

FOOD PROTEIN ANALYSIS

Quantitative Effects on Processing

R. K. Owusu-Apenten

*The Pennsylvania State University
University Park, Pennsylvania*



MARCEL DEKKER, INC.

NEW YORK • BASEL

Copyright © 2002 by Marcel Dekker, Inc. All Rights Reserved.

ISBN: 0-8247-0684-6

This book is printed on acid-free paper.

Headquarters

Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
tel: 212-696-9000; fax: 212-685-4540

Eastern Hemisphere Distribution

Marcel Dekker AG
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
tel: 41-61-261-8482; fax: 41-61-261-8896

World Wide Web

<http://www.dekker.com>

The publisher offers discounts on this book when ordered in bulk quantities. For more information, write to Special Sales/Professional Marketing at the headquarters address above.

Copyright © 2002 by Marcel Dekker, Inc. All Rights Reserved.

Neither this book nor any part may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, microfilming, and recording, or by any information storage and retrieval system, without permission in writing from the publisher.

Current printing (last digit):

10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

FOOD SCIENCE AND TECHNOLOGY

A Series of Monographs, Textbooks, and Reference Books

EDITORIAL BOARD

Senior Editors

Owen R. Fennema University of Wisconsin–Madison

Y.H. Hui Science Technology System

Marcus Karel Rutgers University (emeritus)

Pieter Walstra Wageningen University

John R. Whitaker University of California–Davis

Additives **P. Michael Davidson** University of Tennessee–Knoxville

Dairy science **James L. Steele** University of Wisconsin–Madison

Flavor chemistry and sensory analysis **John H. Thorngate III** University of California–Davis

Food engineering **Daryl B. Lund** University of Wisconsin–Madison

Food proteins/food chemistry **Rickey Y. Yada** University of Guelph

Health and disease **Seppo Salminen** University of Turku, Finland

Nutrition and nutraceuticals **Mark Dreher** Mead Johnson Nutritionals

Phase transition/food microstructure **Richard W. Hartel** University of Wisconsin–Madison

Processing and preservation **Gustavo V. Barbosa-Cánovas** Washington State University–Pullman

Safety and toxicology **Sanford Miller** University of Texas–Austin

1. Flavor Research: Principles and Techniques, *R. Teranishi, I. Hornstein, P. Isenberger, and E. L. Wick*
2. Principles of Enzymology for the Food Sciences, *John R. Whitaker*
3. Low-Temperature Preservation of Foods and Living Matter, *Owen R. Fennema, William D. Powrie, and Elmer H. Marth*
4. Principles of Food Science
Part I: Food Chemistry, *edited by Owen R. Fennema*
Part II: Physical Methods of Food Preservation, *Marcus Karel, Owen R. Fennema, and Daryl B. Lund*
5. Food Emulsions, *edited by Stig E. Friberg*
6. Nutritional and Safety Aspects of Food Processing, *edited by Steven R. Tanenbaum*
7. Flavor Research: Recent Advances, *edited by R. Teranishi, Robert A. Flath, and Hiroshi Sugisawa*
8. Computer-Aided Techniques in Food Technology, *edited by Israel Saguy*
9. Handbook of Tropical Foods, *edited by Harvey T. Chan*
10. Antimicrobials in Foods, *edited by Alfred Larry Branen and P. Michael Davidson*
11. Food Constituents and Food Residues: Their Chromatographic Determination, *edited by James F. Lawrence*

