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FOREWORD

It is a pleasure to provide this foreword to the *Handbook of Mass Spectrometry*, edited by Dr. Mike S. Lee, a PhD graduate of my research group at the University of Florida 25 years ago. Mike is not only an outstanding scientist and a visionary in how mass spectrometry can drive science in a diverse range of disciplines; he is also a master at assembling and leading a team of experts, as he has ably demonstrated with this volume.

Mass spectrometry, although barely a hundred years old, has become the dominant force in modern analytical chemistry. It provides unparalleled levels of sensitivity and selectivity for trace analysis, and an impressive range of capabilities and application. Some of these unique capabilities arise from the unique feature of mass spectrometry (compared to other spectrometric methods) that the sample itself (matter) passes through the spectrometer and is separated and detected. Thus mass spectrometry is both a spectrometric method and a separation method!

Many of the capabilities of modern mass spectrometry arise from the remarkable advances in instrumentation over the past 30 years, many of which are reviewed in this handbook. Advances in ionization techniques have expanded the applicability of mass spectrometry from small, volatile, and thermally stable molecules to large, nonvolatile, and labile molecules, including intact proteins and polymers. The coupling of mass spectrometry with separation techniques (gas chromatography [GC], liquid chromatography [LC], capillary electrophoresis [CE], and even a second stage of mass spectrometry) has established it as the standard for trace mixture analysis. Innovations in mass analyzers continue to bring improved performance in terms of mass resolution, mass range, and sensitivity. And perhaps most impressively, the pace of advances in mass spectrometry instrumentation and methodologies has not slacked off—we continue to see remarkable advances every year.

I often date the “coming of age” of modern analytical mass spectrometry to a 1982 quote from *Chemical & Engineering News*:

> Mass spectrometry has advanced to the point that it’s no longer (as has been said) . . . “the method of choice – if there’s no other way.”

Indeed, mass spectrometry is the method of choice for an amazing range of applications, from structure determination of proteins to forensic toxicology, from fundamental studies of reaction kinetics to imaging tissues. And that breadth of use and dominance of mass spectrometry is well represented in the chapters assembled here.

The remarkable growth of mass spectrometry is well represented in the growth of attendance at the Annual Meeting on Mass Spectrometry and Allied Topics of the American Society for Mass Spectrometry, from 700 attendees in the mid-1970s to 7000 today. This reflects not only the expanding scope of application of the technique, but also the ease with which modern mass spectrometers can be mastered by users new to the field, without needing to understand the underlying fundamentals. This handbook provides in its 13 sections and 52 chapters an excellent overview of that wide range of applications. The breadth of coverage makes this an excellent resource for practicing mass spectrometrists as well as to those new to the field.

Welcome to a hopefully stimulating journey through modern mass spectrometry and its breadth of applications!

RICHARD A. YOST

University of Florida
October 2011
Mass spectrometry is an integral part of modern research in academic, industrial, and clinical laboratories. The Handbook of Mass Spectrometry represents the current state-of-the-art practices in these laboratory settings. The purpose of the handbook is to provide a unique reference that allows for easy access to a variety of applications that involve mass spectrometry. The intent of the handbook is to provide a resource for beginners, practitioners, and experts to obtain vital background, current approaches, and real-world methodology. Further, the handbook can also be viewed as an interactive time capsule to perhaps delineate “where we are,” “where we came from,” and “where we are headed” with regard to these specific applications—current and emerging. Thus, the handbook is not intended to be comprehensive, but rather to provide unique, in-depth information on specific techniques and experiences.

The evolution of mass spectrometry has been both dramatic and fascinating. Trace analytical measurement, specifically the demand for trace mixture analysis, has created an increased demand for this powerful tool. In many cases, the preference for the trace mixture sample type has transformed the mass spectrometer into a gold standard platform for qualitative and quantitative assays.

In its simplest form, a mass spectrometer can be viewed as a molecular weighing machine. Much like we regularly weigh ourselves in the morning to provide an early, facile benchmark for personal health and well-being, mass spectrometers are being used for a similar function. Specifically, a mass spectrometer is routinely used to monitor the “well-being” of a specific analyte. Moreover, the confirmation each analyte (structure or amount), or ensemble of analytes, often provides a surrogate benchmark into a specific process that relates to a biological or chemical condition.

Regardless of the application, mass spectrometry-based methods can be organized into two areas of analytical focus: qualitative (“What is it?”) and quantitative (“How much is there?”) analysis. Similar to the building of a picture puzzle—starting with the edges (the molecular ion!) to define the size of the puzzle and/or set a defined limit to where all remaining subsequent puzzle pieces (fragment ions!) may fit inside the edges—the use of mass spectrometry provides a powerful way to quickly and confidently “define the edges” by providing molecular weight information.

