METHODS IN MOLECULAR BIOLOGY™ 421

Affinity Chromatography

Methods and Protocols

Second Edition

Edited by

Michael Zachariou

Humana Press

METHODS IN MOLECULAR BIOLOGY™

Affinity Chromatography

Methods and Protocols

SECOND EDITION

Edited by

Michael Zachariou

Director Project Management,
BioMarin Pharmaceutical Inc., CA

Humana Press

Editor
Michael Zachariou
Director Project Management,
BioMarin Pharmaceutical Inc., CA

ISBN: 978-1-58829-659-7 e-ISBN: 978-1-59745-582-4

Library of Congress Control Number: 2007930114

Cover illustration: Fig. 4, Chapter 7, "Rationally Designed Ligands for use in Affinity Chromatography: An Artifical Protein L," by Ana Cecilia A. Roque and Christopher R. Lowe

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

Affinity Chromatography

SECOND EDITION

METHODS IN MOLECULAR BIOLOGY™

John M. Walker, SERIES EDITOR

436. **Avian Influenza Virus**, edited by *Erica Spackman, 2008*
435. **Chromosomal Mutagenesis**, edited by *Greg Davis and Kevin J. Kayser, 2008*
434. **Gene Therapy Protocols:** *Volume 2: Design and Characterization of Gene Transfer Vectors*, edited by *Joseph M. LeDoux, 2008*
433. **Gene Therapy Protocols:** *Volume 1: Production and In Vivo Applications of Gene Transfer Vectors*, edited by *Joseph M. LeDoux, 2008*
432. **Organelle Proteomics**, edited by *Delphine Pflieger and Jean Rossier, 2008*
431. **Bacterial Pathogenesis:** *Methods and Protocols*, edited by *Frank DeLeo and Michael Otto, 2008*
430. **Hematopoietic Stem Cell Protocols**, edited by *Kevin D. Bunting, 2008*
429. **Molecular Beacons:** *Signalling Nucleic Acid Probes, Methods and Protocols*, edited by *Andreas Marx and Oliver Seitz, 2008*
428. **Clinical Proteomics:** *Methods and Protocols*, edited by *Antonio Vlahou, 2008*
427. **Plant Embryogenesis**, edited by *Maria Fernanda Suarez and Peter Bozhkov, 2008*
426. **Structural Proteomics:** *High-Throughput Methods*, edited by *Bostjan Kobe, Mitchell Guss, and Huber Thomas, 2008*
425. **2D PAGE:** *Volume 2: Applications and Protocols*, edited by *Anton Posch, 2008*
424. **2D PAGE:** *Volume 1: Sample Preparation and Pre-Fractionation*, edited by *Anton Posch, 2008*
423. **Electroporation Protocols**, edited by *Shulin Li, 2008*
422. **Phylogenomics**, edited by *William J. Murphy, 2008*
421. **Affinity Chromatography:** *Methods and Protocols*, Second Edition, *edited by Michael Zachariou, 2008*
420. **Drosophila:** *Methods and Protocols*, edited by *Christian Dahmann, 2008*
419. **Post-Transcriptional Gene Regulation,** edited by *Jeffrey Wilusz, 2008*
418. **Avidin–Biotin Interactions:** *Methods and Applications*, edited by *Robert J. McMahon, 2008*
417. **Tissue Engineering, Second Edition**, edited by *Hannsjörg Hauser and Martin Fussenegger, 2007*
416. **Gene Essentiality:** *Protocols and Bioinformatics*, edited by *Andrei L. Osterman, 2008*
415. **Innate Immunity,** edited by *Jonathan Ewbank and Eric Vivier, 2007*
414. **Apoptosis in Cancer:** *Methods and Protocols*, edited by *Gil Mor and Ayesha Alvero, 2008*
413. **Protein Structure Prediction, Second Edition,** edited by *Mohammed Zaki and Chris Bystroff, 2008*
412. **Neutrophil Methods and Protocols,** edited by *Mark T. Quinn, Frank R. DeLeo, and Gary M. Bokoch, 2007*
411. **Reporter Genes for Mammalian Systems**, edited by *Don Anson, 2007*
410. **Environmental Genomics,** edited by *Cristofre C. Martin, 2007*
409. **Immunoinformatics:** *Predicting Immunogenicity* In Silico, edited by *Darren R. Flower, 2007*
408. **Gene Function Analysis,** edited by *Michael Ochs, 2007*
407. **Stem Cell Assays,** edited by *Mohan C. Vemuri, 2007*
406. **Plant Bioinformatics:** *Methods and Protocols*, edited by *David Edwards, 2007*
405. **Telomerase Inhibition:** *Strategies and Protocols*, edited by *Lucy Andrews and Trygve O. Tollefsbol, 2007*
404. **Topics in Biostatistics,** edited by *Walter T. Ambrosius, 2007*
