, has led students to foster ownership of the analytical chemistry curriculum. With the inclusion of bioanalytical applications, our students are now considerably more content and happy than what they were 5 years ago. Although primarily designed for the benefit of our students, an interesting aspect of our approach is that it also provides an intellectually stimulating and rewarding experience for the teaching assistants, and the faculty and staff involved. In comparison to other laboratory curricula, we have been delighted by the level of enthusiasm towards SMILE from our chemistry students and the positive attitudes we have observed at the inclusion of a biological focus to many of our analytical labs.

Acknowledgments

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Chapter 7

Bioanalytical Chemistry in Instrumental and Biochemistry Laboratories

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Most science students in liberal arts universities are seeking entrance into health-related professional schools (at least initially). Lipscomb University is particularly blessed with large numbers of such students. As opportunities in analytical chemistry have grown, engaging these pre-professional students in relevant laboratory activities has been a challenge. A number of experiments have been developed in bioanalytical chemistry, both in Instrumental Analysis and Biochemistry Laboratories. Students have performed peptide mapping by HPLC, detected riboflavin by fluorescence, and analyzed for amino acids by capillary electrophoresis. New bioanalytical experiments are being developed for capillary electrophoresis, LC-MS, and cyclic voltammetry. Bioanalytical chemistry has also become an area for exploration in undergraduate research.

Rationale

Many if not most of the undergraduate students at predominantly undergraduate institutions who major in chemistry or biochemistry desire to attend a health related professional school. Students at such institutions are commonly motivated by a desire to serve others in their community, and often

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times the best and the brightest are steered toward medicine. This means that the classrooms and laboratories in chemistry at liberal arts colleges and universities are heavily populated with students more interested in learning about biochemistry and drugs than they are about instruments, chemical analysis, and many of the classic analytical experiments that chemists have performed for many years. The students are not highly motivated to learn the number of waters of hydration in an inorganic salt's crystal structure by thermogravimmetric analysis. In such cases, the health of a chemistry department is often determined by its ability to engage the students. One method of involving these health-related students in analytical chemistry is to emphasize the analysis of biological compounds.

Of course, the range of options available to the instructor is dependent upon the materials and the instruments available to the instructor at the university. Students interested in the health sciences often take numerous biology courses where they perform Northern, Southern, and Western blots, transformations of bacteria with various vectors, and seemingly innumerable gel electrophoresis experiments. And, biology students commonly perform polymerase chain reaction (PCR) experiments. Much of what they do is never quantitated, and generally the instruments that they see and perhaps use are limited to ultraviolet-visible (UV-vis) spectrophotometers, power supplies and gel boxes for electrophoresis and blotting experiments, and thermal cyclers. When health science students come to analytical chemistry, instrumental analysis, and biochemistry labs, it seems natural to expose the students to a much broader array of analytical techniques than they see in cell and molecular biology laboratories. And, they enjoy performing biologically relevant analyses in their chemistry laboratory experiences.

So, many students enjoy biological applications in their chemistry laboratory experience. How can we introduce more biologically relevant experiments into the curriculum? This article seeks to share one instructor's experience at a small school in hopes that it might make this process easier. We have integrated biologically relevant analytical experiments into Analytical Chemistry, the Instrumental Analysis course, and Biochemistry Laboratory. We will discuss these by course and experiment, and then try to share some experiments which are currently under development which others might like to try to perfect and share with the greater bioanalytical community.

Analytical Chemistry

Our analytical chemistry course is largely concerned with aqueous equilibria and the laboratory experiments are dominated by titrations, although we have also included electrochemical topics such as ion selective electrodes and cyclic voltammetry. We also include a potentiometric pH titration of a diprotic acid. Rather than titrate carbonate unknowns or similar inorganic acids, many years ago we went to the titration of an amino acid. This emphasizes the ionic nature of the amino acids as well as helps the students to learn the significance of pK_a data in their analytical and biochemistry textbooks. Another laboratory experiment which illustrates the importance of pK_a values in chemical separations is ion exchange

chromatography. This experiment has the added benefit of exposing students to column chromatography other than the more commonly encountered adsorption chromatography.

Ion Exchange Chromatography

Ion exchange chromatographic separation of amino acids is used to teach students how pH can be controlled to separate amino acids by charge (*1*). In these experiments we give the students a mixture of amino acids as an unknown and ask them to separate the various amino acids by use of a cation exchange resin such as Amberlite IR-120 which was packed into a buret. Each mixture contains an acidic amino acid (either glutamate or aspartate), a neutral amino acid, and a basic amino acid (either lysine or arginine). The unknown mixture is dissolved, added to the ion exchange column the students had poured, and eluted by using a series of buffers which would easily separate the amino acids. Fractions eluting off the column are collected for further analysis. The first buffer is a pH 3 citrate buffer which generally would elute acidic (negatively charged) amino acids from the column first. This would be followed by a pH 6 citrate buffer which would elute neutral amino acids. Basic amino acids were washed off the column with a pH 9 Tris-hydroxymethylaminomethane buffer. Ninhydrin spray was used to identify the presence of an amino acid as it came off the column. The amino acids are then identified by paper chromatography in a suitable solvent system.

There are a number of difficulties with these procedures. The ion exchange process is not the only separation process that is occurring. Viewing any ion exchange chromatogram of amino acids will illustrate that some small polar neutral amino acids elute prior to or alongside some acidic amino acids. The ion exchange columns often have irregularities in them due to poor packing of the chromatography column. Ninhydrin identification of an amino acid is slow and messy, and paper chromatograms do not always yield a clear indication of which amino acid is present due to similar R_f values. This procedure begs for a faster, more sensitive, instrumental method of analysis. Ion exchange HPLC and capillary electrophoresis illustrate the ionic properties of biological molecules much more quickly and are easier to quantitate, but students often lose sight of the molecular interactions that are actually taking place within the instruments.

Instrumental Analysis

As the number, quality, and diversity of chemical instrumentation grows in undergraduate institutions one of the challenges has been to develop successful bioanalytical laboratory procedures which make use of this broad array of weapons in the instrumental arsenal. Fortunately, the *J. Chem. Educ.* and similar journals provide a constantly growing source of inspiration for those seeking relevant, modern bioanalytical procedures as well as a method for the dissemination of innovative laboratory instruction.

Two Component Mixture by UV-Visible Spectroscopy

Many years ago we used a mixture of two transition metal complexes to teach the Beer-Lambert Law and to illustrate how simultaneous equations could be used to determine the concentrations of two species occurring in solution at the same time. However, there was too much liability in the disposal and too great a safety hazard for comfort, so when a simultaneous determination of the components in Dristan Nasal Spray was published, we switched to it (*2*). This procedure worked well. The students got good results, as both components absorbed well at separate wavelengths to yield a good determination. When Dristan was reformulated, we changed to a determination of benzoate and caffeine in soft drinks using Mello Yellow or Mt. Dew (*3*). Students make up standard solutions of caffeine and benzoic acid in 0.01 M HCl, obtain the UV-vis spectra, choose the maximally absorbing wavelengths for both components, and collect data for four Beer's Law plots, two each for benzoic acid and caffeine at both wavelengths. The absorbance of the diluted soft drink is then measured at each wavelength. This experiment certainly separates good students from weaker ones, as some students will not collect all four data sets even though they are explicitly told to do so.

The determination of the two components in Mello Yellow or Mountain Dew does not work quite as well as the Dristan Nasal Spray did, largely because benzoic acid does not absorb very much at the maximally absorbing wavelength for caffeine, leading to much error in that determination. Benzoic acid's UV spectrum is also highly dependent upon pH, so care must be taken in making up its solutions. At some pH values, the absorption maximum at approximately 230 nm is shifted to shorter wavelengths which overlaps with a prominent absorption band of caffeine. If the benzoic acid solution is not make sufficiently acidic, the students will choose a wavelength which does not yield good results.

Riboflavin Fluorescence

When a spectrofluorimeter became available to us, we wanted to use fluorescence to determine the concentration of one component in a complicated matrix of many absorbing materials. We have determined the amount of riboflavin in a multivitamin (*4*). Standards of varying concentrations of riboflavin are used to construct a calibration curve and one is used to determine the optimum excitation and emission wavelengths. A multivitamin tablet is crushed with a mortar and pestle and extracted into acidic solution. The emission of the riboflavin in the resulting solution is measured under the appropriate excitation conditions and compared to the calibration curve. This procedure powerfully demonstrates that fluorescence can allow the determination of a fluorescing species while mixed with a complicated mixture of other UV-vis absorbing species which do not fluoresce in the same region of the spectrum.

Diet Mountain Dew Determination of Benzoate, Caffeine, and Aspartame by High Peformance Liquid Chromatography and Capillary Electrophoresis

The *J. Chem. Educ.* article that describes the simultaneous spectrophotometric determination of benzoate and caffeine in soft drinks also describes the determination of these ingredients along with aspartame in diet soft drinks by reverse phase high performance liquid chromatography (HPLC) and capillary electrophoresis (CE, 3). We have now performed this experiment for several years in the instrumental laboratory. We have occasionally done the determination of detection limits which is given as an optional experiment at the end of the article. A chromatogram illustrating the separation of the three major components by HPLC is shown in Figure 1, and capillary electropherograms of both standards and Diet Mountain Dew are shown in Figure 2.

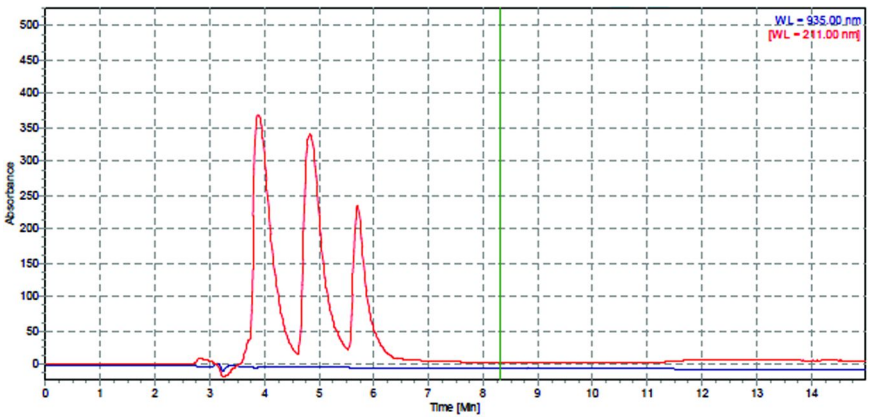

Figure 1. HPLC analysis of a mixed standard solution in the analysis of Diet Mountain Dew® showing the elution of caffeine (retention time of approximately 4 minutes), aspartame (almost 5 minutes in retention time), and benzoate (about 5.75 minutes retention time), respectively from left to right. The photodiode array detector is set at 211 nm for this chromatogram.

Two HPLC solvents are prepared by making mobile phase A 0.025 M phosphate buffer at pH 3.0 and mobile phase B methanol. HPLC grade solvents are used, and the solvents are filtered through 0.45 μm Millipore filters and degassed. The separation is done isocratically using a mixture of 45% methanol and 55% phosphate buffer. The binary gradient HPLC system consists of a Varian ProStar 410 autosampler, two model 210 pumps, a Supelcosil LC18 250 X 4.6 mm HPLC column (and other C18 columns have been used), and a ProStar 335 photodiode array detector.

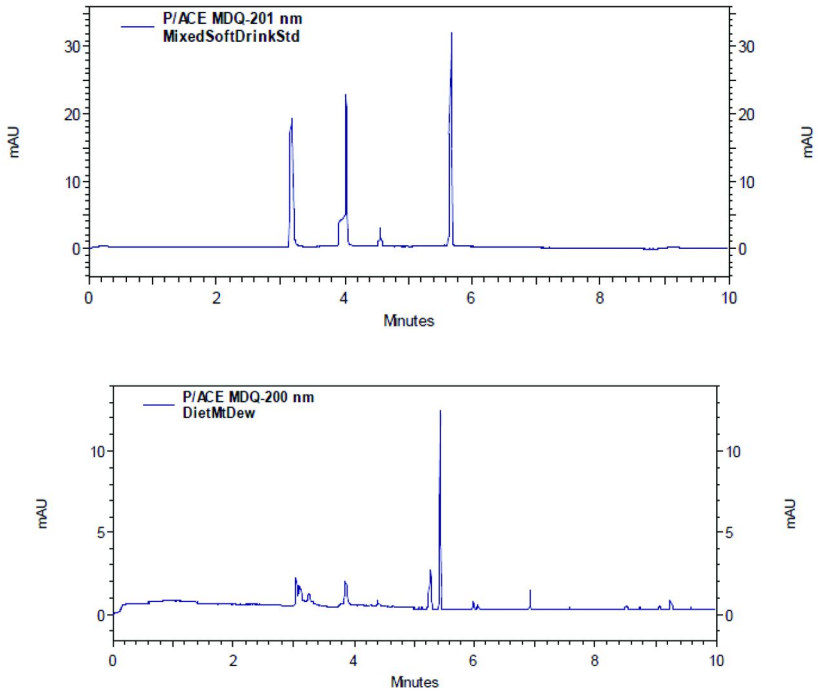

Figure 2. Analysis of caffeine, aspartame, and benzoate (migration time of just over 3 minutes, 4 minutes, and about 5.5 minutes, respectively) from Diet Mountain Dew by capillary electrophoresis.The first electropherogram is a standard mixture and the second electropherogram is an actual diluted sample of Diet Mountain Dew.

The capillary electrophoresis determination is performed using a Beckman P/ACE MDQ capillary electrophoresis instrument equipped with a 50 cm effective length and 57 cm total length eCAP column with a 75 µm inside diameter. The instrument cooling system maintains a 25 degree temperature. The separation is done using 0.10 M borate buffer of pH 8.35, using a hydrodynamic injection for 5.0 seconds at 0.5 psi. The separation voltage is 25.0 kV maintaining a current of 300 µA with a 0.17 minute ramp and a separation time of 10 minutes.

We find that the determinations based upon these separation techniques on a slightly more complicated mixture are more straightforward for the students than the simultaneous UV-vis determination. Therefore, the student generated results are usually better for the separation methods. However, we are hampered by our capacity on these instruments since we have fewer of these more expensive instruments than UV-visible spectrophotometers. The capacity problem is magnified in capillary electrophoresis since the CE column is generally regenerated periodically during a series of analyses unlike HPLC. Both determinations are relatively fast and photodiode array detectors make the analysis quite straightforward.

Biochemistry Laboratory

Peptide Analysis of Cytochrome C by High Performance Liquid Chromatography and Capillary Electrophoresis

As instruments are added, the innovative experiments found in the *J. Chem. Educ.* and elsewhere can be tweaked to make use of new instrumentation. For instance, the pairing of a photodiode array high performance liquid chromatograph with a microwave reactor allows a two laboratory period experiment in peptide mapping by HPLC to be performed in one class period by using the microwave reactor to perform a microwave enhanced enzymatic digestion using trypsin (*5*–*8*). This experiment works particularly well when the protein contains a visible chromophore or a colored prosthetic group. To that end, it has worked well with cytochrome C (we have tried performing this experiment with other colored proteins such as myoglobin and hemoglobin. A small word of warning: the heme group in myoglobin and hemoglobin is not covalently bonded to the polypeptide, so it runs separately from any of the peptides produced by proteases.). All peptides can be detected by their absorbance at about 210 nm, but the peptides containing the chromophore can be identified by the unique absorbance in the visible region where the chromophore absorbs.

In order to perform this experiment in one 3-4 hour laboratory period, solutions of cytochrome C, trypsin, and the HPLC solvents need to be prepared in advance. We have also run SDS-polyacrylamide gel electrophoresis along with the HPLC peptide mapping and we will describe that procedure as well for those who are interested. The gel electrophoresis can be run at the same time as the chromatography and by using pre-stained molecular weight markers, the cutting of the cytochrome C by the protease can be dramatically illustrated simultaneously by both gel electrophoresis and liquid chromatography of the tryptic peptides. As in Kenigsberg, et al. (*5*), we prepare a 4 mg/mL solution of cytochrome C in 1% ammonium bicarbonate. A stock 4 mg/mL trypsin solution is prepared in the same concentration of ammonium bicarbonate. Two HPLC solvents are prepared by making mobile phase A 0.1% trifluoroacetic acid in water and mobile phase B 0.1% trifluoroacetic acid in 2:1 acetonitrile:isopropanol. HPLC grade solvents are used, and the solvents are filtered through 0.45 μm Millipore filters and degassed. A CEM corporation MARS5 microwave reactor is used to accelerate the tryptic digestion. A mixture of 2.0 mL of the stock cytochrome C and 0.15 mL of the stock trypsin are placed in a GreenChem reaction vessel. The reactor is programmed to ramp up to 400 W power over one minute, and then to maintain a temperature of 37 degrees for 20 minutes.

While the microwave-assisted reaction is proceeding, the SDS-gels are prepared and the HPLC column equilibrated. We have used Novex Tris-glycine SDS-polyacrylamide gels and they are prepared according to the directions of the manufacturer (we find that an 18% gel works better than 12% or gradient gels due to the small size of the resulting peptides). Sigma Colorburst molecular weight markers are mixed with an equal volume (no more than 100μL is required) of the 2X SDS sample buffer, as are the digests after waiting for the reaction vessels to approach room temperature. An intact cytochrome C sample is prepared as well for a control. These are heated to 95 degrees for five minutes and loaded

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onto the gel (10-50 μL). The remainder of the microwave-treated samples are processed for chromatography by filtering through a 0.45 μm syringe filter and placed in an HPLC vial. The gel is loaded into a Hoefer Mighty Small II SE260 gel electrophoresis apparatus and connected to a power supply set at 125 volts constant voltage. While the gel is running (about an hour), the peptides can be separated on the HPLC.

We have used a Varian binary gradient HPLC system equipped with a ProStar 410 autosampler, two model 210 pumps, a Varian Microsorb-MV 100-5 C8 150 X 4.6 mm HPLC column, and a ProStar 335 photodiode array detector. The original *J. Chem. Ed.* paper used a C4 column (*5*), but the C8 column works also. The detector is set to monitor 214 and 410 nm, to detect peptide bonds and the heme group respectively. Initially, mobile phase A is pumped at a flow rate of 1.0 mL/ minute for the first two minutes. A gradient is run from 2 minutes to 20 minutes in changing from 100% solvent A (aqueous) to 50% solvent B (acetonitrile and isopropyl alcohol) while maintaining the same flow rate. On a 250 mm C4 or C8 column, 35 minutes of data collection time should be sufficient. At least one peak in the HPLC should absorb at both 214 and 410 nm (see the large red peak in Figure 3). If more than one peak at 410 nm is present, then the proteolytic cleavage was incomplete (there is a small peak at 400 nm in Figure 3).

The gel electrophoresis experiment is complete in about an hour. Since the chromophore is reddish-orange, spotting the heme-containing peptide on the gel is easy. The cleavage of the cytochrome C is readily apparent to the naked eye without staining. Since all of these polypeptides are visible to the eye, staining is unnecessary for the calculation of a molecular mass. Cytochrome C moves to near the bottom of the gel, but the heme-containing peptide of the tryptic digest of cytochrome C moves noticeably faster. The gel can be stained by Coomassie blue or silver stain if desired, but it is simple to estimate the extent of digestion without staining the gel.

The preparation of the HPLC, gel electrophoresis, and microwave-assisted proteolytic digestion takes no more than two hours. It requires about an hour to run two chromatograms on the HPLC (one digested and one not) and the gel electrophoresis experiment. The entire experiment can be completed in three hours unless the instructor wishes to stain the gel or perform some other enhancement. We have not run the semi-preparative experiment and assay for cytochrome C as Kenisberg, et al. (*5*), did.

Another experiment that could easily be added is an analysis of the peptic digest by capillary electrophoresis. We have performed this experiment using a Beckman P/ACE MDQ capillary electrophoresis instrument equipped with a 50 cm effective length and 57 cm total length eCAP column with a 75 μm inside diameter. The instrument cooling system is set to maintain a 25 degree temperature. The separation is done using 0.1 M phosphate buffer of pH 2.5, using a hydrodynamic injection for 5.0 seconds at 0.5 psi. A separation voltage of 25.0 kV maintains a current of 300 μA with a 0.17 minute ramp and a separation time of 30 minutes. From the results in Figure 4, it is clear that the heme containing peptide has a migration time of 9.5 minutes and that the total separation time is really on the order of 24-25 minutes. (The first electropherogram detects any peptide bond at 204 nm while the second electropherogram at the longer 400 nm

wavelength detects only the heme chromophore.) The capillary electrophoresis experiment could be performed at the same time as the gel electrophoresis experiment and high performance liquid chromatography.

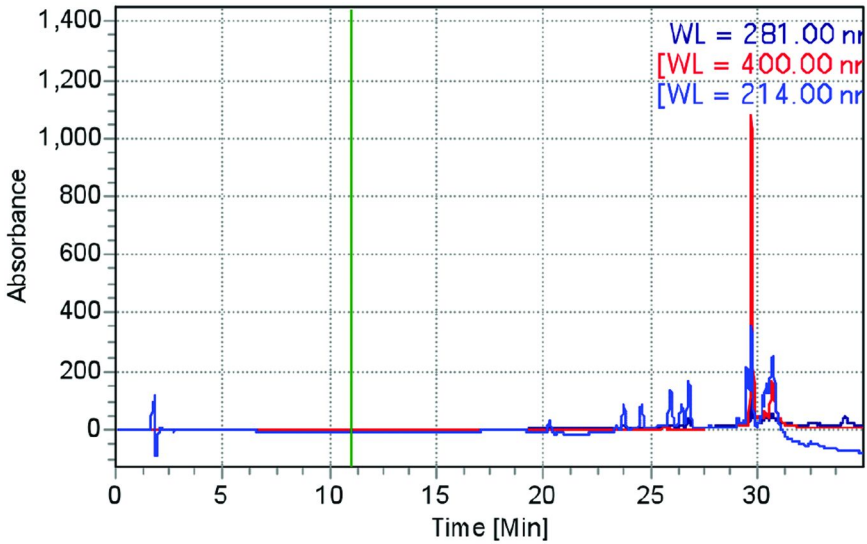

Figure 3. Reverse phase HPLC chromatogram of trypsin digested cytochrome C. Photodiode Array Detector allows the heme containing peptide to be easily seen at 410 nm (in red), while all peptides are visible at 214 nm (blue trace). The digested peptide containing the heme group is seen just before the 30 minutes retention time, and the remaining intact protein is believed to be the small peak shortly after 30 minutes. (see color insert)

By combining the microwave-assisted enzymatic proteolysis with SDS-gel electrophoresis, coupled with peptide mapping utilizing both HPLC and CE, the students have learned a number of important techniques in one laboratory period. Students have carried out an enzymatic reaction which can be tested for the degree of completion by a simple visual analysis of the SDS-PAGE gel. The students have learned to load a SDS-polyacrylamide slab gel, to use a high voltage power supply, and they could be asked to calculate an apparent molecular weight for intact cytochrome C. The HPLC and CE analyses suggest ways that the particular heme-containing peptide could be detected and isolated. Either instrumental technique coupled to electrospray ionization(ESI)-mass spectroscopy would be able to generate molecular mass information for each peptide. A size exclusion HPLC column could also be used to estimate peptide size.

We have plans to extend this peptide mapping experiment by leading into it with a protein isolation laboratory experiment, performing a protease digestion on the isolated protein, and then analyzing the resulting peptides by HPLC and CE using photodiode array detection to detect chromophore containing peptides. Of course, ultimately we plan to identify the individual peptides by LC-MS using electrospray ionization to determine the molecular weight of each peptide.

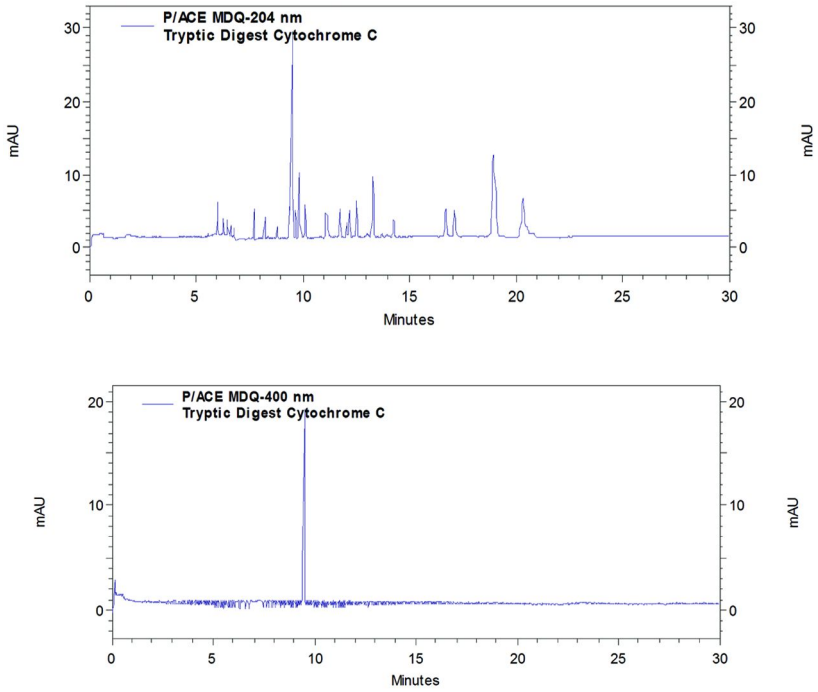

Figure 4. Tryptic peptides of cytochrome C separated by capillary zone electrophoresis detected at 204 nm and 400 nm respectively. These two electropherograms are extracted from the same injection and separation of a tryptic digest of cytochrome C. The heme group is detected at 400 nm so that the heme containing peptide can be seen easily. (see color insert)

Experiments under Development

Capillary electrophoresis allows the illustration of electrophoresis in a very convenient format without the use of large amounts of buffer and long and messy staining procedures. The presence of the electroosmotic flow (EOF, (*9*)), however, allows for more novel separations but essentially requires more discussion of the theory of the technique. A number of biologically relevant separations and quantitations can be performed by CE.

Virtually all of the enzymatic assays that are commonly done in the undergraduate biochemistry laboratory are spectrophotometric assays. We have used many of these: alkaline phosphatase, lactase, α-galactosidase (Beano), and tyrosinase (from a number of different sources). However, many enzymes are not readily assayed spectrophotometrically. Students need to realize that there are many different ways to monitor the progress of a reaction, and many of them require analytical techniqes with which many biologists are not familiar. We are currently working to develop two new experiments which are not quite ready for implementation, but which show promise for the future.

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Amino Acid Decarboxylases

In our laboratories, students have been studying amino acid decarboxylase reactions for a number of years (*10*, *11*). Amino acid decarboxylation reactions can be readily assayed by manometric procedures (*12*), and also by the release of radiolabelled CO_2 from radio-labelled amino acids (*13*), or by CO_2 sensitive electrodes. The manometric methods require equipment (a Warburg apparatus) which is somewhat rare, and takes up much room for those who are not using it often. Two amino acid decarboxylase reactions are illustrated in Figure 5.

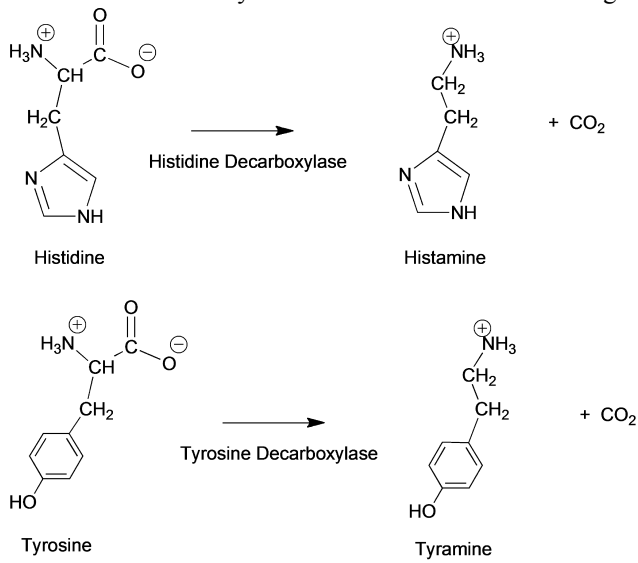


Figure 5. Reactions catalyzed by the enzymes histidine decarboxylase and tyrosine decarboxylase.

The loss of the negatively charged carboxyl group should make a far more significant difference in the electrophoretic migration of a decarboxylated amino acid from that of its precursor amino acid than the difference in retention time for a reverse phase chromatographic analysis. This suggests that capillary electrophoresis could be an extremely valuable method for the determination of the activity of amino acid decarboxylases. Using virtually the same conditions as in the capillary electrophoresis separation of tryptic peptides of cytochrome C, histamine can be readily separated from histidine and tyramine can be readily separated from tyrosine. Please remember that at pH 2.5 where this analysis is being conducted, the imidazole ring of histidine is also protonated, which carries it more rapidly to the negative electrode of the electrophoresis instrument (*14*) (Figure 6). In the first electropherogram, histidine is shown to have a migration time of about 7 minutes in 0.1 M pH 2.5 phosphate. Histamine, due to its greater positive charge migrates to the detection window in about 4 minutes under the same conditions. Tyrosine, with less positive charge, runs much more slowly, with a migration time of about 17 minutes. Its decarboxylation product, tyramine, has a migration time of approximately 6 minutes.

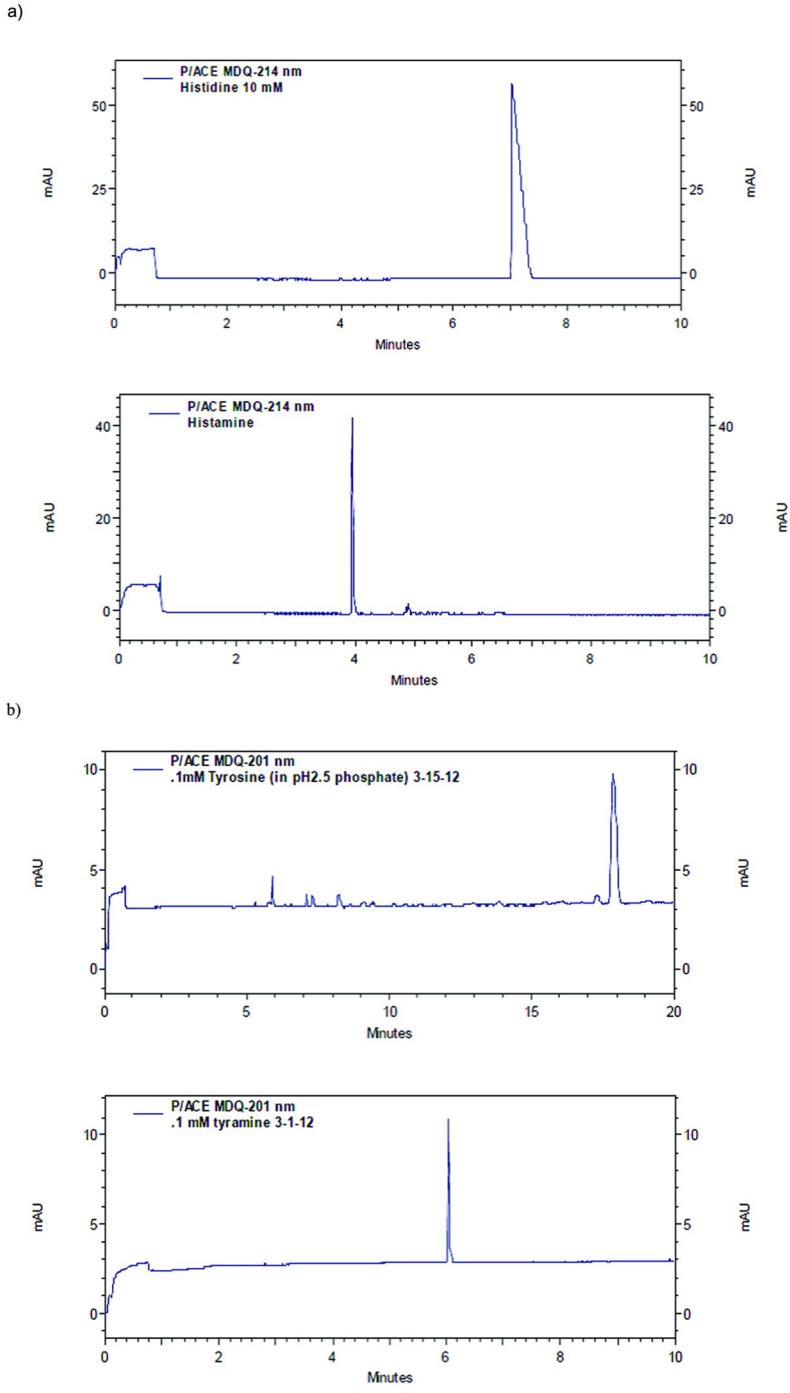


Figure 6. The reactants and products for a) histidine decarboxylase and b) tyrosine decarboxylase are shown in this series of electropherograms.

Electrochemical Methods

Cyclic Voltammetry

Techniques which many life science students almost never see are electrochemical techniques other than ion selective electrodes. With the newly available inexpensive potentiostats now on the market, it is much less expensive and easier to introduce undergraduates to this field. Cyclic voltammetry can be used to determine ibuprofen or ascorbic acid (*15*, *16*), but it can also be used as a method to obtain kinetic parameters in enzymology. Recalling that catecholamines were often determined electrochemically in the past, we investigated the possibility of determining dihydroxyphenylalanine (DOPA) and performing an assay of mushroom tyrosinase via a cyclic voltammetric technique. This reaction is shown in Figure 7.

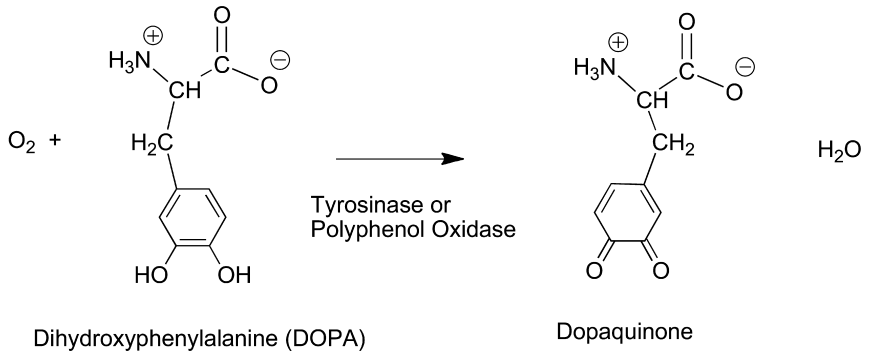
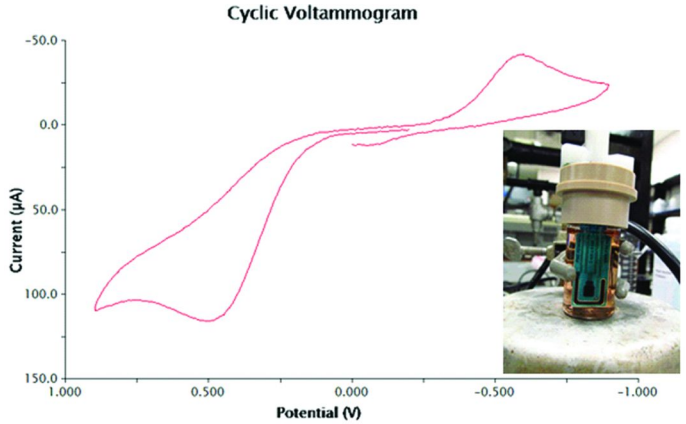
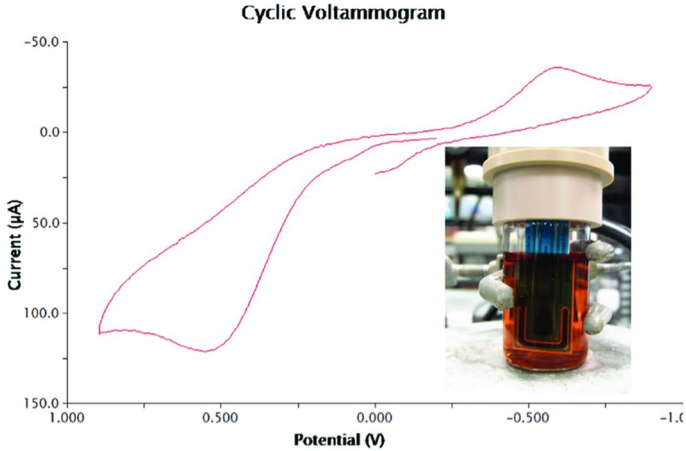


Figure 7. The chemical reaction catalyzed by tyrosinase (also called polyphenol oxidase) when DOPA is used as the substrate.

In a preliminary experiment, we used a Pine Research Instrumentation WaveNow potentiostat equipped with a student voltammetry cell and an inexpensive patterned carbon electrode to attempt this enzyme assay. The electrodes were activated according to the manufacturer's directions. A pH 6.75 0.08 M dibasic sodium phosphate and 0.01 M citrate buffer with 0.1 M potassium chloride was used for the reactions with varying concentrations of DOPA. Please see the sequence of photographs and associated voltammagrams run about the same time in Figure 8 illustrating the progression of the reaction catalyzed by mushroom tyrosinase using 3 mM DOPA as substrate and 1.0 mg/mL mushroom tyrosinase from Sigma as the catalyst. As can be seen in the illustrations, the peak corresponding to the red dopaquinone at first enlarges as the red color intensifies, but then the peak shrinks as the red color fades and the black dopachrome and other melanin related products are formed (*17*). Note that the peak at -500 mV shrinks throughout the analysis, and that the current increases at about +200 mV over time as the red dopaquinone decreases and the darker products increase. The voltammetric settings were an initial potential of -0.2 V, with upper and lower potentials of +0.9 V and -0.9 V. The final potential was 0 V with a sweep rate of 0.05 V/s and an electrode range of 5 mA.