12. Aspartame: Physiology and Biochemistry, *edited by Lewis D. Stegink and L. J. Filer, Jr.*
13. Handbook of Vitamins: Nutritional, Biochemical, and Clinical Aspects, *edited by Lawrence J. Machlin*
14. Starch Conversion Technology, *edited by G. M. A. van Beynum and J. A. Roels*
15. Food Chemistry: Second Edition, Revised and Expanded, *edited by Owen R. Fennema*
16. Sensory Evaluation of Food: Statistical Methods and Procedures, *Michael O'Mahony*
17. Alternative Sweeteners, *edited by Lyn O'Brien Nabors and Robert C. Gelardi*
18. Citrus Fruits and Their Products: Analysis and Technology, *S. V. Ting and Russell L. Rouseff*
19. Engineering Properties of Foods, *edited by M. A. Rao and S. S. H. Rizvi*
20. Umami: A Basic Taste, *edited by Yojiro Kawamura and Morley R. Kare*
21. Food Biotechnology, *edited by Dietrich Knorr*
22. Food Texture: Instrumental and Sensory Measurement, *edited by Howard R. Moskowitz*
23. Seafoods and Fish Oils in Human Health and Disease, *John E. Kinsella*
24. Postharvest Physiology of Vegetables, *edited by J. Weichmann*
25. Handbook of Dietary Fiber: An Applied Approach, *Mark L. Dreher*
26. Food Toxicology, Parts A and B, *Jose M. Concon*
27. Modern Carbohydrate Chemistry, *Roger W. Binkley*
28. Trace Minerals in Foods, *edited by Kenneth T. Smith*
29. Protein Quality and the Effects of Processing, *edited by R. Dixon Phillips and John W. Finley*
30. Adulteration of Fruit Juice Beverages, *edited by Steven Nagy, John A. Ataway, and Martha E. Rhodes*
31. Foodborne Bacterial Pathogens, *edited by Michael P. Doyle*
32. Legumes: Chemistry, Technology, and Human Nutrition, *edited by Ruth H. Matthews*
33. Industrialization of Indigenous Fermented Foods, *edited by Keith H. Steinkraus*
34. International Food Regulation Handbook: Policy • Science • Law, *edited by Roger D. Middlekauff and Philippe Shubik*
35. Food Additives, *edited by A. Larry Branen, P. Michael Davidson, and Seppo Salminen*
36. Safety of Irradiated Foods, *J. F. Diehl*
37. Omega-3 Fatty Acids in Health and Disease, *edited by Robert S. Lees and Marcus Karel*
38. Food Emulsions: Second Edition, Revised and Expanded, *edited by Kåre Larsson and Stig E. Friberg*
39. Seafood: Effects of Technology on Nutrition, *George M. Pigott and Barbee W. Tucker*
40. Handbook of Vitamins: Second Edition, Revised and Expanded, *edited by Lawrence J. Machlin*
41. Handbook of Cereal Science and Technology, *Klaus J. Lorenz and Karel Kulp*
42. Food Processing Operations and Scale-Up, *Kenneth J. Valentas, Leon Levine, and J. Peter Clark*
43. Fish Quality Control by Computer Vision, *edited by L. F. Pau and R. Olafsson*
44. Volatile Compounds in Foods and Beverages, *edited by Henk Maarse*
45. Instrumental Methods for Quality Assurance in Foods, *edited by Daniel Y. C. Fung and Richard F. Matthews*
46. Listeria, Listeriosis, and Food Safety, *Elliot T. Ryser and Elmer H. Marth*
47. Acesulfame-K, *edited by D. G. Mayer and F. H. Kemper*
48. Alternative Sweeteners: Second Edition, Revised and Expanded, *edited by Lyn O'Brien Nabors and Robert C. Gelardi*

49. Food Extrusion Science and Technology, *edited by Jozef L. Kokini, Chi-Tang Ho, and Mukund V. Karwe*
50. Surimi Technology, *edited by Tyre C. Lanier and Chong M. Lee*
51. Handbook of Food Engineering, *edited by Dennis R. Heldman and Daryl B. Lund*
52. Food Analysis by HPLC, *edited by Leo M. L. Nollet*
53. Fatty Acids in Foods and Their Health Implications, *edited by Ching Kuang Chow*
54. *Clostridium botulinum*: Ecology and Control in Foods, *edited by Andreas H. W. Hauschild and Karen L. Dodds*
55. Cereals in Breadmaking: A Molecular Colloidal Approach, *Ann-Charlotte Eliasson and Kåre Larsson*
56. Low-Calorie Foods Handbook, *edited by Aaron M. Altschul*
57. Antimicrobials in Foods: Second Edition, Revised and Expanded, *edited by P. Michael Davidson and Alfred Larry Branen*
58. Lactic Acid Bacteria, *edited by Seppo Salminen and Atte von Wright*
59. Rice Science and Technology, *edited by Wayne E. Marshall and James I. Wadsworth*
60. Food Biosensor Analysis, *edited by Gabriele Wagner and George G. Guilbault*
61. Principles of Enzymology for the Food Sciences: Second Edition, *John R. Whitaker*
62. Carbohydrate Polyesters as Fat Substitutes, *edited by Casimir C. Akoh and Barry G. Swanson*
63. Engineering Properties of Foods: Second Edition, Revised and Expanded, *edited by M. A. Rao and S. S. H. Rizvi*
64. Handbook of Brewing, *edited by William A. Hardwick*
65. Analyzing Food for Nutrition Labeling and Hazardous Contaminants, *edited by Ike J. Jeon and William G. Ikins*
66. Ingredient Interactions: Effects on Food Quality, *edited by Anilkumar G. Gaonkar*
67. Food Polysaccharides and Their Applications, *edited by Alistair M. Stephen*
68. Safety of Irradiated Foods: Second Edition, Revised and Expanded, *J. F. Diehl*
69. Nutrition Labeling Handbook, *edited by Ralph Shapiro*
70. Handbook of Fruit Science and Technology: Production, Composition, Storage, and Processing, *edited by D. K. Salunkhe and S. S. Kadam*
71. Food Antioxidants: Technological, Toxicological, and Health Perspectives, *edited by D. L. Madhavi, S. S. Deshpande, and D. K. Salunkhe*
72. Freezing Effects on Food Quality, *edited by Lester E. Jeremiah*
73. Handbook of Indigenous Fermented Foods: Second Edition, Revised and Expanded, *edited by Keith H. Steinkraus*
74. Carbohydrates in Food, *edited by Ann-Charlotte Eliasson*
75. Baked Goods Freshness: Technology, Evaluation, and Inhibition of Staling, *edited by Ronald E. Hebeda and Henry F. Zobel*
76. Food Chemistry: Third Edition, *edited by Owen R. Fennema*
77. Handbook of Food Analysis: Volumes 1 and 2, *edited by Leo M. L. Nollet*
78. Computerized Control Systems in the Food Industry, *edited by Gauri S. Mittal*
79. Techniques for Analyzing Food Aroma, *edited by Ray Marsili*
80. Food Proteins and Their Applications, *edited by Srinivasan Damodaran and Alain Paraf*
81. Food Emulsions: Third Edition, Revised and Expanded, *edited by Stig E. Friberg and Kåre Larsson*
82. Nonthermal Preservation of Foods, *Gustavo V. Barbosa-Cánovas, Usha R. Pothakamury, Enrique Palou, and Barry G. Swanson*
83. Milk and Dairy Product Technology, *Edgar Spreer*
84. Applied Dairy Microbiology, *edited by Elmer H. Marth and James L. Steele*