403. **Patch-Clamp Methods and Protocols**, edited by *Peter Molnar and James J. Hickman, 2007*
402. **PCR Primer Design**, edited by *Anton Yuryev, 2007*
401. **Neuroinformatics,** edited by *Chiquito J. Crasto, 2007*
400. **Methods in Lipid Membranes,** edited by *Alex Dopico, 2007*
399. **Neuroprotection Methods and Protocols,** edited by *Tiziana Borsello, 2007*
398. **Lipid Rafts,** edited by *Thomas J. McIntosh, 2007*
397. **Hedgehog Signaling Protocols,** edited by *Jamila I. Horabin, 2007*
396. **Comparative Genomics,** *Volume 2,* **edited by** *Nicholas H. Bergman, 2007*
395. **Comparative Genomics,** *Volume 1,* **edited by** *Nicholas H. Bergman, 2007*
394. **Salmonella:** *Methods and Protocols*, edited by *Heide Schatten and Abe Eisenstark, 2007*
393. **Plant Secondary Metabolites**, edited by *Harinder P. S. Makkar, P. Siddhuraju, and Klaus Becker, 2007*
392. **Molecular Motors:** *Methods and Protocols*, edited by *Ann O. Sperry, 2007*
391. **MRSA Protocols,** edited by *Yinduo Ji, 2007*
390. **Protein Targeting Protocols, Second Edition,** edited by *Mark van der Giezen, 2007*
389. ***Pichia* Protocols, Second Edition,** edited by *James M. Cregg, 2007*
388. **Baculovirus and Insect Cell Expression Protocols, Second Edition,** edited by *David W. Murhammer, 2007*
387. **Serial Analysis of Gene Expression (SAGE):** *Digital Gene Expression Profiling*, edited by *Kare Lehmann Nielsen, 2007*
386. **Peptide Characterization and Application Protocols**, edited by *Gregg B. Fields, 2007*
385. **Microchip-Based Assay Systems:** *Methods and Applications*, edited by *Pierre N. Floriano, 2007*
384. **Capillary Electrophoresis:** *Methods and Protocols*, edited by *Philippe Schmitt-Kopplin, 2007*
383. **Cancer Genomics and Proteomics:** *Methods and Protocols*, edited by *Paul B. Fisher, 2007*
382. **Microarrays, Second Edition:** *Volume 2, Applications and Data Analysis*, edited by *Jang B. Rampal, 2007*
381. **Microarrays, Second Edition:** *Volume 1, Synthesis Methods*, edited by *Jang B. Rampal, 2007*
380. **Immunological Tolerance:** *Methods and Protocols*, edited by *Paul J. Fairchild, 2007*
379. **Glycovirology Protocols**, edited by *Richard J. Sugrue, 2007*
378. **Monoclonal Antibodies:** *Methods and Protocols*, edited by *Maher Albitar, 2007*
377. **Microarray Data Analysis:** *Methods and Applications*, edited by *Michael J. Korenberg, 2007*
376. **Linkage Disequilibrium and Association Mapping:** *Analysis and Application*, edited by *Andrew R. Collins, 2007*

To Tina, Emmanuella, Natalie, and Ashez

Preface

Forty years after the term "affinity chromatography" was introduced, this mode of chromatography remains a key tool in the armory of separation techniques that are available to separation and interaction scientists. Affinity chromatography is favored because of its high selectivity, speed, and ease of use. The rapid and selective isolation of molecules using affinity chromatography has allowed a better understanding of biological processes, accelerated the identification of target molecules, and spawned new process areas such as immobilized enzyme reactors. It has had ubiquitous application in most areas of science ranging from small molecule isolation to biopolymers such as DNA, proteins, polysaccharides, and even whole cells. The number of applications of affinity chromatography continues to expand at a rapid rate. For example, more than 60% of purification protocols include some sort of affinity chromatography step, while a database search of PubMed reveals more than 36,000 publications making use of the term "affinity chromatography," more than 3000 of which refer to it in their title. The US patent office reports more than 16,000 references to the term "affinity chromatography", while there are more than 270 references to the same term in the patent title.