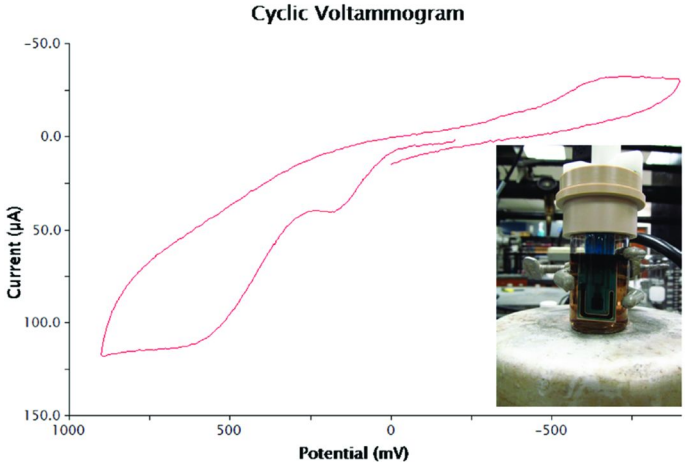


Figure 8. Three cyclic voltammograms and photographs taken illustrating the enzyme kinetics of mushroom tyrosinase using dihydroxyphenylalanine as the substrate. The first two were taken about four minutes apart near the beginning of the analysis and the third near the end. (see color insert)

Conclusion

With the influx of pre-health professional students into chemistry and biochemistry departments continuing, maintaining their interest in the field is very helpful if not essential. One method of engaging the mind of these students is by attempting to find and develop analytical procedures to determine materials of interest to the students. As more instruments become available to instrumental and biochemistry instructors, the creativity of the instructors may lead them to fascinating determinations which have not yet been discovered. The experiments contained in this article are but a few which have been investigated. We have attempted to share a few ideas and perhaps encourage others to explore other options that fit the needs of their students and the techniques available at their institution. The authors look forward to discovering many more fascinating bioanalytical procedures in the literature in the near future.

Acknowledgments

The authors would like to thank all of the students and colleagues who have helped perform the experiments discussed in this article. In particular, Dr. Matthew Vergne of Shimadzu Scientific Instruments has been a great source of assistance to us for several years. Dr. Leslie Hiatt of Austin Peay State University has provided us with guidance via a number of crucial discussions concerning the cyclic voltammetry of DOPA. Among the students who have contributed to this article, the amino acid decarboxylase assays by capillary electrophoresis were pioneered by Dr. Rachel Stephens Crouch while she was an undergraduate. This electrophoretic analysis has been continued recently by Brendon Burke. Dr. Linda Phipps supervised the determination of the detection limits for caffeine and benzoic acid.

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Chapter 8

Bioinstrumental Analysis

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An undergraduate course in bioinstrumental analysis was developed at the University of Washington and taught for six years between 2005 and 2010. The course consisted of 18 lectures, two exams, and eight laboratory experiments during a 10-week quarter. The first half of the course dealt with nucleic acid analysis and the second half dealt with protein analysis. Instrumentation used in the laboratory included gel electrophoresis and a gel reader, a PCR thermocycler, and a capillary electrophoresis system. Access was required to a core protein mass spectrometer and a DNA sequencing facility. Finally, bioinformatics portions of the laboratory require access to both BLAST for nucleic acid analysis and SYQUEST or Mascot for protein analysis. Enrollment was capped at 32 students, which was limited by laboratory space. The class was typically made up of roughly 2/3 biochemistry and 1/3 chemistry majors with an occasional student from biology and computer science.

Introduction

Courses dealing with *Instrumental Methods of Analysis* are commonly included in the undergraduate curriculum of chemistry departments throughout North America. Those courses focus on the operational principles and applications of instrumental methods, such as chromatography, optical spectroscopy, and electrochemistry, which students are likely to see in industrial chemical

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manufacturing, petroleum refining, and environmental laboratories. The courses usually have both lecture and laboratory components, inevitably are taught in a single quarter or semester, typically enroll undergraduates in their fourth year of study, and often are the terminal analytical chemistry course for these students.

Instrumental methods of analysis courses were developed in the 1960s-1970s and focus on the analytical challenges of that era. While those issues are no longer at the cutting edge of research, these instrumental analysis techniques are of great importance in the mature chemical industries.

Over the past decade, there has been an explosive growth of interest in bioanalytical chemistry. This interest has lead to the generation of courses specific to that topic. I developed graduate courses in *Bioanalysis* at both the University of Alberta in the 1990's and at the University of Washington in the 2000's. These graduate-level didactic courses covered both nucleic acid and protein analysis as practiced during that era.

With the encouragement of colleagues at the University of Washington, I drew upon the graduate-level *Bioanalysis* course to develop an undergraduate course in *Bioinstrumental Analysis*, Chemistry 428, and taught the course every year between 2005 and 2010. *Bioinstrumental Analysis* was offered in parallel with a classical *Instrumental Analysis* course. The two courses had both didactic and laboratory components, were primarily taken by chemistry and biochemistry majors, and were capped at 32 students per quarter, which was limited by access to laboratory equipment. To no surprise, the student makeup in the *Bioinstrumental Analysis* course was dominated by biochemistry majors (~2/3), with a significant fraction of chemistry majors (~1/3), and a smattering of other majors, including microbiology, biology, and computer science. Most students enrolled in Chem. 428 in the last quarter before graduation.

An introductory course in biochemistry was the only prerequisite for Chemistry 428. The prerequisite provided students with the basic vocabulary of biochemistry and familiarity with the molecules of life.

Text

At the inauguration of this course, there was no satisfactory *Bioinstrumental Analysis* textbook. The course instead relied lecture notes, web resources, portions of texts that are available on-line at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/books), and the primary literature.

Syllabus

The bioinstrumental course was organized around two lectures and one laboratory per week over the ten-week quarter. The first five weeks dealt with DNA analysis and the second five weeks dealt with protein analysis. Table 1 presents a typical syllabus for the course.

Table 1. Lecture Schedule

Week	*Day*	*Topic*
		Part 1 – nucleic acid analysis
1	M	Introductory materials
	W	Statistics
2	M	Absorbance & fluorescence
	W	Fluorescence part II
3	M	Electrophoresis of DNA
	W	Sanger DNA sequencing & bioinformatics
4	M	Sequencing the human genome
	W	Hybridization arrays & methods
5	M	Hybridization, PCR, & real-time PCR
	W	Midterm I
		Part 2 – protein analysis
6	M	Electrophoresis of proteins
	W	Liquid chromatography
7	M	Mass spectrometry
	W	Mass spectrometry & bioinformatics
8	M	Post-translational modifications
	W	Enzymes and enzyme chemistry
9	M	Receptors, inhibitors, & screening
	W	Antibody, ELISA, & blotting
10	M	Flow cytometry & fluorescence microscopy
	W	Midterm II

Part 1 – Nucleic Acid Analysis

Week 1: Introductory Material

Quantitative analysis was not a prerequisite for this course, and students usually had not covered basic statistics in their earlier courses. *Bioinstrumental Analysis* began with a review of basic statistics and data analysis at the level of Dan Harris' *Quantitative Analysis* textbook (*1*).

That discussion was expanded by introducing method validation and the regulations covering good laboratory practice. Method validation is seldom addressed in conventional instrumental analysis courses. Because many students enrolled in the bioinstrumental analysis course would eventually find themselves in laboratories where they must develop and validate new methods, roughly

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half of one lecture was devoted to the topic. The discussion went beyond the classic presentation of precision, accuracy. The lecture also covered the concepts of method ruggedness, stability of reagents; within-day and between-day reproducibility; and precision, repeatability, and reproducibility.

Accuracy is the key parameter in characterizing an analytical method. Unfortunately, there are very few standard reference materials available for bioinstrumental methods of analysis. Instead, locally generated reference materials are useful tools in characterizing the performance of a method over extended periods, provided that the material is stored properly.

The material on good laboratory practice was taken from a World Health Organization guide (*2*). Wikipedia provided much of the reading material for the statistics lectures. This material included pages on average, standard deviation, confidence interval, regression analysis, and detection limit (*3*–*7*).

Week 2: Optical Spectroscopic Methods of Analysis

The second week of the course considered spectroscopic methods of analysis, first with a brief review of Beer's law and absorbance, followed by extended coverage of fluorescence methods of analysis. Raman spectroscopy was also presented in terms of the background signal that it generates in high sensitivity fluorescence measurements.

Nucleic acid analysis provides several useful examples of fluorescence detection. The performance of a capillary electrophoresis based DNA sequencer formed the foundation for the discussion, as we worked through the effects of photobleaching, optical saturation, collection efficiency, and dye and detector quantum yields on the predicted performance of the laser-induced fluorescence detector.

Several resources were used for the second week. The chapter on fluorescence fundamentals from the *Molecular Probes* handbook was assigned to provide the basics (*8*). A 1989 manuscript written by Shaole Wu and me was used to provide calculations on the expected signals and noise in a high-sensitivity fluorescence measurement (*9*). A perspective article by Shaner, Steinbach, and Tsein provided detailed information on the properties of fluorescent proteins (*10*). Finally, the Wikipedia pages on fluorescence and Raman were used as additional references (*11*, *12*).

Week 3−4: DNA Electrophoresis, Bioinformatics I, and Bioethics

DNA electrophoresis was the first lecture of the term that addressed separations. We began with the basic theory of electrophoresis, providing the relationship between theoretical plates, voltage, and length of the gel. Joule heating was shown to produce a limit to the voltage applied during electrophoresis. Electrophoresis provides a nice example of both diffusion-dominated band broadening as an ultimate limitation in separations, and extra-column band broadening as a practical limit.

Examples were shown of agarose gel electrophoresis for separation of double-stranded DNA and polyacrylamide gel electrophoresis for separation of DNA sequencing fragments. The theory for DNA migration in gel electrophoresis is quite complicated, and far beyond the level of this course. Instead, the effects of gel composition and electric field were presented qualitatively in terms of biased reptation models. The effect of biased reptation and DNA secondary structure on sequence quality and read length were demonstrated by inspection of electropherograms.

As a high point in this portion of the course, Sanger's dideoxynucleotide sequencing protocol was presented. To put the technology in perspective, an ancient autoradiography was shown and students were given the opportunity to manually call sequence from the gel. Enthusiasm dropped after calling the first dozen bases. As an example of automated sequencing, the design of the ABI model 3700 DNA sequencer was revised. The linear algebra tools required to convert raw fluorescence spectra into DNA sequence were briefly presented.

The technology and results of the Human Genome Project were presented. Both Celera's whole-genome shotgun sequencing technology and the public consortium's mapping and sequencing efforts were compared.

BLAST and its variants were described as basic bioinformatics tools used in DNA analysis. One of the laboratory experiments in the course dealt with the use of BLAST to identify sequences related to an experimental DNA sequence.

This portion of the course provided an opportunity to consider bioethics. While whole-genome sequencing provides powerful insights into inherited diseases associated with mutation of a single gene, genome sequencing has provided precious few cures for disease. The ethical issues associated with diagnosis of genetic diseases can be profound, and provide an opportunity for discussion far beyond that which is found in most instrumental analysis courses.

Several sources from the primary literature used to introduce this material. Sanger's PNAS paper that introduced dideoxy sequencing provides an excellent introduction to the steps involved in creating that technology, as did his Nobel Lecture in 1980 (*13*, *14*). The chapter on isolating, cloning and sequencing DNA from *Molecular Biology of the Cell* is available on line, and provides a good overview of technology (*15*). Wikipedia's page on DNA electrophoresis provided a resource for that topic (*16*). Both Celera's *Science* paper and the public consortium's *Nature* paper were used to describe the sequencing of the Human Genome (*17*, *18*). Finally, a review paper published by Jianzhong Zhang and me reviewed the bioanalytical instrumentation used in the human genome project (*19*).

Week 4−5: DNA Hybridization, Aptamers, Molecular Beacons, Hybridization Arrays, and Stock Options

DNA hybridization provides a remarkably powerful tool for unambiguously identifying and isolating target molecules with high fidelity and at extraordinarily low concentration. We began with a discussion of the thermochemistry associated

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with DNA melting to understand hybridization stability as a function of temperature, ionic strength, base composition and mismatches.

Several examples of hybridization analysis were considered. The polymerase chain reaction and real-time PCR were discussed as archetypical examples of qualitative and quantitative assays that rely upon hybridization.

Transcriptomics measures the abundance of mRNA within a sample. An Affimetrix chip was used to demonstrate hybridization for analysis of the transcriptome. The array reader also provided a first example of confocal microscopy and scanning microscopy, which was expanded upon at the end of the course.

Nucleic acid based affinity probes, such as molecular beacons and aptamers, provide powerful tools for detecting trace amounts of analyte. They also provided an opportunity to review methods based on fluorescence quenching and fluorescence resonance energy transfer.

We briefly delved into forensic analysis by considering restriction fragment length polymorphism analysis, short-tandem repeats, and Southern and Northern blotting. This material allowed us to expand our bioethics discussion by describing The Innocence Project (*20*).

A few students are likely to end up being employed in a start-up company. To provide those students with some useful information, each lecture up to this point in the term began by tracking the stock price of Affymetrix. Affymetrix's share price was used to illustrate the concepts of founders' shares, stock options, lockout periods, and risks and benefits associated with stock options

A large amount of material was used to supplement these lectures. Mullis' *Science* paper was used to introduce PCR (*21*). Three pages from the Nobel foundation were used. The education page on PCR provided a lighthearted description of PCR's role in forensics (*22*). The page on the 1978 Nobel Prize in Physiology or Medicine was used to provide an historical view of restriction enzymes and their applications (*23*). The 1980 lecture by Paul Berg on the dissection and reconstruction of genes and chromosomes expanded the topic (*24*). New England Biosystems' pages on restriction digests and forensics were included (*25–27*).

Fodor's spatially addressable parallel synthesis paper (*28*) provided an introduction to the Affymetrix hybridization chip, which was supplemented with Affymetrix's Gene Chip brochure (*29*). The Wikipedia page on stock options provided background (*30*).

Midterm 1

A one-hour in-class midterm exam was given at the midpoint in the class. The exam consisted of a set of free-response questions, most of which dealt with interpretation of a set of data. As examples, one question covered a calibration curve in some detail, a second dealt with fluorescence instruments and theory, a third covered PCR and its application in forensics, and the fourth question required interpretation of a DNA sequencing electropherogram. Often, a question dealing

with stock options was included. The average of the exam was typically between 65% and 70% with a 15% standard deviation of the mean.

Part 2 – Protein Analysis

Week 6: Protein Separation and Labeling

The second half of the course dealt with protein analysis. We began with a discussion of protein separation methods. Protein electrophoresis built from our earlier lectures on DNA electrophoresis. This material is quite rich, with a much larger range of electrophoretic techniques available for protein electrophoresis than is commonly used for nucleic acids. Classical isoelectric focusing, polyacrylamide gel electrophoresis, and two-dimensional gel electrophoresis were discussed, and examples shown. Modern capillary gel electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectric focusing, and capillary-based two-dimensional comprehensive separations were also presented.

Protein stains, such as Coomassie Blue and silver staining, were presented as protein visualization tools in conventional gel electrophoresis. Covalent fluorescent and fluorogenic tags were also described, and typical reaction chemistries for labeling primary amines were shown.

Liquid chromatography was introduced next. Basic chromatographic theory, theoretical plate and resolution calculations, and common sources of on-column and extra-column band broadening were presented. The van Deempter equation was used to understand the effect of mobile phase flow rate on the quality of the separation.

Instrumentation for analytical-scale protein separations was described. Ion exchange, size-exclusion, strong cation/anion chromatography, reversed-phase, and affinity separations were presented. Isocratic and gradient-elution were described. Process chromatography was described, and capacity, sterility, and cost were considered.

Two chapters from the *Molecular Biology of the Cell* were used as resources (*31*, *32*). The first provides an overview of protein structure. The second presents a concise overview of ultracentrifugation, protein chromatography, affinity chromatography, and protein-based electrophoresis including two-dimensional electrophoresis, proteolytic digestion, and tandem mass spectrometry analysis of proteins. The Wikipedia page on chromatography provides a simple introduction to chromatography (*33*). Scott's chapter on preparative chromatography from the Library 4 Science provides a useful view of large-scale purification, which will be of value for students employed in the bio-pharma industry (*34*).

Week 7: Bottom-Up Protein Analysis, Protein Digestion, Mass Spectrometry, and Quantitative Proteomics

The protein analysis portion of the course focused on bottom-up proteomics. The use of proteolytic enzymes for protein digestion was described. Immobilized

and free-solution digestion were considered. Alternative proteolytic enzymes were briefly mentioned.

A generic overview of mass spectrometry was given, which included discussion of ionization sources, mass analyzers, and detectors. Electrospray ionization and matrix-assisted laser desorption and ionization were presented in some detail. Quadrupole mass analyzers were described and used as the first example of tandem mass spectrometry. Ion traps and time of flight instruments were then discussed. Electron multiplier tubes and image-current detection were presented. Tandem mass spectrometry was described as a tool for protein identification.

The second lecture on bioinformatics introduced database searching for protein identification from tandem mass spectral information. The students performed an experiment that generated tandem mass spectra for an unknown protein. As part of this experiment, students explored both SEQUEST and Mascot search tools.

Challenges associated with protein quantitation arise because of the dramatic differences in ionization efficiency of proteins and peptides. Isotope coded affinity tagging was used as an example of an isotopic labeling strategy for determining the changes in protein abundance between two samples. That technology had been developed at the University of Washington, and there was an extensive institutional memory to draw upon while covering this topic.

Four Nobel Prize lectures were used. Sanger's first Nobel Prize lecture in 1958 was used to highlight the challenges faced in determining protein sequence (*35*). Paul's description of the ion trap and Fenn's and Tanaka's lectures were used to provide an introduction to the powerful electrospray and MALDI ionization methods, which revolutionized protein analysis (*36–38*). Yates's Nature Biotech paper was used to introduce SEQUEST as an algorithm for the automated interpretation of tandem mass spectra in bottom-up proteomics (*39*). Aebersold's *Nature Biotech* paper on ICAT was the primary literature resource for isotopic labeling (*40*).

Week 8: Post-Translational Modifications and Enzyme Analysis

Post-translational modifications are important in determining the function and activity of proteins. A lecture was devoted to identifying the challenges in identifying proteolytic processing of an apoprotein into the mature form, the introduction and removal of phosphate groups, and the presence of carbohydrate and lipid modifications. Ion-metal affinity chromatography was the only tool considered for phosphoprotein concentration.

At the request of the biochemistry faculty, a lecture was added to the course to cover the basics of enzyme assays. This discussion included Michaelis-Menton kinetics and competitive and noncompetitive inhibitors.

Fluorescence quenching and resonance energy transfer were revisited in this portion of the course in the context of proteolytic enzyme detection and characterization.

A chapter from the on-line version of *Genomes* was used to describe both translation and post-translational modifications (*41*). A chapter from *Essentials of Glycobiology* was used to discuss structural analysis of glycans (*42*). The page on the 2004 Nobel Prize in Chemistry was used to discuss ubiquitination (*43*). The Wikipedia page on enzyme (*44*) and the Royal Society self-learning page on Enzymes (*45*) were used to provide background material

Week 9: Immunoassays

The penultimate lecture of the course dealt with immunoassays. Basic complexation equilibrium theory was presented. The generation and properties of both polyclonal and monoclonal antibodies were described. Selectivity of antibody binding was considered. The first immunoassay to be considered was Western blotting, which was first briefly mentioned in the protein gel electrophoresis portion of the course. Non-specific binding was introduced with this material. Noncompetitive immunoassays were presented next, along with typical calibration curves. Competitive immunoassays were also considered. The fluorescent polarization rotation assay was used to describe polarization anisotropy and to illustrate a homogeneous assay.

Rosalyn Yallow's Nobel lecture was used to illustrate immunoassays (*46*).

Week 10: Optical Microscopy and Flow Cytometry

The final lectures dealt with flow cytometry and fluorescence microscopy. The sheath-flow cuvette was presented as an elegant low-volume flow cell for optical measurements. The first optical method considered was Rayleigh light scatter. Both right-angle and forward-angle scatter were presented. The flow cytometer was then used to review laser-induced fluorescence, and calculations were performed to understand the signal and noise observed in the measurement. Multiwavelength fluorescence and multiparameter data analysis were considered. Finally, cell sorting was presented as a tool for generating cells with high purity.

Microscopy was used to review optics. The discussion began with numerical aperture, and quickly followed with conventional, epi-illumination, and confocal fluorescence microscopy. The course concluded with a brief discussion of two-photon absorbance and two-photon fluorescence microscopy as a tool for optical dissection of a thick sample.

A chapter from *Molecular Biology of the Cell* was used to remind students of the types and practice of microscopy (*47*). A set of papers from the original literature was used to describe these technologies, including Wheeless and Horn's review of flow cytometry (*48*) and Denk, Strickler, and Webb's original two-photon fluorescence microscopy publication (*49*). The Wikipedia page on confocal microscopy was used to introduce that technique (*50*).

Midterm 2

A one-hour in-class midterm exam was given at the end of the course. Typical questions dealt with the design and application of fluorescence resonance energy transfer, fluorescence anisotropy in homogeneous immunoassays, basis of size exclusion and strong anion exchange chromatography, and details of a flow cytometer. Students tended to do very well on this exam; the average exam score was 80%.

Laboratory Exercise

The laboratory organization followed the lecture material rather closely, Table 2. A set of four experiments was developed for the DNA analysis portion of the course, and another four experiments for the protein analysis. In each section, two labs involved sample manipulation, one lab involved touring a core facility, and one lab involved bioinformatics for data analysis.

Students were provided a lab manual with fairly detailed instructions for each experiment. Each lab had a set of short pre-lab study sections to provide background for the experiment. Students prepared and handed in a semi-formal lab write-up each week.

Modern Biology, Inc. is a great resource for developing laboratories for this course (*51*). We employed the labs titled IND3 and IND7 in the course; these labs are examples of ELISA and PCR analysis, respectively.

Table 2. Laboratory Exercises

Genomics	
Week	*Topic*
1	Restriction digest & PCR
2	DNA Electrophoresis
3	DNA sequencing lab tour
4	Bioinformatics I Use BLAST as database searching tool
Proteomics	
5	ELISA & run, stain, and destain SDS PAGE
6	Tryptic digest
7	Tour mass spec facility – run digest
8	Bioinformatics II - SEQUEST

DNA Analysis

Restriction Digest, PCR, and Agarose Gel Electrophoresis

The first three labs dealt with amplifying a gene, digesting it with a set of restriction enzymes, and then performing agarose gel electrophoresis to separate the components. The β hemoglobin gene was amplified using PCR with a pair of primers

PCR was performed in one lab period. In the same period, lambda DNA was digested with EcoR1 and HindIII. In the next period, the samples were separated using agarose gel electrophoresis. The relationship between migration distance and fragment length was determined using the stained gels.

DNA Sequencing and Bioinformatics I

Due to a lack of time, DNA sequencing samples were not prepared. Instead, the students were given a tour of one of the DNA sequencing cores on the University of Washington campus. This tour was popular with the students, who finally had a chance to see one of the instruments that had been discussed in lecture.

Students were emailed a DNA sequence, which might have been generated if they had performed a DNA sequencing experiment. They were then given instructions to use nucleotide BLAST, with the goal of determining the organism and gene name from which the sequence originated.

Protein Analysis

ELISA and SDS-PAGE

The first labs in the second portion of the course dealt with ELISA and SDS-PAGE of proteins. The students performed an ELISA for immunoglobulins using a peroxidase readout system. The students also prepared an SDS-PAGE gel and separated a set of standards (β-galactosidase and triosephophosphate), and an *E. coli* protein sample. The students determined the relationship between migration distance and protein molecular weight.

Tryptic Digest

The students isolated a band from their gel. The proteins are extracted and digested using sequencing-grade trypsin. Peptides were purified with a Zip-tip and submitted to the protein sequencing facility.

Bioinformatics II

The output from the mass spectrometry facility was then submitted by the students to SEQUEST and Mascot. The students performed a protein-protein BLAST (blastp) search on the data. The results from the three software tools were compared, and the students were asked to identify the protein that they had been given.

Grading

As is common in senior-level laboratory courses, students tended to very well in the labs. The average lab grade was typically ~90%.

Conclusions and Acknowledgements

The bioinstrumental analysis course was quite successful. However, following my departure from the University of Washington, lack of personnel has prevented the course from being offered again. This situation is common in chemistry departments that do not invest a significant fraction of their faculty count to analytical and bioanalytical chemists.

The costs of the course were typical for a senior-level laboratory course. Two TAs were assigned to cover the four laboratory sections associated with the course. Laboratory equipment costs were minimal. Gel boxes and electrophoresis power supplies were the only capital costs. We also relied on my group's thermocycler for the PCR experiment. Finally, we had access to both the DNA sequencing and protein analysis core facilities on the University of Washington campus. Fortunately, costs for remote users of these facilities are not excessive, and most chemistry departments should be able to afford the course.

The course covers a very dynamic field, and will need frequent updates. For example, next-generation DNA sequencing must be included in a revised curriculum, and will likely form a laboratory experiment by the end of this decade, as costs decrease and data analysis tools become more powerful.

The course was taught as two lectures a week during a 10-week quarter. The small number of lectures result in superficial treatment of a number of topics. The course would be better suited to a semester format, which would provide nearly 50% more lectures, with a concomitant increase in coverage. Epigenetics could easily be added to the course, including nucleic acid modifications in the first half of the course and histone post-translational modifications in the second half. Laboratories dealing with flow cytometry, real-time PCR, and microscopy would be wonderful additions to the curriculum, provided appropriate instrumentation or core facilities are available. Finally, in an ideal scenario, the course would expand to three lectures and one lab per week for four credit hours. The expanded course could include clinical, forensic, and pharmaceutical bioinstrumentation, along with oligosaccharide and lipid analyses.

I worked with a number of talented teaching assistants in this course. I wish to highlight the work of Dr. Haley Puglsey, who developed the laboratories and served as the inaugural teaching assistant for the course, and Mr. Tom Leach, who served as the laboratory coordinator.

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Chapter 9

Nature's Medicine Cabinet: An Interdisciplinary Course Designed To Enhance Student Learning by Investigating the Ecological Roles of Natural Products

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Here we describe a new NSF-sponsored course model for introducing undergraduates to interdisciplinary science. This research-intensive course has students work in interdisciplinary teams to investigate the ecological roles of natural products in mediating plant-insect interactions using modern analytical chemistry. The class is co-taught by a chemical ecologist and an analytical chemist. Lectures are designed to expose students to core concepts in the field of chemical ecology; labs reiterate these concepts by having students carry out analyses on the molecules responsible for these interactions. The field of chemical ecology is ripe for this interdisciplinary model because it naturally bridges the "distinctive cultures" of chemistry and biology to investigate the role of natural products in nature (Kornberg, A. The two cultures: Chemistry and biology. *Biochemistry* **1987**, *26* (22), 6888−6891).

In Teaching Bioanalytical Chemistry; Hou, H.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Introduction

Global warming. Overpopulation. Renewable energy. Access to clean water. The grand global challenges of the 21st century are complex and necessitate new educational models for training undergraduate science students. Current undergraduate teaching models compartmentalize student learning within artificially-defined disciplinary boundaries (*1*). These models shortchange students by ignoring fundamental realities about how science is conducted beyond campus walls: in interdisciplinary teams of scientists who bring expertise from multiple fields to bear on an important scientific problem.

While graduate programs place an increasing emphasis on interdisciplinary training programs (*2*) (i.e. the Howard Hughes Medical Institute (HHMI), the National Science Foundation Integrative Graduate Education and Research Traineeship (IGERT), the National Center for Ecological Analysis and Synthesis (NCEAS) Distributed Graduate Seminars (DGS)), undergraduate institutions have been slow to follow suit. At small colleges, barriers to interdisciplinarity remain high for a number of reasons. First, teaching loads are high and there may be a lack of resources allowing a faculty member from one department to "step away" from their teaching duties within that discipline to teach students from outside that discipline. Secondly, there may be a lack of complementary expertise within a single institution that is necessary to teach a course of this nature. Thirdly, the course must fulfill a requirement within multiple majors in order to incentivize student enrollment in the course.

Here we describe the development of an upper-level undergraduate course in authentic interdisciplinary research experiments intended to foster student appreciation for the excitement of collaborative science.

Nature's Medicine Cabinet Overview

Course Format

The course is designed as an advanced training program in chemical ecology focusing on the role of natural products in ecology, physiology, and medicine. The course consists of two 75-minute lecture/discussions and one three hour lab per week. The course is co-taught by a chemical ecologist housed in the biology department (Arnold), and an analytical chemist housed in the chemistry department (Witter). The first two iterations of the course followed the structure outlined in Table 1, reaching a total of 66 students (73% first-year students, 27% upper-level students).

The intended audience for the course is upper-level science students who have completed organic chemistry, although we did teach a version of the class to introductory chemistry and biology students. Students with multiple science backgrounds including: chemistry, biology, biochemistry and molecular biology, neuroscience, and environmental science comprise the majority of the class, and the class fulfills an upper-level seminar credit for chemistry and biochemistry majors.

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The lecture portion of the course seeks to introduce students to the broad interdisciplinary field of chemical ecology and the roles of natural products in plants, animals, and human health (a detailed syllabus is given below). The laboratory portion of the course is comprised of four modules (of approximately three weeks each) that utilize multiple analytical methods to investigate the molecule(s) responsible for important chemically-mediated interaction(s) in nature. These four modules include the analysis of terpenes, phenolics, alkaloids, and volatile organic compounds (VOCs) (a detailed syllabus is provided below).

Table 1. Overview of course organization and lessons learned from the first two iterations of the course (Fall 2011, Fall 2012)

Component	*Description*	*Future improvements*
Course preparation	One month in summer and 1 meeting per week during semester	Weekly communication is important for co-teaching purposes
Lecture course	13 lectures (75 minutes each), 3 literature readings	Increase reading of primary literature?
Videos	3 clips from the *Botany of Desire*	None indicated
Quizzes, Exams	3 reading quizzes 2 formal exams	None indicated
Outside speakers	2 short lectures (30 minutes) on physiology and neuroscience of addiction	Lectures should be taped for future use
Oral presentations	10 minute PowerPoint presentations on topic of interest by students	Provide oral and written instructions on giving good presentations
Local field trips	1 trip to College Farm to collect samples for analyses	Inclusion of an additional trip to a PA winery
Laboratory work	Four modules built around the analysis of a specific molecule important in nature	A lab manual should be developed for use by the students
Blog post	Formal written report posted to the internet describing results of one of the four modules	Time should be allotted for revision of blog posts

Learning Goals

The learning goals specific to the lecture portion of the course are as follows:

1. To develop an advanced understanding of scientific methods that students may analyze and critically evaluate scientific literature, apply laboratory, field, and computational methods specific to the discipline, and interpret empirical data;
2. To develop an advanced ability to communicate effectively as scientists, both in written and oral formats, with other scientists as well as the general public;
3. To develop a detailed understanding of chemical ecology and the role that natural products play in ecological interactions, in physiology, and in medicine.

The learning goals specific to the laboratory portion of the course are:

1. To develop observational and critical thinking skills necessary for answering scientific questions;
2. To help students gain confidence in the use of modern chemical equipment;
3. To have students work in interdisciplinary teams;
4. To help students communicate laboratory results in a meaningful way.

Nature's Medicine Cabinet Lecture Syllabus

An example of the lecture syllabus from our course is given in Table 2. Given the lack of an undergraduate text in Chemical Ecology, course materials and readings were developed/assembled by Tom Arnold, and are available on request from the authors.

Table 2. An example of the lecture syllabus from *Nature's Medicine Cabinet*

	Lecture	
Week	*T*	*Th*
Natural Products in plants and animals		
1	Introduction to the course and chemical ecology	Survey of bioactive natural products Pre-lab time
2	Types of species interactions	*The Botany of Desire:* Potatoes and GMO Crops
3	Plant-herbivore interactions	Journal Club 1 Pre-lab time
4	Plant-microbe interactions	*The Botany of Desire:* Apples and Alcohol Pre-lab time
5	Insect chemical ecology	Journal Club 2 Pre-lab time
6	Vertebrate chemical ecology	Journal Club 3 Pre-lab time
7	Marine chemical ecology	Outside seminar speakers
8	**Fall Pause**	**Exam 1**
Natural Products and Human Health		
9	Human diets, olfaction, taste, and digestion	Student presentations Pre-lab time
10	Reproduction, hormones, and human pheromones	Student presentations Pre-lab time
11	Muscles, the nervous system and venoms	Student presentations Pre-lab time
12	Animal behavior; addiction and abuse	Student presentations Pre-lab time
13	**Exam 2**	**Thanksgiving**
14	Drug discovery in nature	*The Botany of Desire:* Psychoactive plant substances
15	Writing workshop: improving your first drafts	Final assessment and course evaluation

Nature's Medicine Cabinet Laboratory Syllabus

An example of the laboratory syllabus from Fall 2012 is presented in Table 3. Laboratory handouts and class instructional materials are available upon request from the authors.

Table 3. Example of the laboratory syllabus from *Nature's Medicine Cabinet*

Week	*Lab Activity*
Module 1: Terpenoid Labs	
1*	Trip to Dickinson College Farm, sample collection, introduction to chemical ecology in the field
2	Thin-layer chromatography of cucurbitacins from cucumbers with beetle bioassays
3	Cucurbitacin analyses using reverse-phase HPLC
Module 2: Phenolics Labs	
4*	Vineyard visit and grape collection for resveratrol analyses
5	Grape taste testing and micro-Folin Denis assay for polyphenols
6	Total antioxidant capacity of grape juice and grape skins
7	Reverse-phase HPLC analyses of resveratrol in grape skins
Module 3: Alkaloid Labs	
8	Bioactive properties of alkaloids: heart-rate monitoring after caffeine ingestion
9	Nicotine analysis of cigars by reverse-phase HPLC
10	Nicotine analysis of cigars by hydrophilic interaction chromatography (HILIC)
11	Nicotine analysis of cigars by solid phase microextraction (SPME) GC-MS
Module 4: Volatile organic compound (VOCs) Labs	
12	Volatile terpene analysis of insect-damaged versus healthy hemlocks using GC-MS
Communicating Science	
13	Orientation and guidance on writing a research post to the class blog
14	Peer-revision of 1st drafts of class blog posts
15	Course evaluations and wrap-up; final blog posts due

* Denotes days we will be travelling off campus.

Laboratory Results

Module 1: Cucurbitacin Analyses Using Reverse-Phase HPLC

In module 1, students collect cucurbits from the Dickinson College Farm, and extract and separate cucurbitacins using thin-layer chromatography and high-pressure liquid chromatography, as shown in Figure 1.

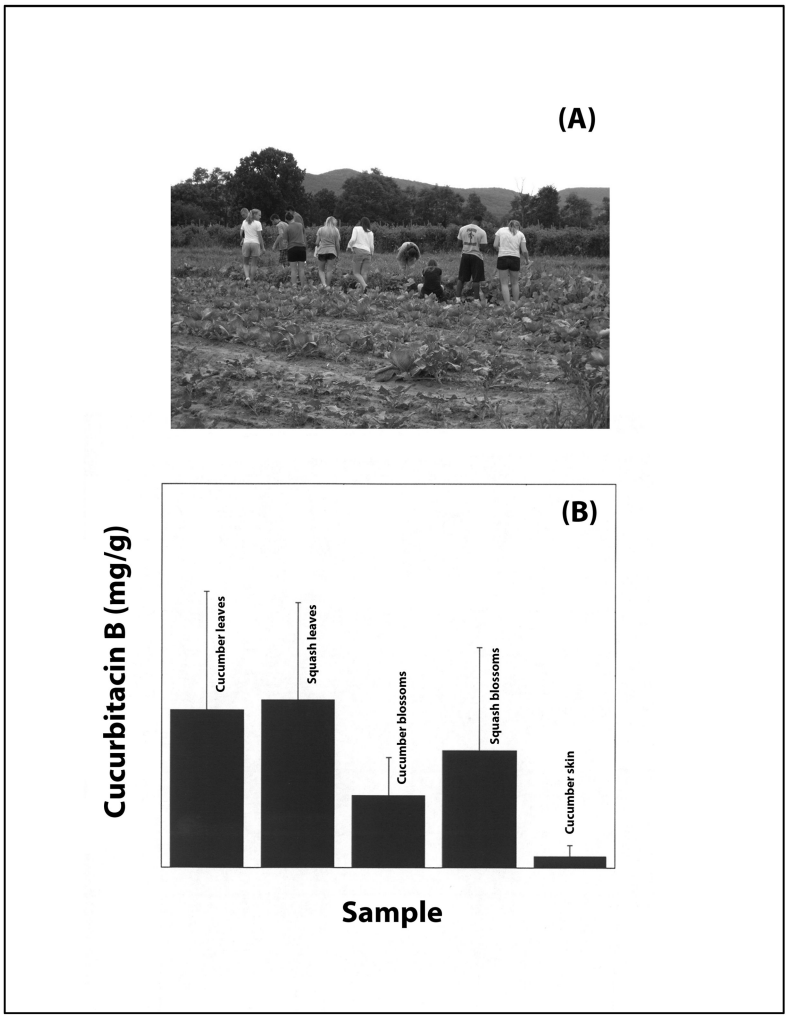

Figure 1. (A) Students enrolled in Nature's Medicine Cabinet collecting cucumber and squash leaves, blossoms, and fruit from the Dickinson College Farm for chemical analysis. (B) Bar graph depicts the amount of cucurbitacin B contained in various plant parts as determined by students (n = 36) using HPLC. Mobile phase: methanol–water.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Module 2: Resveratrol in Grape Skins

In module 2, students perform taste tests on five varieties of grapes, and we measure the sugar content using a Brix refractometer. We extract total phenolics from the grapes and perform the Folin-Cocalteu assay to measure the total phenols. We also measure the total antioxidants in the extract using the TEAC assay. Finally, we perform HPLC analysis to measure the concentration of resveratrol in each grape extract, as shown Figure 2.