Molecular weight can then become a surrogate for confirmation or even be used for the identification of a targeted compound, particularly when used in conjunction with an authentic standard or chromatographic technique, for example. Advanced studies that involve two or more dimensions of mass analysis can also be used to obtain specific structural detail (fragment ions that correspond to specific pieces of the picture puzzle!) or more selectivity to enable powerful approaches for high throughput quantitation. Moreover, similar to how high-definition televisions are improving our entertainment experience, the higher resolution mass spectrometry (and chromatography!) technologies are poised to provide a benefit to the scientific community in perhaps a highly routine manner.

Thus, the diverse contributions to the handbook are essentially unified based on the puzzle analogy. Confident and definitive “What is it?” and/or “How much is there?” information is obtained via molecular weight measurements provided by the mass spectrometer. The specific mass spectrometer and, of course,
specific chemistries (i.e., sample preparation, chromatography, ionization) help to define the analytical method.

Although the handbook is not necessarily designed to be comprehensive, the contributions represent an impressive array of critical work from diverse areas ranging from biological studies to food analysis to environmental analysis to archaeology. Each chapter in the handbook contains several compulsory elements: (1) essential background and history of the application; (2) detailed analytical methodology; and finally, (3) valuable references for more in-depth study.

Each contributor has provided critical updates in their respective field of expertise. Both current and emerging trends are highlighted. Perhaps a distinguishing feature of the handbook is that nearly all of the chapters provide a detailed description of the actual methodologies used in their respective laboratory—specifically intended so that others may initiate similar work in their respective laboratory. We hope that this unique feature will allow broad base interest and use for all scientists!

Certainly, the handbook is quite diverse in scope and application. The handbook is organized into 13 sections—starting with life sciences and culminating with specialized analytical techniques. Section I provides an exciting perspective on the recent applications of mass spectrometry for the identification of proteins and peptides. These methods represent the emerging role of mass spectrometry in biology-related fields to assist with the determination of both process and function. The section also features the recent methodology used for imaging studies on biological systems as well as the profiling of microorganisms and viruses. The current state-of-the-art work performed in the pharmaceutical industry is featured in Section II. A continuum of work that begins with drug discovery activities such as pharmacokinetics (surrogate studies to determine dosing regimen in humans) as well as mass spectrometry methods for screening, characterization, and imaging are featured in Section II. The pharmaceutical section concludes with perspectives into drug development with the use of accelerator mass spectrometry. Exciting growth and, perhaps, a renaissance, is currently experienced in the field of clinical analysis. Section III provides a timely and critical update on the use of mass spectrometry for the screening of inborn errors and steroid analysis in a clinical laboratory setting. The distinct criteria and features necessary for a clinical laboratory—as opposed to a research setting—are powerfully represented and easily understood. Forensics is indeed a challenging area of focus that requires diverse analytical tools as well as a strict protocol of analysis—from sampling to preparation to analysis to reporting. Section IV contains two important applications of mass spectrometry in this field. The use of isotope ratio mass spectrometry is highlighted followed by a specific application that describes the analysis of the explosive triacetone triperoxide. Section V addresses the important role of mass spectrometry in programs involved with space exploration. A fascinating perspective on the use of mass spectrometry for solar system exploration is provided. This chapter is followed by work that features the use of gas chromatography (GC)/gas chromatography–mass spectrometry (GC-MS) for the characterization of extraterrestrial organic matter. Travel and safety has been greatly impacted over the past decade. Section VI contains the recent work that describes the various uses of mass spectrometry for homeland security. Specific methods are detailed along with the requirements and challenges for this specialized application.