85. Lactic Acid Bacteria: Microbiology and Functional Aspects, Second Edition, Revised and Expanded, *edited by Seppo Salminen and Atte von Wright*
86. Handbook of Vegetable Science and Technology: Production, Composition, Storage, and Processing, *edited by D. K. Salunkhe and S. S. Kadam*
87. Polysaccharide Association Structures in Food, *edited by Reginald H. Walter*
88. Food Lipids: Chemistry, Nutrition, and Biotechnology, *edited by Casimir C. Akoh and David B. Min*
89. Spice Science and Technology, *Kenji Hirasa and Mitsuo Takemasa*
90. Dairy Technology: Principles of Milk Properties and Processes, *P. Walstra, T. J. Geurts, A. Noomen, A. Jellema, and M. A. J. S. van Boekel*
91. Coloring of Food, Drugs, and Cosmetics, *Gisbert Otterstätter*
92. *Listeria*, Listeriosis, and Food Safety: Second Edition, Revised and Expanded, *edited by Elliot T. Ryser and Elmer H. Marth*
93. Complex Carbohydrates in Foods, *edited by Susan Sungsoo Cho, Leon Prosky, and Mark Dreher*
94. Handbook of Food Preservation, *edited by M. Shafiur Rahman*
95. International Food Safety Handbook: Science, International Regulation, and Control, *edited by Kees van der Heijden, Maged Younes, Lawrence Fishbein, and Sanford Miller*
96. Fatty Acids in Foods and Their Health Implications: Second Edition, Revised and Expanded, *edited by Ching Kuang Chow*
97. Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality, *edited by Norman F. Haard and Benjamin K. Simpson*
98. Safe Handling of Foods, *edited by Jeffrey M. Farber and Ewen C. D. Todd*
99. Handbook of Cereal Science and Technology: Second Edition, Revised and Expanded, *edited by Karel Kulp and Joseph G. Ponte, Jr.*
100. Food Analysis by HPLC: Second Edition, Revised and Expanded, *edited by Leo M. L. Nollet*
101. Surimi and Surimi Seafood, *edited by Jae W. Park*
102. Drug Residues in Foods: Pharmacology, Food Safety, and Analysis, *Nickos A. Botsoglou and Dimitrios J. Fletouris*
103. Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, *edited by Luis M. Botana*
104. Handbook of Nutrition and Diet, *Babasaheb B. Desai*
105. Nondestructive Food Evaluation: Techniques to Analyze Properties and Quality, *edited by Sundaram Gunasekaran*
106. Green Tea: Health Benefits and Applications, *Yukihiko Hara*
107. Food Processing Operations Modeling: Design and Analysis, *edited by Joseph Irudayaraj*
108. Wine Microbiology: Science and Technology, *Claudio Delfini and Joseph V. Formica*
109. Handbook of Microwave Technology for Food Applications, *edited by Ashim K. Datta and Ramaswamy C. Anantheswaran*
110. Applied Dairy Microbiology: Second Edition, Revised and Expanded, *edited by Elmer H. Marth and James L. Steele*
111. Transport Properties of Foods, *George D. Saravacos and Zacharias B. Maroulis*
112. Alternative Sweeteners: Third Edition, Revised and Expanded, *edited by Lyn O'Brien Nabors*
113. Handbook of Dietary Fiber, *edited by Susan Sungsoo Cho and Mark L. Dreher*
114. Control of Foodborne Microorganisms, *edited by Vijay K. Juneja and John N. Sofos*
115. Flavor, Fragrance, and Odor Analysis, *edited by Ray Marsili*
116. Food Additives: Second Edition, Revised and Expanded, *edited by A. Larry Branen, P. Michael Davidson, Seppo Salminen, and John H. Thorngate, III*