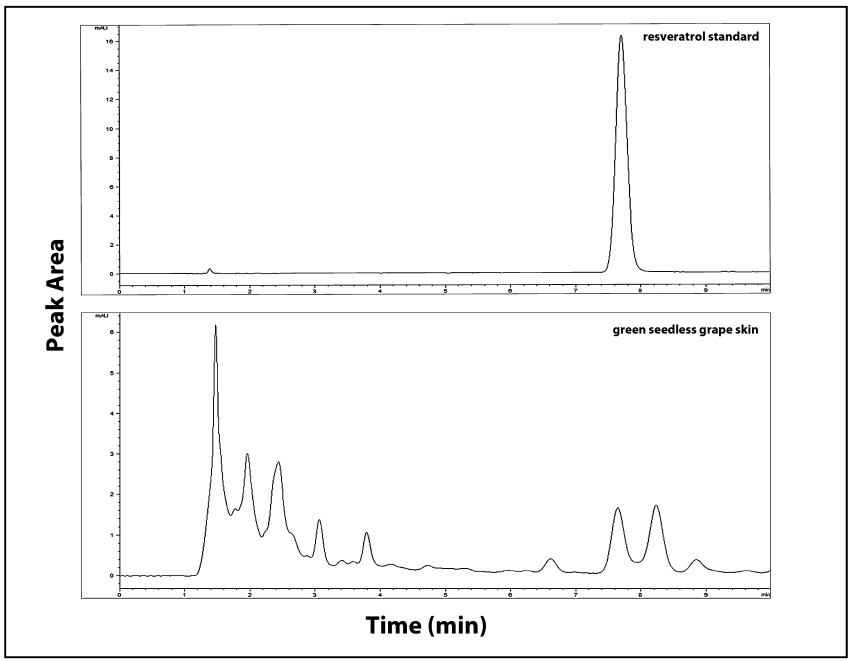

Figure 2. Representative reverse-phase HPLC chromatogram showing a 5 ug/mL resveratrol standard (top) and a grape skin extract containing resveratrol (bottom). Mobile phase: Methanol–water with 0.1% formic acid.

Module 3: Nicotine in Cigar Products

In module 3, we measure the nicotine content in cigar products using RP-HPLC, HILIC-HPLC, and solid-phase microextraction (SPME) gas-chromatography/mass spectrometry (GC/MS). We have the students compare the nicotine concentrations they obtain using the three methods and comment on their results. The RP-HPLC chromatogram of nicotine is shown in Figure 3.

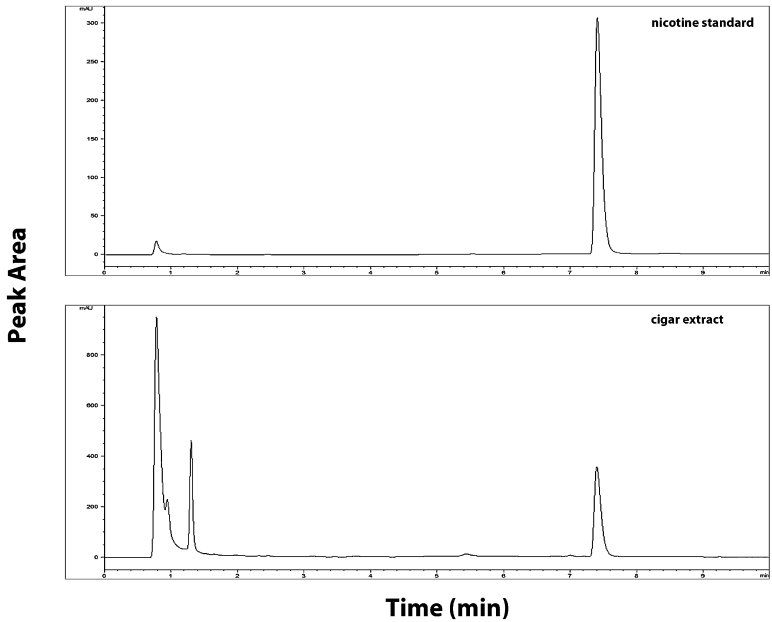

Figure 3. Representative reverse-phase HPLC chromatogram showing a 100 ug/mL nicotine standard (top) and a cigar extract containing nicotine (bottom). Mobile phase: acetonitrile–ammonium bicarbonate (pH 9.8).

Module 4: Volatile Organic Chemical Analyses of Terpenes Extracted from Hemlock Needles

In module 4, students collect needles from healthy and insect-infested hemlock trees on campus and we extract the needles to determine the relative concentrations of terpenes in each sample and control, as shown in Figure 4. Students are tasked with finding information as to why specific terpenes are up- or down-regulated.

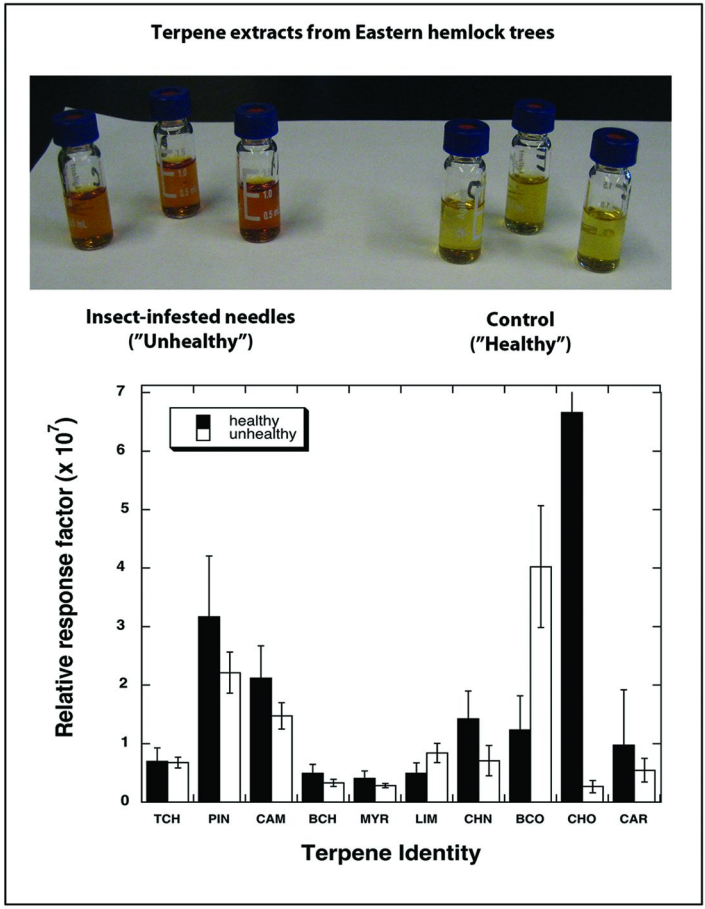

Figure 4. Methanol extracts from hemlock needles collected from insect-infested versus "healthy" trees (top). The bar graph shows the relative concentrations of terpenes measured in infested versus control samples. Volatile terpene abbreviations: TCH – tricycloheptane; PIN – alpha pinene; CAM – camphene; BCH – bicycloheptane; MYR – beta myrcene; LIM – limonene; CHN – 2-cyclohexanone; BCO – bicycloheptanol; CHO – 2-cyclohexanol; CAR – caryophyllene.

Course Assessment

The class was assessed using the Student Assessment of Learning Gains website accessible at www.salgsite.org. A student questionnaire was administered at the start and finish of the class, and the learning gains were compared, as self-reported by the students. Results from the first iteration of the course (taught to first year students) are presented in Table 4:

Table 4. SALG Assessment questionnaire developed for *Nature's Medicine Cabinet*

Item	*Questions*	*Pre-survey (n = 40)*	*Post-survey (n = 38)*
Presently, I understand…			
1	The relationship between a chemical's molecular structure and its biological effects in nature	3.60 ± 1.00	4.60 ± 0.89
2	How chemistry complements the study of biology and vice-versa	4.20 ± 1.00	5.10 ± 0.93
3	The various roles that chemicals play in ecology	3.70 ± 1.09	5.00 ± 0.79
4	How ideas we explore in this class relates to ideas I have heard about in the news	3.50 ± 0.99	4.50 ± 0.83
5	How chemists choose an analytical method for identifying an unknown molecule or measuring the amount of a molecule in nature	3.40 ± 0.84	4.80 ± 0.98
6	The importance of proper sample collection for obtaining accurate data	4.70 ± 0.97	5.20 ± 0.82
7	How to perform instrument calibration	3.80 ± 1.33	4.80 ± 0.98
8	How to use statistical tests to understand the limits of one's data	3.60 ± 1.22	4.60 ± 1.08
9	The types of natural products	3.50 ± 0.88	4.60 ± 1.17
10	The drug discovery process	3.00 ± 0.88	4.20 ± 1.10
Presently, I can…			
20	Find articles relevant to a particular problem in professional journals or elsewhere	4.00 ± 1.12	5.10 ± 0.78
21	Critically read a scientific article	4.20 ± 1.12	4.90 ± 0.84
22	Identify patterns in data	4.40 ± 0.81	5.10 ± 0.87
23	Write documents in a discipline appropriate style and format	4.00 ± 0.86	4.90 ± 0.91
24	Work effectively with others	5.00 ± 0.86	5.40 ± 0.82
25	Prepare and give oral presentations	4.50 ± 0.93	4.90 ± 1.11
26	Use scientific instruments without overt guidance	4.10 ± 0.88	4.80 ± 0.83
27	Use thin layer chromatography to separate a mixture	3.40 ± 1.34	5.10 ± 0.88
28	Use HPLC to separate a mixture	2.50 ± 1.04	5.10 ± 0.88
29	Use gas chromatography to separate a mixture	2.50 ± 1.01	4.80 ± 1.10

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Table 4. (Continued). SALG Assessment questionnaire developed for *Nature's Medicine Cabinet*

Item	*Questions*	*Pre-survey (n = 40)*	*Post-survey (n = 38)*
30	Use UV-Vis to measure the amount of a substance	2.50 ± 1.04	4.70 ± 1.15
31	Use a bioassay to identify an unknown substance	2.50 ± 0.91	4.80 ± 1.23
Presently, I am…			
32	Enthusiastic about science	5.10 ± 1.01	5.20 ± 1.18
33	Interested in or planning to take additional classes in science	5.20 ± 1.21	5.00 ± 1.57
34	Confident that I understand science	4.70 ± 1.22	4.90 ± 1.26
35	Confident that I can do science	5.00 ± 1.09	5.10 ± 1.23
36	Comfortable working with complex ideas	4.90 ± 1.01	5.10 ± 1.01
37	Willing to seek help from others (TA, peers, instructors) when working on academic problems	5.40 ± 0.68	5.30 ± 0.77
38	Confident in my ability to use new instrumentation to solve a scientific problem	4.70 ± 1.06	4.90 ± 1.16
Presently, I am in the habit of…			
39	Connecting key ideas I learn in my classes with other knowledge	4.80 ± 0.83	5.20 ± 0.83
40	Applying what I learn in classes to other situations	4.70± 0.91	5.30 ± 0.76
41	Using systematic reasoning in my approach to problems	4.60 ± 1.03	5.20 ± 0.71
42	Using a critical approach to analyze data	4.50 ± 0.99	5.10 ± 0.90
43	Communicating science to non-scientists	4.10 ± 1.05	4.90 ± 1.04
What best characterizes your major in college?		Yes/ No (%)	Yes/No (%)
44	I plan to major in chemistry, biology, or biochemistry and molecular biology	47/47	55/45
45	I'm not a science major	26/67	24/76
46	I'm undecided at this time	40/53	42/58
47	I plan on majoring in physics, math, computer science, astronomy, geology or environmental science	23/70	32/68
48	I plan on majoring in an area outside science	30/63	29/71
49	I plan to major in neuroscience	7/86	5/95

Continued on next page.

Table 4. (Continued). SALG Assessment questionnaire developed for *Nature's Medicine Cabinet*

Item	*Questions*	*Pre-survey (n = 40)*	*Post-survey (n = 38)*
How much did each of the following aspects of class help your learning?			
50	Interacting with professors during class	4.30 ± 0.76	4.70 ± 0.67
51	Interacting with professors outside of formal class hours	4.00 ± 0.92	4.10 ± 1.00
52	Interacting with professors from two different fields of study	3.80 ± 0.89	4.20 ± 1.06
53	Working with peers during class	4.00 ± 0.88	4.20 ± 0.89
54	Working with peers outside of class	3.70 ± 0.96	4.20 ± 0.97
55	Working with the teaching assistants	3.90 ± 1.06	3.60 ± 1.27
As a result of this class, what gains did you make in the following skills?			
56	Reading peer-reviewed scientific articles	3.00 ± 1.17	3.50 ± 1.18
57	Ability to understand connections in biology and chemistry	3.70 ± 1.34	4.30 ± 0.84
58	Identifying patterns in data	3.50 ± 1.26	4.30 ± 0.96
59	Thinking mathematically	3.50 ± 1.31	3.80 ± 1.02
60	Designing and carrying out experiments to deepen your understanding of chemical ecology	3.30 ± 1.22	4.00 ± 0.94
61	Working effectively with others	3.50 ± 1.36	4.00 ± 1.03
62	Understand methods of calibration and accurate data collection for chemical measurements	3.10 ± 1.25	4.20 ± 0.93
63	Your confidence level with using analytical equipment	3.00 ± 1.12	4.10 ± 0.86
As a result of working in this class, what gains did you make in the following?			
64	Interest in the interdisciplinary aspects of science	3.80 ± 1.37	4.20 ± 1.09
65	Thinking differently about the contributions from other fields of science	3.60 ± 1.27	4.20 ± 0.98
66	Confidence that you understand the proper use of equipment	3.70 ± 1.37	4.10 ± 0.97
67	Confidence that you understand the chemistry involved in chemical ecology	3.40 ± 1.26	4.20 ± 1.04
68	Your comfort level in working with ideas from multiple disciplines	3.60 ± 1.32	4.20 ± 1.06

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Table 4. (Continued). SALG Assessment questionnaire developed for *Nature's Medicine Cabinet*

Item	*Questions*	*Pre-survey (n = 40)*	*Post-survey (n = 38)*
69	Thinking of approaches to learn about natural phenomena	3.40 ± 1.17	4.10 ± 0.98
70	Your ability to solve problems	3.70 ± 1.45	4.10 ± 1.06

Challenges/Rewards of Interdisciplinary Teaching/Learning

The rewards of providing students with meaningful undergraduate lecture and lab experiences are great. Students report a deeper appreciation of the chemically-mediated interactions that occur around them in nature, and a richer understanding of the synergy between disciplines that highlight these interactions. Students also mention that they enjoy the open-endedness of real research, where the answers aren't known in advance. From a faculty perspective, co-teaching a course allowed me to learn new material from an expert colleague, which will in turn enrich my own teaching within my discipline by providing new examples for me to use in other courses. Still, barriers to realizing the full potential of a course of this nature exist. Here, we mention some of these that exist at our institution that might be similar at institutions of other sizes.

Having NSF-funding to support the development of this course was integral to its creation. While the authors had discussed a course of this type several years prior, the reality of having reassigned time in order to: teach the course, develop the lecture materials and labs, and for purchasing standards and supplies was tantamount for the course's success. The departments also had to allow the course to be taught for three years in a row, which meant that other courses were not being taught in its place. We experienced some unforeseen problems that we had not thought through in the original design of the course. Initially, we wanted to offer the course as two three-hour lecture/lab periods per week. However, the constraints of the college schedule did not allow for that model to materialize. Also, in our first year of the grant, we received funding after students had already chosen their courses for the upcoming fall semester. Thus, despite our best intentions, we could not fill the class with upper-level students. To compensate, we offered the class and lab to incoming first year students. While the enthusiasm level was high (mostly) for the material, the students didn't have an understanding of basic organic chemistry which is necessary for understanding the many extractions carried out in lab.

An Annotated Bibliography for Chemical Ecology Curricular Materials

Books

Algal Chemical Ecology. Charles D. Amsler (Editor), 2008, Springer-Verlag.

Chemical Communication in Crustaceans. Thomas Breithaupt and Martin Thiel (Editors) 2011. Springer-Verlag.

Chemical Ecology in Aquatic Systems. Christer Brönmark and Lars Anders Hansson (Editors) 2012.

Chemical Ecology: The Chemistry of Biotic Interaction. Thomas Eisner and Jerrold Meinwald (Editors), 1995, National Academies Press. Available as an e-book through the library or through the National Academies web site. Each chapter was also published as a paper in *Proceedings of the National Academy of Sciences* volume 92, issue 1 (1995). The chapters cover pages 1-82 in the journal and are exactly the same as what is reprinted in the book. You can download each of these as a pdf.

Chemical Ecology of Vertebrates. Dietland Müller-Schwarze. 2006. Cambridge Univ. Press.

Induced Plant Resistance to Herbivory. Andreas Schaller (Editor). 2010. Springer.

Induced responses to herbivory (Interspecific interactions). Richard Kaplan and Ian Baldwin. 1997. University of Chicago Press.

Marine Chemical Ecology. James B. McClintock and Bill J. Baker (Editors), 2001, CRC Press.

Methods in Chemical Ecology. Kenneth F. Haynes and Jocelyn G. Millar (Editors), 1998. Springer.

Pheromones and Animal Behaviour: Communication by Smell and Taste. Tristram D. Wyatt, 2003, Cambridge University Press.

Textbooks

Hands-on Chemical Ecology: Simple Field and Laboratory Exercises. Dietland Muller-Schwarze. 2009. Springer.

Journals

Journal of Chemical Ecology

Chemoecology

Internet-Based Resources

International Society for Chemical Ecology (www.chemecol.org)

Max Planck Institute for Chemical Ecology (www.ice.mpg.de)

Chemical Ecology of Insects (www.chemical-ecology.net)

Ideas for Student Experiential Learning

We take the student to the Dickinson College Farm to observe first-hand some of the chemically-mediated interactions between insects and plants.

Conclusions

Educating the next generation of scientists capable of addressing the challenges of the 21st century requires new educational models for training undergraduate scientists. These new models require that students are comfortable collaborating as part of a team, and that they can speak a common language of science that crosses disciplinary boundaries. Additionally, students need to experience the open-endedness of the scientific process, which leads to many dead ends, as well as to novel insights. Finally, students need to recognize that solutions to many of society's more compelling global issues requires collaboration across disciplines. As educators, our role is to provide meaningful educational experiences earlier in the careers of undergraduate scientists. The course described here provides one such model.

References

1. Kornberg, A. The two cultures: Chemistry and biology. *Biochemistry* **1987**, *26* (22), 6888–6891.
2. Wagner, H. H.; Murphy, M. A.; Holderegger, R.; Waits, L. Developing an interdisciplinary, distributed graduate course for twenty-first century scientists. *BioScience* **2012**, *62*, 182–188.

Chapter 10

Bioanalytical Chemistry and Chemical Sensors: An Advanced Elective Course for Undergraduates

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Teaching chemical concepts and process skills in a context that is interesting, stimulating, and relevant to students' lives is an effective pedagogical approach. The ultimate goal is to produce students in the STEM disciplines who are able to think and communicate like expert scientists. Described below is an advanced undergraduate course in Bioanalytical Chemistry and Chemical Sensors that was developed at a primarily undergraduate institution. Bioanalytical Chemistry provides a rich context for practicing skills such as problem solving, data interpretation, oral communication, and critical analysis of primary literature. This chapter covers the rationale for the pedagogical approach, course structure, course content, learning objectives, reference resources, testing using context based questions, and course evaluation. Emphasis is given to the use of primary literature in developing a rich conceptual framework that covers a variety of science disciplines.

Introduction

The vast improvements and developments in the instrumental analysis methods during the recent decades have enabled the study of very small samples of macromolecules such as proteins and nucleic acids. This has led to the rapid development of the bioanalytical chemistry that has allowed major progress in genomics and proteomics. Furthermore, many of these methods have been adapted into easy-to-use bench top instruments that allow computer controlled

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analysis of biological samples, or that have resulted in commercially available test kits based on robust and clever assay designs. As a rapidly developing, highly interdisciplinary area of chemistry that provides many interesting contexts for learning chemical principles, bioanalytical chemistry should be included in the upper level chemistry curricula more often. It has applications in medicine, pharmacy, technology, industry, national defense, forensic science etc. This interdisciplinary field also provides a rich context for practicing science process skills such as problem solving, data interpretation, oral communication, and critical analysis of primary literature.

Despite its current popularity among the research community, only a relatively small number of universities offer a course dedicated to bioanalytical chemistry, especially at the undergraduate level. Several textbooks or reference books that are suitable for a bioanalytical chemistry course have become available in the recent years. These include *Bioanalytical Chemistry* by Andreas Manz et. al. (*1*) and *Bioanalytical Chemistry* by Susan Mikkelsen and Eduardo Corton (*2*). A new introductory book on chemical and biological sensors by Florinel-Gabriel Banica may also be a good fit for some special topics courses (*3*). However, textbooks struggle to keep up with such a rapidly developing research field. This means that review articles that are accessible to undergraduate students and the general scientific community are an invaluable educational resource. This type of use of primary literature in teaching science students has been shown to improve their critical thinking skills (*4–8*).

Teaching Chemistry in Context

Context-based approaches to teaching chemistry have gained great popularity over the past two decades (*9*). These help provide the students with "relevance" that improves their attitudes and motivation towards science and chemistry in particular. Students are often drawn to bioanalytical chemistry, because it relates chemical principles and methods to applications involving real, biologically relevant samples. The increased student interest in applied science courses may also result from instructors who have a tendency to appear more energetic and enthusiastic when teaching topics related to their own research interests. This could be in part because they view the topics being taught as more important (*10*). The instructor's attitudes towards science and their own discipline has been shown to be critical to student learning and the consideration of future careers in science especially at the primary school level (*10*). The same can be said at the university level.

Teaching in context has also been extended to theme-based testing (*11*). These tests provide a single general context for a broad set of scientific concepts that are brought together under a unifying theme throughout the exam. The themes often come from current events, popular culture such as TV shows and movies, sports, and "real life" applications of science. These exams include some traditional content type questions, in addition to more involved, open-ended problems that give the students the opportunity to apply, synthesize, integrate, and evaluate information in a specified context (*11*). This promotes the complex

understanding of course material, inquiry skills on which scientists base their work, and long term retention of information as well as flexibility, critical thinking skills, willingness to take risks, and persistence (*11*). Context-based questions, where students are asked to describe, design, or troubleshoot methods, are of great value to courses such as bioanalytical chemistry. These courses often include context-based questions involving real life scenarios or applications of methods for graded assignments and exams.

Although higher education is gradually changing to include more progressive pedagogy, Chemistry, as a discipline, still has a culture with firmly established educational traditions. Most instructors are still more comfortable teaching in the way they were taught. This equates to traditional science teaching, where well established information is learned from a large textbook followed by testing with a content driven emphasis. Many faculty members focus on the delivery of content to prepare students for later courses in their curriculum. Most science majors also follow a vertical course structure throughout their studies with very little emphasis on interdisciplinary aspects of science and broader learning objectives. As a part of the larger discussion on curriculum design and course revisions, it has been suggested that it is time to examine what is taught and how it affects student learning. This is separate to how material is taught, which has been the main focus for many years (*12*). Chemical education research has focused on the teaching of and learning for elementary- and secondary-school students, followed by research commonly done with first-year chemistry students at the tertiary level (*13*). In the past several years the number of studies and conference presentations of chemical education research and practice focused on upper-level undergraduate courses has increased significantly. This is important as advanced or in-depth courses often present different learning objectives as well as different pedagogical and cognitive challenges (*13*). The instructors, regardless of the level at which they teach, are encouraged to develop a navigable path to mastering core scientific ideas and skills based on the student's current level of mastery (*12*). It is important that college graduates are scientifically literate with the ability to apply the scientific method, to critically read published results (literature), and to evaluate relevant information in their field. This can be addressed by incorporating several assignments and teaching practices that help strengthen science process skills into the courses.

Providing the motivation for the hard work required to master a subject and to develop science process skills is also critical. Analytical chemistry can seem dry or overly technical. Presenting it in an applied context with biological relevance helps students to see the connection between the material and their future careers or everyday life (*11*). This is a strong motivational factor for students. It also helps them to see the "big picture" and making connections between concepts and various science courses (*11*). This is the time in their college career that students make important decisions regarding their future plans. At the end of their college experience, the new graduates should have a set of skills that includes effective writing ability, creative problem solving, being able to argue their point, being able to constructively evaluate the work of others, being able to adapt to their changing environment, and being able to learn new things independently (*14*). In addition to having the content knowledge within their discipline, these are critical skills for both graduate level training and future career success.

Bioanalytical Chemistry and Chemical Sensors

This course was developed to help students improve their science process skills. The course material provides a solid foundation in modern bioanalytical methods and applications. This is especially important to students interested in pursuing graduate studies in chemistry or employment in the chemical industry. The course is entirely based on primary literature and review papers. As the course is not dependent on a textbook, it is possible to frequently update the course content to align with the most recent developments in the field. Unlike many introductory or lower level courses, there is no fixed amount of content that has to be covered during the semester, which allows more time to be spent on assignments and activities that teach students how to "think like a scientist". The course content continues to evolve. Every time it is taught the course is quite different. Preparing to teach the course is, therefore, quite time consuming, requiring significant changes in content every time it is offered. However, it is very rewarding to teach and allows new groups of students to be introduced to this important field. Furthermore, it has been suggested that primary-literature based teaching benefits undergraduate science majors by increasing their scientific literacy and significantly helps them with the transition to graduate and professional programs (*15*).

The course material has an emphasis on biosensors and immunoassays. Biosensors is an actively growing area of research in analytical chemistry which uses devices with selective biological recognition components to measure a physical or chemical change in signal in the presence of the analyte of interest. Biosensors have applications in national security, health care, food and beverage safety, environmental monitoring, the pharmaceutical industry, technology, and materials science which are used as examples when designing the course content. Biosensors, as a discipline, draws from a broad range of other fields such as biochemistry, molecular biology, clinical chemistry, environmental chemistry, material science, chemical engineering, and nanotechnology.

The course was developed at a primarily undergraduate institution that has a strong presence in the sciences. Most of the science majors are interested in pursuing graduate or professional studies in health related or STEM fields. Many of the chemistry graduates also opt for careers in the chemical industry. The chemistry and biochemistry and molecular biology (BMB) programs are still relatively small. Most of the students that take the bioanalytical chemistry lecture course are chemistry, BMB, or health science majors. The course satisfies one of their advanced (in-depth) course requirements. Some students also take the course to complete the requirements for a minor in chemistry. A typical class size for this course is 10-16 students. The relatively small class size allows experimentation with different pedagogical approaches, exam formats, and types of graded assignments. Most students are juniors or seniors and, therefore, have already completed many foundation science courses and are well prepared to meet the expectations of the 300-level in-depth course. Students taking this course have, at a minimum, already completed general chemistry, organic chemistry, an instrumental analysis lecture and lab courses (which are offered as 200-level sophomore fall semester courses), and many have also taken

a biochemistry course although that is not a prerequisite. Since many institutions offer instrumental analysis lecture and lab as 300-level courses, these do not necessarily have to be prerequisites for a course in bioanalytical chemistry and chemical sensors.

In addition, physical science and BMB students are required to complete an undergraduate research experience. Therefore, the bioanalytical course has incorporated in it, the training of undergraduate research students in essential bioanalytical concepts (learning goals 1-4 in Table 1) as well as scientific and other process skills (broader learning goals 1-9 in Table 1). The bioanalytical course teaches critical thinking and problem solving skills that are used in analyzing and interpreting chemical data. These help provide a foundation for undergraduate research. The course also includes aspects of graduate level training such as abstract writing, presenting research results, independent review of scientific studies/presentations, and critically reading and reviewing chemical literature. These skills also greatly benefit the senior undergraduate students, who are required to do a public presentation of their research results and to write a paper that is reviewed by faculty during their last semester. The course-specific learning goals are outlined in Table 1.

Table 1. Learning Goals

In general the objective is to understand:

(1) the theory of each method or technique;

(2) the instrumentation discussed;

(3) advantages and limitations of the method: and

(4) selected applications.

Other broader learning goals include:

(1) Strengthening analytical thinking and problem solving skills

(2) Applying course content to real life situations

(3) Interpreting graphs, diagrams, and tables

(4) Ability to compare and contrast multiple methods

(5) Ability to make connections among several course units

(6) Ability to gather information from multiple sources

(7) Ability to effectively read and interpret primary literature

(8) Ability to describe and critically evaluate research results

(9) Developing written and oral scientific communication skills

Students are also expected to learn the common acronyms encountered in Bioanalytical Chemistry for example ELISA (Enzyme-Linked Immunosorbent Assay).

Course Structure

The fifteen week long one semester course consists of 37.5 student contact hours in class and a two hour final exam. The class time is divided between introductory lectures by the instructor, literature reviews, discussions, content exams, and oral presentations by the students. The students are also expected to spend a minimum of six hours each week outside of class on reading, completing assigned problems, studying for exams, and preparing their presentations. The course does not currently include a lab.

Throughout the semester PowerPoint presentations are prepared based on chemical literature, review papers, online resources, and books. New material is integrated through introductory lectures on the topics supported by these presentations. Students are given lecture handouts of the presentations as reference material. The topics are divided into seven units which are listed in the lecture topics outline (Table 2) below. Reading assignments are given at the end of the class period once the introduction to the topic is complete. The assigned reading consists of journal articles, communications, and review papers based on the topics covered in lecture. Most of the journal articles describe research related to the techniques covered in the lecture. One or two articles are assigned at a time, depending on their length and difficulty level. The students read the articles outside of class and come prepared to discuss them at the beginning of the next class. Often the instructor has to ask the students to summarize the paper and to answer some questions about key points in the article in order to get the discussion started. Because it is important to expose students to how scientists think and analyze scientific results, the discussions and question and answer sessions may take an entire class period. The seven units and corresponding details have been listed numerically in the following outline of course topics (Table 2).

Table 2. Lecture Topics Outline in a One-Semester Bioanalytical Chemistry and Chemical Sensors Course

1. Enzymes in Bioanalytical Chemistry
   - Nomenclature
   - Enzyme structure and stability
   - Enzyme reactivity and selectivity
   - Enzyme activation and inhibition, catalytic properties
   - Enzyme kinetics
   - The effect of pH, temperature, solution concentration on enzyme activity
   - Immobilized enzymes and common enzyme immobilization methods
   - Cofactors
   - Quantitation of enzymes and their substrates
   - Electrochemistry of redox enzymes

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Table 2. (Continued). Lecture Topics Outline in a One-Semester Bioanalytical Chemistry and Chemical Sensors Course

- Mediators and wired enzymes
- Direct electron transfer

2. DNA and RNA in Bioanalytical Chemistry
   - Nucleic acids (structure, forms, function, stability in solutions)
   - DNA base pairing and analyte binding in DNA biosensors
   - Aptamers (specificity, affinity, and characteristics)
   - Structural changes in aptamers upon ligand binding
   - SELEX process
   - DNA hybridization biosensors and their applications
   - Electrochemical DNA assays and biosensors
3. Biosensors
   - Transducers, labels, detectors, and common analytes
   - Examples of biosensor configurations
   - Biocatalytic vs. affinity sensors
   - Detection methods
     - Amperometric sensors
     - Potentiometric sensors
     - Fluorescence sensors
     - Microcantilever sensors
   - Glucose biosensors
   - Historical development of enzyme electrodes
   - Design: disposable, continuous, and implantable sensors
   - Applications: Clinical and food & fermentation
   - Construction: mediators, immobilization of GOx and membranes
   - Receptor and organism based biosensors
     - Tissue, whole-cell, and bacteria electrodes
     - Bananatrode
     - Bacteria electrodes
     - Microbial fuel cells
4. Antibodies and Immunoassays
   - Antibodies (Abs) and their production
   - Structural and functional properties of Abs and antigens (Ag)

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Table 2. (Continued). Lecture Topics Outline in a One-Semester Bioanalytical Chemistry and Chemical Sensors Course

- Monoclonal vs. polyclonal Abs
- Antigen-antibody interactions
- Vaccination
- Immobilization methods of Abs onto sensor/assay surfaces
- Immunoassay development steps
- Nonspecific binding
- Immunoassay formats
  - Direct binding reactions
  - Competitive binding reactions
  - Avidin-biotin
  - Enzyme-Linked Immunosorbent Assay (ELISA)
  - Enzyme Multiplied Immunoassay Technique (EMIT)
- Immunoassay labels (enzymatic, fluorescent, radiochemical)
- Applications of immunoassays
  - Pregnancy test and other home testing kits
  - HIV testing
  - Pesticides and herbicides in the environment and produce
  - National defense (toxins, spores, bacteria and viruses)
  - Forensic toxicology testing for drugs, toxins, and poisons
  - Initial test for presence of drugs (clinical, workplace, and rehabilitation drug testing)
  - Sports, antidoping laboratories (recombinant human growth hormone)

5. Microfluidic systems, micro total analysis systems (μTAS), and lab on a chip devices (LOCs)
   - Common materials for fabrication
   - Fabrication and designs of the devices
   - Sample and reagent introduction and movement through the system
   - Applications (cell counting, immunosensors, biomedical and chemical screening)
6. Nanomaterials
   - Nanomaterials commonly used in biosensors
     - Carbon nanotubes (CNTs)
     - Graphene
     - Gold nanoparticles

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Table 2. (Continued). Lecture Topics Outline in a One-Semester Bioanalytical Chemistry and Chemical Sensors Course

	Ferromagnetic beads for immunoassays
	Applications of nanomaterials in medicine (drug delivery), electronics, engineering and industry
	Potential health and environmental risks of nanomaterials
7.	Validation and development of new biosensors and assays
	Proof of concept
	Sensitivity and selectivity
	Reproducibility
	Regeneration of the sensing surface and stability
	Reliability, interferences, and sample matrix effects
	Response time
	Cost, manufacturing, storage, and longevity
	Ease of operation and portability
	Calibration, limit of detection, dynamic and linear ranges
	Limitations of different sensor designs

Reference Materials

Electrochemical Biosensors (*16*) is one example of a review article that is regularly used as reference material in the course. Other tutorial and critical review papers (*17*) from *Chemical Society Reviews* and review papers from *Biosensors and Bioelectronics* (*18*) have been used, since they are typically written at a level suitable for senior students and are relevant for the course content. They provide a suitable introduction into a research area for someone who has no prior knowledge about the subdiscipline.

Other assigned reference reading materials include book chapters such as "Electrochemical Immunoassays and Immunosensors" (*19*) in *Immunoassay and Other Bioanalytical Methods*. Other book chapters on biological applications of sensors and bioelectrochemistry that have been used extensively in lecture preparation include *Sensors for Chemical and Biological Applications* (*20*) and *Bioelectrochemistry Fundamentals, Experimental Techniques and Applications* (*21*). Additionaly, examples featuring interesting applications of nanomaterials in biosensors can be found in *Nanomaterials for Biosensors* (*22*).

Problem Sets

Graded problem sets are assigned each time a major unit has been completed, which is about once a month. Students are expected to work individually on the graded problem sets, which are due in 10 days. They are turned in electronically.

There is usually a theme for each problem set that is based on a set of interrelated journal articles. Most of the journal articles are referenced on the assignment. Different sets of problems may be assigned to individual students. Sometimes, all of the students complete the same set of problems. Completing the problems without thoroughly reading the articles and, thus, understanding the key ideas, is difficult. This forces the students to read the articles in detail. The assigned articles typically cover an application, development of a method or a detection scheme that was not discussed in class. Therefore, students are challenged to utilize what they have learned during class in a new context. Some of the questions on the problem set are designed to help students distinguish between the technical and/or experimental details in the articles as opposed to the big picture; including main outcomes, conclusions, limitations of the technique/study, advantages over other existing methods, practical applications, interpreting graphs and tables, and broader impact on the field. Examples of problem set questions from the 2007 course are included in Table 3. below. Some questions require the students to perform additional independent literature searches using SciFinder or Web of Science which are available through our school library and to provide multiple references for their answers.

Table 3. Examples of Graded Problem Set Questions

You are expected to work on this assignment *alone*. You may use any reference sources (such as journal articles, books, and research websites) you wish as long as you *site* them properly. Questions 1-8 are related to the article: Farrell, S.; Ronkainen-Matsuno, N.; Halsall, H. B.; Heineman, W. R. Bead-based immunoassays with microelectrode detection. *Anal. Bioanal. Chem.* **2004**, *379*, 358−367.