The safety of our food and subsequent food supply is of critical worldwide importance. The role of mass spectrometry for food analysis is highlighted in Section VII. A perspective on agriculture, food and flavors is provided to give the reader some historical perspectives and background in food analysis. The recent mass spectrometry application of “top-down” proteomic methods for the identification of biomarkers of foodborne pathogens highlights future direction and analysis formats. Perhaps a cornerstone of commercial applications of mass spectrometry is in the field of environmental analysis. Section VIII contains the recent work that details how mass spectrometry is used to monitor targeted analytes such as fungicides, commercial by-products, and targeted carcinogens. Section IX focuses on geology. In this section, the authors provide their unique perspective on mass spectrometry applications that address the analysis of oil and gas, geochronology, and hydrocarbon processing. The section concludes with a chapter on the current status and prospects for renewable energy. Mass spectrometry methods have made significant contributions to archaeology. Section X focuses on recent work to give the reader historical and background information as well as specific studies that require careful field work (collection of the actual samples!) along with trace analysis using mass spectrometry-based methods. Surface analysis is a challenging area of study with very specific criteria for analysis. Section XI provides perspective and recent methods in the area of semiconductor research, organic film analysis, and characterization of ceramic materials. Section XII provides perspective on the role and uses of mass spectrometry in polymer research. Background and methodology are highlighted from three leading laboratories. Specialized analytical techniques are presented in Section XIII. The section begins with a chapter on the approaches used for the measurement of metals and alloys followed by a variety
of interesting techniques that involve the use of thin layer chromatography, laser ionization, steady-state isotopic transient kinetic analysis, and proton transfer reaction mass spectrometry.

It is my sincere hope that the handbook provides the information and details to assist scientists with current work as well as inspire future studies. Also, because of the vast content of work, it is hoped that seemingly unrelated applications provide helpful insight into novel uses of mass spectrometry and promote new areas of research.

Finally, I wish to acknowledge the contributions of many—authors, collaborators, editors, and families—who made this handbook possible. Also, along with the terrific editorial staff at John Wiley & Sons, I would like to give a special acknowledgment to Gladys Mok, Managing Editor at John Wiley & Sons, for her significant contributions and premier support during this project.

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SECTION I

BIOTECHNOLOGY/PROTEINS
TARGETED PROTEOMICS USING IMMUNOAFFINITY PURIFICATION

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1.1 INTRODUCTION

Proteins are multimodular and multifunctional, interacting in complex networks that drive cellular function. Pathological alterations in signaling networks are thought to result in a number of diseases, particularly cancer. Understanding the roles and consequences of protein–protein interactions is therefore a fundamental goal in systems biology. The two-hybrid approach [1] emerged in the early 1990s as the first method to assay whether two proteins interact in a pair-wise fashion. A number of bait–target strategies were subsequently developed [2], including techniques exploiting affinity purifications coupled to mass spectrometry (MS) to rapidly identify potentially novel protein interactions. Initial studies were performed in yeast [3,4] and were subsequently expanded to mammalian models [5]. Since MS-based proteomics is not necessarily limited to specific sites or to specific proteins, it represents an unbiased and direct approach to studying cellular processes [6].

As recently described [6,7], immunoaffinity purification has emerged as the most frequently employed method for multiprotein complex purification. Its success is based on the principle that multiple members of a complex may be captured when one complex member is enriched, regardless of whether the complexed proteins are directly bound to the target protein. Additionally, purification of posttranslational modifications has been used extensively to globally profile modified proteins throughout cellular networks [8,9] and provides invaluable insights into signal transduction mechanisms.

A summary of typical steps employed to generate samples using an immunoaffinity-based approach is illustrated in Figure 1.1 and described in detail for two example applications below. Following purification, peptide mixtures resulting from the digestion of bands or eluates are analyzed using tandem mass spectrometry (MS/MS) and proteins are identified by database searching and spectral matching.

A gel-based approach may be useful when two conditions are being compared—bands exhibiting visual differences can be excised to yield data most likely to contrast biologically significant results (note that interesting low abundance proteins may be covered up by more abundant nonspecific proteins). Another useful method, gel-enhanced liquid chromatography–tandem mass spectrometry (GeLC-MS/MS), has also emerged for the analysis of complex protein mixtures [10] and can be applied to the separation of immunoaffinity eluates. In this approach, a protein-containing gel lane is chopped into equivalent sections, digested, and peptide mixtures analyzed. When complexed protein levels are extremely low or sample is limited, elution followed by in-solution digestion may provide a better option, as less protein is lost to sample handling. An important caveat to note is that a protein of interest may be “covered up” by comigrating background or nonspecific proteins. This is a particular concern for proteins that may comigrate with immunoglobulin (Ig)
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heavy and light chains. Using a cross-linker such as DMP (dimethyl pimelimidate) to bind the primary antibody to Protein A will suppress elution of Ig chains when nonreducing conditions are used for elution. However, cross-linking may result in loss of affinity; an optimization workflow should ideally include both cross-linked and noncross-linked trials.