117. Food Lipids: Chemistry, Nutrition, and Biotechnology: Second Edition, Revised and Expanded, *edited by Casimir C. Akoh and David B. Min*
118. Food Protein Analysis: Quantitative Effects on Processing, *R. K. Owusu-Apenten*
119. Handbook of Food Toxicology, *S. S. Deshpande*
120. Food Plant Sanitation, *edited by Y. H. Hui, Bernard L. Bruinsma, J. Richard Gorham, Wai-Kit Nip, Phillip S. Tong, and Phil Ventresca*
121. Physical Chemistry of Foods, *Pieter Walstra*
122. Handbook of Food Enzymology, *edited by John R. Whitaker, Alphons G. J. Voragen, and Dominic W. S. Wong*
123. Postharvest Physiology and Pathology of Vegetables: Second Edition, Revised and Expanded, *edited by Jerry A. Bartz and Jeffrey K. Brecht*
124. Characterization of Cereals and Flours: Properties, Analysis, and Applications, *edited by Gönül Kaletunç and Kenneth J. Breslauer*
125. International Handbook of Foodborne Pathogens, *edited by Marianne D. Miliotis and Jeffrey W. Bier*

Additional Volumes in Preparation

Handbook of Dough Fermentations, *edited by Karel Kulp and Klaus Lorenz*

Extraction Optimization in Food Engineering, *edited by Constantina Tzia and George Liadakis*

Physical Principles of Food Preservation: Second Edition, Revised and Expanded, *Marcus Karel and Daryl B. Lund*

Handbook of Vegetable Preservation and Processing, *edited by Y. H. Hui, Sue Ghazala, Dee M. Graham, K. D. Murrell, and Wai-Kit Nip*

Food Process Design, *Zacharias B. Maroulis and George D. Saravacos*

For Mum and Dad, Elizabeth, James, Richard, Candida, and Afia

Preface

There is no book dealing with food protein analysis exclusively, that is, with the analysis of proteins in the food system. This book attempts to fill this niche. Protein analysis comes in two forms: 1) Quantitative analysis, and 2) fractionation and characterization. The first activity is described here. This publication provides a reference for planning, performing and interpreting assays for food proteins. Many approved methods derive from the late-19th century, but they have undergone rigorous testing and modernization. This book does not focus on reviewing the latest research methods for protein analysis. With the exceptions of Chapters 6 and 7, each of the 14 self-contained chapters describes one protein assay—principles, practices, and expected results.

This book describes the effect of food processing on protein assay results with the emphasis on how to analyze proteins in real foods. A number of “Methods” sections provide instructions for specific tests. Sample pretreatment and clean-up procedures are described. General pretreatment strategies help in the avoidance of interference. More specific clean-up methods apply to particular protein assays and are described along with these. Example results, performance characteristics, case reports, and practical problems and solutions related to a wide range of foods are detailed in numerous figures, tables, and references.

Food protein analysis is a hugely important activity performed by thousands worldwide. The book should appeal to professionals interested in food proteins and anyone working in the food system formerly called the food chain. This includes researchers and workers in agricultural production, food processing, and wholesale and/or retail marketing. It provides information for the grain or dairy farmer, extension worker, agricultural scientist, food scientists and technologists, or college professor. Some techniques described in this book were first used by clinicians, nutritionists, and veterinary scientists. The book may also be of interest to those in small businesses, private or government laboratories, research institutes, colleges, and universities. It will be useful to undergraduate, postgraduate, or postdoctoral students. Sections dealing with mechanisms assume graduate level chemistry and/or analytical biochemistry.

Any shortcomings of this project are wholly my responsibility. I thank all those colleagues worldwide whose research is reported here. My thanks to Anna Dolezal, Mr. DeSouza and Professor Arthur Finch for teaching me to think for myself. I am grateful to my past students: Drs. Yetunde Folawiyo, Despina Galani, Michael Anaydiegwu, Kiattisak Duangmal, Pitaya Adulyatham, Kwanele Mdluli, Halima Omar and Sripaarna Banerjee for raising my awareness of protein assay issues and for reading parts of the manuscript. Thanks to Dr. Bob Roberts (The Pennsylvania State University) for his advice on combustion methods. I am grateful to Dr. S. Khokhar and Marcel Dekker, Inc., for their commitment. I am also grateful to my family for their support.