1. What was the objective of the work described in the paper?

2. What are the advantages of using microelectrodes for the electrochemical detection step?

3. What is an RDE? What are the advantages of using RDE detection?

4. Why are paramagnetic beads considered to be a better solid support for the capture antibody than polystyrene microwells or capillaries?

5. The paper describes a series of experiments done to optimize a new immunoassay and detection combination. List all the parameters that were optimized in the described work.

6. Describe the detection set-up used in the manuscript.

7. Based on the data provided in the paper what was the optimal drop volume?

8. What was the effect of moving the microelectrode further away from the paramagnetic beads?

Questions 9-24 are related to the article: Dimcheva, N.; Horozova, E.; Jordanova, Z. An amperometric xanthine oxidase enzyme electrode based on hydrogen peroxide electroreduction. *Z. Naturforsch. C* **2002**, *57*, 883−889.

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Table 3. (Continued). Examples of Graded Problem Set Questions

9. What is the systematic name of Xanthine oxidase? What is the common abbreviation for Xanthine oxidase?

10. Write a balanced chemical equation for the reaction catalyzed by Xanthine oxidase?

11. What is the electroactive product of the enzymatic reaction?

12. What is the most common application for Xanthine sensors? What is the sample matrix for the enzyme electrode in this study?

13. Does the paper describe a two- or three-electrode detection system? What were the working, reference and auxiliary electrodes used in the experiments? (*Auxiliary electrodes* are also sometimes called *counter electrodes*.)

14. How was the enzyme immobilized onto the biosensor?

15. What substances were indicated as potential interfering species in biological samples? Was interference from these species seen in the studies reported in this publication?

16. Over how many days was the lifetime of the enzyme electrode studied?

17. What was the optimal detection potential for xanthine? How can you tell?

18. Define Michaelis constant, K_M. How is it determined? Why was K_M determined in the paper? Should K_M be high or low ideally? What are the advantages of having a high or low K_M?

19. What type of electrochemical detection method was used for quantitative studies using the biosensor?

20. What is the purpose of reporting r^2 values in the paper?

21. What substance can be used to stabilize Xanthine oxidase?

22. Why were lower temperatures more optimal for the xanthine biosensor? What is the optimum temperature for Xanthine oxidase based on research literature?

23. Define detection limit and signal to noise ratio. What were those experimental figures of merit in this study?

24. What was perceived to be the limiting factor in the sensor response?

Presentations

At the end of the semester, each student is asked to prepare a 25 minute PowerPoint presentation on a topic preapproved by the instructor. The last few weeks of the class are reserved for these student presentations. These help provide the class with a sampling of current ongoing research areas related to the course material. It also allows the students to further study a course topic that was of particular interest for them personally. Some examples of past presentation topics are included below. The presenting student will turn in a 250 word (maximum) abstract for their chosen presentation topic and the main journal article or reference two weeks prior to their presentation date. The presentations are usually based on topics chosen from journal articles that are relevant to the course material and must have been published within the last two years. Students choose a topic that they

are not familiar with. The instructor meets with each student individually during the two weeks leading up to their presentation to help answer any questions they may have on the material. The in-class presentations are followed by 10 minutes of questions from the fellow students and the instructor. This is usually the part that the students dislike the most, as it reveals how well they have prepared for the presentation and their understanding of the topic. This also forces the students to think and respond to questions and criticism like a scientist.

The presentations are evaluated by the instructor and fellow students. Peer review is commonly used in writing and other higher education courses as it has been shown to be effective in helping students improve their writing skills (*23*). It has also been used successfully in many science courses with emphasis on writing (*24–26*). Some students feel uncomfortable about giving detailed feedback or criticism to their peers. However, taking some time to articulate to students how peer review can be done in an informative and constructive way usually remedies that initial hesitation. The peer review of oral presentations in this class helps the students learn more about themselves and their current abilities by identifying strengths and weaknesses in their classmate's presentations. Peer review is a valuable tool as it illustrates how science is practiced by researchers and educators (*27*, *28*). The oral presentations follow the usual scientific presentation format and are graded on content, understanding of the subject matter, organization, delivery, and format. The evaluation sheets are returned to the student after the instructor has reviewed them and assigned the presentation grade. Table 4. contains selected examples of student presentation topics.

Table 4. Examples of Student Presentation Topics in Bioanalytical Chemistry and Chemical Sensors Course

1. Anthrax biosensors
2. Carbon nanotube biosensors
3. Cholesterol biosensors
4. Cocaine biosensors
5. Whole cell living biosensors
6. Microfluidic biosensors
7. Biosensors for detection of explosives
8. Biosensors for pathogenic bacteria in food
9. Detection of influenza A virus using a magnetic biosensor
10. Screen printed electrodes for biosensor applications
11. Electronic noses and tongues
12. Electrochemical assay for early HIV detection
13. Detection of toxins in seafood by SPR

Continued on next page.

Table 4. (Continued). Examples of Student Presentation Topics in Bioanalytical Chemistry and Chemical Sensors Course

14. DNA biosensor for drug studies
15. Enzyme immobilization methods onto Au nanoparticles
16. Pesticide immunoassays
17. Fluorescent labels in immunoassays

Exams

There are two 75 minute content tests and a cumulative final exam. These motivate students to keep up with the course material and to master the discipline specific content covered in lectures and selected journal articles. These exams include many question types from fill in the blank, define, compare, explain, short answer, to more open ended questions that tie in multiple course topics and require a written narrative. Essay questions (Table 5) are used for this course because the instructor can get feedback about student understanding of these advanced topics. These more open ended questions sometimes have many "correct" or reasonable answers. They are also a good indication of how well the students understand the underlying concepts. In addition, context-based questions are used for the exams. Students should be able to solve problems in multiple contexts including some that they may not have seen before. The question types vary depending on the material and the types of knowledge being assessed for each unit. Many of the questions on the final exam expect students to combine information and concepts learned in multiple units. This allows students to integrate information from multiple topics into broader scientific concepts. Example 1 of the context-based exam questions (Table 6) was used on a final exam and combines concepts learned in four different units. Examples 2 and 3 (Table 6) require the students to interpret graphed data (Figures 1−4).

Table 5. Examples of Traditional Exam Questions

1. Why are experiments that are done in dilute aqueous buffer solutions not representative for most proteins?
2. What is the main difference between biocatalytic sensors and affinity biosensors?
3. Discuss the oxygen dependence of first generation glucose biosensors and errors in sensor response.
4. Describe routes for facilitating the electrical communication between the redox center of glucose oxidase and electrode surfaces.
5. Why do biosensor test strips and biosensor devices have to be stable for over 130 days after manufacturing for the device to be commercially viable?
6. Why is electrochemical detection so common in biosensors vs. other methods such as spectroscopy?

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Table 5. (Continued). Examples of Traditional Exam Questions

7. What is a major limitation of impedance biosensors?

8. What gives carbon nanotubes their incredible strength?

9. How are reagents and samples typically introduced into and how do they move through the microfluidic system?

10. What are the two most common sample matrices when analyzing for trace levels of pesticides and herbicides in the environment using immunoassays?

11. When are competitive immunoassay formats used instead of sandwich immunoassay format and why?

12. What is nonspecific binding and how can it be minimized?

13. What is cross-reactivity of antibodies? Is that a good or bad thing for the immunosensor? Explain why?

14. How does a disposable home pregnancy test work?

15. Why is it sometimes beneficial to use disposable biosensors?

Table 6. Examples of Context-Based Exam Questions

1. Medicinal chemist, Dr. Karhunen, recently developed a new drug (with FDA approval) called Leenastatin (MW = 406 amu) that helps minimize plaque formation on the walls of the arteries. Plaque buildup eventually may lead to heart disease or stroke. You are working for a biotechnology company and your boss assigns you to develop a new aptamer biosensor for the measurement of Leenastatin levels in the patient at ng/mL concentrations.

A) What is the sample matrix for Leenastatin in which the biosensor has to function?

B) What makes this sample matrix more problematic to work with than for example aqueous environmental samples?

C) Why do you think your boss told you to use aptamers instead of antibodies or enzymes as the biorecognition element in the sensor? Give at least two reasons for why aptamers would be better in this case than either antibodies or enzymes.

D) Once the biosensor is fully developed, what types of parameters would you have to determine, test, and consider before it is launched for clinical use. Give at least 5 things that you would determine and report to your boss.

2. As a part of an NIH funded research project at Northwestern University you have developed a new peroxide assay for hydrogen peroxide (H_2O_2) measurement in protein samples. Hydrogen peroxide is usually difficult to detect and quantify because of its short lifetime.

A) Identify a problem with the calibration curve that may affect the quantitative results for determination of unknown sample concentrations of H_2O_2.

B) What is the most likely cause of this phenomenon?

C) How would you remedy the problem?

Continued on next page.

Table 6. (Continued). Examples of Context-Based Exam Questions

D) Are all of the concentrations of H_2O_2 chosen in the linear range for this calibration plot?

E) How would you evaluate the correlation between the actual data points and the data model-fitting?

3. Enzyme electrodes combine the sensitivity of electrochemical detection with the bioselectivity of enzymes. You and your lab colleagues are developing a new electrochemical detection method using a rotating disk electrode (RDE) modified with glucose oxidase (GOx) for amperometric measurement of β-D-glucose in energy drinks. While optimizing the new method your team has collected the following data.

A) What seems to be the optimum detection potential for the new electrochemical method?

B) Do you expect to see interferences from other sample components at that detection potential? Why or why not?

C) What are the ideal rotation rates of the RDE according to your data?

D) Explain the initial increase in relative enzyme activity with increasing rotation rate. What is the likely reason for the increase?

E) Make a statement about the reproducibility between trials while optimizing the RDE rotation rates.

F) What is the realistic life span of the biosensor that you have prepared?

G) Give some possible reasons for decreases in GOx enzyme activity over time.

H) What may help with increasing the life span of the enzyme electrode?

4. Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) often found in cigarette smoke, heavily polluted air, water, soil, and charbroiled meat. BaP (Figure 5) is highly toxic and carcinogenic, and therefore an important analyte in clinical analysis and environmental monitoring.

BaP is one of over 100 PAH's that are formed by burning fossil fuels and organic substances in food, garbage or cigarettes. The U.S. Environmental Protection Agency (EPA) has identified 17 harmful PAH's that are of interest in water, and established detection limits for them. Many PAH's are very similar in their molecular structure, molecular weight, lack of side chain groups, and electron density. Highly sensitive electrochemical immunoassays have been developed for the measurement of PAH's at ng/L to μg/L levels.

A) Why do you think that it is impossible to produce Ab's that are specific for only one PAH?

B) Define and explain cross reactivity of Ab's.

C) How would it still be possible to evaluate the presence of PAH's and their levels using immunoassays in clinical and environmental settings?

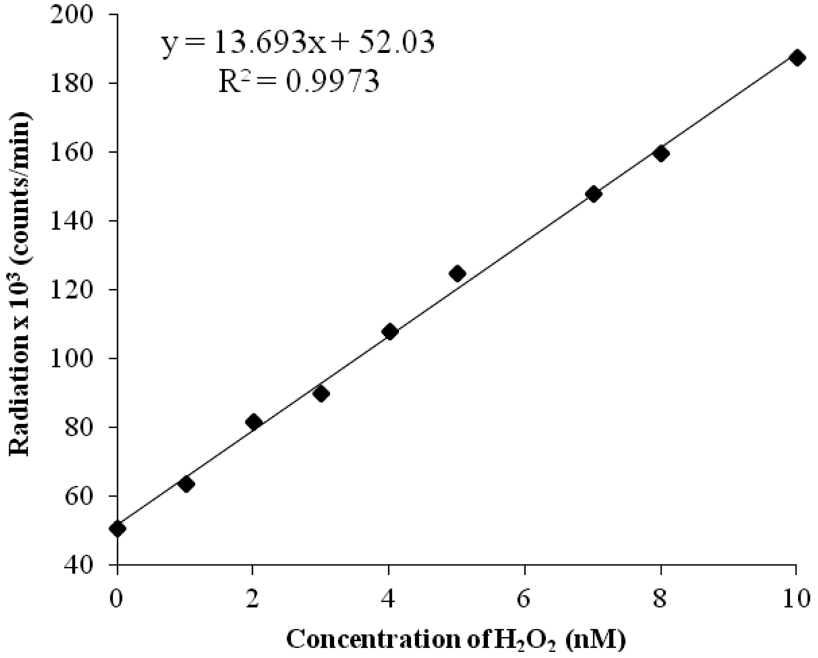

Figure 1. Standard calibration curve for quantitation of hydrogen peroxide in the nanomolar concentration range in 10^{-3} M phosphate buffer, pH 6.8, obtained by competitive ELISA with radioactive labels.

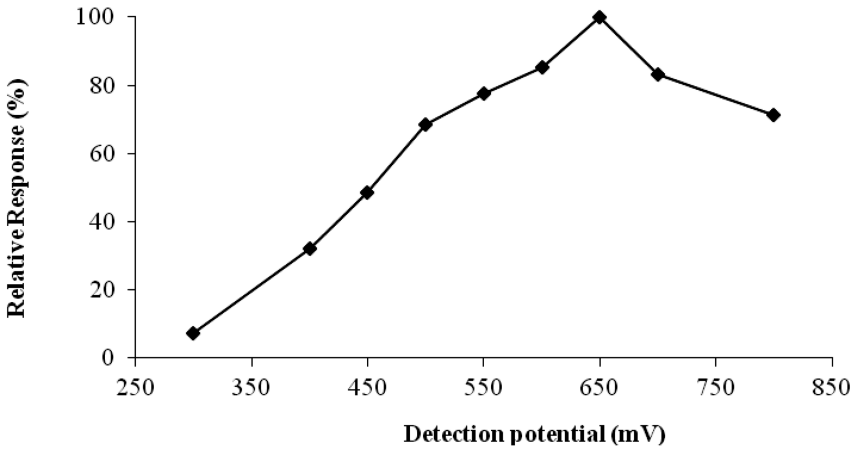

Figure 2. Plot showing the relationship between the relative current response and the detection potential of glucose using Pt GOx RDE vs. Ag wire pseudo reference electrode.

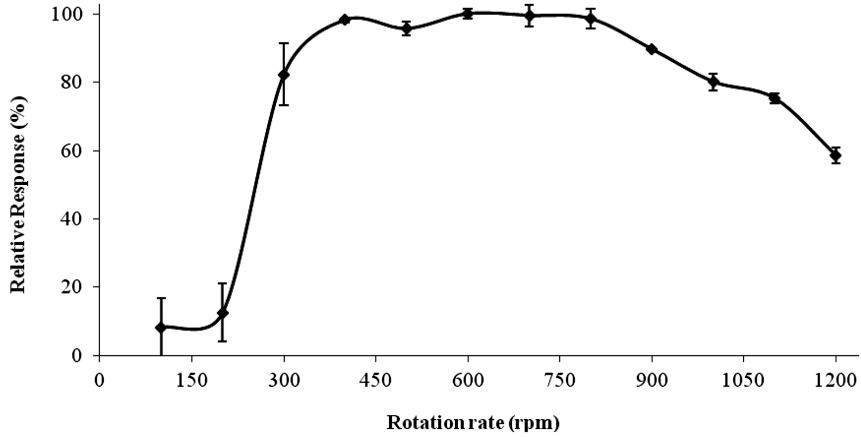

Figure 3. Plot showing the relationship between the relative current response resulting from GOx catalyzed oxidation vs. the rotation rate of a GOx-modified RDE. Each rotation rate was tested five times in 5 mL samples.

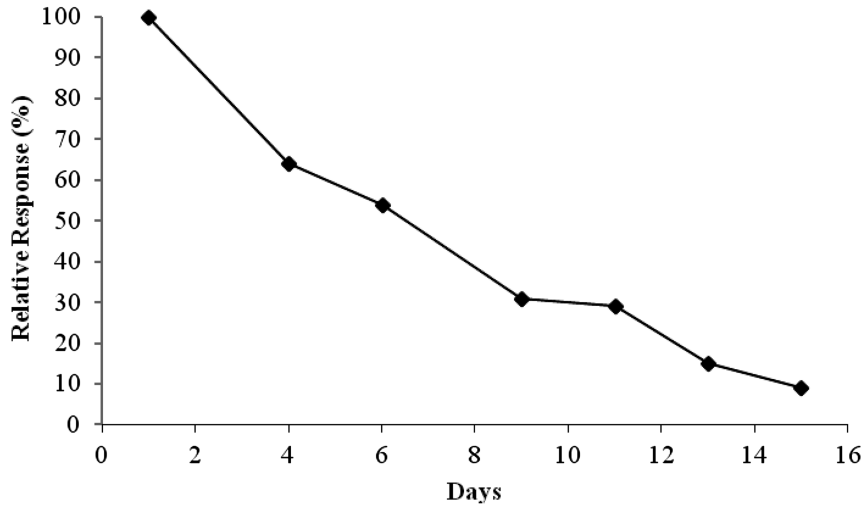

Figure 4. Shelf life of GOx RDE. Relative enzyme activity for a GOx-modified RDE over a period of 15 days at pH 7.4 and 24 °C. Each measurement was done three times.

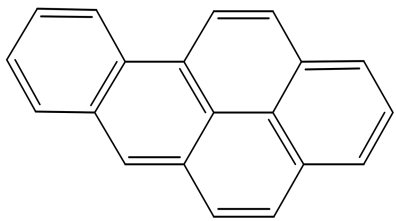

Figure 5. The molecular structure of Benzo[a]pyrene.

Student Feedback and Course Assessment

The open-ended student responses below reflect their thoughts about the course, teaching methods, and assignments. This formative assessment was done at the end of the spring 2011 semester. Although qualitative, the responses give some idea about the student satisfaction, motivation, and interest in the course material.

Sample Comments from Students

"The course was useful in that it allowed me to see what type of research projects are out there. Usually in a class only basic concepts are covered. This course took it a step further than that."

"I like that a lot of the course was based in the primary literature. I think that the selected articles were very good and provided adequate supplemental information to what we learned in class."

"Using primary literature is more interesting and closer to a real world research setting."

"The presentations allowed us to do additional research on topics that we were interested in. The professor gave us the materials ahead of time to work on our presentations early."

"The presentation at the end was a nice attribution because it allowed us to research our own interests and also allowed us to go more in depth."

"The course definitely increased my interest in the field. I feel that whenever market applications for any concept are introduced, I am more interested to learn about the technological developments and limitations of the field."

An IDEA survey is administered for all courses with all ratings evaluated on a 5-point scale. This survey emphasizes student ratings of instruction, and improving teaching effectiveness. Eight out of 11 students (73% response rate) who took the course during the spring 2011 semester completed the survey. Sections of the IDEA report that are related to the improvement of student interest in the subject include active student involvement in learning, relating the course material to real life, and structuring the course assignments and testing. These categories are all related to the teaching style, course format, and course objectives that were created ahead of time. To get more information about this evaluation tool go to http://www.theideacenter.org. Table 7 includes the relevant IDEA ratings.

Table 7. Results for the IDEA Survey on Teaching Methods and Styles (Rating Scale: 0-5)

1. Stimulating Student Interest	
a. Stimulated students to intellectual effort beyond that required by most courses	4.6
b. Introduced stimulating ideas about the subject	4.8

Continued on next page.

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Table 7. (Continued). Results for the IDEA Survey on Teaching Methods and Styles (Rating Scale: 0-5)

c. Inspired students to set and achieve goals which really challenged them	4.8
d. Demonstrated the importance and significance of the subject matter	4.8
2. Encouraging Student Involvement	
a. Gave projects, tests, or assignments that required original or creative thinking	4.8
b. Encouraged students to use multiple resources (e.g. data banks, library holdings, outside experts) to improve understanding	5
c. Related course material to real life situations	4.6
d. Involved students in "hands on" projects such as research, case studies, or "real life" activities	5
3. Structuring Classroom Experiences	
a. Made it clear how each topic fit into the course	4.8
b. Explained course material clearly and concisely	4.8
c. Scheduled course work (class activities, tests, projects) in ways which encouraged students to stay up-to-date in their work	4.8
d. Gave tests, projects, etc. that covered the most important points of the course	5

Future Directions

In the future, this interdisciplinary course will be a recommended course for the biochemistry and molecular biology majors. That major is currently undergoing curricular revision and is likely to include more upper level elective courses after the revision is complete.

It is intended that a graded project be added where students will be asked to develop a model for a biosensor or an assay using aspects of existing sensors and methods. The students will also be asked to plan a series of experiments for optimizing the device or assay. This will be a valuable exercise, as many advances in modern research are made by revising or combining existing methods or techniques. It also reinforces designing experiments for a new study. The project will allow for student creativity which is rarely a component of a science course at the university level. The project will be due at the end of the semester and will be presented to the entire class as a scientific poster presentation for feedback, peer review, and shared learning. A small poster session will be scheduled where each student will give a short 5-10 minute overview of their new sensor or an assay to their peers, and any interested faculty. Each poster will be carefully peer reviewed by three students and the instructor using an evaluation form that will be provided before a grade will be assigned for the project and presentation. The evaluators will also have to write a short synopsis of the main points of the project that they evaluate. Those will also be reviewed and graded.

A more detailed assessment is planned for the course, the assignments, and the teaching methods the next time it is taught. Since the course is primarily based on

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scientific literature, it is different every time it is offered and therefore continues to evolve over time. The intent is to use an assessment tool, which shows great promise for customized course assessment, called SALG (Student Assessment of their Learning Gains), to assess the student learning and attitudes towards the course material and the teaching methods.

Conclusion

Teaching analytical chemistry in a biologically and clinically relevant context can generate great success with learning difficult content specific material in bioanalytical chemistry as well as developing scientific process skills. The intention was to get students excited about learning technical information and to improve their scientific literacy and scientific thinking in order to make the transition into graduate and professional studies easier. The students who enter the chemical industry after completing their degree also greatly benefit from learning how to find, interpret, and evaluate information in primary literature. Student response to this teaching approach has been overwhelmingly positive. Since the introduction of teaching science in context, it has been noted that the motivation level of students has improved noticeably.

The assignments, discussions, and presentations help students develop a conceptual framework in this highly interdisciplinary field with deeper understanding of the underlying scientific principles and improve their scientific communication skills. The open ended test questions and the context based questions on the final exam require students to draw knowledge from multiple course units. Based on the student's answers, they seem to understand the concepts at a deeper level and are better able to describe experiments and results, identify the major limitations or advantages of a given approach, devise a series of experiments to optimize a new method, and to scientifically justify their conclusions.

Although the teaching objectives and expectations with lower level science courses are somewhat different, a few sections of the General Chemistry and the sophomore level Analytical Chemistry lecture courses are being modified to draw from successful aspects of the advanced course in Bioanalytical Chemistry and Chemical Sensors. The small, upper level in depth course provides a great window into how students learn and think about science. It also allows the instructor to experiment with new and different pedagogical approaches, assignments, and assessment without having a major impact on later undergraduate courses and the overall curriculum in the science majors at the institution.

It is clear that using primary literature and applied context as the foundation for teaching these courses result in a better motivated student with a deeper and richer understanding of the topics in the course. This leads to an improvement in critical thinking and problem solving skills that can then be used in analyzing and interpreting data and this leads to a good foundation for students, especially those who want to work in research.

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Chapter 11

A Lab-Intensive Bioanalytical Chemistry Course

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Bioanalyical chemistry is an interdisciplinary mixture of analytical chemistry and biochemistry. It focuses on the analysis of biological molecules. While a lecture course will provide beneficial training to students, incorporation of lab experiences is crucial for maximal student learning. A lab-intensive course is described here with thirteen lab experiences focused on the analysis of protein samples. These experiments are designed for students to complete individually, but small groups could be used to allow more student throughput.

Introduction

As the name implies, bioanalytical chemistry is an interdisciplinary combination of analytical chemistry and biochemistry or molecular biology. Numerous different varieties of a bioanalytical course could be envisioned, including courses focused on pharmaceutical analysis, proteomics, toxicology or genomics. Previous reports have described some of the approaches to teaching a bioanalytical chemistry course (*1–3*). Offerings include both undergraduate and graduate level courses.

While interdisciplinary courses and experiences are quite popular, they should not replace a solid fundamental course. In order for students to understand how various disciplines can be applied to an interdisciplinary problem, they must first have a firm grasp of the disciplinary topics. Only after understanding the individual disciplines are students ready to see how these disciplines can be combined to probe even deeper and more interesting questions. Thus, while replacing a traditional analytical chemistry or biochemistry course with a bioanalytical course might sound like an attractive option, in order to cover the

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interdisciplinary topics, coverage of some of the fundamental topics will most likely have to be reduced or eliminated entirely. In addition, not all students will likely be interested in a given interdisciplinary topic. For example, if a traditional analytical chemistry course is replaced with a bioanalytical chemistry course, students interested in environmental analytical chemistry, or forensic chemistry, or industrial chemistry will not have their interests addressed.

However, to be competitive in the modern scientific community, students need to be trained in, and have an appreciation for, interdisciplinary topics. Bioanalytical chemistry is especially crucial due to increased research in biomedical and biological areas. New methods of analysis are continually opening up new areas of research and allowing scientist to study more complex problems. As chemical instrumentation becomes cheaper and easier to use, more scientists are able to incorporate them into their research programs. Students need to understand the principles behind the instruments so that they can determine not only the best method and instrument for a particular analysis, but also be able to interpret data, determine if it is valid data, and if not determine the issues affecting the results. This requires an understanding of the individual disciplinary topics as well as how they are related to one another.

With limited faculty resources, and numerous topics that could be taught, a natural question is why and how should a bioanalytical course be offered? A large portion of modern research is done at interdisciplinary boundaries, including bioanalytical, biophysical, environmental, forensic, or material science.

A bioanalytical chemistry course could include a laboratory component or be purely lecture based. Here, a lab-intensive bioanalytical course is described. While this course was originally designed and is taught to undergraduates, it was inspired by a graduate-level course that the author used to teach. It could easily be modified to be taught at the graduate level, or as a hybrid undergraduate-graduate course.

Lab-Intensive Format

While learning about the various bioanalytical techniques, instruments, protocols, limitations, and abilities available to researchers is important, actual hands on laboratory experience is crucial. While it is important for students to gain an understanding of the instruments and techniques from lectures and readings, nothing can substitute for actually getting into the lab to perform the protocols and use the instruments. There are many "tricks of the trade" that are difficult to discuss in a lecture format, but can be demonstrated to students quite easily in the lab. These "tricks" can be the difference between a successful experiment and a failed attempt. For example, describing to students how to get a PAGE gel off of the plates without tearing is difficult, but by showing them once, they will quickly be able to move gels off the plates, into a staining chamber, or onto an imager or gel dryer.

There are two major impediments to incorporating bioanalytical chemistry laboratory experiments into the curriculum. The first is the availability of instrumentation for experiments. While a lot of interesting experiments can be

done with relatively common instrumentation, such as 1-D gel rigs, UV/VIS spectrometers, and HPLC systems. Other experiments might require more exotic and expensive instruments, such as 2-D gel systems, capillary electrophoresis systems and mass spectrometers. Most instructors should be able to put together a very instructive laboratory program using existing equipment, or equipment that can be obtained fairly inexpensively. Another consideration is the cost of reagents. Depending what experiments are done, the analytes can be fairly expensive. Careful choice of proteins can limit the cost, but there will still likely be substantial cost. Some reagents, such as enzymes and antibodies have limited storage life and will likely have to be purchased each time the course is offered.

The second impediment to offering a bioanalytical chemistry course is the amount of time needed for some experiments. While some fit very nicely into a three- or four-hour laboratory period, others require significantly more time. A 1-D gel electrophoresis experiment may only take a few hours, while a 2-D experiment might require all day, or even overnight. An enzymatic digest of a protein might require overnight incubation. An instructor must decide how to use the allotted lab time. One choice is to only incorporate labs that can be completed within the single lab period. This is the easiest, but also limits which experiments can be done. The instructor could prepare some of the experiments before the students arrive in lab, or come in the next day to work with student samples. For example, an instructor could have students prepare an enzymatic digest one lab period, allow the digest to incubate overnight, and come in the next day to quench the reaction and store it in the freezer for students to use the next lab period. This opens up additional experiments, but also requires more involvement outside of lab by the instructor. The instructor can also ensure that many of the solutions needed are available in the lab for students. This allows students to focus on the actual analysis and data interpretation rather than creating needed solutions. This also means that either the instructor needs to prepare solutions outside of lab time, or a stockroom worker, if available, needs to do so. This represents a trade off in what students can accomplish during the lab and having students making solutions, which although they should be able to do by the time they take this course extra practice never seems to hurt.

Another solution, available at some institutions, is to increase the amount of lab time available. This could be accomplished by having lab twice a week or more. Some institutions have a concentrated period of study for a single course. This is generally accomplished by students taking one course for approximately one month. A few schools offer all of their courses in this manner, but it is more common for an institution to offer a single-month course either in January (often called J-term, or Interim) or in May (May-mester). Here, the Bioanalytical Chemistry course is offered during our January Interim course. This allows students to take a single course for the four week term. With a single course, students do not need to attend other lectures or focus on other courses. This allows extended lab time to be incorporated into the course. At St. Olaf, students in the Bioanalytical Chemistry course attend a one and a half to two hour lecture every day, with the rest of the day open for lab work. Students are allowed to self-schedule their lab time, but most will spend three to four hours each day in

lab. This allows students to undertake labs that require extended periods of time, or span more than one day.

Students could be arranged in groups to complete each experiment, but this will limit the pedagogical impact of each experiment. Students learn best be doing an activity, not watching another student do it. Depending on enrollment, and equipment available, allowing students to complete each experiment individually maximizes their hands-on experience and their learning opportunities. This of course increases the total number of experiments that must be completed during the term. Another factor is likely the number of each instrument available. Depending on which experiments are incorporated into the curriculum and the total enrollment, there likely won't be enough of any one instrument available for each student to use. For more expensive instruments, such as capillary electrophoresis units, HPLCs, and mass spectrometers, even if students work in groups there likely won't be enough instruments for each group to use simultaneously. This necessitates different students to be working on different experiments at the same time, rotating through all the experiments over the course of the term. Initially this leads to more work for the instructor, as multiple experiments must be setup and prepared at the same time, and students will be working on different experiments simultaneously, asking questions about various procedures. Over the course of the term, however, the instructor's role diminishes as students can help train each other on experiments that they have already completed. This lessens the instructor's role in the lab, but even more importantly it aids in student learning. By helping to train other students on an experiment they have already completed, the student doing the teaching will reinforce his or her own understanding of the experiment.

Course Topics

As described previously, there are numerous options for teaching a bioanalytical chemistry course including how to structure a lab component, which lab experiences to include, and what topics to cover. The course described here is taught during a four week January term. Students work individually to complete the thirteen laboratory experiences described below. Each day a two hour lecture is given, with the rest of the day an open laboratory. Students schedule their own time in the lab, rotating between the thirteen experiences. Course topics are listed in Table 1.

This is not an exhaustive list of all possible topics that could be covered in a bioanalytical course, but covers many of the most commonly used techniques. As described below, several of these course topics have associated laboratory experiences to allow the students to experience hands-on what they have learned in lecture. Labs are not opened to students until the topic has been covered in the classroom to ensure that students have a fundamental understanding of how the technique works and what the results should look like before they attempt to actually perform the analysis in the lab.

Table 1. Topics Taught in Bioanalytical Chemistry Lectures

Biomolecular Structures	Protein Fingerprinting
UV/VIS and Fluorescence Spectroscopy	Peptide Sequencing (Edman and Mass Spectrometry based)
Chromatography (size-exclusion, ion-exchange, affinity, partition)	Analysis of Post-Translational Modifications
Gel Electrophoresis	Other Mass Spectrometry Applications (DNA, Bacteria Identification, Tissue Imaging)
Isoelectric Focusing and 2D-Gel Electrophoresis	Dialysis
Capillary Electrophoresis	Centrifugation
Blotting (Northern, Southern, Western)	Electrochemistry
Assays	Surface Plasmon Resonance
Mass Spectrometers	Clinical Applications
MALDI and Electrospray Ionization	

Labs

Over the course of the term, students must individually complete thirteen different laboratory experiences successfully. Students are allowed to decide when they want to work on each experiment, with the caveat that all of them must be completed by the end of the term with a lab report for each experiment due on the last day of class. Lab reports consist of an experimental section, the data collected, the analysis performed by the student and some discussion of what the results show. Most lab reports are a few pages, plus any data (spectra, printouts, graphs, pictures, etc.) collected by the student. The experiments are not available for the students to work on until the corresponding topic has been discussed in the lecture portion of the course. Some of the experiments can be completed in under an hour, some require a few hours, and others are multiple day experiences. A few of the experiments build on previous experiments, so students are asked to complete these in order. A brief description of each experience is given below, along with the protocol that students are given. While students do have to make some solutions, others have been made for them by stockroom personnel. Since the genetics and biochemistry courses offered here are heavily focused on genomic analysis, the experiences built into the Bioanalytical Chemistry course described below are centered on proteomics. This is only one particular set of experiments that could be envisioned as a bioanalytical lab course. Other institutions may incorporate a number of the described experiments into their biochemistry course and thus might want to incorporate other laboratory experiences.

Lab A: Quantitation of Proteins Using Bradford Analysis

The first laboratory experience that students do, used as an introduction to the lab, is a Bradford analysis to determine the total amount of protein in a given sample. Students create a working curve using a standard (2000 μg/mL) solution of bovine serum albumin (BSA) and then analyze three unknown protein samples. The three unknowns used are BSA at 10 μg/ml and cytochrome c at 10 μg/ml and 25 μg/ml. The latter two unknowns allow students to get a feel, once they have completed the analysis and are told the identity of the unknowns, for how accurate results might be when different proteins are used for the standardization curve and unknown analysis. Students can easily complete this experiment in an afternoon, even sharing spectrometers.

Lab A Learning Objectives

Students will learn how to use the UV/VIS spectrometer, make solutions, graph data, and calculate results.

Protocol:

1. Obtain one ampule of bovine serum albumin (BSA) standard and 14 fresh test tubes.
2. Create 2 samples of each of the 7 standard solutions as shown in Table 2.
3. Obtain 3 unknown proteins and 20 clean micro cuvettes.
4. Pipette 1.0 mL of each standard or unknown into a fresh test tube, making two replicates of each sample.
5. Add 1.0 mL of the Coomassie Plus™ reagent to each test tube and mix well.
6. Incubate samples for 10 minutes at room temperature.
7. Set the UV/VIS spectrometer to monitor 595 nm and zero the instrument on a nanopure water blank. Within 10 minutes, record the absorbance of all solutions.
8. Subtract the average absorbance for the BSA blank solution from all other solutions.

Table 2. Standard Protein Solutions for Bradford Analysis

Vial	*Volume of nanopure water*	*Volume and source of BSA*	*Final BSA Concentration*
A	3,555 μL	45 μL stock solution	25 μg/ml
B	6,435 μL	65 μL of stock solution	20 μg/ml
C	3,970 μL	30 μL of stock solution	15 μg/ml
D	3,000 μL	3,000 μL of Vial B solution	10 μg/ml

Continued on next page.

In Teaching Bioanalytical Chemistry; Hou, H.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Table 2. (Continued). Standard Protein Solutions for Bradford Analysis

Vial	*Volume of nanopure water*	*Volume and source of BSA*	*Final BSA Concentration*
E	2,500 µL	2,500 µL of Vial D solution	5 µg/ml
F	1,700 µL	1,700 µL of Vial E solution	2.5 µg/ml
G	4,000 µL	0	0 µg/ml

Prepare a working curve based on the average absorbance values and determine the concentration of the three unknown proteins.

Lab B: Using Various % SDS-Gels To Analyze Proteins

Gel electrophoresis is a major analysis technique used in the analysis of biological species. This includes agarose gels for DNA and RNA analysis and Polyacrylamide (PAGE) gels, both denaturing and non-denaturing, for proteins. Since the lab focus of this bioanalytical chemistry course is proteins, students use sodium dodecylsulfate (SDS) PAGE gels. While gels can be purchased commercially, and in fact students do use a commercially available gradient gel for the 2D gel experience (Lab C), students learn how to cast their own gels in this experiment. As gel analysis will be used in several subsequent experiments, students need to complete this lab fairly early in the term. In order for students to gain an appreciation for the ability of different percentage gels to separate different sized molecules, they cast four different percentage gels and analyze the same mixture on each gel. The protein samples analyzed span a fairly wide range and allow students to observe large proteins not migrate very far in a higher percentage gel while small proteins separate well, and larger proteins separate nicely on lower percentage gels while small proteins run off the bottom of the gel.

Lab B Learning Objectives

Students learn how to cast and run SDS-PAGE gels and estimate molecular weights of observed bands.

Casting of the Gel

1. Clean and completely dry the glass plates, combs, and any other parts needed.
2. Place a short plate on top of a spacer plate. Insert both plates into the green casting frame on a flat surface. Clamp the casting frame and make sure that the plates are level on the bottom.
3. Clamp the green casting frame into the clear casting stand assembly making sure there is a snug seal along the bottom rubber gasket.
4. Prepare four gels using the recipes in Table 3, combining the ingredients in a small beaker.