The primary challenge of immunoaffinity-based workflows lies in the difficulty of separating true low abundance interactors from nonspecifically binding proteins. Use of negative controls, such as preimmune sera or antibodies against other proteins, or, if the model allows, using a knockout or knock down of the protein of interest, can help separate out these background proteins. As described below, cross-linking the antibody, minimizing incubation times and antibody concentrations, optimizing wash buffer stringency, and other approaches may help mitigate the extent of nonspecific binding. Assessing the utility of at least a few of these parameters should be included in the optimization workflow. A good way to begin optimizing the protocol is to immunoprecipitate the protein of interest and probe for a known interactor using Western blot. Begin by titrating the primary antibody and beads to find the minimum amount required to effectively immunodeplete the sample. Then experiment with incubation times. Fewer beads and shorter incubation times will help reduce nonspecific binding. Ultimately, orthogonal techniques such as co-immunoprecipitation (IP) with Western blot should be used to validate a subset of the potential interactors, whenever possible.

In this work we present examples of workflows in which immunoaffinity-purified proteins were either separated using gel electrophoresis and bands exhibiting significant change from control were analyzed, or complexed proteins were eluted from the beads, digested in solution, and analyzed. It is important to note that these protocols provide general guidelines and that several optimization steps with multiple iterations of MS will likely be required for purification of a protein complex of interest.

As discussed in more detail below, data analysis and mining are critical for gleaning relevant information from proteomics studies. Careful extraction of peptide signals, determination of properly stringent search engine parameters [11], and reversed or scrambled
database searching leads to an output data set where significance of the identifications may be established with score or probability cutoffs. Although a false discovery rate of ~1% is often employed in more global approaches [12], establishing criteria for two peptide “hits” to a protein with peptide probabilities of ~95% is sufficient to provide a false discovery rate approaching 0% for immunoaffinity purification applications. Following identification, data mining is employed to obtain functional information about the proteins to begin to decipher mechanisms that may be triggered by the interaction. The tools used often include those described below.

1.2 EXPERIMENTAL PROTOCOLS

Materials and Solutions

- **Cell Lysis Buffer.** 0.33% 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS); 150 mM NaCl; 10 mM sodium pyrophosphate; 10 mM Tris-HCl pH 7.4; 1 mM phenylmethylsulfonyl fluoride (PMSF); 0.4 mM ethylenediaminetetraacetic acid (EDTA); 1.8 mg/mL iodoacetamide (IAA); 10 mM NaF; 2 mM Na,VO₃; and 1 µg/mL each of aprotinin, leupeptin, and pepstatin.

- **Tissue Lysis Buffer.** Buffer A (10 mM HEPES pH 7.9, 1.5 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.1% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO), and 0.5 mM PMSF) and Buffer B (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 4 µM leupeptin). *(Note: These buffers were selected for the analysis of liver proteomes, as described below. Other lysis buffers may be more appropriate for the sample/proteins under investigation. A search of recent literature should provide some direction regarding appropriate buffer selection.)*

- **Reagents for preparation of magnetic beads as described below.**

- **Protein A or protein G beads.** *(Note: Either Sepharose [GE Healthcare, Piscataway, NJ] beads or magnetic beads may be used; however, magnetic beads are preferred as they tend to exhibit less non-specific binding than Sepharose beads.)*

- **Pipette tips cut 5 mm from the top (will avoid damaging the beads if using Protein A/G Sepharose beads).**

- **Laemmli buffer.**

- **High performance liquid chromatography (HPLC)-grade water (Honeywell Burdick and Jackson [Morristown, NJ] or other high quality liquid chromatography–mass spectrometry [LC/MS]-grade water).**

- **Desalt spin columns.**

- **Reagents for in-gel or in-solution digestion as listed below.**

**Equipment**

- Refrigerated centrifuge

- Rotary mixer

- Vacuum centrifuge

- Gel electrophoresis apparatus and 10% polyacrylamide gel

- Nanoscale HPLC, tandem mass spectrometer

**Lysis**

Note that the cell numbers and tissue amounts presented here are a guideline. These numbers should be increased if complexed proteins are of low abundance.

**Cell Lysis**

1. Lyse ~5 × 10⁸ cells using 500–1000 µL cell lysis buffer at 4°C overnight. Note that if low abundance or weakly interacting proteins are to be analyzed, increase the cell numbers to as much as 10¹⁰ (as shown by Malovannaya and coworkers [17]).

2. In the morning, centrifuge lysates at 12,000 g at 4°C for 20 min. Remove supernatants to a clean tube.

**Tissue Lysis**

1. Homogenize ~100 mg of tissue using a mortar and pestle over liquid nitrogen. For identification of potentially weakly binding complexes, increase tissue amount to 10–20 g (following Moresco et al. [18]) and increase lysis buffer volume to 5–10 mL.