R. K. Owusu-Apenten

Contents

Preface

Part 1. Fundamental Techniques

- Chapter 1. Kjeldahl Method, Quantitative Amino Acid Analysis and Combustion Analysis
1. Introduction to Food Protein Analyses
 2. Kjeldahl Analysis
 3. Colorimetric Analysis of Kjeldahl Nitrogen
 4. Quantitative Amino Acid Analysis
 5. Combustion Nitrogen Analyzers
- References

Part 2. Copper Binding Methods

- Chapter 2. The Alkaline Copper Reagent: Biuret Assay
1. Introduction
 2. The Alkaline Copper Reagent Protein Assay
 3. Chemistry of the Alkaline Copper Reagent Protein Assay
 4. Interference Compounds

5. Sample Pretreatment and Avoiding Interferences
6. The Micro-Biuret or Ultraviolet Biuret Protein Analysis
7. Applications of the ACR Solution for Food Protein Analysis

References

Chapter 3. The Lowry Method

1. Introduction
2. The Lowry Protein Assay
3. Chemistry of the Lowry Assay
4. Calibration Features
5. Interference Compounds
6. Sample Pretreatment, Avoiding Interferences, and Ensuring Accuracy
7. Applications of Lowry Assays to Food Protein Analysis

References

Chapter 4. The Bicinchoninic Acid Protein Assay

1. Introduction
2. The BCA Protein Assay
3. Chemistry of the BCA Protein Assay
4. Calibration Features
5. Interference Compounds
6. Sample Pretreatment, Avoiding Interference, Ensuring Accuracy
7. Automated BCA Protein Assays
8. Applications of the BCA Assay to Food Protein Analysis

References

Part 3. Dye Binding Methods

Chapter 5. The Udy Method

1. Introduction
2. The Udy Method
3. Solid-Phase Dye-Binding Assays
4. The Chemistry of Dye-Binding Protein Assays
5. Interference Compounds and Their Avoidance
6. Applications of Dye-Binding Assays for Food Protein Analysis

References

Chapter 6. The Bradford Method—Principles

1. Introduction

2. Theory of the Bradford Assay
 3. Effect of Protein-Dye Binding Parameters on the Bradford Assay
 4. Linearization Plots for the Bradford Assays
 5. Assay Sensitivity and the Maximum Number of Dye Binding Sites
 6. Solid-Phase Dye-Binding Assays
 7. Interference Compounds and Sample Pretreatment
- References

Chapter 7. Bradford Assay—Applications

1. Introduction
 2. Coomassie Brilliant Blue Dye-Binding Assays
 3. Performance Characteristics of CBBG Dye-Binding Assays
 4. Applications to Food Protein Analysis
- References

Part 4. Immunological Methods for Protein Speciation

Chapter 8. Immunological Assay: General Principles and the Agar Diffusion Assay

1. Introduction
 2. Immunological Methods
 3. Speciation of Proteins by Agar Gel Double Immunodiffusion Assay
- References

Chapter 9. Speciation of Meat Proteins by Enzyme-Linked Immunosorbent Assay

1. Introduction
 2. Raw Meat Speciation by Indirect ELISA
 3. Raw Meat Speciation by Sandwich ELISA
 4. Muscle Protein Antigens for ELISA
 5. Cooked Meat Analysis by ELISA
 6. Monoclonal Antibodies for Meat Speciation
 7. Fish and Seafood Identification by ELISA
 8. Performance Characteristics for Different ELISA Formats
 9. Meat Testing for Transmissible Spongiform Encephalopathy Agents
- References

- Chapter 10. Speciation of Soya Protein by Enzyme-Linked Immunoassay
1. Introduction
 2. Sample Pretreatment and Analysis of Soy Protein
 3. Structure, Denaturation, and Renaturation of Soybean Proteins
 4. Solvent-Extractable Soybean Protein
 5. Thermostable Antigens for Soybean Protein Analysis
 6. Other Nonmeat Proteins
- References

- Chapter 11. Determination of Trace Protein Allergens in Foods
1. Introduction
 2. Soya Bean Protein Allergens
 3. Peanuts
 4. Wheat and Related Cereals
- References