5. When you are ready to pour the gels, quickly add the TEMED, mix by swirling gently, draw the solution into a 10 mL syringe and gently dispense the solution between the glass plates completely filling the space between the plates to just below the green line (about an inch from the top).
6. Eject the remaining acrylamide solution back into the beaker. Polymerization of this solution will indicate complete polymerization of the gel between the plates.
7. Gently overlay the top of the cast gel with nanopure water, making sure to keep the gel level.
8. Allow to polymerize (~30 minutes).
9. Once the gels have polymerized, gently pour out the water covering the gels.
10. Prepare a 4% stacking gel solution using the following recipe, combining in a small beaker:

    a. Water, 6.2 mL
    b. 0.5 M Tris, pH 6.8, 2.6 mL
    c. 10% SDS, 100 µL
    d. 40% acrylamide, 1.0 mL
    e. 10% APS, 100 µL
    f. TEMED, 10 µL (**Do not add until ready for polymerization)**

11. When you are ready to pour the stacking gel, quickly add the TEMED, mix by swirling gently, draw the solution into a 10 mL syringe and gently dispense the solution between the glass plates of each gel completely filling the space between the plates. Insert the well forming comb into the opening between the glass plates.
12. Allow to polymerize, again monitoring polymerization be the remaining gel solution in the beaker.
13. Once the gel has polymerized, the combs can be gently removed.

Table 3. Recipes for Various Percentage SDS-PAGE Gels

	7.5% Gel	*10% Gel*	*15% Gel*	*18% Gel*
Water	5.325 mL	4.7 mL	3.45 mL	2.7 mL
1.5 M Tris, pH 8.8	2.6 mL	2.6 mL	2.6 mL	2.6 mL
10% SDS	100 µL	100 µL	100 µL	100 µL
40% acrylamide	1.875 mL	2.5 mL	3.75 mL	4.5 mL

Continued on next page.

Table 3. (Continued). Recipes for Various Percentage SDS-PAGE Gels

	7.5% Gel	*10% Gel*	*15% Gel*	*18% Gel*
10% Ammonium persulfate (APS)	100 µL	100 µL	100 µL	100 µL
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	10 µL	10 µL	10 µL	10 µL

(**Do not add TEMED until ready for polymerization**)

Preparing the Sample

1. Create a boiling water bath.
2. Add 19.3 mg of dithiothreitol (DTT) to 1.0 mL of Protein Loading Buffer (PLB).
3. Obtain 100 pmol/µL stock solutions of Apomyoglobin, Aprotinin, BSA, Cytochrome c, Lysozyme, Ubiquitin.
4. Obtain the Prestained SDS-PAGE standards.
5. Obtain 9 new 1.5 mL vials
6. Make the 9 sample solutions listed in Table 4, thoroughly mix by repeated aspiration and dispensing all ingredients.
7. Boil all samples for 5 minutes.

Table 4. Samples for Gel Electrophoresis

Sample	*Volume of DTT-PLB*	*Volume of Protein Stock*	*Volume of Protein Stock*	*Volume of Protein Stock*
A	40 µL	15 µL Apomyoglobin	15 µL BSA	15 µL ubiquitin
B	40 µL	40 µL cytochrome c		
C	40 µL	15 µL Aprotinin	15 µL Apomyoglobin	15 µL cytochrome c
D	40 µL	40 µL ubiquitin		
E	40 µL	40 µL apomyoglobin		
F	40 µL	40 µL BSA		
G	40 µL	40 µL Aprotinin		

Continued on next page.

Table 4. (Continued). Samples for Gel Electrophoresis

Sample	*Volume of DTT-PLB*	*Volume of Protein Stock*	*Volume of Protein Stock*	*Volume of Protein Stock*
H	40 µL	40 µL Lysozyme		
I	40 µL	15 µL Aprotinin	15 µL BSA	15 µL Lysozyme

Electrophoresis

1. Remove the gel cassette from the casting stand and place it in the electrode assembly with the short plate on the inside. Place the buffer dam plate opposite the gel cassette assembly.
2. Provide a slight upward pressure on the gel cassette and buffer dam while clamping the frame to secure the electrode assembly. This step is important to minimize the potential for leakage during the electrophoresis experiment.
3. Place the assembly into the electrophoresis tank.
4. Prepare 500 mL of 1X electrophoresis buffer.
5. Completely fill the inner chamber with 1X electrophoresis buffer. Check for leaks. Using a gel-loading tip or syringe, gently clean each sample well with buffer.
6. Briefly centrifuge all protein samples.
7. Once the wells are clean, slowly pipette 10 µL of sample or SDS-PAGE marker solution into individual wells. Make sure you note which well contains which sample.
8. Add enough 1X electrophoresis buffer to the region outside the frame to cover the bottom of the gel cassette assembly.
9. Cover the tank with the lid aligning the electrodes (red or black) correctly.
10. Connect the electrophoresis tank to the power supply.
11. Run the gel at 200 V until the dye front reaches the bottom of the gel. This may take up to 1 hour, depending on the gel.
12. When electrophoresis is complete, turn off the power supply, disassemble the equipment and clean all items.

Staining

Coomassie Staining Protocol

1. Soak the gel in the Coomassie staining solution on a gently rocking platform shaker for at least 15 minutes. Increased staining time may allow fainter bands to be visualized.
2. Recycle the Coomassie stain and cover the gel with destain solution, again gently rocking. Allow to destain for at least an hour. Decant off the destaining solution and replace with fresh destaining solution. If

destaining is not complete, it can be allowed to proceed overnight. A few Kimwipes, or small piece of wool may be placed in the destaining container to soak up released dye overnight.

3. Once the gel has destained adequately, capture a gel image using the digital camera or the Lumi-Imager. If desired, the gel can be dried on the gel dryer.
4. Using the known molecular weights of the SDS-PAGE standards (6.6 kDa, 19.3 kDa, 28.5 kDa, 36.8 kDa, 52.2 kDa, 77.8 kDa, 117.0 kDa, and 203.8 kDa), determine the experimental molecular weights of the five protein samples.
5. Compare the results from the different gels.

A representative result of each gel percentage is shown in Figure 1.

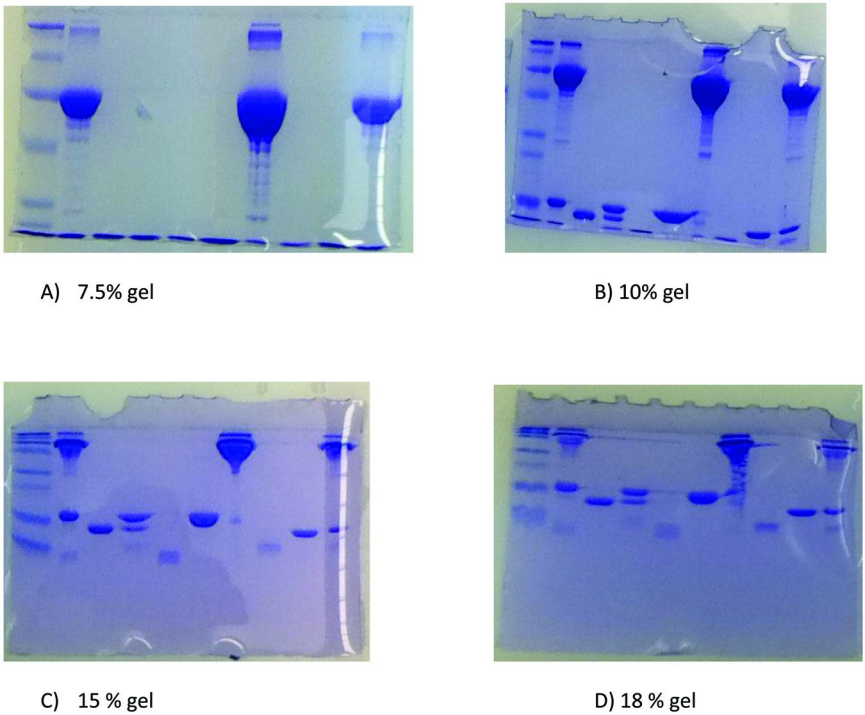

Figure 1. Separation of protein mixtures on various percentage gels.

Solution Recipes

Protein Loading Buffer: Add 12 mL of 0.5 M Tris (adjusted to pH 6.8) as prepared above to 25 ml glycerol, 2 gm SDS, 10 mg bromophenol blue and fill to 100 mL with nanopure water.

5X Tris/Glycine/SDS Runing Buffer: 15.1 gm Tris Base, 94 gm Glycine, 5.0 gm SDS, dilute to 1 L with nanopure water.

Coomassie Stain solution: 450 mL water, 450 mL methanol, 100 mL acetic acid, 2.5 g Coomassie Brilliant Blue R-250.

Coomassie Destaining Solution: 540 mL nanopure water, 360 mL methanol, 100 mL acetic acid.

Lab C: 2D Gel Analysis of *E. coli* Sample

Once students have successfully analyzed protein mixtures using 1D SDS-PAGE, they analyze a mixture of *E coli* proteins using two dimensional (2D) gel electrophoresis. The first step in a 2D gel electrophoresis separation is an isoelectric focusing step. Rather than casting their own pH gradient tube gels, students use commercially available immobilized pH gradient (IPG) strips. Once the protein mixture has been separated based on isoelectric point, the IPG strip is placed on a SDS-PAGE gel to allow separation based on size. For this experiment, students use a commercially available gradient PAGE gel. As the sample is allowed to hydrate the IPG strip overnight, this experiment requires at least two days. The isoelectric focusing step requires several hours, followed by several hours for the SDS-PAGE separation, gel staining and destaining steps. These can be accomplished in a single, long day, or spread over two lab periods.

Lab C Learning Objectives

Students learn how to perform isoelectric focusing, and how to couple IEF strips with SDS-PAGE gels to perform 2D-gel electrophoresis.

Protocol:

Sample Loading

1. Remove an Immobilized pH Gradient (IPG) strip from the freezer.
2. Obtain an equilibration tray of the correct size.
3. Pipet 125 µL of the *E. coli* sample as a line into one of the channels on the equilibration tray. The line should cover the length of the channel except for ~1cm on each end. Be careful not to introduce bubbles.
4. Using a forceps, remove the coversheet from the IPG strip. Place the IPG strip gel side down onto the sample. Make sure not to get sample on the plastic backing or trap air bubbles under the gel.
5. Carefully overlay the strip with 2-3 mL of mineral oil by dripping from a pipet to prevent evaporation.
6. Cover the tray with the plastic lid and let the tray sit overnight to load the *E. coli* sample.

Isoelectric Focusing

1. Obtain an Isoelectric focusing (IEF) focusing tray of the correct size.
2. Using forceps, place a paper wick at both ends of the channel, covering the wire electrodes.
3. Pipet 8 μL of nanopure water onto each wick.
4. Carefully remove the IPG strip from the equilibration tray and let the mineral oil drain for 10 seconds.
5. Place the IPG strip, gel side down, in the IEF focusing tray. Make sure that the "+" end of the strip is placed at the "+" end of the tray as marked.
6. Cover the IPG strip with 2-3 mL of fresh mineral oil. Remove any bubbles. Place the lid onto the tray, again making sure that the "+" end is aligned correctly.
7. Place the focusing tray into the PROTEAN® IEF cell and close the cover.
8. Program the PROTEAN® IEF cell as shown in Table 5:
   - Default cell temperature of 20 °C
   - Maximum current of 50 μA/strip
   - No Rehydration
9. If you do not plan to be in lab when the IPG strip finished focusing, you can add a "hold" step as step #4. This should have a voltage of 500 V and a time long enough such that you will be back in lab before the time expires. This "hold" will keep the bands focused and counter diffusion.
10. Press Start to begin the isoelectric focusing.
11. Once the focusing step is complete, remove the IPG strip, allow mineral oil to drain for 10 seconds and then transfer to a clean equilibration tray.
12. Add 2.5 mL of 2D Gel Equilibration Buffer 1 to the IPG strip in the channel.
13. Gently shake on the orbital shaker for 10 minutes making sure the speed is low enough to prevent solution sloshing out.
14. Obtain 10 mL of 2D Gel Equilibration Buffer 2 and add 0.25 gm of iodoacetamide.
15. After the 10 minute equilibration, decant off the solution from the IPG strip.
16. Add 2.5 mL of 2D Gel Equilibration Buffer 2 (with the iodoacetamide) to the IPG strip in the channel.
17. Gently shake on the orbital shaker for 10 minutes again making sure the speed is low enough to prevent solution sloshing out.
18. During the incubation, melt the overlay agarose in a microwave by loosening the cap and microwaving on high for 30 seconds. Swirl the solution. Continue to heat for 15 seconds followed by swirling until agarose is melted.
19. After the 10 minute incubation, decant the solution off the IPG strip.

Table 5. Isoelectric Focusing Program

	Voltage	*Time*	*Volt-Hours*	*Ramp*
Step 1	250	20 minutes	--------	Linear
Step 2	4000	2 hours	--------	Linear
Step 3	4000	-------	10,000 V-hr	Rapid
Total		5 hours	14,000 V-hr	

SDS-PAGE

1. Obtain a precast polyacrylamide gradient gel from the refrigerator.
2. Remove the IPG comb from the gel and rinse the well carefully with nanopure water.
3. Remove any excess water with blotting paper.
4. Fill a 100 mL graduated cylinder with 1X SDS-PAGE Running Buffer.
5. Remove the IPG strip and briefly dip into the graduated cylinder filled with SDS-PAGE Running Buffer.
6. Lay the strip gel side up onto the back plate of the SDS-PAGE gel.
7. Place the gel in a vertical stand with the short plate facing you.
8. Pipet overlay agarose solution into the IPG well of the gel.
9. Using forceps, carefully push the strip into the well, being careful not to trap any air bubbles beneath the strip. Make sure you push on the plastic backing and not the gel itself.
10. Allow the agarose to solidify (~ 5 minutes).
11. Mount the gel into the SDS-PAGE cell.
12. Fill the cell reservoirs with 1X Tris/glycine/SDS Running Buffer.
13. Run the gel at a constant 200 V for approximately 40 minutes. The Bromophenol Blue migration can be monitored during the separation.

Staining

1. Stain SDS-PAGE gel with Coomassie or other stain.
2. Obtain digital picture of gel. If desired, dry the gel on the gel dryer.

A representative 2D gel separation of the *E coli* protein mixture is shown in Figure 2.

Solution Recipes

2D gel sample buffer: Dissolve 4.8 g urea, 0.2g 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.077g DTT, 0.04ml Bio-Lyte® 3/10 ampholytes, 0.001 g bromophenol blue in 10 mL nanopure water.

2D Gel Equilibration Buffer 1:Add 12.5 mL of 1.5 M Tris (adjusted to pH 8.8) as prepared above to 18.0g urea, 1.0g SDS, 1.0g DTT, 10 mL glycerol and dilute to 50 mL nanopure water.

2D Gel Equilibration Buffer 2: Add 12.5 mL of 1.5 M Tris (adjusted to pH 8.8) as prepared above to 18.0g urea, 1.0g SDS, 10 mL glycerol and dilute to 50 mL nanopure water.

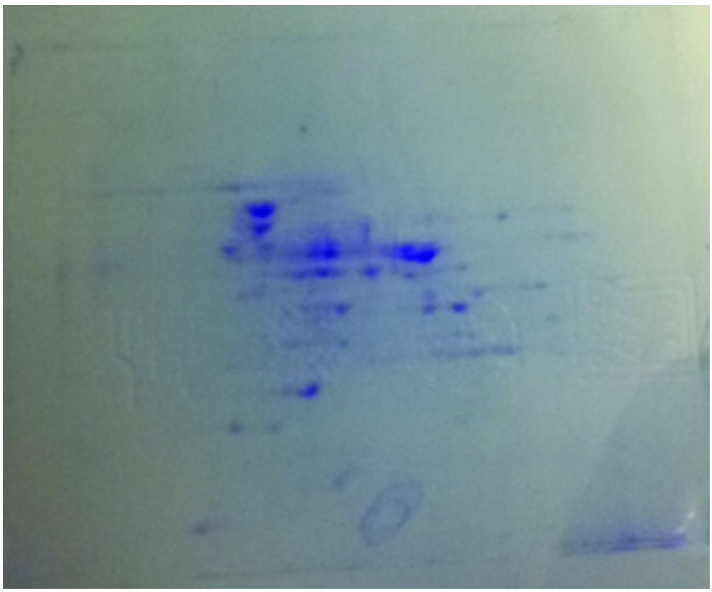

Figure 2. 2D Gel separation of E. coli mixture.

Lab D: Affinity Purification of His-Tagged Protein Using Magnetic Beads

Several different affinity separations are discussed in the lecture portion of the course. For lab experience with affinity purification, students use magnetic beads with immobilized nickel to selective capture polyhistidine-tagged proteins. The magnetic beads allow for easy, rapid collection of the beads and easy washing and/or elution. A mixture of lysozyme and histidine-tagged ubiquitin is allowed to interact with the beads. The wash solution, which should contain all of the lysozyme, is removed and kept for analysis. Any proteins bound to the beads (which should be the polyhistidine-tagged ubiquitin) are then eluted and kept. Students concentrate the wash and elution solutions and then analyze each using an SDS-PAGE gel in order to determine if the magnetic beads did selectively capture the histidine-tagged protein. This experiment can be completed in an afternoon.

Lab D Learning Objectives

Students learn how to perform affinity separation, how to use magnetic beads, and how to lyophilize samples.

Protocol:

1. Shake beads before using to disrupt aggregation.
2. Transfer 50 μL magnetic beads into new 1.5 mL tube.
3. Magnetically separate the beads and aspirate the solution to waste.
4. Add 300 μL of His-tagged protein mixture (Lysozyme and His-tagged Ubiquitin) sample to washed beads. Gently agitate and incubate at room temperature for 20 minutes.
5. Magnetically separate the beads and aspirate the solution to a new collection tube (washes).
6. Wash magnetic beads four times with 300 μL Wash and Binding solution with gentle agitation after each wash, magnetically separating beads and aspirating solution to waste.
7. To elute the protein, add 100 μL Elution buffer and gently agitate at room temperature for 20 minutes.
8. Aspirate and collect the elution solution into a new tube.
9. Separately lyophilize the wash sample from step 5 and the elution solution to approximately 15-20 μL each. Be careful not to lyophilize to dryness.
10. Analyze 10 μL of the wash solution,10 μL of the elution solution, and 10 μL of the original protein mixture using SDS-PAGE. Make sure you follow the SDS-PAGE protocols you learned earlier (i.e. add DTT-PLB, boil, add a lane of Prestained standards). Use a gel of appropriate acrylamide percentage, based on previous observations. Separate and stain to determine if the separation was successful.

A representative result of the gel following affinity separation is shown in Figure 3.

Solution Recipes

Wash and Binding solution: In 25 mL of nanopure water, add 0.178g disodium phosphate (anhydrous) 0.438g NaCl, 2.5 μL Tween 20, adjust to pH 8.0 with HCl.

Elution solution: In 25 mL of nanopure water, add 0.255g Imidazole, 0.178g disodium phosphate(anhydrous), 0.438g NaCl, 2.5 μL Tween 20, adjust to pH 8.0 with HCl.

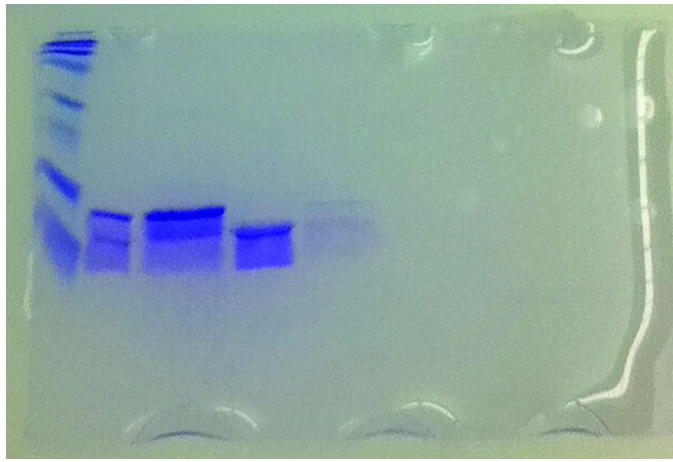

Figure 3. From left to right, lane 1-protein size markers, lane 2-mixture of lysozyme and His-ubiquitin, lane 3-wash solution (mainly lysozyme), lane 4-elution solution (His-ubiquitin).

Lab E: Size Exclusion Chromatography of Proteins

Students pack a size exclusion column and use it to analyze several different proteins of varying mass. Each protein is introduced individually and the elution time is monitored. A calibration curve relating elution time to the log of the protein mass is created and used to estimate the molecular mass of an unknown protein sample. This experiment can be done in a single day.

Lab E Learning Objectives

Students learn how to perform size-exclusion chromatography, including how to pack a size exclusion column and use the low-pressure LC system.

Protocol:

1. Weigh out 4.5 gm of Bio-Gel® P-10 Size Exclusion Chromatography (SEC) Media.
2. Heat 60 mL of 100 mM ammonium acetate just to boiling.
3. Remove buffer from heat and gradually add SEC gel.
4. Let gel hydrate for 2 hours.
5. Decant half of supernatant.
6. Transfer solution to a vacuum filtration flask and attach to vacuum. Degas for 10-15 minutes with occasional swirling of the flask.
7. Add 60 mL of degassed buffer and gently swirl.
8. Allow gel to settle until ~90% of particles have settled. Decant supernatant. Repeat 3 times to remove small particles.
9. Place funnel in top of SEC column and plug bottom of column.

10. Fill 20% of column with buffer.
11. Pour the slurry into the column. Avoid splashing.
12. Once 2-5 cm of gel has packed, open the column plug and allow to flow until packed.
13. Plug the column outlet and attach the flow adapter.
14. Open the column outlet and flow 2 bed volumes of buffer through column at desired flow rate (1.0 mL/min).
15. Close outlet and adjust flow adapter to level of the gel bed.
16. Mix 50 µL of a 1nmol/µL solution of ubiquitin with 350 µL of degassed buffer and inject sample onto column with a flow rate of 1.0 mL/min. Observe elution time.
17. Mix 50 µL of a 1nmol/µL solution of bombesin with 350 µL of degassed buffer and inject sample onto column with a flow rate of 1.0 mL/min. Observe elution time.
18. Mix 50 µL of a 1nmol/µL solution of lysozyme with 350 µL of degassed buffer and inject sample onto column with a flow rate of 1.0 mL/min. Observe elution time.
19. Mix 50 µL of a 1nmol/µL solution of cytochrome c with 350 µL of degassed buffer and inject sample onto column with a flow rate of 1.0 mL/min. Observe elution time.
20. Mix 50 µL of unknown protein solution with 350 µL of degassed buffer and inject sample onto column with a flow rate of 1.0 mL/min. Observe elution time.
21. Determine the molecular weight of the unknown protein.
22. If you are the last person to use the LC, run 50:50 Methanol:water through the LC at 1 mL/min for 30 minutes.

Lab F: Dot Blot of Proteins

In preparation for Western Blotting (Lab G), students perform two dot blots. They spot five different proteins on two polyvinylidene fluoride (PVDF) membranes and treat one with an anti-ribonuclease A antibody and the other with an anti-ovalbumin antibody, both rabbit IgG. After incubation with the primary antibody, the membranes are incubated with a goat anti-Rabbit IgG secondary antibody with an enzyme that reacts with 4-Chloronaphthol and hydrogen peroxide to form a colored product allowing the detection of the protein with which the primary antibody reacts. This experiment can be completed in an afternoon.

Lab F Learning Objectives

Students learn how to spot samples on PVDF membranes, how to use antibodies and how to develop blotting assays in preparation for performing a Western Blot.

Protocol:

Preparing the Membrane

1. Obtain two strips of PVDF membrane (approximately 1/3 of a sheet will be adequate). Since PVDF is very hydrophobic, it must first be prewet before aqueous solutions can be blotted onto it. It is important to limit the handling of the membrane and especially avoid skin contact. You should handle the membrane using a tweezers at the corners as much as possible to avoid potential contamination.
2. Immerse each membrane in 100% methanol until the entire membrane is translucent.
3. Transfer the membranes to a dish containing water and soak until membranes can be submerged.
4. Once the membranes have been wetted, do not allow to dry out.

Applying Sample

1. Obtain 100 pmol/μL of ovalbumin, cytochrome c, ribonuclease A, α-lactalbumin, and ubiquitin.
2. Prepare 100 mL of 4% BSA in Tris-buffered Saline with Tween (TBST).
3. Using a micropipette, spot 1 μL of each protein in a different spot on the membrane. Make sure you know where each spot is located. Once each spot has dried, apply two more μL drops.
4. To saturate nonspecific binding sites, incubate the membrane in TBST + 4% BSA for 60 minutes with gentle shaking.

Primary Antibody Binding

1. Obtain the Rabbit IgG fraction of anti-Ovalbumin (primary antibody #1) and add 50 μL to 50 mL of TBST to make the primary antibody #1 solution.
2. Obtain the Rabbit IgG fraction of anti-Ribonuclease A (primary antibody #2) and add 50 μL to 50 mL of TBST to make the primary antibody #2 solution.
3. Replace the blocking solution with TBST containing primary antibody and gently shake for 60 minutes. Make sure a different antibody is used for the two membrane strips.
4. To remove unbound antibody, wash the membrane three times in TBST for 5-10 minutes each.

Secondary Antibody Binding

1. Obtain the secondary antibody and add 10 μL of the secondary antibody to 50 mL of TBST.
2. Transfer the membrane to TBST containing the secondary antibody and incubate for 30 minutes with gentle shaking.

3. Wash the membrane in TBST three times for 5-10 minutes each to remove unbound secondary antibody.
4. Rinse briefly twice in Tris-buffered Saline (TBS) to remove Tween 20 from the membrane.

Color Development (makes about 50 mL, scale accordingly)

1. Get 4-Chloronaphthol powder from fridge. Let warm up to room temperature before opening bottle.
2. Take 15 mg of 4-Chloronaphthol, dissolve in 5 mL methanol and add 50 mL of TBS.
3. Immediately before using, add 100 μL of 30% hydrogen peroxide. This solution must be made fresh right before you use it.
4. Add enough 4-Chloronaphthol/H_2O_2 reagent to just cover membrane and incubate with gentle shaking until color develops.
5. Take a picture of the blot before the color fades.

Figure 4 shows a representative dot blot membrane.

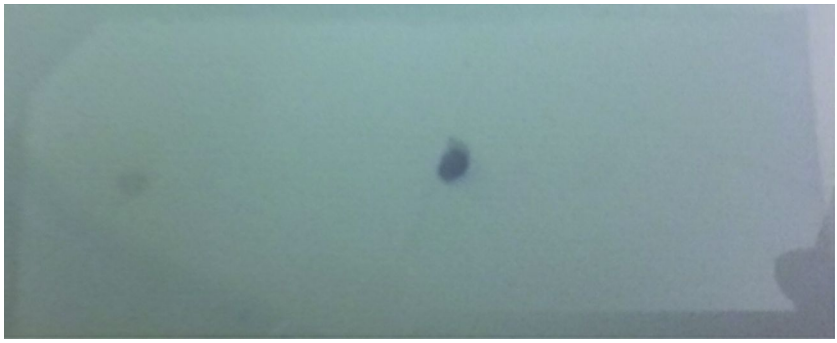

Figure 4. Dot blot membrane using an anti-ribonuclease A primary antibody. The dark spot corresponds to the location of the ribonuclease A protein with the lighter spot corresponding to the ovalbumin protein location (possibly indicating contamination with the other primary antibody).

Solution Recipes

TBS: Dissolve 2.42 g Tris and 8.77 g NaCl in ~800 mL nanopure water, adjust to pH 7.5 with HCl, fill to 1L with nanopure water.

TBST: Dissolve 9.69 g Tris and 35.1 g NaCl in ~800 mL nanopure water, adjust to pH 7.5 with HCl, add 2 mL Tween 20 and fill to 4L with nanopure water.

Lab G: Western Blot of Proteins

Once students have successfully completed the dot blot lab experience, they perform a Western blot experiment using the same two antibodies. A mixture of proteins is separated using an SDS-PAGE gel which is then blotted onto a PVDF

membrane using a semi-dry blotting system. One membranes is then treated with the rabbit IgG anti-ribonuclease A antibody and the other with a rabbit IgG anti-ovalbumin antibody. After incubation with the primary antibody, the membranes are incubated with a goat anti-Rabbit IgG secondary antibody with an enzyme that reacts with 4-Chloronaphthol and hydrogen peroxide to form a colored product allowing the detection of the protein with which the primary antibody reacts. This experiment can be completed in a long afternoon, or split into two lab periods.

Lab G Learning Objectives

Students learn how to perform a Western Blot and how to use the semi-dry blotting apparatus.

Protocol:

Running the Gel

1. Cast two 15% SDS-PAGE gels with 4% stacking gels.
2. Obtain 100 pmol/μL of ovalbumin, cytochrome c, ribonuclease A, α-lactalbumin, and ubiquitin.
3. Make the sample solutions listed in Table 6, thoroughly mix by repeated aspiration and dispensing all ingredients.
4. Boil all samples for 5 minutes.
5. Load 10 μL of each of the 7 samples into separate wells on each gel. Make sure you note which sample is in which well.
6. Electrophorese at 200 V.

Table 6. Samples for Western Blots

Sample	*Volume of DTT-PLB*	*Volume of Protein Stock*	*Volume of Protein Stock*
A	20 μL	10 μL Ribonuclease A	10 μL cytochrome c
B	20 μL	20 μL cytochrome c	
C	20 μL	5 μL ovalbumin	5 μL α-lactalbumin
D	20 μL	20 μL ubiquitin	
E	20 μL	10 μL ovalbumin	
F	20 μL	10 μL α-lactalbumin	
G	20 μL	20 μL ribonuclease A	

Electroblotting onto PVDF

1. Obtain two PVDF membranes. Make sure you are wearing gloves whenever handling the membrane or use forceps. **(see bottom of protocol for second option)**
2. Wash the gels three times in Towbin transfer buffer for 5 minutes each.
3. Cut the membrane to the size of the gel.
4. Prewet the membrane in 100% methanol until they turn gray.
5. Slowly immerse each membrane in Towbin transfer buffer and let equilibrate for 15-30 minutes.
6. For each gel, cut two pieces of extra-thick filter paper to the size of the membrane. Completely saturate the filter paper with Towbin buffer.
7. Remove the lid/cathode from the semi-dry blotting unit.
8. Place a pre-soaked sheet of filter paper onto the anode. Roll a pipet or test tube over the surface of the filter paper to get rid of air bubbles.
9. Place the pre-wetted membrane on top of the filter paper, again rolling out air bubbles.
10. Carefully place the equilibrated gel on top of the membrane, again rolling out any air bubbles.
11. Place the other piece of pre-soaked filter paper on top of the gel, again removing air bubbles.
12. Carefully place the cathode on top of the stack. Be careful you do not disturb the stack.
13. Place the safety cover on the semi-dry blotting unit. Plug the unit into the correct power supply, making sure the polarity is correct.
14. Turn on the power supply. Blot for ~75 minutes at 15V.
15. Once transfer is complete, turn off the power supply. Remove the cover and cathode. Discard the filter paper.
16. Stain the gel in Coomassie to monitor the efficiency of protein transfer.

Blocking the Membrane

1. Prepare 100 mL of 4% BSA in TBST (Tris-buffered Saline with Tween).
2. To saturate nonspecific binding sites, incubate the membranes in TBST + 4% BSA for 60 minutes with gentle shaking.

Primary Antibody Binding

1. Obtain the Rabbit IgG fraction of anti-Ovalbumin (primary antibody #1) and add 50 μL to 50 mL of TBST to make the primary antibody #1 solution.
2. Obtain the Rabbit IgG fraction of anti-Ribonuclease A (primary antibody #2) and add 50 μL to 50 mL of TBST to make the primary antibody #2 solution.
3. Replace the blocking solution with TBST containing primary antibody and gently shake for 60 minutes. Make sure a different antibody is used for the two membranes.

4. To remove unbound antibody, wash the membranes three times in TBST for 5-10 minutes each.

Secondary Antibody Binding

1. Obtain the secondary antibody and add 10 µL of the secondary antibody to 50 mL of TBST.
2. Transfer the membranes to TBST containing the secondary antibody and incubate for 30 minutes with gentle shaking.
3. Wash the membranes in TBST three times for 5-10 minutes each to remove unbound secondary antibody.
4. Rinse briefly twice in TBS to remove Tween 20 from the membranes.

Color Development (makes about 50 mL, scale accordingly)

1. Get 4-Chloronaphthol powder from fridge. Let warm up to room temperature before opening bottle.
2. Take 15 mg of 4-Chloronaphthol, dissolve in 5 mL methanol and add 50 mL of TBS.
3. Immediately before using, add 100 µL of 30% hydrogen peroxide. This solution must be made fresh right before you use it.
4. Add enough 4-Chloronaphthol/H_2O_2 reagent to just cover membrane and incubate with gentle shaking until color develops.
5. Take a picture of the blot before the color fades.

Second Option

Only blot one gel onto PVDF and probe with one of the primary antibodies. Stain the other gel with Coomassie. This will allow you to visualize the position of the bands on the gel and let you compare these bands to the bands observed with the Western blot.

Solution Recipe

Towbin transfer buffer: 3.03 g Tris, 14.4 g glycine, 0.2g SDS dissolved in water, add 200 mL methanol, dilute to 1 L final volume with water.

Lab H: Peptide Identification via Molecular Weight Determination

In this experiment, students are given an unknown peptide and use an electrospray ionization mass spectrometer to determine the molecular weight of the peptide and thus the identity from a given list of possibilities. This experiment has previously been published (*4*). Due to the multiple charges that the electrospray ionization process can impart on analytes, students cannot rely solely on the observed mass-to-charge (*m/z*) ratio. They need to look at the *m/z* spacing of the isotope peaks in order to first determine the charge state of the

observed peaks and then use the observed *m/z* to calculate the molecular mass of the unknown peptide. Many of the unknown peptide samples will yield peaks with more than a single charge state, allowing students to confirm their calculations with a second peak. Once they have been shown how to use the instrument and software, students can complete this lab experience in about thirty minutes.

Lab H Learning Objectives

Students learn how to use the electrospray ionization mass spectrometer, how to obtain mass spectra, and how to determine the charge and mass of an analyte using isotope distributions.

Protocol:

1. Obtain an unknown peptide at a concentration of 100 pmol/μL.
2. Using direct infusion at 2-5 μL/min, and positive ion electrospray, optimize the signal for the unknown peptide.
3. Using the isotope patterns observed, determine number of charges on each ion peak and determine the molecular weight of the unknown peptide.
4. If increased resolution is needed to observe the isotope patterns, use the highest resolution mode for the mass spectrometer.
5. Clean the syringe and capillary by flushing with 2-3 syringe volumes of acetonitrile.
6. Based on your results, and the peptide list in Table 7, determine the identity of the unknown peptide.
7. Turn in a copy of your data and a brief description of how you determined the molecular weight and identity of the unknown peptide.

Table 7. Possible Peptide Identities

Peptide	*Monoisotopic Molecular Weight*
Bombesin	1618.8
RHYNSGHFWFLGP	1616.8
Substance P	1346.7
RPKPQQFFGLK	1344.8
Angiotensin 1	1295.7
GDFYWPFR	1086.5
Arg^8-Vasopressin	1083.4
RPPGFSVFR	1061.6
Bradykinin	1059.6

Continued on next page.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Table 7. (Continued). Possible Peptide Identities

Peptide	*Monoisotopic Molecular Weight*
ACTH fragment 4-10	961.4
RKRSRKE	958.6
RERAERE	944.5
RKRARKE	942.6
EARSRER	902.5
RKRSRAE	901.5
TVFGLR	691.4
YIGSR	594.3

An example mass spectrum is shown in Figure 5.

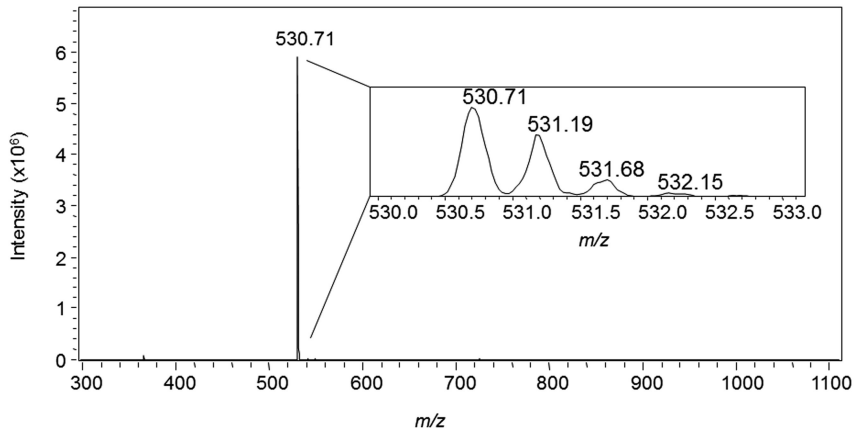

Figure 5. ESI mass spectrum of bradykinin. Reproduced with Permission from Reference #(4). Copyright 2009, American Chemical Society.

Lab I: Peptide Sequencing Using Tandem Mass Spectrometry

Once students have completed Lab H (Peptide Identification via Molecular Weight Determination), they can use the tandem mass spectrometry capabilities of the electrospray mass spectrometer to obtain a collision-induced dissociation (CID) fragmentation spectrum for an unknown peptide, different than the one they analyzed in Lab H. After determining the molecular mass of this unknown peptide, they can isolate it in the mass spectrometer and induce fragmentation. Based on the observed fragmentation spectrum, students attempt to sequence their unknown peptide, which is covered as part of the lecture portion of the course. This sequencing experiment has previously been published (*5*). The data collection

portion of this experience can be completed in thirty minutes, but the data analysis and peptide sequencing portion will take considerable time outside of the lab.