2. Add homogenized tissue to 0.5 mL of tissue lysis buffer A (ice cold) and ultrasonicate three times, 15 s each. Place sample tubes in an ice bath for at least 1 min between sonications.

3. Incubate samples on ice for 30 min, then centrifuge at 14,000 × g, 4°C for 10 min. Remove the supernatant (cytoplasmic fraction) to clean tube.

4. Resuspend the membrane/organell fraction pellet in 0.2 mL ice-cold tissue lysis buffer B and
incubate on ice for 30 min. Following centrifugation at 14,000 × g for 30 min at 4°C, remove the supernatant to clean tube.

Note: For all subsequent steps, be sure to keep samples on ice or at 4°C, however freezing lysates before the immunoaffinity purification should be avoided [18]. If necessary, store lysates at −80°C prior to use.

Total Protein Quantification
Use either the Bradford or bicinchoninic acid (BCA) method to quantify total protein concentrations following the manufacturer’s instructions for a microwell plate assay. Make sure that the lysis buffer components are compatible with the manufacturer’s stated levels. Try several dilutions to ensure the sample concentration is within the linear range of the assay.

Immunoadfinity Purification
Immunoadfinity purification may be accomplished using either soluble antibodies or antibodies cross-linked to beads. Generally the first step of the optimization should be done using cross-linked antibodies; cross-linking significantly reduces contaminating signals from Ig light and heavy chains. Procedures for both approaches are provided below.

Immunoadfinity Purification Using Magnetic Beads
Reagents for Magnetic Bead Preparation (Dynabeads Are Typically Used)

- Citrate Phosphate Buffer, pH 5.0, 25 mM citric acid, 50 mM sodium phosphate (Na₂HPO₄)
- 0.2 M Triethanolamine (TEA), pH 8.2, 3.71 g triethanolamine-HCl/100 mL water
- 20 mM DMP, 5.4 mg DMP-2HCl per milliliter of TEA buffer
- 50 mM Tris pH 7.5
- PBS-T, 0.01% Tween-20 (Thermo Fisher Scientific, Waltham, MA) in phosphate buffered saline
- 0.1 M glycine pH 2.5–2.7
- Storage Solution, PBS-T with 0.02% sodium azide

Dynabeads (Invitrogen Corp., Carlsbad, CA) are packaged as a 5% slurry. Prepare 0.5–1.0 mL of slurry to obtain 25–50 µL of packed beads. A rule of thumb is that 1 mL of slurry binds ~300 µg of antibody. Incubate with about 400 µg of the primary antibody.

Equilibrate Dynabeads
Centrifuge beads briefly, place tube in a magnetic rack, and remove the supernatant. Add 1 mL citrate phosphate buffer, vortex, spin briefly in a minifuge (1 s to remove bead solution from cap), and place tubes in a magnetic rack. Remove supernatant. Repeat two more times.

Incubate with Primary Antibody
Prepare 400 µg of primary antibody in 1 mL citrate phosphate buffer and add to beads. Reducing the volume may improve binding and may be included in subsequent optimization steps. Rotate tube end over end for 2–3 h at room temperature.

Wash
Centrifuge briefly, place tubes in a magnetic rack, remove supernatant, and add 1 mL citrate phosphate buffer and wash three times as described in the section “Equilibrate Dynabeads.” Wash two times more with 1 mL 0.2 M triethanolamine-HCl.

Cross-Link
Remove final TEA wash from the beads using the magnet and add 1 mL of DMP solution. Incubate 30 min at room temperature, rotating end over end.

Clean Up
Using the magnet, remove the DMP solution and incubate beads with 50 mM Tris for 15 min to remove free cross-linking reagent.

Wash
Wash beads three times with PBS-T.

Remove Free Antibody
Incubate the beads with 0.1 M glycine for 5 min, rotating end over end at 4°C.

Wash
Wash beads three times with PBS-T.

Store Beads
Bring beads back to original packaged volume in storage solution for storage at 4°C. For a 1 mL stock, use 950 µL of storage solution.

To use, wash beads three times using 1 mL PBS, then three time with 1 mL lysis buffer. Use Western blots and bead titration to determine the minimum amount of beads to use and the minimum amount of time to incubate. A starting point for optimization may be 20 µL of packed beads and 2 h of incubation at 4°C. Elute as described below.

Immunoadfinity Purification Using Protein A/G Sepharose Beads
Incubation with Primary Antibody
Add 3–20 µg of primary antibody to the supernatant and incubate for 2 h to overnight at 4°C. This step