Part 5. Protein Nutrient Value

- Chapter 12. Biological and Chemical Tests for Protein Nutrient Value
1. Introduction
 2. Human and Other In Vivo Assays for Protein Nutrient Value
 3. Small Animal Bioassays for Protein Nutrient Value
 4. In Vitro Methods for Assessing Protein Nutrient Value
 5. Protein Digestibility
- References

- Chapter 13. Effect of Processing on Protein Nutrient Value
1. Introduction
 2. Milk and Milk Powders
 3. Infant Formulas
 4. Feedstuffs and Concentrates for Livestock
 5. Legumes and Oilseeds
 6. Cereal and Cereal Products
 7. Improving Cereal Protein Quality by Screening
- References

- Chapter 14. Protein Digestibility–Corrected Amino Acid Scores
1. Introduction
 2. Protein Digestibility
 3. Protein Denaturation
 4. Chemical Deterioration of Protein Ingredients

5. Matrix Effects on the Rate of Deterioration of Protein Ingredients
6. Protein Digestibility–Corrected Amino Acid Scores (PDCAAS)

References

Kjeldahl Method, Quantitative Amino Acid Analysis and Combustion Analysis

1. INTRODUCTION TO FOOD PROTEIN ANALYSES

Protein analysis is a subject of enormous economic and social interest. The market value of the major agricultural commodities (cereal grains, legumes, flour, oilseeds, milk, livestock feeds) is determined partly by their protein content. Protein quantitative analysis is necessary for quality control and is a prerequisite for accurate food labeling. Proteins from different sources have varying aesthetic appeal to the consumer. Compliance with religious dietary restrictions means excluding certain protein (sources) from the diet. The variety of protein consumed is also extremely important in relation to food allergy. Detecting undeclared protein additives and substitutions is a growing problem. Proteins show differing nutritional quality or ability to support dietary needs. In summary, protein analysis has legal, nutritional, health, safety, and economic implications for the food industry (1).

The estimated global food production total for 1988 was 4 billion metric tons. Allowing an average of 10% protein in foodstuffs yields 400 million metric tons of protein annually (2). Nonetheless, sensitivity is a major consideration for protein analysts. Some immunological methods can detect nanomole (10^{-9} mole) amounts of protein. Other important considerations when choosing a method for food protein analysis include

TABLE 1 Approximate Chronology for Methods for Food Protein Analysis

Date	Technique
1831	Dumas ^a
1843	Nessler's reagent ^a
1849	Biuret method
1859	Alkali-phenol reagent or Bethelot's method ^a
1883	Kjeldahl ^a
1927	Folin-Ciocalteau
1944	Dye binding ^a
1951	Lowry
1960	Direct alkaline distillation
1960	Near-infrared reflectance (NIR) ^a
1971	Modified Berthelot reaction
1975	Modified Lowry method (Peterson)
1976	Bradford method (Coomassie Blue binding method)
1985	Bicinchoninic acid (BCA) method

^aTechniques for which semiautomated or fully automated apparatus has been manufactured.

high sample throughput, simplicity, and low capital costs. Some of the most significant methods (Dumas, Kjeldahl, and biuret assays) date from the late 1800s (Table 1). Techniques for food protein analysis are described in this book. I will focus on the techniques that feature most often in the food science literature. Infrared analysis of food proteins is not discussed here.

1.1. Characteristics of Food Protein Assays

Techniques for food protein analysis need to be robust. This means one of several things. Foremost is compatibility with fresh produce (cereals, fruits, vegetables, meat, milk) and processed foods. Samples in various physical states (powders, slurries, dilute liquids, emulsions, gels, pastes) should be analyzable. A robust assay will also deal effectively with foods from either animal or plant sources. Such techniques are unaffected by the presence of dyes or pigments that absorb infrared, visible, or ultraviolet light. A robust protein assay needs minimal sample pretreatment, which increases error and decrease analytical precision. Sample cleanup also increases the time per analysis (reduces sample throughput) and adds to costs. In the worst-case scenario, pretreatment can be too invasive, thereby invalidating results. In summary, a robust protein assay is simple, quick, sensitive, and reliable. It is also compatible with a diverse range of foods. The economic imperative

leads to a preference for techniques requiring low capital expenditure and minimum training. Laboratories handling more than 8000 analyses per year tend to select techniques on the basis of their speed and ease of operation. A high sample throughput is usually achieved by automation or continuous flow analysis (CFA). A rough “time line” for some food protein assays is given in Table 1. Common descriptive terms for protein analysis are defined in Table 2.