The aim of this edition of Methods in Molecular Biology, *Affinity Chromatography: Methods and Protocols, Second Edition* is to provide the beginner with the practical knowledge to develop affinity separations suitable for various applications relevant to the post-genomic era. This second edition expands on the first edition by introducing more state-of-the-art protocols used in affinity chromatography. This current edition also describes protocols that demonstrate the concept of affinity chromatography being applied to meet the modern high throughput screening demands of researchers and development scientists, while expanding on some more traditional affinity chromatography approaches that have become of greater interest to separation scientists. This volume begins with an overview of affinity chromatography authored by one of the pioneers of affinity chromatography, Professor Christopher Lowe. Part I expands on affinity chromatography techniques that currently enjoy frequent citation in the literature from those purifying biomolecules. These affinity chromatography techniques include immobilized metal affinity chromatography, immunoaffinity chromatography and dye-ligand chromatography.

Affinity tags for purification of proteins have become useful and common tools in academic and industrial research laboratories for rapid protein isolation. The sequencing of the human genome along with a multitude of prokaryotic genomes has forced research laboratories and biotechnology companies to find rapid and high-yielding approaches to screen for protein targets. Affinity chromatography techniques allow for high-yielding, rapid approaches to target identification. Part II presents a number of protocols describing the use of various fusion tags as well as how to cleave them, so as to allow the scientists to study the native phenotype of the protein. This section also discusses methods for selecting ligands through rational combinatorial design and phage display for use in affinity chromatography. Part III ventures into diverse applications of affinity chromatography such as its use in catalytic reactions, DNA purification, whole cell separations, and for the isolation of phosphorylated proteins. Protocols are also presented on analytical applications of affinity chromatography, such as in capillary electrophoresis and quantitative affinity chromatography.

Affinity Chromatography: Methods and Protocols, Second Edition is aimed at those interested in separation sciences, particularly in the pharmaceutical and biological research sectors that have an interest in isolating macromolecules rapidly, quantitatively, and with high purity.

Michael Zachariou

Contributors

Marie-Isabel Aguilar • *Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia*
José Arnau • *Unizyme Laboratories A/S, Hørsholm, Denmark*
Katja Benčina • *BIA Separations d.o.o., Ljubljana, Slovenia*
Mojca Benčina • *Laboratory of Biotechnology, National Institute of Chemistry, Ljubljana, Slovenia*
Natalie A. Betz • *University of Wisconsin, Madison, WI*
Joanne L. Casey • *Cooperative Research Center for Diagnostics, Department of Biochemistry, La Trobe University, Victoria, Australia*
Adam Charlton • *Industrial Biotechnology, CSIRO Molecular and Health Technology, Australia*
Andrew M. Coley • *Cooperative Research Center for Diagnostics, Department of Biochemistry, La Trobe University, Victoria, Australia*
Laurie Engel • *Proteomics R&D, Promega Corporation, Fitchburg, WI*
Michael Foley • *Cooperative Research Center for Diagnostics, Department of Biochemistry, La Trobe University, Victoria, Australia*
Gareth M. Forde • *Department of Chemical Engineering, Monash University, Clayton, Victoria, Australia*
Igor Yu. Galaev • *Department of Biotechnology, Centre for Chemistry and Chemical Engineering, Lund University, Lund, Sweden*
Stuart R. Gallant • *Process Sciences Department, BioMarin Pharmaceutical Inc, Novato, CA*
Becky Godat • *Proteomics R&D, Promega Corporation, Fitchburg, WI*
Danette Hartzell • *PBI R&D, Promega Biosciences Inc., San Louis Obispo, CA*
Niels H. H. Heegaard • *Department of Autoimmunology, Statens Serum Institut, Copenhagen S, Denmark*
Dieter H. Klaubert • *PBI R&D, Promega Corporation., Fitchburg, WI*
Tonny M. Johnson • *Proteomics R&D, Promega Corporation, Fitchburg, WI*
Vish Koppaka • *BioMarin Pharmaceutical Inc, Novato, CA*
Ashok Kumar • *Department of Biological Sciences and Bioengineering, Indian Institute of Technology Kanpur (IITK), India*
Conni Lauritzen • *Unizyme Laboratories A/S, Hørsholm, Denmark*