Lab I Learning Objectives

Students learn how to perform tandem mass spectrometry using the electrospray ionization ion trap mass spectrometer, how to isolate and fragment an ion of choice, and how to use the resulting fragmentation pattern to sequence a short peptide.

Protocol:

1. Obtain an unknown peptide at a concentration of 100 pmol/μL.
2. Using direct infusion at 2-5 μL/min, and positive ion electrospray, determine the molecular weight of the unknown peptide.
3. Select a characteristic *m/z* ion peak and isolate the signal in MS/MS mode.
4. Confirm that the requested ion peak is isolated.
5. Fragment the selected ion peak, making sure that the majority of the precursor ion signal has been eliminated.
6. Clean the syringe and capillary by flushing with 2-3 syringe volumes of acetonitrile.
7. Print out the peptide MS/MS product ion spectrum and determine the sequence (offline) of the unknown peptide.
8. Using the GPMAW program, compare the fragment ions observed for your peptide with the theoretical fragment ions expected for the sequence you propose.

An example mass spectrum is shown in Figure 6.

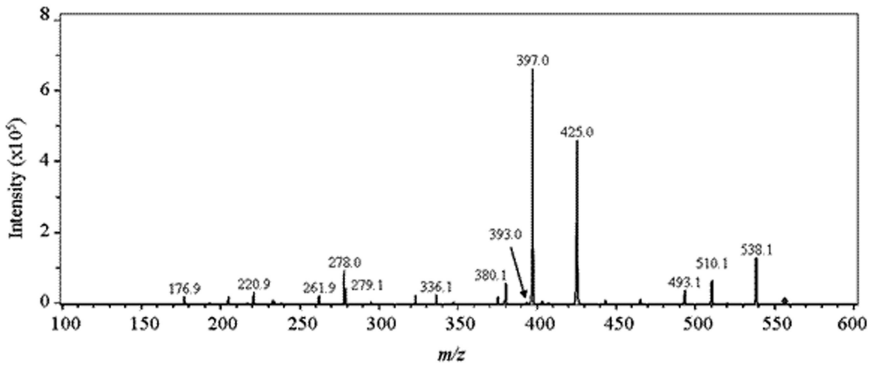

Figure 6. Tandem mass spectrum from fragmentation of m/z 556.3 from Leucine Enkephalin. Reproduced with Permission from Reference #(5). Copyright 2009, American Chemical Society.

Lab J: Protein Molecular Weight Determination

The electrospray ionization mass spectrometer is also used to determine the molecular mass, and thus the identity, of an unknown protein (*6*). In this experiment, students analyze a protein and obtain the charge-state envelope. Due to the larger mass of the proteins, and the fact that they have multiple basic sites that can accept a proton, the analytes in this lab all have multiple (>6) charges. These larger charge states prevent the determination of charge state based on *m/z* separation of isotopes that was used in Lab H. Students use the *m/z* separation between neighboring charge state peaks in order to determine the charge state of each peak, and thus the molecular mass of the unknown protein. This experiment can be completed in less than thirty minutes.

Lab J Learning Objectives

Students learn how to use electrospray ionization mass spectrometry to analyze a protein, how to obtain a charge envelope for the protein, how to use the data to determine the charge of each peak, and how to calculate the mass of the analyte.

Protocol:

1. Obtain an unknown protein at a concentration of 100 pmol/μL.
2. Using direct infusion at 2-5 μL/min, and positive ion electrospray, optimize the signal for the unknown protein.
3. Using the multiple charge state pattern observed, determine number of charges on each ion peak and determine the molecular weight of the unknown protein.
4. Clean the syringe and capillary by flushing with 2-3 syringe volumes of acetonitrile
5. Based on your results, and the protein list shown in Table 8, determine the identity of the unknown protein.
6. Turn in a copy of your data and a brief description of how you determined the molecular weight and identity of the unknown protein.

Table 8. Possible Protein Identities

Protein	*Molecular Weight*
β-Galactosidase	465,000
Phosphorylase b	97,200
Bovine Serum Albumin	66,000
Protein A	45,000
Ovalbumin	44,287

Continued on next page.

Table 8. (Continued). Possible Protein Identities

Protein	*Molecular Weight*
Trypsin inhibitor, soybean	20,036
Apomyoglobin	16,951
Lysozyme	14,300
α-Lactalbumin	14,178
Ribonuclease A	13,700
Cytochrome c	12,384
Ubiquitin	8,565
Aprotinin	6,500

An example protein mass spectrum is shown in Figure 7.

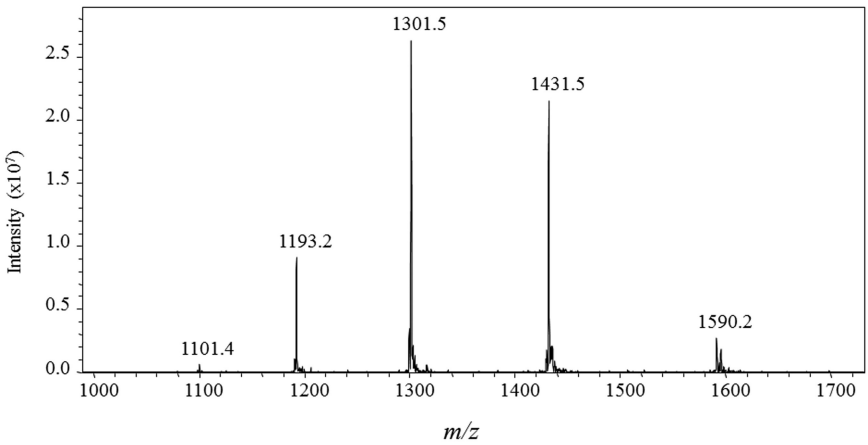

Figure 7. ESI mass spectrum of lysozyme. Reproduced with Permission from Reference #(6). Copyright 2007, American Chemical Society.

Lab K: MS of Digested Protein

As an introduction to protein digestion and identification using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer, students digest and analyze a cytochrome c sample. Modified trypsin is used as the digestion enzyme to limit the amount of trypsin auto-lysis and the digestion is allowed to proceed overnight to ensure significant digestion. A digestion cleanup step is included to get rid of salts and contaminants prior to mass spectral analysis. Students use either a commercial mass spectral database searching program, or ProteinProspector, a free online database searching

program to identify the protein based on the masses of the digestion fragments observed (*7*, *8*). This experiment requires two days due to the overnight digestion step.

Lab K Learning Objectives

Students learn how to digest a protein sample using an enzyme, how to clean up the digest using C18 pipet tips, use MALDI-TOF mass spectrometry to obtain a spectrum of the resulting enzymatic peptides and how to use database searching software to identify the protein based on the mass spectral results.

Protocol:

Protein Digestion

1. Obtain a sample of cytochrome c at a concentration of 100 pmol/μL.
2. Obtain a sample of alkylated trypsin (0.2 μg/μl) from the -80 °C freezer.
3. Pipette 100 μL of 100 mM ammonium bicarbonate (pH 8.5) into a new 1.5 mL microcentrifuge tube.
4. Add 2 μL of 100 pmol/μL cytochrome c to the ammonium bicarbonate buffer.
5. Add 4 μL of the trypsin solution to the tube.
6. Indicate on the trypsin tube that you have used the enzyme.
7. Vortex and centrifuge the cytochrome c/trypsin/buffer tube to ensure good mixing.
8. Place the digest tube in the 37 °C constant temperature block and let incubate overnight.
9. If you are around, remove the tube from the 37 °C constant temperature block every couple of hours and vortex and centrifuge before putting the tube back in the 37 °C constant temperature block.
10. Once digestion is complete, remove the tube from the 37 °C constant temperature block, add 1-2 μL of glacial acetic acid, and freeze for later analysis, or proceed to the next step.
11. Reduce the final solution volume to ~10 μL using the lyophilizer/vacuum centrifuge unit.

Spotting on the MALDI plate

1. Add 0.5 μL trifluoroacetic acid to peptide sample.
2. Obtain a ZipTip® C_{18} pipette tip. Set pipettor to 10 μL.
3. Aspirate 10 μL acetonitrile into the ZipTip®. Dispense to waste. Repeat 2 times (3 times total).
4. Aspirate 10 μL 0.1% trifluoroacetic acid into the ZipTip®. Dispense to waste. Repeat.
5. Set pipettor to 5 μL. Bind peptides to ZipTip® by aspirating 10 times in peptide sample.

6. Aspirate 5 µL 0.1% trifluoroacetic acid into the ZipTip®. Dispense to waste. Repeat 2 times (3 times total).
7. Dispense 5 µL 50:50 acetonitrile:0.1% trifluoroacetic acid into a clean 1.5 mL microcentrifuge tube using a standard pipette tip.
8. Aspirate and dispense eluent through ZipTip® 10 times.
9. Add 2 µL α-cyano matrix solution to peptide sample using a standard pipette tip.
10. Spot 1 µL sample on the stainless steel MALDI plate.
11. Put MALDI plate into mass spectrometer and obtain spectrum.
12. Use Biotools™ database software and ProteinProspector software to identify the protein

Figure 8 shows the MALDI-TOF mass spectrum for the digest of cytochrome c.

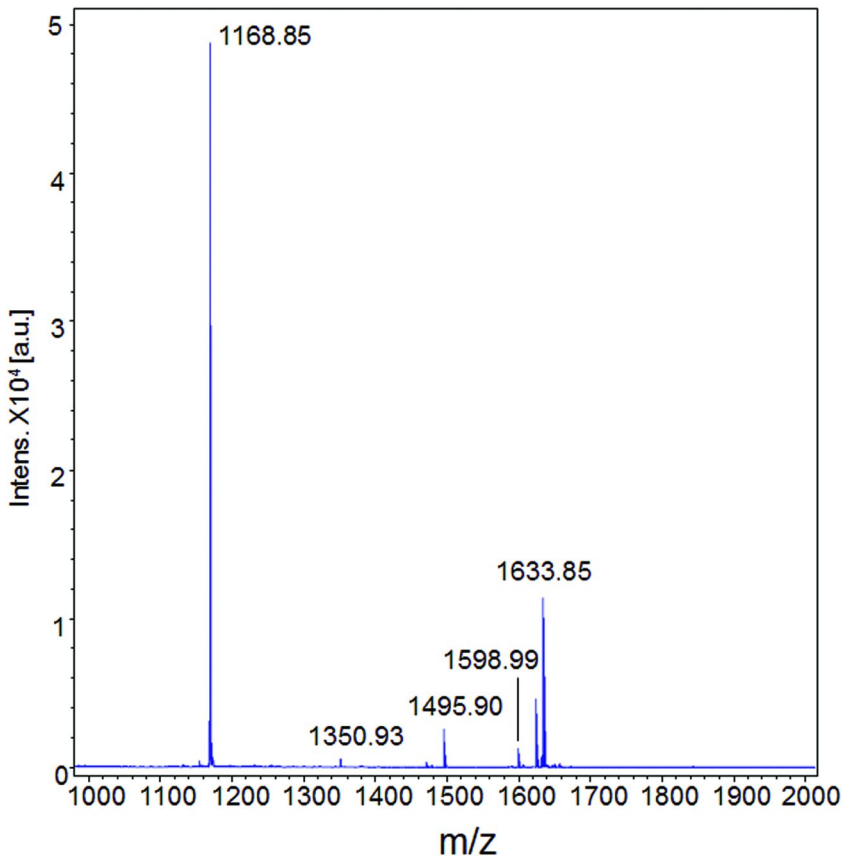

Figure 8. Mass spectrum of tryptic peptides from cytochrome c.

Lab L: Identification of Gel-Separated Protein

Once students have successfully digested and identified a sample of a cytochrome c solution, they use the same protein fingerprinting to analyze and identify an unknown protein separated on an SDS-PAGE gel, as previously described (*9*). Students are given a protein mixture, usually cytochrome c and lysozyme, which they must first separate using gel electrophoresis. Alternatively, students can chose to analyze one of the spots from the 2D gel separation of the *E coli* sample (Lab C). Students select and excise a protein band (or spot), reduce the protein to break disulfide bonds, alkylate the reduced bonds to prevent reforming of disulfides and an overnight digestion using trypsin. The resulting mixture of tryptic peptide fragments are cleaned up prior to analysis using MALDI-TOF. The same database searching software used in Lab K is used for the analysis of results. This experiment requires two days due to the overnight digestion step.

Lab L Learning Objectives

Students learn how to excise a protein band from a gel, how to reduce and alkylate the protein, perform in-gel digestion, and analyze the resulting peptide fragments using MALDI-TOF mass spectrometry.

Protocol:

In-Gel Protein Digestion

1. Locate a well-defined protein band or spot.
2. Using a clean razor blade, cut out the band/spot of interest and place it in a new 1.5 mL microcentrifuge tube.
3. Add 25 µL (or enough to just cover the gel piece) of 50:50 acetonitrile:100 mM ammonium bicarbonate and shake for 15 minutes at room temperature.
4. Remove and discard the wash solution and add 25 µL (or enough to just cover gel) of fresh 10 mM DTT in 100 mM ammonium bicarbonate. Incubate at 50-55 °C for one hour.
5. Add 25 µL of fresh 50 mM iodoacetamide in 100 mM ammonium bicarbonate.
6. Wrap the tube completely in aluminum foil.
7. Shake the tube at room temperature in the dark for one hour.
8. Remove the solution and wash twice for 10 minutes each while shaking with 50 µL of 50:50 acetonitrile:100 mM ammonium bicarbonate.
9. Remove the solution and completely dry the gel piece using the lyophilizer/vacuum centrifuge unit.
10. Obtain a sample of alkylated trypsin from the -80 °C freezer.
11. Add 0.4 µg of modified trypsin to 6 µL of 0.01% SDS (w/v) in 50 mM ammonium bicarbonate and let the gel rehydrate for 10 minutes.

12. Add enough 50 mM ammonium bicarbonate to just cover the gel piece and place in a 37 °C constant temperature block overnight.

Peptide Fragment Recovery

1. Obtain a new 1.5 mL microcentrifuge tube.
2. Transfer the supernatant from the in-gel digestion to the new tube.
3. Add enough 50:50 acetonitrile:0.5% trifluoroacetic acid to just cover the gel piece and wash with shaking for 20 minutes.
4. Remove the supernatant and add it to the digestion solution from step #2.
5. Add a second aliquot of 50:50 acetonitrile:0.5% trifluoroacetic acid to just cover the gel piece and wash with shaking for 20 minutes.
6. Again remove the supernatant and add it to the digestion solution from step #2.
7. Freeze liquid sample for future analysis or proceed with the next step.
8. Reduce the solution volume to ~10 µL using the lyophilizer/vacuum centrifuge unit.

Spotting on the MALDI plate

1. Add 0.5 µL trifluoroacetic acid to peptide sample.
2. Obtain a ZipTip® C_{18} pipette tip. Set pipettor to 10 µL.
3. Aspirate 10 µL acetonitrile into the ZipTip®. Dispense to waste. Repeat 2 times (3 times total).
4. Aspirate 10 µL 0.1% trifluoroacetic acid into the ZipTip®. Dispense to waste. Repeat.
5. Set pipettor to 5 µL. Bind peptides to ZipTip® by aspirating 10 times in peptide sample.
6. Aspirate 5 µL 0.1% trifluoroacetic acid into the ZipTip®. Dispense to waste. Repeat 2 times (3 times total).
7. Dispense 5 µL 50:50 acetonitrile:0.1% trifluoroacetic acid into a clean 1.5 mL microcentrifuge tube using a standard pipette tip.
8. Aspirate and dispense eluent through ZipTip® 10 times.
9. Add 2 µL α-cyano matrix solution to peptide sample using a standard pipette tip.
10. Spot 1 µL sample on the stainless steel MALDI plate.
11. Put MALDI plate into mass spectrometer and obtain spectrum.
12. Use Biotools™ database software and ProteinProspector software to identify the protein

Lab M: Capillary Electrophoresis of Peptides

As an instrumental alternative to gel electrophoresis, students use capillary electrophoresis (CE) to determine the identity of and quantitate an unknown peptide. Students are given an unknown and four standard solutions. They are free to devise their own method for identifying the unknown peptide, with most eventually deciding to run each standard and the unknown individually and use

migration time of each to identify the unknown. In some cases, the migration time of the unknown is very close (within a few seconds) to two of the standards. When this happens, students are generally unsure as to which of the two peptides the unknown is, but with some prompting they realize that if they rerun the separation mixing the unknown with one of the two standards in question they will either observe a single peak, indicating the standard used matches the unknown, or two peaks if the unknown is different from the standard used. Once the identity of the unknown has been established, students can make a concentration calibration curve using the standard and compare peak heights and/or areas to that observed for the unknown in order to quantitate the unknown. While the entire experiment does require several hours, the instrument has an autosampler so students can set up and start the analysis and leave to work on other experiments.

Lab M Learning Objectives

Students learn how to use the capillary electrophoresis instrument, how to identify an analyte based on migration time and how to create a calibration curve to quantitate an analyte.

Protocol:

Capillary Conditioning (check with instructor to see if done previously)

1. Set capillary temp at 25 °C.
2. Place an empty vial in position 6.
3. Flush capillary with acetonitrile (vial #1) for 10 minutes.
4. Flush capillary with 0.1 N HCl (vial #2) for 10 minutes.
5. Flush capillary with 1.0 N NaOH (vial #3) for 10 minutes.
6. Flush capillary with nanopure water (vial #4) for 10 minutes.
7. Flush capillary with run buffer (50 mM acetate) (vial #5) for 20 minutes.

Setting up CE Instrument

1. Fill vials 7, 8 and 9 with run buffer (50 mM acetate).
2. Place an empty outlet vial in position 6.
3. Create a new method – default values can be used except as noted below.
4. Set capillary temperature at 25 °C.
5. Set Inlet Home vial to position 7, Outlet Home vial to position 8.
6. Select no replenishment, but select Preconditioning and edit the table – Flush capillary for five minutes with buffer inlet 9 and outlet 6.
7. Set pressure injection of 50 mbar for 6 seconds.
8. Set separation voltage at 30 kV, positive potential, 100 μA current, power at system limit, 0 μA low current limit.
9. Store current and set stop time of 12 minutes.
10. Store Signal A: 192 nm, 4 nm bandwidth
11. Select Store all in peak, and prerun autobalance

12. Add 192 nm signal to method
13. Once method is complete, select "Save method As" from method pulldown and provide a method name.

Running Peptide Samples

1. Obtain 1 nmol/μL stock samples of angiotensin 1, bradykinin, Leu-enkephalin and Met-enkephalin.
2. Obtain unknown sample from instructor.
3. The linear working range for each peptide is 0.01 nmol/μL to 1.0 nmol/μL.
4. Determine the peptide(s) in your unknown and the concentration(s).
5. You can run samples individually (click on the single vial icon in the upper left corner) by selecting which vial contains your sample or you can set up a sequence to run multiple samples (click on the three vial icon in the upper left corner) by filling out the sequence table. For quantitation, the unknown should be run close to the same time as the standards used for the calibration curve (i.e. don't use the area for the unknown if it was run several hours or days before).

Conclusions

Bioanalytical chemistry is an important and growing subfield. While it shouldn't replace a traditional analytical or biochemistry course, adding a bioanalytical chemistry course to the curriculum is well worth the effort. There will likely be significant student interest in this interdisciplinary course and will prepare students for research in graduate programs or in an industrial setting. When possible, adding a laboratory component to a bioanalytical chemistry course will provide students an opportunity to experience hands-on the techniques they are learning about in the course. Many of the techniques used for the analysis of biological samples are best learned by actually performing the analysis, and many have "tricks of the trade" that have to be experienced. There are numerous different ways to incorporate bioanalytical lab experiences into the curriculum. One such example of a lab-intensive course has been presented here. The experiences described here have successfully been used several times by different offerings of the course. While the lab-intensive format presented here, where students individually complete each lab rotating through the labs and helping train other students in the class, works with the one-month January term offered here, other formats could also be adopted depending on the number of students in the course, the amount of instrumentation available, the academic calendar, and the experiences the instructor wishes to provide. Ultimately, any coverage of bioanalytical chemistry will be useful for students at any academic level.

Acknowledgments

The 8-16 students that enroll in this course each time it is offered have been instrumental in making improvements to the course format, topics, and lab experiences. The results presented above were collected by students in the 2013 offering of the course. In addition, Isaac Arnquist helped develop many of the lab experiences described here.

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Chapter 12

Student-Assisted Research-Focused Experiential Learning in the Bioanalytical Chemistry Curriculum

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Given that academic success is irrefutably correlated to student preparation and motivation, the emphasis on innovative pedagogy bodes well with the expectation that students will gain observational and applied experiences in the classroom setting. This chapter details the use of experiential educational concepts; in particular, research-focused curricular tools as means to promoting student learning and motivation for rigorous exploration in the field of analytical chemistry. Parallel efforts to recruit and retain chemistry students from diverse backgrounds, with careful attention paid to their matriculation into graduate programs and industrial careers, are examined.

Introduction

At the core of any traditional analytical chemistry course is an expectation that students will develop a basic understanding of the principles, instrumentation and application of chemical analysis. In this context, instructors and program coordinators have begun to reach similar conclusions—an emerging unanimity—about the positive role of research-enriching experiences in bringing innovation to the analytical chemistry curriculum. Moreover, when examining and applying the pioneering concepts of Dewey, Piaget, and Kolb among others, it is evident that experiential learning activities—those that promote learning from

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observational and/or applied experiences—can lead to improving the interest and academic achievement of students in the physical sciences (*1–11*).

Karukstis, in a number of reports addressing efforts by the Council on Undergraduate Research (CUR), highlights the role of research in supporting chemistry curricula, as well as in the development of targeted, one-on-one faculty student collaboration (*12–14*). In a review of the book *Engaging Student Voices in the Study of Teaching and Learning*, Ahern-Rindell states: "…faculty-student partnering decentralizes the power in the classroom and in turn, gives students more responsibility for their own learning and that of their peers. After all, how can we so-called 'educators' leave out the most important voices in the learning process? The voices of our students in the chemical sciences (*15*)." And when considering the different learning styles of students, Towns in her informative article outlining Kolb's Experiential Learning Theory (ELT), describes two lessons from a physical chemistry course (*16*). Her work illustrates how the ELT framework is utilized to bolster student comprehension and appeal to a wider range of learning styles, which may ultimately help to attract and retain undergraduate STEM majors.

Instructors need to be deliberate in the design and implementation of analytical chemistry curriculum. Structuring of high quality, high-impact courses must begin with concentration on the learning outcomes expected and with appreciation of the various learning styles of student cohorts. After all, affirmation for learning and conditions for learning are imperative for student success. Herein, research-focused experiential learning concepts are presented as tools for the development of a laboratory exercise demonstrating a complicated analytical task allocation problem. Specifically, undergraduate research students identified based on Kolb's defined learning styles were asked a series of questions related to a published journal article to facilitate understanding of advanced computational and chemical separation concepts. This research-based exercise will be featured in a subsequent classroom setting. Through curricular intentionality and attention to diverse student needs, we feel that we have presented a significant step towards increasing high-impact practices in the analytical chemistry classroom.

An Overview of Kolb's Experiential Learning Theory (ELT)

Kolb, drawing on a knowledge base developed by earlier educational theorists and social/developmental psychologists, theorized that "experience" is a key component of learning (*1*). Experiential learning, although broadly defined in the literature, includes the following abbreviated steps:

1. Study and preparation;
2. Direct observation and/or active engagement;
3. Reflection and distillation;
4. Creative application and action; and
5. Performance assessment and evaluation.

For the vast majority of individuals involved in higher education—students, faculty, staff, administrators, not to mention parents—Kolb's model of the underlying structure of learning represents a mechanism through which students can reach their full academic potential, regardless of the discipline. Four distinct learning styles based on a four-stage learning cycle were postulated (Table 1) (*1*). Regardless of the style of learning, a viewpoint shared by most educators in the physical sciences calls for experiences that develop critical thinking skills and confidence and assurance about one's growth as a young scientific investigator. Given the diversity of learning practices outlined above, the quality and direct application of these practices is particularly important. One of the high-impact practices discussed in this chapter, undergraduate research, works to increase problem solving and critical thinking through personal interaction with faculty and peers.

Table 1. Kolb's identified learning styles based on a four-stage learning cycle

Kolb's Learning Styles	*Description/Characteristics*
Divergers	Students who respond to concrete situations from multiple perspectives, adapting by observation rather than by action. Cooperative group learning and brainstorming are key components of this learning process. Ultimately, divergers ask, "Why is this concept/study important?"
Assimilators	Students who reason inductively and are good at creating and interpreting theoretical models and underlying concepts. Project design and implementation are key components of this learning process.
Convergers	Students who enjoy decision-making, problem solving, and the practical application of concepts and ideas. They learn best by emphasizing the practical application of ideas through well-defined tasks.
Accommodators	Students who solve problems at an intuitive level in a trial-and-error manner. They learn best by applying problem solving strategies to real-world situations. They are keen to know how concepts would apply if the problem at hand were slightly different.

Experimental Approach and Project Assessment/Evaluation

Background

The motivation for this work stems from a complicated chemical separation problem outlined in Page et al. (*17*). In this context, efficient optimization of experimental parameters is crucial to the success of separating complex chemical compounds and biological macromolecules. More specifically, the implementation of a particle swarm optimization (PSO) algorithm on a neural network platform (Figure 1) for multiparameter optimization of multiplexed 24-capillary electrophoresis technology with UV detection is featured. PSO is

an effective search metaheuristic motivated by the social behavior of movement organisms (e.g., birds flocking and fish schooling), with the original algorithm modeling a problem space by a population of individuals or particles (*18*). Figure 2 displays the pseudo-code, which is described in detail in the original study (*17*). When combined with neural networks, computational learning systems inspired by the brain's neural structure (*19*), PSO provides a framework to solve advanced linear and non-linear optimization problems. This algorithm generates possible experimental solutions and measures their quality by using a forward propagation through the neural network to obtain a minimized error function. By introducing PSO, we have developed a neural network platform whose architecture matches the requirements of predicting optimal experimental conditions, where the need to configure and test multiple parameters on a parallel separation format is critical. For faculty and students, this symbolizes a way to routinely perform optimization protocols that were previously impractical due to time, algorithm convergence, model inaccuracy, high output error, and computational cost. Moreover, by encouraging the study of such concepts, students will no doubt gain a more complete picture of how hybrid computational platforms function and how powerful they can be in solving complex chemical problems.

Two experimental systems were examined: 1) separation of purified rabbit metallothioneins (MT's), and, 2) separation of model toluene urinary metabolites and selected organic acids. MT's are a class of sulfhydryl-rich proteins that bind a wide range of metals with high affinity. While our understanding of the role of MT's and methods for the separation and characterization of MT isoforms has grown considerably over the past few decades, there is still a critical lack of insight over the use of MT's as potential biomarkers for metal contamination. Although there have been a number of studies investigating ways in which to optimize separation conditions for CE-related applications, only a limited few have centered on evaluating experimental conditions for the separation of MT isoforms. Of these, none reported the use of intelligent optimization metaheuristics as described in our previously published study (*17*).

The second model application examined the separation of toluene urinary metabolites and selected organic acids. Toluene exposure has been shown to lead to acute and chronic respiratory effects, functional alteration of the central nervous system and chromosome aberrations. Given that two metabolic pathways exist—each resulting in major and minor metabolites—several potential biomarkers are available for assessing human exposure to toluene. Both pathways are highlighted in Figure 3. As shown, the major pathway is driven by the hydroxylation of toluene to benzyl alcohol, which is subsequently metabolized to benzylaldehyde (*17*). The latter is metabolized to benzoic acid which, upon conjugation with glycine, results in hippuric acid—a major urinary metabolite. Benzylmercapturic acid, produced from benzylaldehyde, has also been shown to be a minor metabolite of toluene. Hydroxylation to cresols (Fig. 3b) is considered a minor pathway in the metabolism of toluene. Thus, several potential biomarkers are available for assessing internal exposure to toluene. Increased throughput, particularly by optimizing higher-order multiplexing CE technology, is an important tool for making electrophoretic separations competitive for time- and dose-dependent urinary metabolite screening.

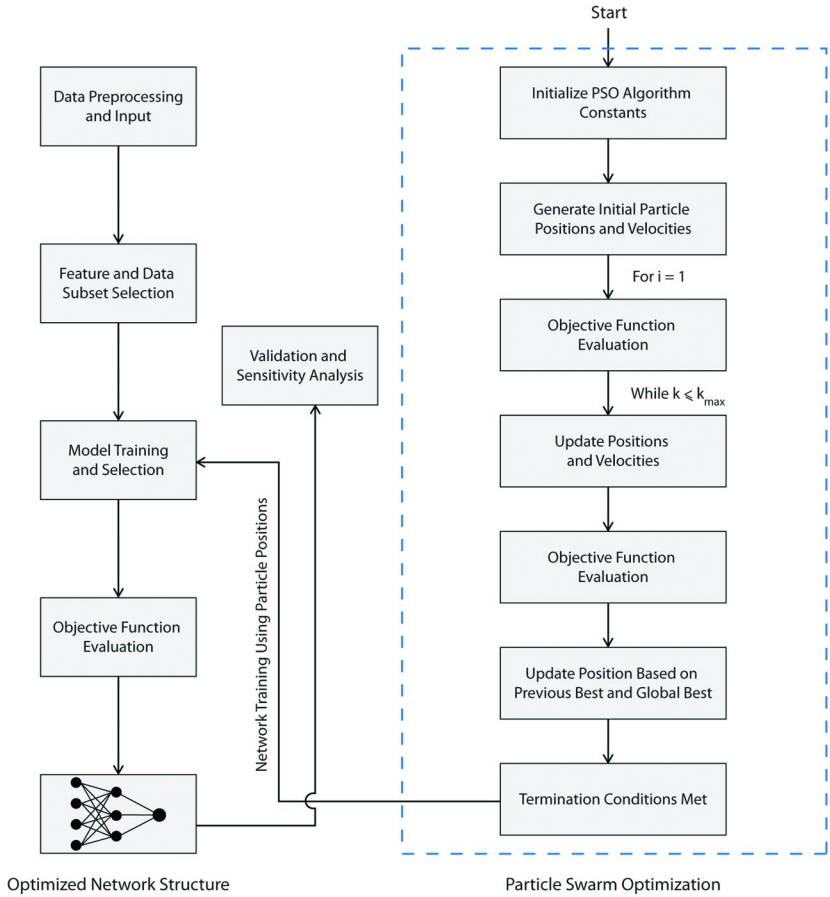

Figure 1. Generalized schematic of the neural network platform employing the PSO algorithm. The heuristic is implemented to search for multiple parameters of the model including the best sample weighting of the vectors and optimal neural architecture. Reproduced with permission from reference (17). Copyright 2012; Springer.

Begin Particle Swarm Optimization (PSO)

Do:

1. Initialize algorithm constants and define termination conditions, e.g., k_{max}, v_{max} and P.
2. Generate a random initial swarm of particles using positions x_k^i and velocities v_k^i in the multidimensional search space.

For $i = 1$

3. Objection function $f(x_k^i)$ evaluation.
4. Best positions $p_k^i = x_k^i$ assigned with $f(p_k^i) = f(x_k^i)$.
5. Find $f_k^{best}(p_k^{best}) = \min\{f(p_k^1),..., f(p_k^i),..., f(p_k^P)\}$.
6. Initialize $p_k^g = p_k^{best}$ and $f(p_k^g) = f^{best}(p_k^{best})$.

While $k \le k_{max}$

7. Update x_k^i using Equation (3).
8. Update v_k^i using Equation (4).
9. Objection function $f(x_k^i)$ evaluation.
10. Update particle best position if $f(p_k^i) > f(x_k^i)$ then $p_k^i = x_k^i$ with $f(p_k^i) = f(x_k^i)$.
11. Find $f_k^{best}(p_k^{best}) = \min\{f(p_k^1),...,f(p_k^i),...,f(p_k^P)\}$. If $f(p_k^g) \rangle f(p_k^{best})$ then $p_k^g = p_k^{best}$ and $f(p_k^g) = f^{best}(p_k^{best})$.
12. Termination conditions defined in step 1 satisfied.

End while

Figure 2. Pseudo-code for the basic PSO algorithm used in reference (17). Note: P represents the population of solutions at k iterations. Reproduced with permission from reference (17). Copyright 2012; Springer.

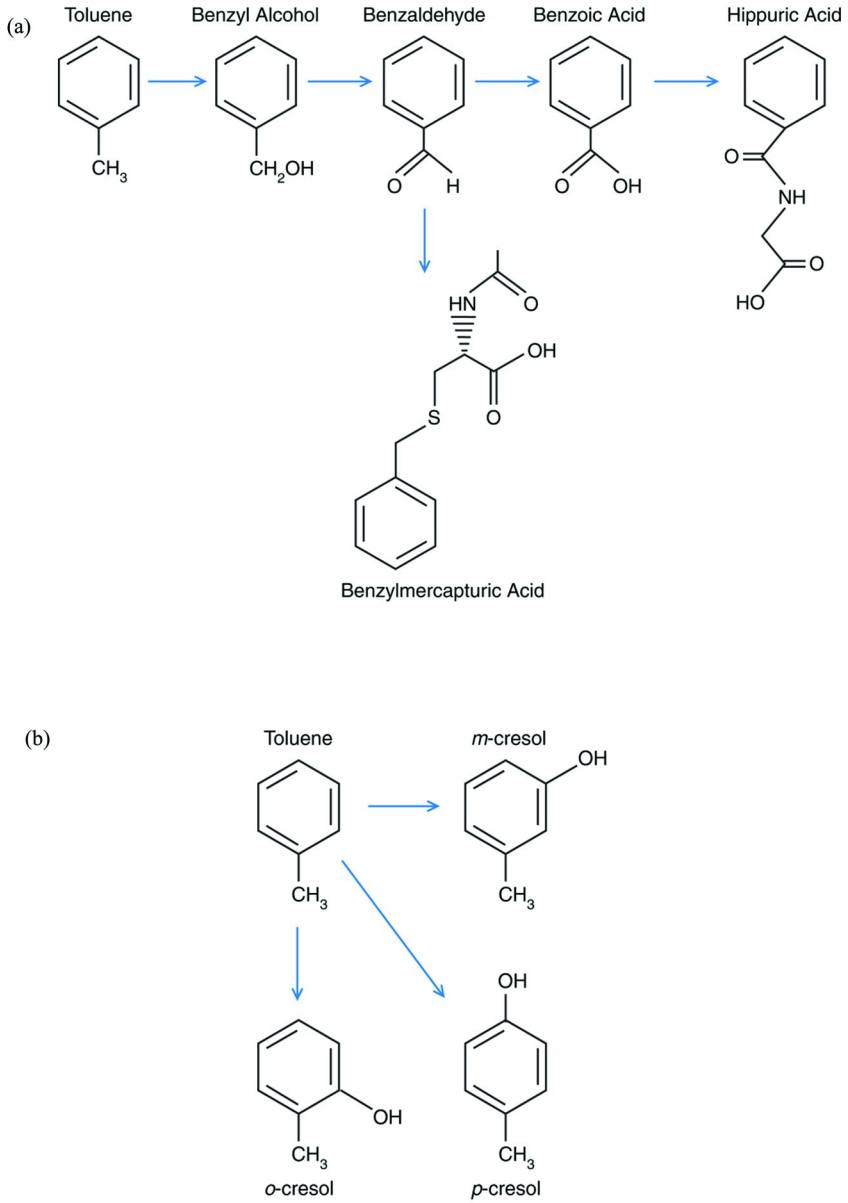

Figure 3. Two associated metabolic pathways of toluene. (a) Major pathway driven by the hydroxylation of toluene to benzyl alcohol. Hippuric acid is formed upon the conjugation of benzoic acid with glycine. Benzylmercapturic acid is shown as a minor metabolite. (b) Minor pathway involving hippuric acid hydroxylation to cresols. Reproduced with permission from reference (17). Copyright 2012; Springer.

Method and Outcomes

In our approach, we used both direct and indirect methods to evaluate student-learning styles; and thus, developed a comprehensive way of identifying and classifying students according to Kolb's learning model. Direct methods included the review of prior course and homework assignments, examinations, quizzes, and lab-based performance activities including research projects and reports. Grading was linked to clear learning goals/outcomes defined in our quantitative chemical analysis course syllabi. Indirectly, feedback provided by end of class questionnaires and instructor observations of students laboratory skills allowed for further examination and classification. All evaluation methods were designed with reliability and validity considerations in mind. In addition, we helped the students explore and analyze their own learning through self-reflection exercises.

The four identified summer research students were asked to read and summarize the work of Page et al. (*17*)—a project completed and published by our laboratory a year earlier. They were subsequently asked a series of questions as outlined in Table 2. The goal of this exercise was to charge the students with the task of identifying the importance of advanced modeling concepts in relation to optimizing CE experimental conditions. Comprehension of advanced optimization concepts is especially important for applications in analytical chemistry given the unpredictable availability, quality, representativeness, and size of input data sets. Moreover, the relationships between given input variables and the output properties afford a plenitude of available information from which to model non-linear chemical and instrumental processes. These efforts are catalyzed by student exposure to increases in computational power and availability, as well as the incorporation of modern software, algorithms, and related methodologies into the analytical chemistry classroom/laboratory setting. Nevertheless, despite the demonstrated success of incorporating statistical and low-end modeling techniques into the curriculum, extending knowledge to cover advanced computational concepts has been languid at best.