Kjeldahl analysis gives accurate protein readings no matter what the physical state of the sample. This technique has approved status and is *the* reference method adopted by many national and international organizations. However, the use of hazardous and potentially toxic chemicals in Kjeldahl analysis is creating concern. The Dumas combustion method is comparatively quicker, cheaper, easier to perform, safer, and more environment friendly; it is now considered on equal terms with Kjeldahl analysis in the United States, Canada, and Western Europe. Dye binding is another robust test for proteins (3,4). The biuret method is widely used,

TABLE 2 Some Important Calibration Indices and a Brief Explanation of Their Meaning

Calibration feature	Explanation
Linear dynamic range	Range over which signal is proportional to analyte concentration
Sensitivity	Slope of the calibration graph; analytical response per unit change in protein concentration. cf. parameters a , a' in Eqs. (1)–(4)
Accuracy	Degree of agreement of results with a true value
Precision, repeatability, or reproducibility	Agreement between repeated measurements taken with a single sample or with different paired samples
Specificity	Ability to discriminate between protein and interfering substance. Ratio of sensitivity for the analyte and interference
Reliability	A composite parameter combining specificity, accuracy, precision, and sensitivity
Lower limit of detection (LLD)	Minimal protein concentration detectable above background noise
Sample throughput (time per analysis)	Numbers of samples analyzed per unit time, speed of analysis

especially for cereal proteins (5). Procedures involving copper-based reagents (Lowry and bicinchoninic acid assays) continue to be important. Finally, a range of empirical (viscosity, refractive index, specific gravity) measurements are used for protein quantitation within industry.

1.2. Calibration and Statistical Principles

The two common forms of calibration are (a) method calibration and (b) sample calibrations. With *method calibration* a set of food samples are analyzed using a new test method and a *reference* method that has been validated by a committee of the Association of Official Analytical Chemists (AOAC). A calibration graph is then drawn by plotting results from the reference method (% Kjeldahl protein) on the *Y*-axis and the test results on the *X*-axis. The X_i and Y_i observations are usually related by an equation for a straight line:

$$Y_i = aX_i + b \quad (1)$$

where a is the gradient and b is the intercept for the calibration graph. For each X_i result we can determine the *calculated* % Kjeldahl protein value (Y_{calc}) via Eq. (2).

$$Y_{\text{calc}} = aX_i + b \quad (2)$$

Values for Y_i and Y_{calc} can be compared in order to evaluate the test method (see later). Some investigators choose to plot the Kjeldahl results on the *X*-axis. Therefore, rather than Eq. (1) we get

$$Y_i^* = a'X^* + b' \quad (3)$$

where X_i^* is % Kjeldahl protein and Y_i^* is the test result. To compare Eq. (1) and Eq. (3), notice that $a' = 1/a$ and $b' = Y_i - (X_i / a)$.

For *sample* calibration, the assay technique is assumed to be valid. We analyze a set of (standard) samples containing known amounts of protein. In Eq. (1), X_i now represents a range of known protein concentrations and Y_i are the corresponding instrument responses. Calibration factors (a , b , etc.) can be determined from simple algebra or statistical analysis of paired (X_i , Y_i) results. From the principles of least-squares analysis,

$$a = \frac{\sum(X_i - X_m)/(Y_i - Y_m)}{\sum(X_i - X_m)^2} \quad (4)$$

and

$$b = Y_m - aX_m \quad (5)$$

where X_m and Y_m are the mean values for all X_i and Y_i observations.

Agreement between the reference and test results is measured by the correlation coefficient (R); $R \approx 1$ shows excellent agreement. When Y_i and Y_{calc} observations are poorly correlated, $R \approx 0$. The squared correlation coefficient (R^2) can be calculated from Eq. (6). Most handheld calculators can perform this operation automatically.

$$R^2 = 1 - \left[\frac{(Y_i - Y_{\text{calc}})^2}{\sum (Y_i - Y_m)^2} \right] \quad (6)$$

Precision is another measure of the (dis)agreement between Y_i and Y_{calc} values. This can be expressed as the standard deviation (SD) or coefficient of variation (CV). High-precision methods produce low values for the SD and CV.

$$(\text{SD})^2 = \frac{\sum (Y_i - Y_{\text{calc}})^2}{n - 2} \quad (7)$$

$$\text{CV} = (\text{SD}/Y_m) \times 100 \quad (8)$$

We can also measure precision (commonly called error) from n -replicate (Y_i) measurements on a single test sample. Thereafter, the numerator in Eq. (7) becomes $(Y_i - Y_m)^2$, which is the square of the differences between individual observations and the mean for all observations. A low CV implies good agreement between successive test results.

1.3. Assay Performance

Calibration parameters can provide a great deal of other information about assay performance (Table 2). The *linear dynamic range* is the concentration range over which a linear relationship exists between the instrumental response and protein concentration. *Sensitivity* is the slope of the calibration graph, and the lower limit of detection (LLD) is smallest quantity of sample that triggers an instrumental response above the background noise. The LLD is dependent on the instrument baseline quality and assay sensitivity.