TZONG-HSIEN LEE • *Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia*
CHRISTOPHER R. LOWE • *Department of Biotechnology, Institute of Biotechnology, University of Cambridge, Cambridge, UK*
BO MATTIASSON • *Centre for Chemistry and Chemical Engineering, Lund University, Lund, Sweden*
JACQUI MENDEZ • *Cellular Proteomics, R&D, Promega Corporation, Fitchburg, WI*
JESPER ØSTERGAARD • *Department of Autoimmunology, Statens Serum Institut, Copenhagen S, Denmark*
LEONARD K. PATTENDEN • *Department of Biochemistry and Molecular Biology, Monash University, Clayton Victoria, Australia*
JOHN PEDERSEN • *Unizyme Laboratories A/S, Hørsholm, Denmark*
GITTE EBERT PETERSEN • *Unizyme Laboratories A/S, Hørsholm, Denmark*
ALEŠ PODGORNIK • *BIA Separations d.o.o., Ljubljana, Slovenia*
MARJETA URH • *Cellular Proteomics, R&D, Promega Corporation, Fitchburg, WI*
ANA CECÍLIA A. ROQUE • *Faculdade de Ciéncias e Tecnologia, Universidade Nova de Lisboa, Portugal*
CHRISTIAN SCHOU • *Department of Autoimmunology Statens Serum Institut, Copenhagen S, Denmark*
ALEŠ ŠTRANCAR • *BIA Separations d.o.o., Ljubljana, Slovenia*
WALTER G. THOMAS • *Baker Heart Research Institute, Melbourne, Victoria, Australia*
GRIGORIY S. TCHAGA • *Clontech Laboratories, Inc., Mountain View, CA*
NIHAL TUGCU • *Bioprocess R&D, BioPurification Development, Merck, Rahway, NJ*
KEITH WOOD • *Cellular Proteomics, Promega Corporation, Fitchburg, WI*
MICHAEL ZACHARIOU • *Director Project Management, BioMarin Pharmaceutical Inc. Novato, CA*
NICK ZECHERLE • *Process Sciences Department, BioMarin Pharmaceutical Inc, Novato, CA*

Contents

1

Affinity Chromatography

History, Perspectives, Limitations and Prospects

Ana Cecília A. Roque and Christopher R. Lowe

Summary

Biomolecule separation and purification has until very recently steadfastly remained one of the more empirical aspects of modern biotechnology. Affinity chromatography, one of several types of adsorption chromatography, is particularly suited for the efficient isolation of biomolecules. This technique relies on the adsorbent bed material that has biological affinity for the substance to be isolated. This review is intended to place affinity chromatography in historical perspective and describe the current status, limitations and future prospects for the technique in modern biotechnology.

Key Words: Affinity; chromatography; biomimetic; ligands; synthetic; proteins; purification; design; combinatorial synthesis.

1. Introduction

Traditional techniques for biomolecule separation based on precipitation with pH, ionic strength, temperature, salts, solvents or polymers, ion exchange or hydrophobic chromatography are slowly being replaced by sophisticated chromatographic protocols based on biological specificity. Affinity techniques exploit highly specific biorecognition phenomena and are ideally suited to the purification of biomolecules. In affinity chromatography, the specific adsorption properties of the bed material are realized by covalently attaching the ligand complementary to the target biomolecule onto an insoluble matrix. If a crude

From: *Methods in Molecular Biology, vol. 421: Affinity Chromatography: Methods and Protocols, Second Edition*
Edited by: M. Zachariou © Humana Press, Totowa, NJ

cell extract containing the biologically active target is passed through a column of such an immobilized ligand, then all compounds displaying affinity under the given experimental conditions will be retained by the column, whereas compounds showing no affinity will pass through unretarded. The retained target is then released from the complex with the immobilized ligand by changing operational parameters such as pH, ionic strength, buffer composition or temperature. Conceptually, the technique represents chromatographic nirvana: Exquisite selectivity combined with high yields and the unparalleled simplicity of a 'load, wash, elute' philosophy. However, experience over the last 3–4 decades has shown that there is a very high penalty to pay for the implicit specificity and simplicity of affinity chromatography, which has important ramifications for commercial use and process development.