Referring back to Table 2, Kolb-categorized summer research students were asked a series of questions related to the applications outlined above. For example, the student classified as a diverger was asked to identify the overall significance of this work, as well as considering its importance to the field of analytical chemistry. This student was highly reflective—not only was he able to articulate the broad scope of this project, his keen perception of the significance of advanced optimization techniques related to chemical separations was apparent. He understood that by developing research methods that integrate computational analysis and chemistry, many chemical problems can be studied accurately and efficiently.

The assimilator demonstrated an active intellectual curiosity and took it upon herself to try to improve on the standard PSO process outlined in the published study. To address the limitations of using proprietary modeling software and to assess the effects of changing internal model variables on instrument response, she utilized the PSO pseudo-code presented in Figure 2 to develop user-friendly working code on a parallel computing framework. This meant that, unlike existing commercial software options, this new approach made it possible to manipulate

the range of experimental variables more freely. She understood scientific computing as a body of methods and as a means for framing research questions. In addition, through her interactions in the summer learning community, she was able to educate the other students on basic scientific computing concepts. Such an approach allows students to integrate perspectives from computer science, mathematics, and the physical sciences to articulate solutions to complex, multidisciplinary, real-world problems.

Table 2. Research-focused questions posed to students identified based on Kolb's Experiential Learning Theory (ELT) and reference (*17*)

Student Learning Styles	*Questions/ Situations Posed*
Divergers	1. What is the importance/significance of this work? 2. What are the implications of this work? 3. How does this study help the field of interest?
Assimilators	1. Describe the modeling concepts presented. 2. How would you improve on the model structure and performance?
Convergers	1. How were the model concepts applied to real chemistry situations? 2. What other strategies could be used to optimize capillary electrophoresis parameters?
Accommodators	1. Refer to Figure 4. If Figure 4a were the only graph produced, how would this change the outcome of this study? The performance of the model? 2. In Table 4, what if the r^2 values were lower? What if the RSD's presented were higher? What would this say about the model performance?

The converger, like the assimilator, was asked to reflect on how the underlying modeling concepts were applied to the given problems at hand. She approached this learning situation as an assimilative process rather than a simple "trial and error" activity. She used insight, competence and deliberation as methods of establishing a framework by which to conceptualize difficult modeling concepts. She drew upon her mathematical training to propose new methods of optimization. This effort, as it reached its climax during the summer research period, helped delineate the rigors of chemical separation and how to solve limitations through alternative modeling procedures. The student's interpretation of the basic mathematical principles together with real applications in the field of chemical separations makes the comprehension of complex analytical problems more accessible.

The final area of investigation deserves more attention than we normally afford it, no matter how in depth we teach. This area is comprehended in the answers to standard questions posed in the analytical chemistry classroom:

1. How do we effectively plan and design experiments?
2. How do we interpret the results of analytical measurements/statistical models?
3. How do analytical figures of merit serve as indicators for the characteristics of an analytical technique?

Realize that this exercise was an investigation that went beyond assessment of student learning as a means of simple answerability. More significantly, the student identified as the accommodator was given the complicated task of data interpretation. Inadequate interpretations of graphs and tabulated data pose a significant barrier to the ability of students to problem solve in analytical chemistry. These representations are a principal mode of communication among analytical chemists, and are used in part to classify their unity of practice.

To bring focus to this area of concern, refer to the electropherogram displayed in Figure 4 showing the separation of MT isoforms before (a) and after (b) model optimization of experimental parameters. The accommodator reflected on the importance of well-planned and effectively executed experimental designs, especially with regard to selecting instrumental variables for subsequent optimization attempts. She was cognizant of the fact that the MT-2 sub-isoform (shown in Figure 4b) would not have been identified without proper optimization leading to complete separation. In addition, she tested the performance of the PSO optimized neural network by examining model parameters and the measure of fit by use of the mean square error (*MSE*), defined by:

$$MSE = \frac{SSE}{n} \tag{1}$$

where *SSE* is the sum of squared error, given by:

$$SSE = \mathrm{Sum}_{(i=1 \text{ to } n)}\{w_i (y_i - f_i)^2\} \tag{2}$$

where y_i *is* the observed data value, f_i is the predicted value from the fit, and w_i is the weighting applied to each data point (w_i=1). As shown in Table 3, the hybrid PSO-neural network (PSO-NN) model proved to be superior to both multiple linear regression (MLR) and standard neural network models in terms of correlation and *MSE* values for both training and test data sets. Finally, when considering tabulated data from the second model application (Table 4), it was apparent that she understood the significance of the determination of analytical figures of merit for the separation of toluene urinary metabolites and associated organic acids. She demonstrated knowledge of regression models, in large part, the correlation between two variables and the line of best fit. In addition, she understood that

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relative standard deviation was a measure of precision, not accuracy. Ultimately, she realized that the execution of the optimization model was directly linked to instrument performance and measurement error.

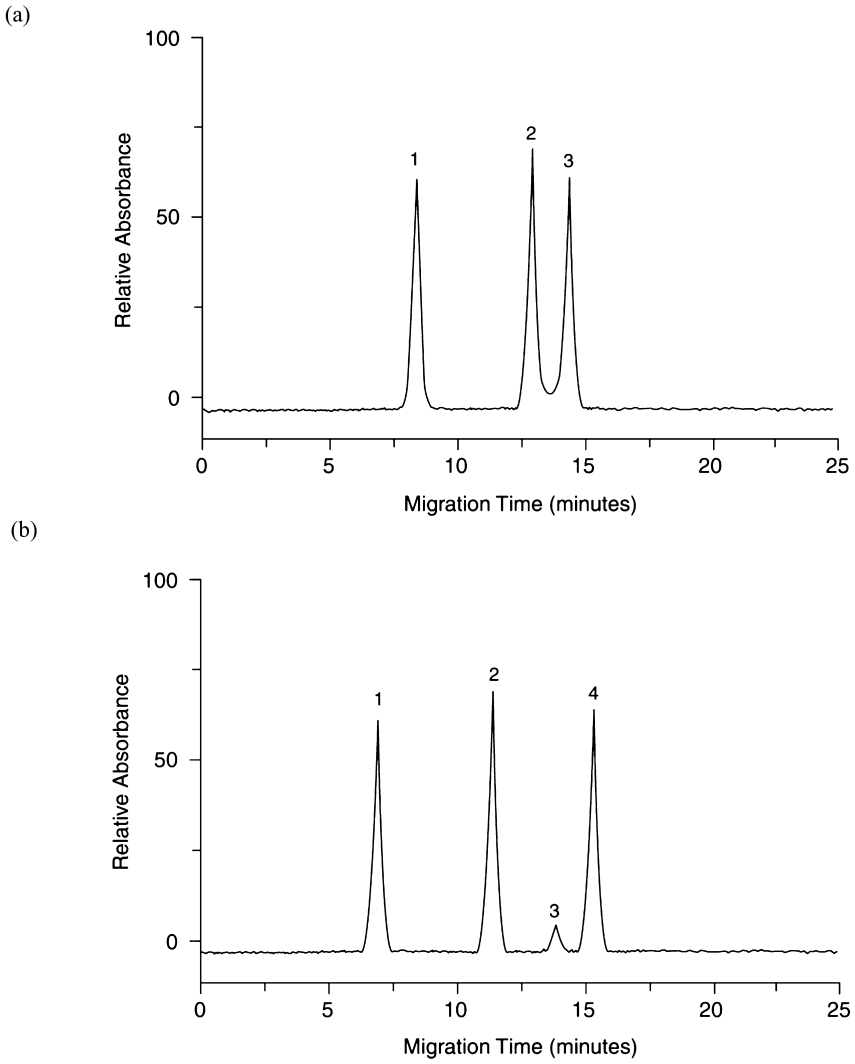

Figure 4. Representative electropherograms obtained before (a) and after (b) model optimization (validation run 1, capillary 1). Peaks 1, 2 and 3 in (a) represent the neutral marker (DMSO), MT-1 and MT-2, respectively. Peaks 1, 2, 3, and 4 in (b) represent DMSO, MT-1, a possible sub-isoform of MT-2, and MT-2, respectively. Optimized conditions (b) were as follows: 10 mM borate buffer (pH = 7.8), +10.8 kV applied voltage, injection time = 3.0 seconds, and an effective capillary length of 55-cm. All runs were carried out at 20°C. Reproduced with permission from reference (17). Copyright 2012; Springer.

Table 3. Mean training and test set results of the PSO-NN approach compared to standard NN and MLR models

Model	*Training r^2 (n=10)*	*Training MSE (n=10)*	*Test r^2 (n=10)*	*Test MSE (n=10)*
MLR	0.83	0.259	0.82	0.211
Standard NN	0.88	0.188	0.84	0.202
PSO-NN	0.96	0.010	0.95	0.023

Table 4. Analytical figures of merit for toluene urinary metabolites and selected organic acids. Reproduced with permission from reference (*17*). Copyright 2012; Springer.

Analyte	*r^2*	*Migration Time (%RSD) (n=10)*	*Peak Area (%RSD) (n=10)*	*LOD (μg/mL)*
Creatine	0.996	1.7	1.4	0.09
m-cresol	0.993	3.1	2.5	0.21
p-cresol	0.995	4.9	3.6	0.17
o-cresol	0.992	5.4	4.6	0.35
Benzoic acid	0.998	2.6	3.2	0.10
Benzylmercapturic acid	0.989	3.5	1.5	0.48
Hippuric acid	0.994	1.9	1.4	0.10
Uric acid	0.996	1.8	2.0	0.07

Evaluation and Development of a Subsequent Laboratory Exercise

Campus surveys conducted as part of our newly adopted strategic plan revealed that experiential learning was an organic, widespread and common endeavor at the university. Undergraduate research is a major emphasis of this movement. In fact, during the last academic year (2011-2012), more than 300 students presented original research work at our annual Festival of Scholars event in fields ranging from STEM disciplines to the performing arts.

Anticipating similar success in incorporating research in a future quantitative chemical analysis course, we tracked the progress of the above mentioned summer research students in helping develop a computational-driven, separation-based laboratory exercise. It was clear that research, as an experiential learning tool, contributed to student engagement. The expectation was that by the end of the summer research activity, the students would be able to reflect upon their experience, and by the start of the academic term, be able to help translate this work into a fully integrated laboratory exercise. All four students presented their results at our Summer Research Symposium, a testament emphasizing their acquisition of effective communication skills and comprehension of difficult

subject matter. Self-assessment and positive instructor feedback resulted in an increase in self-efficacy—the scale to which a student believes that he/she is capable of achieving an explicit objective or goal. It was also apparent that the community-learning group formed resulted in students being motivated toward helping one another achieve and better understand and articulate experimental results.

Many of the educational tactics described above will be utilized in an upcoming quantitative chemical analysis course. By using the research exercise outlined in this chapter as a model, we expect that students will demonstrate an increase in knowledge, cognitive ability, confidence, and motivation for learning. Moreover, by further refining and implementing this innovative approach, we expect to help ensure the retention and educational progress of student cohorts. Evaluation will be formative (as the laboratory exercise is ongoing) and summative (at the conclusion of the course). Monitoring will involve the continuous assessment of exercise implementation and the academic progress of students during and after their enrollment in the course. In addition, multiple benchmarks and assessment tools will be utilized to track the long-term educational progress of students including ongoing contact with faculty mentors and personal reflections. Finally, the use of inter-student comparisons will enhance our efforts to compare student abilities and to assess whether questions asked to students in one category apply to students in other categories. For example, it may be that students with similar preparedness and similar socioeconomic profiles exhibit parallels in learning style. However, given that experience and aptitude play major roles in learning, we generally expect that students will exhibit distinct learning styles

Underrepresented Populations in STEM Education: The Role of Experiential Learning

Dedicated efforts in the STEM field are underway to recruit, train and retain students from first generation, low-income and underrepresented backgrounds, with deliberate efforts geared towards their matriculation into graduate programs and subsequent careers. With an emphasis on experiential learning, we can expand the breadth and depth of student inclusion and participation in academically rigorous activities such as research. Student involvement includes training on research design, qualitative and quantitative data analysis, methodological approaches common in the physical sciences, and critical analysis of relevant literature in the analytical chemistry field. By adopting a faculty-directed research approach, one can capitalize on the area of expertise and professional contacts of faculty mentors and more closely align with the model of research that is most common in graduate programs, particularly in the STEM fields of study. Leveraging the guidance of faculty mentors and harnessing the power of engaged experiential learning would be essential to student success in graduate degree enrollment, persistence and completion.

We have shown that exposing this population of students to real-world learning experiences directly affects their performance in our quantitative chemical analysis course. Applied experiences not only promote classroom

inclusivity, but also help create a true "community of scholars" by integrating faculty-led, group research with standard classroom techniques. From 2007-2012, just over 80% of underrepresented students enrolled in this course participated in our summer research program. This is significant given that this population faces more barriers than the general student cohort does—for example, the need to earn money for school during the summer months. Our instructors continuously design and implement innovative activities to ensure the retention, good academic standing and educational progress of all students regardless of pre-course preparation. Instructors routinely determine effectiveness in meeting course objectives using benchmarks, and incorporate evaluation results to inform good laboratory practice.

Summary

Prior evidence suggests that experiential learning activities, including undergraduate research, are routinely used to foster student learning and bolster interest in scientific disciplines. By involving undergraduate research students in curricular development activities—such as those described in this chapter—a genuine learning community is possible. The reflective exercises demonstrated will be incorporated into a future laboratory exercise for a quantitative chemical analysis course. Student success in this course will improve, in large part by the incorporation of holistic, active learning approaches such as undergraduate research. Students will benefit from close and sustained faculty interaction—not just transparent communication—and are expected to make a smooth transition to graduate school or industrial careers. Moreover, when examining the possible challenges of engaging low-income, minority and first-generation STEM students, we expect that an emphasis on active experiential learning will provide the right environment for student achievement and degree attainment.

Acknowledgments

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Chapter 13

Guided Inquiry and Project-Based Learning in Biophysical Spectroscopy

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Based on a graduate-level course offered at Yale University, we discuss using guided inquiry and project-based teaching strategies to teach a survey of biophysical spectroscopic methods to graduate students. By demonstrating the applications of biophysical spectroscopy to address fundamental questions in the field of biology at the molecular level, students learn both the basic principles of these methods and their applications to problem-solving in research.

Introduction

The materials described in this chapter are based on a graduate-level course titled *Biophysical Spectroscopy*, offered in the spring semester from 2008 to 2012 in the Department of Chemistry at Yale University. In 2013, the course was renamed *Biophysics II*.

Goals of the Course

The primary goal of the course is to introduce several spectroscopic methods commonly used in biophysical chemistry to beginning graduate students. By demonstrating, exploring, and discussing the applications of the selected methods to answer fundamental questions in the field of biology at the molecular level, students can later apply these methods in order to solve problems in their individual research projects.

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By the end of the course, students are expected to be able to describe how each of 6-10 spectroscopic methods may be applied to study the structure, function, or mechanism of a biomolecular system. For each method, the fundamental principle of operation and basic instrument design are discussed to provide foundational knowledge for these techniques. Students are also expected to develop critical reading skills required for graduate-level researchers in order to be able to identify the implicit question that inspires a biophysical study. Furthermore, students learn to evaluate how effectively the methods discussed in class are used to answer questions in the recent literature.

The secondary goals of this course are to expose students to examples of the important systems currently being investigated in the biophysical chemistry literature and to develop scientific writing and presentation skills through the assignment of short reading reports and a longer proposal project.

Scope of the Course

Throughout the course, the applications of specific spectroscopic methods to biomolecules are discussed. Topics include Raman spectroscopy, fluorescence spectroscopy, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), optical ultrafast spectroscopy, and circular dichroism spectroscopy. Emphasis is placed on designing experiments and interpreting spectroscopic data to obtain structural and dynamic information and to answer biological questions at the molecular level. Students are expected to have previous knowledge of undergraduate level physical chemistry as well as protein composition and structure.

Structure of the Course

The course is divided into modules and each module covers one spectroscopic method, e.g. fluorescence spectroscopy. The course can cover 6-10 spectroscopic methods depending on the length of the semester or the quarter. Each module consists of three parts and is covered by three to four lectures.

- Part I: Basic principles of a selected spectroscopic technique. This part includes one to two lectures to be delivered either by the instructor or invited local experts.
- Part II: Applications of the technique to a model system. In particular, we chose rhodopsin as the model system to illustrate the applications of the spectroscopic techniques. The advantages of using a single model system throughout the course and the reason for choosing rhodopsin as the model system will be discussed in detail later. This lecture is delivered by the course instructor.
- Part III: Panel discussion on the applications of the technique to other biological molecular systems. This part requires all students to read one or two assigned research articles and submit a report before the discussion class. A student volunteers or is chosen to lead the discussion. Detailed

format and requirements for report and panel discussion will be described in the next section.

The course is completed by a final project in which each student chooses a biomolecular system that was not covered in the course and defines a specific question about the structure or mechanism that may be answered using one or more of the spectroscopic techniques discussed in class. The student writes a two-page proposal outlining the experimental design he or she would apply to answer the question and the expected results. At the end of the course, the proposals are presented to the class in a symposium format for discussion.

Assessment

At the end of the semester, 50% of the assessment of each student is based on the reports he or she submits for the panel discussions as well as participation in the panel discussion sections. In the one-page reading report, the student should be able to articulate the fundamental question asked by the assigned literature study and summarize how the method discussed in class was used to answer this question. The grading is based on the instructor's subjective assessment of each student's understanding of the fundamental concepts according to his or her writing. During the class discussion, the student is expected to contribute by asking additional questions and responding to others' questions about the study.

In addition, each student is required to submit a two-page independent research proposal that accounts for the other 50% of the course grade. This proposal should include novel questions about a biomolecular system chosen by the student, proper biophysical spectroscopic methods to approach the questions, and the expected outcome. The independent research proposal offers an opportunity for students to apply the spectroscopic methods they learned during the semester to answer questions in research. Each student defends his or her proposal in the form of an oral presentation in class.

Teaching Strategies

There are primarily three strategies used to teach this course:

- A single model system is used throughout the course to illustrate the applications of various spectroscopic methods.
- A panel discussion led by students is used to introduce the latest studies on the spectroscopic techniques discussed in class to other biomolecular systems.
- An independent proposal assignment allows students to apply the newly learned methods to a new system.

Below, we provide more details about these strategies.

Model System

In this course, a single model system is used to introduce the basic theoretical background and applications of each biophysical spectroscopic method. The advantage of using only one model system throughout the course to teach several spectroscopic methods is that the students do not need to spend extensive time reading related background information about various molecular systems. Consequently, they can focus on the underlying theories and applications of the spectroscopic methods. Moreover, by systematically studying the same model system with various techniques, students learn to appreciate the methodology of investigating a molecular system with the assistance of a variety of spectroscopic methods. This understanding will lay a solid foundation for experimental design to solve problems in their future research.

There are several criteria for choosing the model system. Because the aim of the course is to demonstrate the power of biophysical spectroscopic methods to address fundamental questions in biology at the molecular level, the model system must be extensively studied so that there is a wealth of literature that can serve as examples to support teaching. Moreover, the model system should be representative enough so that it is possible for the students to extend the applications of the methods from the model system to other biomolecular systems. Finally, the chosen model system should carry scientific significance and biomedical implications that will interest students in the topic. In the "Course Materials" section (Part II), we will discuss how these criteria are met by using rhodopsin as the model system.

Panel Discussion

The second strategy is to implement a student-led discussion of a recent publication reporting the application of the same biophysical technique to a biomolecular system different from the model system. The discussion immediately follows the lecture on the applications of the technique to the model system. For the panel discussion sections, students are required to read the selected paper before class and submit a reading report to the instructor and the assigned discussion leader. The leader moderates the discussion based on the collected reading reports as the rest of the class discusses how the technique provides insight into the structure or function of the chosen system as well as what additional questions are generated. When necessary, the instructor clarifies points and answers questions during the panel discussion. The student discussion leader may be assigned or volunteer, and the publication may be chosen by the instructor or suggested by the class based on common interests.

There are several advantages to adding a panel discussion to each module. The discussion not only keeps students updated on the latest research in the field but also gives them a chance to learn how the spectroscopic method may be generally applied to address biological problems at the molecular level of systems other than the model system. Because research articles are highlighted during the discussion, students learn how techniques explained in textbooks are related to questions being

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asked in research laboratories. The interactive nature of a student-led discussion also promotes active learning. Each student in the panel has an opportunity to lead a discussion, and all the students are assessed on their participation and thus encouraged to offer their opinions. Finally, this strategy can also inject research components into classroom teaching so that students can have an opportunity to explore individual research interests that, in part, will help them to make informed decisions in their future research directions in biophysics.

Proposal Project

The final assignment of the course is an independent proposal project. For this project, students choose a biomolecular system and propose the application of one or more of the techniques discussed in class to produce new insight into the structure or mechanism of the chosen system. Though the students are not expected to complete their proposed research, the process of identifying a question and suggesting an experiment demonstrates how techniques discussed in the classroom relate to future research directions.

The assignment is completed in a series of parts. Several weeks before the end of the course, each student presents the biological question that his or her proposal intends to answer. Students are encouraged to ask very specific questions consistent with the types of studies discussed in the literature articles used for the model system and panel discussions. Then, the student proposes one or several of the spectroscopic methods discussed in the course with which to study that biomolecular system. The aims and background as well as the experimental design are described in a two-page written report. In addition, the student makes a brief presentation to the class describing his or her proposal. During this presentation, the student must defend his or her objective against the questions from the other students, who are now familiar with the spectroscopic methods.

For this assignment, students are challenged to identify an existing deficiency in the present understanding of a biomolecular system. While the remainder of the course is focused on a broad survey of a variety of methods, this project encourages students to study one method and one biomolecular system more deeply. Moreover, this strategy allows students to see how their knowledge of a variety of biophysical spectroscopic methods can be turned into proposed research projects.

In addition to reinforcing students' understanding of the spectroscopic methods, this assignment familiarizes students with the proposal process. The written report includes specific aims, background, and experimental design sections that mirror the grant proposals submitted to the National Institutes of Health and other funding agencies. Through this assignment, students are exposed to the format and style of writing grant applications on a small scale. Scientific presentation skills are also introduced during the class presentation section. By teaching these communication skills in the context of biophysical spectroscopic methods, students develop the transferrable skills required to become successful graduate researchers.

Thus far, we have discussed the general practice and advantages of using the three teaching strategies—(1) a single model system for teaching all spectroscopic methods, (2) a panel discussion for teaching each spectroscopic method, and (3) an independent proposal project applying one spectroscopic method to a new system. In the "Course Materials" section (Parts II and III), we will provide more details on implementing the first two strategies.

Course Materials

Part I: Principles of Spectroscopic Techniques

Textbooks and References

The principles of various spectroscopic techniques serve as a basis for their applications to biological questions at the molecular level. Hence, the principles should be covered by 1-2 lectures when each technique is first introduced. There are excellent resources and references available to the course instructor during the preparation of the lectures. A few are given below:

Banwell, C. N. *Fundamentals of Molecular Spectroscopy,* 2nd ed.; McGraw-Hill: New York, 1972.

Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry: Part II: Techniques for the Study of Biological Structure and Function;* Freeman: San Francisco, CA, 1980.

Fasman, G. D. *Circular Dichroism and the Conformational Analysis of Biomolecule;* Plenum Press: New York, 1996.

Templer, R. H.; Leatherbarrow, R. J. *Biophysical Chemistry: Membranes and Proteins;* Royal Society of Chemistry: Cambridge, UK, 2002.

Lakowitz, J. R. *Principles of Fluorescence Spectroscopy,* 3rd ed.; Springer: New York, 2006.

Mispelter, J.; Lupu, M.; Briguet, A. *NMR Probeheads for Biophysical and Biomedical Experiments: Theoretical Principles & Practical Guidelines;* Imperical College Press: London, 2006.

Weil, J. A.; Bolton, J. R. *Electron Paramagnetic Resonance: Elementary Theory and Practical Applications,* 2nd ed.; John Wiley & Sons: Hoboken, NJ, 2007.

Hagen, W. R. *Biomolecular EPR spectroscopy;* CRC Press: Boca Raton, FL, 2008.

Allen, J. P. *Biophysical Chemistry;* Wiley-Blackwell: Hoboken, NJ, 2008.

Campbell, I. D. *Biophysical Techniques;* Oxford University Press: Oxford, United Kingdom, 2012.

Mély, Y.; Duportail, G. *Fluorescent Methods to Study Biological Membranes;* Springer Series on Fluorescence: Methods and Applications 13; Springer-Verlag Berlin Heidelberg: Berlin, German, 2012.

Online Materials

The materials in the above books and references can serve as a basis for the lectures; however, the instructor can also supplement these materials with online information and materials. Here are a few examples of excellent websites:

Analytical Chemistry I and II at the University of Akron
http://ull.chemistry.uakron.edu/analytical/
Online course materials presenting a general overview of analytical techniques at the advanced undergraduate level.

Online NMR course from Queens University
http://www.chem.queensu.ca/facilities/nmr/nmr/webcourse/
Thorough coverage of NMR theory and basic NMR techniques, including two-dimensional NMR.

IR spectroscopy tutorial from the University of Colorado at Boulder
http://orgchem.colorado.edu/Spectroscopy/irtutor/tutorial.html

Online course materials in Biophysical Spectroscopy from the Indian Institute of Technology, Kharagpur
http://www.chem.iitkgp.ernet.in/faculty/SDG/course.php
Useful lecture notes about biological applications of various spectroscopic techniques, especially electronic absorbance, fluorescence, and infrared spectroscopies as well as circular dichroism.

Organic Spectroscopy course from the University of California, Irvine
http://www.youtube.com/course?list=ECC86CC98DDF0CDDAC
Videos of lectures on a variety of topics, including infrared spectroscopy and NMR spectroscopy.

Part II: Model System

As discussed earlier, a well-chosen model system provides the foundation for exploring the spectroscopic techniques and is critical for the success of the course. When the course is offered in the Department of Chemistry at Yale University, rhodopsin is used as the model system.

A major reason why we have chosen rhodopsin as the model system is due to its important role in revealing the molecular mechanism of G-protein-coupled receptors (GPCRs) over the past few decades. GPCRs are a group of proteins involved in signal transduction across cell membranes, and are major targets for drug discovery. Rational drug design targeting GPCRs relies on a fundamental understanding of the molecular mechanism of signal transduction. Consequently, the link between spectroscopic methods applied to rhodopsin and the future of drug discovery motivates students to learn the fundamentals of spectroscopic techniques. Here, we focus first on the significance of GPCRs and then on the role

of rhodopsin as a prototypical model system to reveal a molecular understanding of GPCRs.

GPCRs

GPCRs are proteins expressed in the plasma membrane, and are also referred to as seven-transmembrane (7TM) receptors because the polypeptide chain passes seven times through the membrane (*3*). About 950 human genes code for GPCRs (*4*). Examples include the adrenergic receptors, dopamine receptors, histamine receptors, and odor and taste receptors, as well as the light receptor, rhodopsin.

As major portals of intracellular communication, GPCRs are widely involved in sensing signals from the endoplasmic side and conducting the signal transduction to cytoplasmic side. Upon binding a ligand on the extracellular or transmembrane domain, GPCRs transmit the signal via conformational changes to the cytoplasmic side, which then couple with G-proteins to start signaling cascades (*5*). A graphical description of the signaling pathway is shown in Figure 1. GPCRs respond to extracellular stimuli such as a change in concentration of peptides, hormones, lipids, neurotransmitters, ions, odorants, tastants, or photons.

The ligands of GPCRs may be classified as agonists, inverse agonists and antagonists. Agonists bind to a GPCR and stabilize a conformation that activates the G-protein on the cytoplasmic side. Inverse agonists bind to and stabilize the inactive form of the receptor. Antagonists compete with the agonist by blocking the agonist-binding site. By preventing the conformational change that activates the G-protein, antagonists inhibit GPCR-mediated signaling. This serves as the basis for extensive pharmaceutical development and makes GPCRs promising drug targets. Since the ligand-binding site for each GPCR is highly specific, small molecules can be designed to target selected receptors and modulate physiological processes to achieve therapeutic effects (*6*).

GPCRs are classified into five families (A-E) based on function and amino acid sequence (*4*). Families A, B, and C are the best studied with respect to ligand binding, function, and G-protein coupling. Class A is the largest class, and its members account for 85% of GPCR genes. Many drugs have been developed to target class A GPCRs.

Despite much interest in studying the mechanism of GPCR activation, the structure and activity were not well understood until the publication of recent crystal structures. Before the techniques of expression and purification were developed such that many GPCRs could be purified in the quantity large enough for spectroscopic studies, rhodopsin has been used as a model system for all GPCRs. This is because rhodopsin can be directly purified in high purity and large quantity (milligram) from a natural source—bovine retinas. Almost all spectroscopic methods available have been applied to study rhodopsin to reveal its molecular functions. Thus, rhodopsin has a unique role in the history and application of biophysical spectroscopy.

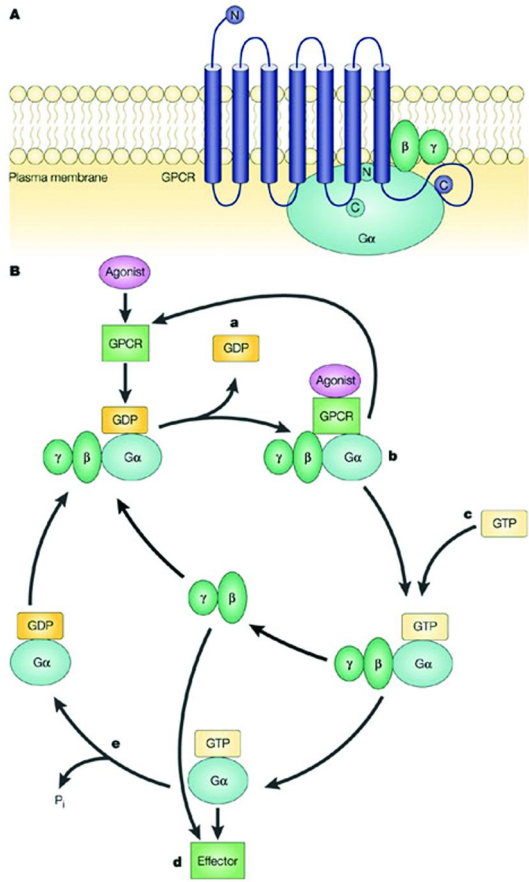

Figure 1. (A) Schematic of a GPCR. (B) G-protein cycling. Reprinted by permission from Nature Publishing Group: Nature Reviews Drug Discovery (2), copyright 2004.

Rhodopsin was first identified as a photosensitive pigment in the 1870s, and its covalent ligand, retinal, was reported in 1933 (*7*). The first view of GPCRs was a projection structure of rhodopsin using cryo-electron microscopy (*8*, *9*). Higher resolution and three-dimensional structures on well-diffracting crystals were achieved by Tetsuji Okada and coworkers by developing a purification strategy (*10*). The first crystal structure of any GPCR was reported by Palczewski and Okada, who solved the three-dimensional structure of non-activated rhodopsin, providing a more detailed view of the helices in the membrane, the loops and the binding pocket for retinal (*11*, *12*).

Rhodopsin has also served as a pioneer for methods development in recombinant expression and protein purification for GPCRs. The first GPCR purified in functional form was rhodopsin achieved by Ruth Hubbard in vesicles (spherical model membranes) (*13*). Later, recombinant expression of rhodopsin in mammalian cells enabled the studies on the role of individual amino acids by site mutation.

Owing to the significance of GPCRs in drug development and extensive volume of studies on GPCRs, many Nobel Prizes were awarded to work related to rhodopsin and GPCRs in physiology or medicine and in chemistry. In 1967, Ragnar Granit, Haldan Keffer Hartline, and George Wald were awarded the Nobel Prize for their discoveries concerning the primary physiological and chemical visual processes of rhodopsin. The Nobel Prizes for 1971, 1988, 1994, 2004, and 2012 were all related to achievements in GPCRs.

In summary, GPCRs have significant biomedical implications. Because rhodopsin has been studied extensively with a variety of techniques, it is an ideal model system for introducing spectroscopic methods.

Rhodopsin

After introducing the biomedical implications of GPCRs and the role of rhodopsin as a model system in the GPCR field, we now introduce our model system—rhodopsin. Rhodopsin is a member of class A, the largest class of GPCRs. It is found in the retina of the eye, where different visual pigments found in rods and cones are responsible for vision. Rhodopsin is responsible for dim light vision and is located in the disc membranes of the rod outer segments (Figure 2) (*14–16*). Rhodopsin is composed of two parts: opsin and a chromophore. Opsin consists of 348 amino acids that form a seven α-helical transmembrane tertiary structure under physiological conditions. 11-*cis* retinal serves as the chromophore and connects to opsin via a protonated Schiff base at Lys 296 (*17*). Upon absorption of a photon, the C_{11}=C_{12} double bond of 11-*cis* retinal undergoes isomerization to form the all-trans configuration, resulting in the active state, Metarhodopsin II (Meta II) (Figure 3). Simultaneously, the Schiff base is deprotonated. These changes induce further conformational changes in the opsin protein and trigger the downstream signal transduction cascade for vision (*18*, *19*).

Rhodopsin is among the most widely studied GPCRs because it is amenable to experimental constraints. Rhodopsin has a high quantum yield of 0.65, indicating that 65% of the incident photons result in activation. The high quantum yield results in high sensitivity to spectroscopic measurements. The photo-isomerization process is amplified by the coupling to the conformational changes in the G-protein transducin. In a spectroscopic measurement, background or dark isomerization events can complicate interpretation, but rhodopsin has very low dark noise associated with thermal isomerization (*19*, *20*).

Rhodopsin has been studied since the 1950s—long before the successful purification of other GPCRs. For decades, researchers in various fields of spectroscopy used their expertise to understand the structures and functions of rhodopsin. Almost all the spectroscopic methods have been applied to studying rhodopsin in hope of revealing the mechanism of other GPCRs. Consequently, plenty of spectroscopic studies are available in determining the structures and functions of rhodopsin, making rhodopsin a good model system from the instructor's point of view.

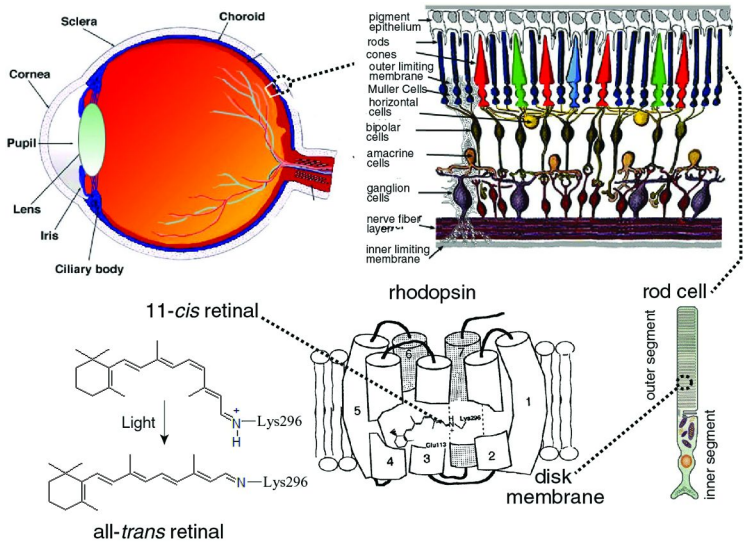

Figure 2. Schematic diagrams of a human eye, vertical section of a retina, a rod cell, rhodopsin, 11-cis retinal and the cis-to-trans isomerization in the photoactivation process. Reproduced from reference (1).

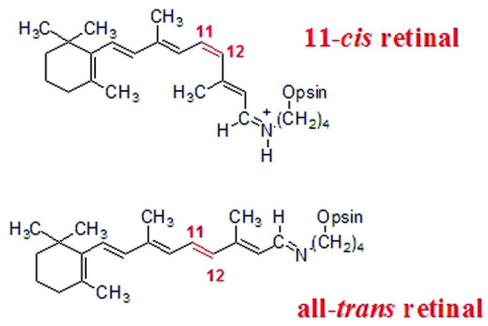

Figure 3. Structure of 11-cis retinal bound to opsin (rhodopsin) and all-trans retinal bound to opsin (Meta II).

Many excellent studies were performed on rhodopsin using various biophysical spectroscopic methods. Below are some research articles that can serve as references for the lectures on the applications of the spectroscopic methods to rhodopsin.

FTIR Spectroscopy

- Fahmy, K.; Sakmar, T. P.; Siebert, F. *Biochemistry* **2000,** *39*, 10607-10612.
- Mahalingam, M.; Martinez-Mayorga, K.; Brown, M. F.; Vogel, R. *Proc. Natl. Acad. Sci. U. S. A.* **2008,** *105*, 17795-17800.

Raman Spectroscopy

- Pan, D. H.; Ganim, Z.; Kim, J. E.; Verhoeven, M. A.; Lugtenburg, J.; Mathies, R. A. *J. Am. Chem. Soc.* **2002,** *124*, 4857-4864.
- Palings, I.; Vandenberg, E. M. M.; Lugtenburg, J.; Mathies, R. A. *Biochemistry* **1989,** *28*, 1498-1507.