2. Historical Perspective

Affinity chromatography is a particular variant of chromatography in which the unique biological specificity and reversibility of the target analyte and ligand interaction is utilized for the separation *(1)*. It is possible to distinguish four phases in the development of the technique (*see* **Fig. 1**) starting from the early

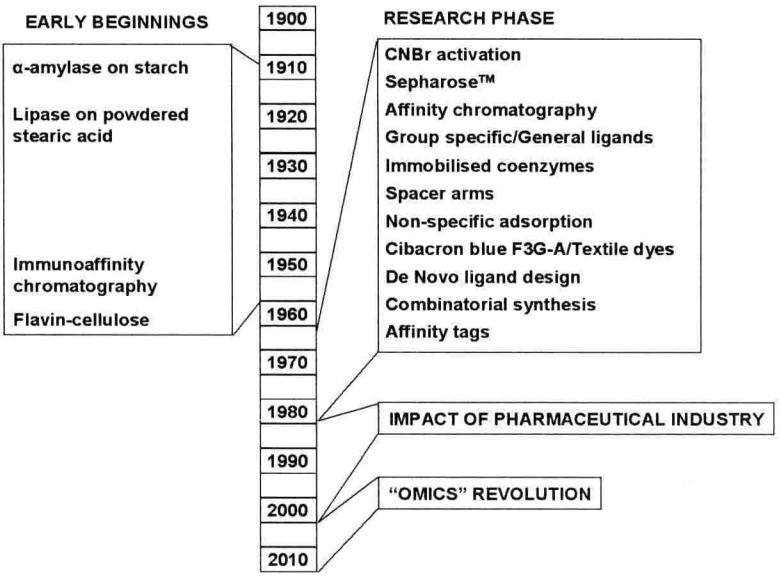

Fig. 1. Development of affinity chromatography as a technique: (i) Early beginning; (ii) Research phase; (iii) Impact of pharmaceutical industry and (iv) 'Omics' revolution.

realization of the technique, through the research phase, the impact of the nascent biopharmaceutical industry to the likely effect of the 'omics' revolution.

2.1. Early Beginnings

The concept of resolving complex macromolecules by means of biospecific interactions with immobilized substrates has its antecedents reaching back to the beginning of the 20th century. The German pharmacologist Emil Starkenstein (1884–1942) in a paper published in 1910 ***(2)*** on the influence of chloride on the enzymatic activity of liver α-amylase was generally considered to be responsible for the first experimental demonstration of the biospecific adsorption of an enzyme onto a solid substrate, in this case, starch. Not long after, Willstätter et al. ***(3)*** appreciably enriched lipase by selective adsorption onto powdered stearic acid. It was not until 1951, however, that Campbell and co-workers ***(4)*** first used the affinity principle to isolate rabbit anti-bovine serum albumin antibodies on a specific immunoadsorbent column comprising bovine serum albumin coupled to diazotised *p*-aminobenzyl-cellulose. This technique, now called immunoaffinity chromatography, became established before the development of small-ligand selective chromatography, where Lerman ***(5)*** isolated mushroom tyrosinase on various *p*-azophenol-substituted cellulose columns, and Arsenis and McCormick ***(6,7)*** purified liver flavokinase and several other FMN-dependent enzymes on flavin-substituted celluloses. Insoluble polymeric materials, especially the derivatives of cellulose, also found use in the purification of nucleotides ***(8)***, complementary strands of nucleic acids ***(9)*** and certain species of transfer RNA ***(10)***.

2.2. Research Phase

The general notion of exploiting strong reversible associations with highly specific substrates or inhibitors to effect enzyme purification was evident in the literature in the mid-1960s ***(11)***, although the immense power of biospecificity as a purification tool was not generally appreciated until 1968 when the term 'affinity chromatography' was coined ***(12)***. It was recognized that the key development required for wider application of the technique was that the solid-phase adsorbent should have a number of desirable characteristics:

> ... the unsubstituted matrix or gel should show minimal interactions with proteins in general, both before and after coupling to the specific binding group. It must form a loose, porous network that permits easy entry and exit of macromolecules and which retains favourable flow properties during use. The chemical structure of the supporting material must permit the convenient and extensive attachment of the specific ligand under relatively mild conditions, and through chemical bonds that are stable to the conditions of adsorption and elution. Finally, the inhibitor

groups critical in the interaction must be sufficiently distant from the solid matrix to minimise steric interference with the binding process ***(12)***.