Fluorescence

- Marin, E. P.; Krishna, K. G.; Zvyaga, T. A.; Isele, J.; Siebert, F.; Sakmar, T. P. *J. Biol. Chem.* **2000,** *275*, 1930-1936.
- Farrens, D. L.; Khorana, H. G. *J. Biol. Chem.* **1995,** *270*, 5073-5076.

Fluorescence Correlation Spectroscopy

- Kriegsmann, J.; Gregor, I.; von der Hocht, I.; Klare, J. P.; Engelhard, M.; Enderlein, J.; Fitter, J. *Chembiochem* **2009,** *10*, 1823-1829.

Fluorescence Anisotropy

- Krishna, A. G.; Menon, S. T.; Terry, T. J.; Sakmar, T. P. *Biochemistry* **2002,** *41*, 8298-8309.

Circular Dichroism Spectroscopy

- Fujimoto, Y.; Ishihara, J.; Maki, S.; Fujioka, N.; Wang, T.; Furuta, T.; Fishkin, N.; Borhan, B.; Berova, N.; Nakanishi, K. *Chem-Eur J* **2001,** *7*, 4198-4204.

Second-Order Surface Specific Spectroscopy

- Shen, Y. R. *Nature* **1989,** *337*, 519-525.

Transient Absorption Spectroscopy

- Hug, S. J.; Lewis, J. W.; Einterz, C. M.; Thorgeirsson, T. E.; Kliger, D. S. *Biochemistry* **1990,** *29*, 1475-1485.

NMR

- Patel, A. B.; Crocker, E.; Eilers, M.; Hirshfeld, A.; Sheves, M.; Smith, S. O. *Proc. Natl. Acad. Sci. U. S. A.* **2004,** *101*, 10048-10053.

EPR

- Farrens, D. L.; Altenbach, C.; Yang, K.; Hubbell, W. L.; Khorana, H. G. *Science* **1996,** *274*, 768-770.
- Altenbach, C.; Kusnetzow, A. K.; Ernst, O. P.; Hofmann, K. P.; Hubbell, W. L. *Proc. Natl. Acad. Sci. U. S. A.* **2008,** *105*, 7439-7444.

Part III: Panel Discussion

According to the defined goals of the course, students are expected to develop a basic understanding of the principles of each spectroscopic technique and its application to rhodopsin after the first two to three lectures. The goal of the panel discussion section is the application of the technique to another biomolecular system in the literature.

Panel discussion sections are governed by students under the support and guidance of the course instructor. Each panel discussion has a discussion leader who can be acted by students in turn. At the very beginning of the semester, the course instructor functions as the discussion leader until the students are familiar with the format of the panel discussion. Before each panel discussion, students are assigned a paper, and each student is required to submit a reading report based on their understanding of the paper to both the discussion leader and the course instructor. The selection of publications for the panel discussion sections is flexible, either assigned by the instructor or chosen by students. In general, the literature references are chosen as high quality examples of each technique. The discussion leader is responsible for opening the discussion and for managing the time within the scheduled class time. During the panel discussion, all students of the class are encouraged to offer their points of view on the basis of their reports, including both the merits and shortcomings of the study. The instructor can help clarify concepts and confusion if needed.

To help students familiarize themselves with the format of the panel discussion, a list of suggested questions is provided to assist in writing the report. In our experience, the discussion often began with a discussion of these questions but led to deeper discussions of the students' interests.

- What is the system being studied? How is it different from the model system discussed in class?

- What are the biological questions being investigated? What are the purposes of the study?
- What techniques are used in the paper? How can the biological questions be answered by these techniques?
- What are the results and corresponding conclusions?
- Do you have any comments on the experimental procedure in the paper? Are the conclusions solidly supported by the experimental results?
- Do you have any questions/thoughts about the paper?
- How does the lecture on the model system help you understand the system discussed in this paper?

By answering all the questions above, students should have a better understanding of how the spectroscopic method could be used to address questions in a new system. No doubt there are many systems suitable for panel discussion, but some suggested papers are listed below.

FTIR Spectroscopy

- Pan, K. M.; Baldwin, M.; Nguyen, J.; Gasset, M.; Serban, A.; Groth, D.; Mehlhorn, I.; Huang, Z. W.; Fletterick, R. J.; Cohen, F. E.; Prusiner, S. B. *Proc. Natl. Acad. Sci. U. S. A.* **1993,** *90*, 10962-10966.

Raman Spectroscopy

- Bell, A. F.; He, X.; Wachter, R. M.; Tonge, P. J. *Biochemistry* **2000,** *39*, 4423-4431.

Fluorescence

- Overton, M. C.; Blumer, K. J. *Curr Biol* **2000,** *10*, 341-344.

Fluorescence Correlation Spectroscopy

- Rhoades, E.; Ramlall, T. F.; Webb, W. W.; Eliezer, D. *Biophys J* **2006,** *90*, 4692-4700.

Fluorescence Anisotropy

- Kwok, R. P. S.; Lundblad, J. R.; Chrivia, J. C.; Richards, J. P.; Bachinger, H. P.; Brennan, R. G.; Roberts, S. G. E.; Green, M. R.; Goodman, R. H. *Nature* **1994,** *370*, 223-226.

Circular Dichroism Spectroscopy

- Munishkina, L. A.; Henriques, J.; Uversky, V. N.; Fink, A. L. *Biochemistry* **2004,** *43*, 3289-3300.

Second-Order Surface Specific Spectroscopy

- Liu, J.; Conboy, J. C. *J. Am. Chem. Soc.* **2004,** *126*, 8376-8377.

Transient Absorption Spectroscopy

- Lim, M. H.; Jackson, T. A.; Anfinrud, P. A. *Nat Struct Biol* **1997,** *4*, 209-214.

NMR Spectroscopy

- Petkova, A. T.; Ishii, Y.; Balbach, J. J.; Antzutkin, O. N.; Leapman, R. D.; Delaglio, F.; Tycko, R. *Proc. Natl. Acad. Sci. U. S. A.* **2002,** *99*, 16742-16747.

EPR Spectroscopy

- Jao, C. C.; Der-Sarkissian, A.; Chen, J.; Langen, R. *Proc. Natl. Acad. Sci. U. S. A.* **2004,** *101*, 8331-8336.

An Example: A Module for Fluorescence Spectroscopy

After providing the general course materials, we will use fluorescence spectroscopy as an example to show how the course materials and teaching strategies can be implemented as a module. We will first provide the materials about the basic principles of fluorescence spectroscopy (Lecture 1). Then, we will discuss the work of Marin et al. to illustrate how fluorescence spectroscopy can be used to determine the role of the fourth cytoplasmic loop in modulating the rhodopsin-transducin interaction (Lecture 2). Finally, by providing a sample reading report, we demonstrate the materials for the panel discussion and expectations for student reading reports. A detailed description about the format of the panel discussion can be found in the "Course Materials" section (Part III).

Lecture 1: Principles of Fluorescence Spectroscopy

Physical Basis of Fluorescence Spectroscopy

Fluorescence is a spectroscopic method in which the molecules of an analyte are excited by irradiation at a specific wavelength and then usually emit radiation of a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis.

Fluorescence spectroscopy is related to electronic and vibrational states. Molecules of the same analyte may have various energetic states called energy levels. The lowest energy state is referred to as the ground state, and higher energy states are called excited states. For each electronic state, there are also vibrational ground and excited states. When an electron is excited to a higher

energy electronic state by absorbing a photon, it can relax back either to the ground state or to an excited state with lower energy. The energy difference may be emitted as a photon, and this process is called luminescence. If the electron is excited to an excited state with opposite spin orientation relative to the electron in the lower orbital, then the excited electronic state is called the singlet excited state (S1, S2, S3…). On the contrary, if the electron in a higher energy orbital has the same spin orientation relative to the electron in a lower orbital, the excited electronic state is called the triplet excited state (T1, T2, T3…). Luminescence may occur from either the singlet or triplet excited states, leading to either fluorescence or phosphorescence.

Fluorescence spectroscopy can provide structural information of analytes based on the wavelengths of emitted photons along with the intensities. In fluorescence spectroscopy, the analyte absorbs a photon of a particular energy, which results in excitation from its ground electronic state to one of many vibrational levels in the excited electronic states, usually the first excited singlet state, S1 (Figure 4). Molecules at higher vibrational levels in the excited states collide with other molecules and lose the vibrational energy until they reach the lowest vibrational state of the excited electronic state. The molecule may then go back to one of the many vibrational levels of the ground electronic state again, leading to the emission of a photon that is detected by the instrument. Because molecules may drop down into any of the vibrational levels in the ground state, the emitted photons will have different energies. Therefore, by analyzing the different wavelengths of light emitted along with their relative intensities, the energies of the different vibrational levels can be known.

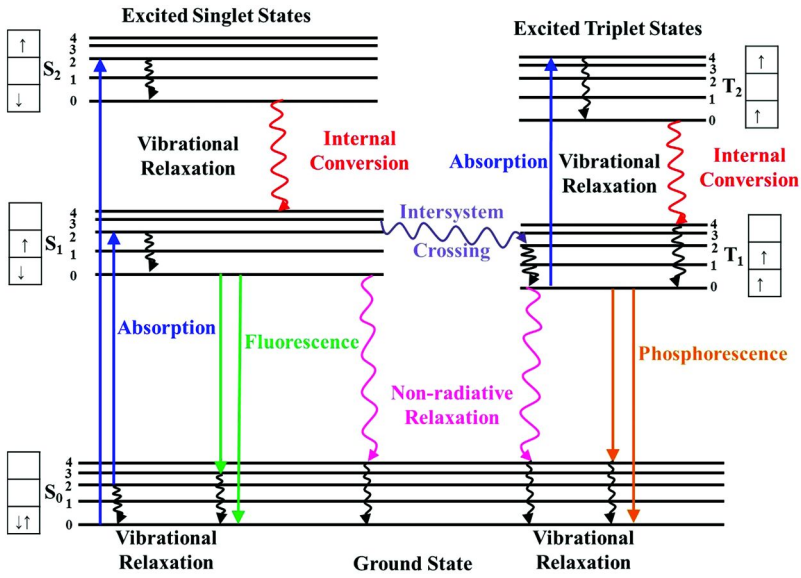

Figure 4. Electronic transition energy level diagram.

Jablonski Diagram

Once the molecule is in the excited state, relaxation can happen through several processes. Fluorescence is one of these relaxation processes. A visualization of all the possible relaxation processes is shown in the Jablonski diagram in Figure 4. The major processes and their timescales are described as follows:

- Vibrational relaxation ($<10^{-12}$ s): Molecules rapidly transfer the energy in a non-radiative manner and relax to lower vibrational levels within the same electronic excited state.
- Internal conversion ($\sim10^{-12}$ s): Molecules at different excited electronic states with the same multiplicity can transfer energy without the emission of radiation. Here, the same multiplicity means singlet-to-singlet or triplet-to-triplet states. The internal conversion is more efficient when two electronic energy levels are close enough that two vibrational energy levels can overlap.
- External conversion: Molecules after excitation can interact and transfer energy to the solvent or solute, leading to non-radiative relaxation. Low temperature and high viscosity can enhance fluorescence because they reduce the frequency of intramolecular collision and thus slow the deactivation process.
- Intersystem crossing ($\sim10^{-9}$s): In this process, the vibrational levels of the singlet state overlap with those of the triplet state. Molecules in the singlet excited state (S1) can cross over to the triplet excited state (T1). Intersystem crossing is one of non-radiative relaxation processes. Enhanced intersystem crossing results in a decrease of fluorescence.
- Phosphorescence (10^{-4} to 100 s): This is the relaxation of the molecule from the triplet excited state to the singlet ground state with emission of photons. During the process, the spin orientation of the electron is changed. Because this is a classically forbidden transition, depletion of the triplet state happens slowly, resulting in a long lifetime for the triplet state and a slow rate for phosphorescence (10^{-4} to 100 s).
- Fluorescence ($\sim10^{-8}$ s): This is relaxation of the molecule from the lowest vibrational energy level in the singlet excited state to the singlet ground state with emission of photons. Fluorescence has a short lifetime ($\sim10^{-8}$ s), so it can compete favorably with internal conversion, intersystem crossing, and phosphorescence.

Among these six relaxation processes, vibrational relaxation, internal conversion, external conversion, and intersystem crossing are non-radiative processes, while phosphorescence and fluorescence are radiative processes accompanied by an emitted photon.

The fluorescence energy is always less than the absorption energy for a given molecule due to the energy loss in vibrational relaxation or solvation. Thus the emitted photon is observed at longer wavelength than the excitation photon, referred to as the Stokes shift. Figure 5 shows the absorption and emission

spectra of a series of commercially available dyes. As an example, the excitation maximum of the fluorophore Alexa 488 is 488 nm, while the emission maximum is 520 nm (Figure 5). The absorption and emission spectra of other Alexa dyes shown in Figure 5 follow the same trend. The difference between absorption and emission spectra enables the use of fluorescence spectroscopy as an analytical method (*21*).

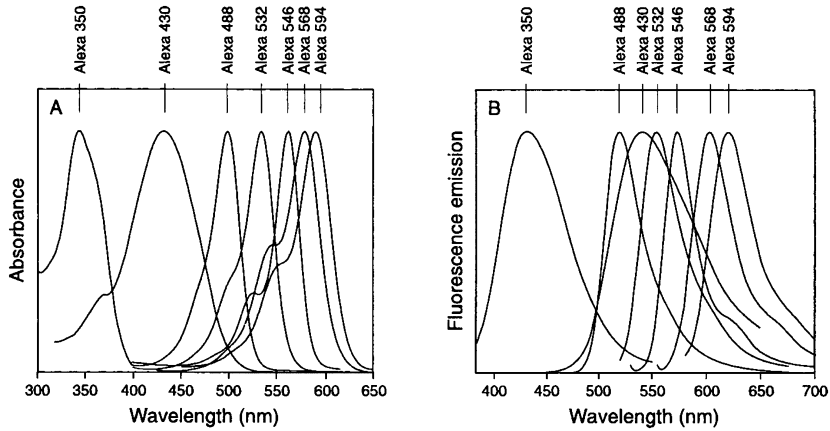

Figure 5. (A) Normalized absorption and (B) emission spectra of the Alexa dyes. Reprinted by permission of SAGE Publications from reference (21). Copyright © 1999 by SAGE Publications.

Fluorescence Quantum Yield

The quantum yield (quantum efficiency) for fluorescent species is the ratio of the number of molecules that fluoresce to the total number of molecules that are excited, ranging from 0 to 1. Highly fluorescent molecules have a quantum efficiency near 1. For molecules that do not fluoresce, the quantum efficiencies approach zero. Fluorescence quantum yield (φ) for a compound is determined by the equation (1):

$$\varphi = \frac{k_f}{k_f + k_i + k_{ic} + k_{pd} + k_d + k_{ec}}. \quad (1)$$

The numerator of this equation is the rate of fluorescence, k_f, while the denominator is the sum of all possible relaxation processes, including k_f, the rate of fluorescence; k_i, the rate of intersystem crossing; k_{ic}, the rate of internal conversion; k_{pd}, the rate of pre-dissociation; k_d the rate of dissociation; and k_{ec}, the rate of external conversion. The quantum yield provides a quantitative interpretation of how structural and environmental factors influence the intensity of the fluorescence. Other processes that compete with fluorescence include excited state isomerization, photoionization, photo-dissociation and acid-base equilibria.

Based on the lifetime of fluorescence (~10^{-8} s), a series of molecular events happening on similar time scales can be detected by fluorescence spectroscopy. These processes may include collisions with quenchers, rotational or translational diffusion, and solvation.

- The fluorescent molecules in the excited state can collide with quenchers, lose energy to the quenchers, and relax back to the ground state without emitting photons. Since collision is dominated by diffusion, fluorescence spectroscopy is only sensitive to the environment within around 100 Å.
- The orientation dependence of rotational diffusion is the fundamental principle for fluorescence anisotropy.
- Solvation can happen not only in vibrational relaxation but also after the molecule has reached the lowest vibrational level of the excited state. The excited molecule can further lose energy to solvent by solvation (Figure 6). As a result, the energy difference for fluorescence becomes smaller, increasing the Stokes shift.

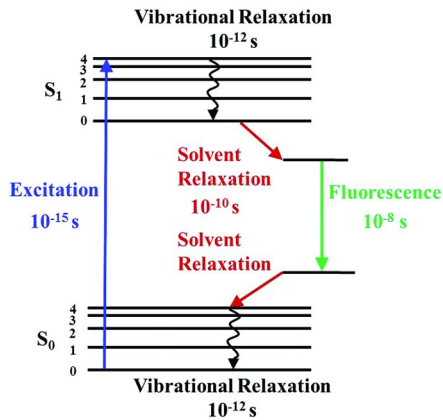

Figure 6. Scheme for solvation.

Photobleaching

Another important aspect about fluorescence spectroscopy is photobleaching. Photobleaching describes the observation that the fluorescence intensity gradually decreases if the fluorophore is continuously irradiated. As phosphorescence occurs much more slowly than intersystem crossing, molecules become trapped in triplet states. With populated triplet states, fewer molecules stay in the ground state, leading to weaker fluorescence intensity. Generally, there are two kinds of photobleaching: reversible photobleaching and irreversible photobleaching. Reversible photobleaching can be restored by blocking the excitation, allowing molecules trapped in triplet states to relax back to the ground state. Irreversible

In Teaching Bioanalytical Chemistry; Hou, H.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

photobleaching occurs when photochemical reactions happen during the excitation process resulting in degradation of the initial structure. Because new products are formed, the ground state cannot be restored by blocking the excitation.

Instrumentation

A typical fluorometer includes a light source, excitation filter/monochromator to select the excitation wavelength, emission filter/monochromator to select the emission wavelength, sample holder, amplifiers to normalize and enhance the fluctuation of signals, and a detector. A scheme for fluorometer is depicted in Figure 7.

Two types of fluorescence experiments are commonly used. In a fluorescence emission experiment, the excitation wavelength is fixed, and the emission wavelength is scanned using a monochromator. Alternatively, the emission wavelength may be held constant while the excitation wavelength is scanned to produce an excitation spectrum. The emission spectrum reflects the structure of vibrational levels in the ground state, and the excitation spectrum reflects the structure of vibrational levels in the excited state. Usually, emission spectrum and excitation spectrum are mirror images of each other. An emission map is produced by combining the emission spectra resulting from a range of excitation wavelengths.

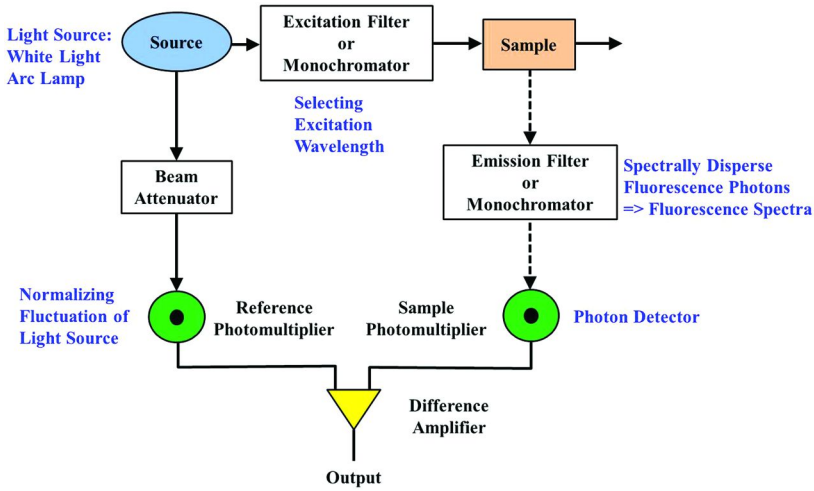

Figure 7. Scheme for a fluorometer.

Förster (Fluorescence) Resonance Energy Transfer (FRET)

In addition to providing information about the electronic structure of a fluorophore, fluorescence spectroscopy can also be used to analyze the spatial relationship of cofactors in a biomolecular system by using Förster Resonance

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Energy Transfer (FRET). FRET is the distance-dependent energy transfer from an excited donor fluorophore to an appropriate acceptor fluorophore in a radiationless way. Due to its sensitivity to distance, FRET has been used to investigate changes in nanometer scale and molecular level interactions.

The energy transfer in FRET occurs via long-range dipole-dipole interactions in a radiationless mechanism. Assuming the excited donor fluorophore as an oscillating dipole, the excited donor fluorophore can exchange energy with an adjacent dipole of the acceptor fluorophore having a similar resonance frequency. The energy transfer only happens between singlet states of the two fluorophores, and there is neither emission nor reabsorption of photons during the process ("non-radiative donor energy transfer" and "non-radiative acceptor excitation" in Figure 8). In order to have effective energy transfer between the excited donor fluorphore and the acceptor fluorophore in a FRET experiment, two fluorophores must have similar resonance frequencies. The primary requirements that need to be met in FRET are:

- The donor and acceptor molecules must be spatially close to each other (typically 10-100 Å) to ensure effective interaction.
- The emission or excitation spectrum of the acceptor must have sufficient overlap with the emission spectrum of the donor (Figure 8).
- The donor and acceptor transition dipoles must be approximately parallel to each other.

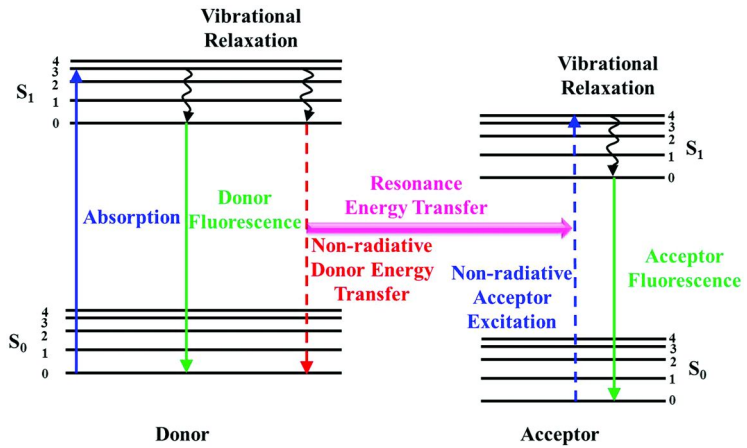

Figure 8. Scheme for electron vibrational energy states that occur during FRET.

Since the efficiency of resonance energy transfer between the excited donor fluorophore and the acceptor fluorophore is not 100%, the electrons on the donor's lowest vibrational level in the excited states can still relax back to the ground state emitting fluorescence photons ("donor fluorescence" in Figure 8). Compared with emission and excitation spectra in fluorescence spectroscopy, FRET involving the resonance energy transfer can reveal structural information regarding the donor-acceptor pair. The intensity of FRET decays proportionally to the inverse distance to the sixth power and to the angle difference between the

dipoles, so very small distance or orientation changes result in a large change in FRET intensity. Consequently, FRET can be useful in studying small angle or distance changes in the system.

Lecture 2: Application of Fluorescence Spectroscopy to the Model System

After introducing the principles of the spectroscopic technique, students are prepared for application of the spectroscopic technique to the model system. In our case, the course instructor chose a representative study on rhodopsin utilizing the fluorescence spectroscopy discussed above. In our experience with this course, we use a paper by Marin et al. (*22*) about the role of the fourth cytoplasmic loop in coupling to the G-protein transducin.

Marin, E. P.; Krishna, K. G.; Zvyaga, T. A.; Isele, J.; Siebert, F.; Sakmar, T. P. *J. Biol. Chem.* **2000,** *275*, 1930-1936.

Background about the Model System

Basic information about the model system, rhodopsin, can be found in the "Model System" section. As described in the "Model System" section, rhodopsin is a seven-helical transmembrane protein. However, on the cytoplasmic side between L321 and N310, there is an eighth α-helix, also referred to as the fourth loop (C4 loop). The C4 loop is parallel to the membrane surface because the C322 and C323 are post-translationally modified resulting in two palmitoyl tails that penetrate into the cell membrane. Due to the location of the C4 loop, it has been suggested to participate in binding the G-protein transducin as part of the downstream signaling pathway. However, there is also evidence indicating that the C4 loop is not important in binding transducin.

There are three types of subunits for a G-protein, called Gα, Gβ, and Gγ. In the inactive state, the Gα subunit is coupled with GDP and may also bind Gβ or Gγ. Once activated by external stimuli, GDP is replaced by a binding motif of the GPCR to form a complex of Gα, Gβ, Gγ, and the GPCR. The specific binding mode and the location of binding on the Gα, Gβ, or Gγ subunits are not well-understood. Next, GTP replaces the GPCR in binding to the Gα subunit. The Gβ and Gγ subunits dissociate from Gα, and the Gα-GTP complex binds effectors in the downstream signaling cascade.

Motivation of the Study

Since all research is problem-oriented, it is important for the instructor to define the specific questions that are posed by the study of the model system in order to understand and evaluate how the questions are answered. There are two aims for this study: to understand the function of C4 loop and to evaluate the contradictory results about C4 loop published previously in the field. A summary of the contradictory results about the role of C4 loop are shown in Table I.

Table I. Contradictory Results Regarding the C4 Loop

C4 loop is important	*C4 loop is not important*
C4 loop-peptide disrupts Meta II-Gt interaction (*23*)	Truncation of rhodopsin N315 does not impair Gα activation-N315 in the middle of C4 loop (*24*)
C4 loop-peptide prevents Gβγ-rhodopsin interactions (*25*)	Mutations from N310 to N315 do not disrupt Gα activation (*26*)

Experimental Design and Results

In the lecture on the model system, the instructor describes how the experimental methodology of the paper addresses the questions asked. In particular, the instructor should highlight how a proper experiment rules out opposing possibilities and that solid logic is used in forming conclusions. Specifically, the experimental design of the study by Marin et al., on rhodopsin and a description of the results is summarized as follows:

- To test whether there is binding between C4 loop and Gα, a synthetic C4 loop-peptide (C4 310-321) was used to assess the binding to Gα.
    - Because there are 2 Trp in Gα and 8 Trp in Gβ & Gγ, any changes in environment can induce either a shift of the peak position or a change in the peak intensity for the intrinsic fluorescence given by Trp.
    - The binding between synthetic C4 310-321 and Gα was measured by fluorescence spectroscopy. A 4-nm shift was observed, suggesting that C4 310-321 binds to Gα.
    - The fluorescence spectrum of Gβ & Gγ shifted by 1 nm after synthetic C4 310-321was added to the system. This could indicate either that C4 310-321 does not bind to Gβ & Gγ or that the binding between C4 310-321 and Gβ & Gγ does not affect the intrinsic fluorescence.

- To study the effect of C4 loop in the activation of transducin, synthetic C4 loop-peptide was used to measure the rate of activation of transducin.
    - The second loop peptide (C2 132-144), the third loop peptide (C3 240-252), and C4 310-321 were synthesized. The relative activation rates of transducin for these three peptides were comparable.
    - Based on the previous result that C2 and C3 loops can couple to Gα, it is concluded that the C4 loop is as important as the C2 and C3 loops.

- To explore the role of each part on the C4 loop in the activation of transducin, the activation of transducin by rhodopsin with mutations on C4 loop was performed.

    - Mutations on the first three amino acids, the middle part from β2 adrenergic receptor and M1-muscarinic receptor, and two palmitoyl tails (C322 & C323) were introduced separately. The activation of transducin was assessed by fluorescence spectroscopy. The rate of activation is determined by the initial slope of the curve. As GTPγS replaces GDP in Gα, the intrinsic Trp fluorescence increases.
    - The C4 mutants changed the rate of transducin activation, and consequently the C4 loop affects the rate of transducin activation.
    - Mutants that have changed the tripeptide 310-312 significantly changed the rate of activation. By comparing the activation rate of various mutants, the tripeptide 310-312 is a determining factor in transducin activation.
    - Mutants of the palmitoyl groups do not alter the rate of activation significantly. The palmitoyl tails are not important in the transducin activation.

Conclusions of the Studies

Based on the experimental design and the results, the following conclusions can be reached:

- The fourth loop directly binds to Gα.
- The fourth loop is important in activating transducin.
- The tripeptide N310K311Q312 is a determining factor in binding to Gα.

This second lecture demonstrates how a specific spectroscopic technique can be used to solve problems in real research. While the details of the rhodopsin system are learned as a model system, the larger goal is that students would be able to apply this technique to a problem encountered in other research. After this one-semester course, students are expected to know the rules for designing experiments and the logic of drawing conclusions.

Lecture 3: Panel Discussion

After the lectures on the principles of fluorescence spectroscopy and the applications of the technique to rhodopsin, students should have a basic idea of fluorescence spectroscopy. The panel discussion section on a different molecular system extends understanding of the spectroscopic technique. Below is a sample report illustrating the expectations of the students and how the panel discussion should proceed.

Paper:
Overton, M. C.; Blumer, K. J. *Curr Biol* **2000,** *10*, 341-344.

Biological questions being investigated:
In intact cells & membranes, do alpha-factor receptors function as monomers or oligomers?

How can the questions be addressed by the technique?

- FRET signals were observed between SteΔ2-CFP (donor) and SteΔ2-YFP (acceptor) due to stable association.
- Collisional interaction was ruled out by absence of FRET signal between SteΔ2-CFP and YFP-tagged glucose transporters.
- Fluorescence microscopy was used to monitor whether endocytosis defect of SteΔ2-CFP could be corrected by co-expressing WT receptors. This demonstrated that alpha-factor receptors oligomerize regardless of the presence of agonist.

Results:

- FRET signals were observed between SteΔ2-CFP (donor) & SteΔ2-YFP(acceptor) at WT levels while no signal between SteΔ2-CFP & YFP-tagged glucose transporters.
- No FRET signal was observed between full length CFP and full length YFP.
- No FRET signal was observed when untagged receptors were overexpressed. This inhibition was also specific.
- FRET had a high efficiency (18+-1%) indicating a stable interaction between alpha-receptors.
- FRET was not affected when actin cytoskeleton was disassembled.
- Monomer-oligomer equilibrium was not affected by agonist or antagonist.
- The endocytosis defect of GFP-tagged tailless receptors could be corrected by co-expressing untagged wild-type receptors.

Conclusions:

- Alpha-factor receptor is oligomeric in intact cells and membranes.
- Co-expression of receptors tagged with the cyan or yellow fluorescent proteins (CFP or YFP) results in efficient fluorescence resonance energy transfer (FRET) due to stable association rather than collisional interaction.
- Monomer-oligomer equilibrium is unaffected by binding of agonist, antagonist, or G-protein heterotrimers.
- Oligomerization is further demonstrated by rescuing endocytosis-defective receptors with co-expressed wild-type receptors.

- Dominant-interfering receptor mutants inhibit signaling by interacting with wild-type receptors rather than by sequestering G-protein heterotrimers. Oligomerization likely governs GPCR signaling and regulation.

Further Questions:

- How can the possibility that oligomers are induced by CFP and YFP be ruled out? How large are CFP and YFP? Are they comparable in size to the absent tail? Is there any other structural information about these two tails?
- Why do the authors use FRET instead of cross-linking experiments to study receptor oligomerization? Could these two types of experiments be used together?

Summary

In this chapter, we discussed three teaching strategies: 1) using a single model system to demonstrate applications of various spectroscopic techniques, 2) introducing panel discussion on recent related research in the field, and 3) writing an independent research proposal implementing the newly learned methods to a new system. We demonstrate the advantages of these strategies using a case study on fluorescence spectroscopy. The goal of the course is to illustrate the power of spectroscopic techniques to answer fundamental questions in biology at the molecular level. Moreover, critical reading and scientific writing skills are introduced. After completing this course, students are prepared to apply spectroscopic methods to research problems.

Appendix

The syllabus for the graduate course "Biophysical Spectroscopy" in the spring semester of 2012 is presented below. The class met three times per week for twelve weeks with each class lasting fifty minutes, but this schedule could be rearranged to suit other academic calendars.

Table A. Syllabus for the Course

SPRING 2012		
BIOPHYSICAL SPECTROSCOPY		
Course Description: A discussion of the application of spectroscopy to biomolecules. Topics include Raman, fluorescence, FTIR, NMR, EPR and optical ultrafast spectroscopy. Emphasis is placed on interpreting spectroscopic data to gain structural and dynamic information in order to answer biological questions at the molecular level.		
Class Activity: Each spectroscopic technique will be covered by 3-4 classes.		
1 or 2 Lectures: Basic principles given by the instructor or experts in the field.		
1 Lecture: Applications of the technique to a model system—rhodopsin.		
1 Discussion: Applications of the technique to other molecular biological systems.		
Course Requirement and Grading:		
50%	Reading assignments/ reports/ discussions	For each spectroscopic technique, you read one research article related to the application. You will participate in and have a chance to lead a discussion of the article in class. You are required to submit a 1-page report to the course instructor & discussion leader a day before the discussion. In the report, you will address: What are the biological questions being investigated? (State the question as *specifically* as possible.) How can the questions be addressed by the technique? What are the results and conclusions? Are the conclusions supported by the results? Have the biological questions been answered? Give your comment. List 2 questions worth discussing in class.

Continued on next page.

Table A. (Continued). Syllabus for the Course

50%	Proposal and oral defense	You will choose a biological question (again, as *specific* as possible) of your own and write a 2-page proposal to solve the problem using spectroscopic techniques. As you develop your proposal, you are required to give two presentations in class. First, after the spring break, you will give a presentation to define the biological question. Second, at the end of the semester, you will give another presentation to defend your proposal. The proposal is due after you present the research problem and right before you defend your proposal. After everyone has a chance to present the research problem, 1-2 lectures will be given on proposal writing.
Classes Schedule		
Date	Topics	References
Lecture 1	Course introduction	
Lecture 2	Introduction to the model system—rhodopsin	
Lecture 3	Basic principle of infrared (IR) spectroscopy	
Lecture 4	Application of IR spectroscopy to rhodopsin	Fahmy et al. & Mahalingam et al.
Lecture 5	Discussion-IR spectroscopy	Pan et al. (1993)
Lecture 6	Basic principle of Raman spectroscopy	
Lecture 7	Application of Raman spectroscopy to rhodopsin	Pan et al. (2002) & Palings et al.
Lecture 8	Discussion-Raman spectroscopy	Bell et al.
Lecture 9	Basic principle of fluorescence spectroscopy	

Continued on next page.

Table A. (Continued). Syllabus for the Course

Lecture 10	Application of fluorescence spectroscopy to rhodopsin	Marin et al. & Farrens et al. (1995)
Lecture 11	Discussion-fluorescence spectroscopy	Overtone et al.
Lecture 12	Basic principle of fluorescence correlation spectroscopy	Guest lecturer: **Dr. Abhinav Nath**
Lecture 13	Application of fluorescence correlation spectroscopy to rhodopsin	Kriegsmann et al.
Lecture 14	Discussion-fluorescence correlation spectroscopy	Rhoades et al.
Lecture 15	Basic principle of fluorescence anisotropy	Guest lecturer: **Dr. Jian Liu**
Lecture 16	Application of fluorescence anisotropy to rhodopsin	Krishna et al.
Lecture 17	Discussion-fluorescence anisotropy	Kwok et al.
Lecture 18	Basic principle of circular dichroism	
Lecture 19	Application of circular dichroism to rhodopsin	Fujimoto et al.
Lecture 20	Discussion-circular dichroism	Munishkina et al.
Lecture 21	Proposal: presentation-research problem (15-20 min x 2)	
Lecture 22	Proposal: presentation-research problem (15-20 min x 2)	

Continued on next page.

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Table A. (Continued). Syllabus for the Course

Lecture 23	Proposal: writing a proposal	
Lecture 24	Basic principle of laser	
Lecture 25	Lab: building a laser	
Lecture 26	Application of transient absorption spectroscopy to rhodopsin	Hug et al.
Lecture 27	Discussion-transient absorption spectroscopy	Lim et al.
Lecture 28	Basic principle of sum frequency generation spectroscopy	Shen
Lecture 29	Discussion-sum frequency generation spectroscopy	Liu et al.
Lecture 30	Basic principle of NMR	
Lecture 31	Application of NMR to rhodopsin	Patel et al.
Lecture 32	Discussion-NMR	Petkova et al.
Lecture 33	Basic principle of EPR	
Lecture 34	Application of EPR to rhodopsin	Farrens et al. (1996) & Altenbach et al.
Lecture 35	Discussion-EPR	Jao et al.
Lecture 36	Proposal Defense (30 min x 4)	
References		
Listed under the "Course Materials" section (Parts II and III).		

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Editor's Biography

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Harvey J. M. Hou (Ph.D., Peking University) is currently an Associate Professor in the Department of Physical Sciences at Alabama State University. He has joined the faculty at the Chinese Academy of Sciences, Gonzaga University, and University of Massachusetts Dartmouth since 1995. Over the years he has worked on projects involving biochemistry, analytical chemistry, forensic science, plant biology, material science, and environmental science, which have been supported by the US government agencies and private foundations. His research interests are photosynthesis, forensic drug/DNA analysis, microbial forensics, and G protein-coupled receptors. He has authored or co-authored 69 publications in books and journals including *Journal of the American Chemical Society*, *Proceedings of the National Academy of Sciences*, and *Biochemistry*. He is the Editor of *NanoPhotoBioSciences*, Topic Editor of *Frontiers in Plant Physiology*, Associate Editor of Academic Book Publishing Program in Physics in Versita Publisher, and serves on the Editorial Board of *Journal of Photochemistry and Photobiology B: Biology*, *Air & Water Borne Diseases*, and *International Journal of Business and Systems Research*. He has organized or co-organized 15 conferences/symposia in photosynthesis and chemical education since 2004.

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