In this seminal paper, the general principles and potential application of affinity chromatography were enunciated and have largely remained unchanged until the present date. The paper contained several important contributions. First, it generalized the technique to all potential enzyme purifications via immobilized substrates and inhibitors and exemplified the approach by application to staphylococcal nuclease, α-chymotrypsin and carboxypeptidase A. Second, it introduced for the first time a new highly porous commercially available 'beaded' matrix of agarose, Sepharose, which displayed virtually all of the desirable features listed above ***(13)*** and circumvented many of the issues associated with conventional cellulosic matrices available at that time. Agarose is a linear polysaccharide consisting of alternating 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose units ***(13)***. Third, the report exploited the activation of Sepharose by treatment with cyanogen bromide (CNBr) to result in a derivative that could be readily coupled to unprotonated amino groups of an inhibitory analogue to generate a highly stable Sepharose-inhibitor gel with nearly ideal properties for selective column chromatography ***(14,15)***. The use of CNBr activation chemistry was a milestone in the development of the technique, because the complex organic chemistry required for the synthesis of reliable immobilized ligand matrices had previously prevented this technique from becoming generally established in biological laboratories. Fourth, the report introduces the notion of spacer arms to alleviate steric interference and exemplifies the concept by showing the dramatically stronger adsorption of α-chymotrypsin to the immobilized inhibitor D-tryptophan methyl ester when a 6-carbon chain, ε-amino caproic acid, was interposed between the Sepharose matrix and the inhibitor. When the inhibitor was coupled directly to the matrix, incomplete and unsatisfactory resolution of the enzyme was observed. Fifth, the report emphasizes the importance of selective affinity for the immobilized inhibitor by demonstrating the absence of adsorption of chemically inhibited enzymes such as DFP-treated α-chymotrypsin or CNBr-treated nuclease to their respective adsorbents ***(12)***. Finally, this paper emphasizes the efficacy of relatively low-affinity inhibitors and suggests that unusually strong affinity constants are not an essential requirement for utilization of these techniques for the rapid single-step purification of proteins.

Affinity chromatography caught the eye of many researchers worldwide and there followed a spate of publications purporting to purify proteins and other biomolecules by every conceivable class of immobilized ligand. However, troubling issues relating to the chemistry of the ligand attachment still remained. For example, there was much debate on how adsorbents should be synthesized ***(16)***; the 'solid-phase assembly' approach was more facile and advocated the

attachment of ligands to spacer arms already present on the pre-activated affinity matrix, whereas the 'pre-assembly' approach uses conventional organic chemistry to modify the ligand with a suitably derivatized spacer arm, after which the whole assembly is coupled to the matrix. The solid-phase assembly approach lead to inhomogeneity problems where there were multiple sites on the target ligand or the coupling chemistries were incomplete, whereas the pre-assembled ligand spacer arm unit could be pre-characterized by conventional chemical techniques and studies in solution to yield useful advance information on binding specificity and kinetic constants. The present authors believe that a combination of both strategies represents an effective means of developing new and well-characterized affinity adsorbents for the purification of target proteins.

A further key development introduced in the early 1970s was that of 'group-specific' ***(17)*** or 'general ligand' ***(18)*** adsorbents. An important advantage of ligands with a broad bioaffinity spectrum, such as the coenzymes, lectins, nucleic acids, metal chelates, Protein A, gelatine and heparin, is that it was not obligatory to devise a new organic synthetic strategy for every projected biospecific purification. However, a possible disadvantage of the group-specific approach is that the broad specificity of the adsorption stage required a compensatory specific elution step to restore the overall biospecificity of the chromatographic system. Nevertheless, of the thousands of enzymes that have been assigned a specific Enzyme Commission number, approximately one-third involve one of the four adenine coenzymes (NAD^+, $NADP^+$, CoA and ATP), and not surprisingly, these classes of enzymes were the first to be targeted by this approach ***(17–19)*** and subsequently extensively exploited in the purification of oxido-reductases by affinity chromatography and in enzyme technology ***(20–22)***.

Until this point in time, most of the studies had generated rules-of-thumb on how to apply the technique of affinity chromatography to selected purifications. However, it became apparent on even a rudimentary examination of the theoretical basis of the technique ***(23)*** that the implicit assumption that the observed chromatographic adsorption of the target protein to the immobilized ligand was due exclusively to biospecific enzyme–ligand interactions was misguided. The large discrepancies observed between what was anticipated on the basis of the biological affinity for the immobilized ligand and what was observed experimentally to be the case were found to be due to the largely unsuspected interference by non-biospecific adsorption, which, in many cases, completely eclipsed the biospecific adsorption ***(24–25)***. O'Carra and co-workers ***(24–25)*** demonstrated that spacer arms do not always act simply as passive links between biospecific ligands and the polymer matrix and described methods for the control of interfering non-specific adsorption effects and for the optimization of affinity chromatography performance by a logical and systematic appraisal