It is common to refer to “sensitivity” when we mean the LLD. We differentiate between sensitivity and LLD via the following exercise. Measure the instrument baseline noise by recording the output (Y_o) and

the standard deviation (SD_o) using a sample *blank*. The smallest instrumental response that can be distinguished from “random noise” in 95% of all cases is $Y_o \pm 2.326SD_o$. Now substitute for $Y_i (= Y_o + 2.326 SD_o)$ and $X_i (= LLD)$ in Eq. (1), leading to the following expression:

$$LLD = \frac{(Y_o + 2.326SD_o) - b}{a} \tag{9}$$

Usually Y_o and b are both set to zero when the analyst sets the instrument baseline response to zero. Consequently, Eq. (9) becomes

$$LLD = 2.326SD_o/a \tag{10}$$

This relation shows that LLD *decreases* with increasing assay sensitivity and with increasing baseline quality (see decrease in the value for SD_o). In order to ensure high sensitivity, it is important to obtain a stable instrumental baseline.

1.4. Calibrating Protein Assays

The Kjeldahl method is used for calibrating other protein assays. Duda and Szot (6) evaluated six methods for analyzing porcine plasma protein during its manufacture. The techniques are simple and therefore of wider interest (Table 3). The protein content of porcine plasma was 5.58% (w/v). All techniques showed a good correlation with Kjeldahl results ($R=0.905\text{--}0.952$). The precision for density and Kjeldahl assays was the same ($CV=10.8\%$). The sensitivity of the former method was better. With appropriate calibration, density or viscosity measurements could be suitable for the routine analysis during the manufacture of plasma proteins.

TABLE 3 Some Simple Methods for Evaluating Porcine Plasma Protein

Method	Instrument
Densitometry	Standard picnometer
Refractometry	Laboratory refractometer
Modified refractometry	Laboratory refractometer
UV absorbance (215/225 nm)	UV spectrophotometer
UV absorbance (241 nm)	UV spectrophotometer
UV absorbance (280 nm)	UV spectrophotometer

Williams et al. (7) calibrated beer protein analyses using quantitative sodium dodecyl sulfate polyacrylamide gel electrophoresis (QSDS-PAGE). A range of test methods were investigated including biuret, bicinchoninic acid (BCA), Bradford, Kjeldahl, Lowry, and pyrogallol-red molybdate (PRM) assays. QSDS-PAGE revealed that beer has between 0.5 and 1 mg mL⁻¹ protein. Only the Bradford and PRM assays gave accurate results (Fig. 1). The main sources of error were low-molecular-weight interferences. Beer contains plant pigments, starch, sugars, alcohol, and natural dyes of barley origin. Both Kjeldahl and combustion analyses were subject to interferences by nonprotein nitrogenous (NPN) compounds. Dialysis did not improve accuracy for BCA, Lowry, and biuret assays, which were affected by high-molecular-weight Cu- reducing agents such as pectin and starch.

Calibration issues are discussed in two articles by Pomeranz and co-workers (8,9). They considered the *reliability* of several test methods (biuret, dye binding, infrared reflectance, alkaline distillation method) for analyzing proteins in hard red winter wheat varieties from the American Great Plains. The test methods were highly correlated with the Kjeldahl assay ($R=0.976-0.992$). The order of precision was Kjeldahl > biuret > dye binding > infrared analysis. Pomeranz and More (9) also considered the reliability of four “rapid” methods for barley or malt protein analysis.* A summary of assay performance statistics is given in Table 4. For barley samples, the precision and sensitivity of analysis were highest for the Kjeldahl and infrared analyses. The use of Kjeldahl analysis to calibrate protein assays for dairy products was discussed by Luithi-Pent and Puhan (10) and also Lynch and Barbano (11).

2. KJELDAHL ANALYSIS

Johan Kjeldahl was born on August 16, 1849 in the town of Jaegerpris in Denmark. In 1876 he was employed by the Carlsberg brewery to develop an improved assay for grain protein. The Kjeldahl method was published in 1883. The original technique has been extensively modified. Key steps for the assay are (a) sample digestion, (b) neutralization, (c) distillation and trapping of ammonia, and (d) titration with standard acid. An exhaustive

* For the purposes of calibration, 44 samples of barley and 49 samples of malt were analyzed with biuret, dye binding, infrared, alkaline distillation, and Kjeldahl tests. Such results were the basis for deriving calibration relations between Kjeldahl and each test method. Then a further 76 samples of barley and 72 samples of malt were analyzed using only the rapid test methods. Each X_i result gave rise to a corresponding Kjeldahl protein (Y_{calc}) value.

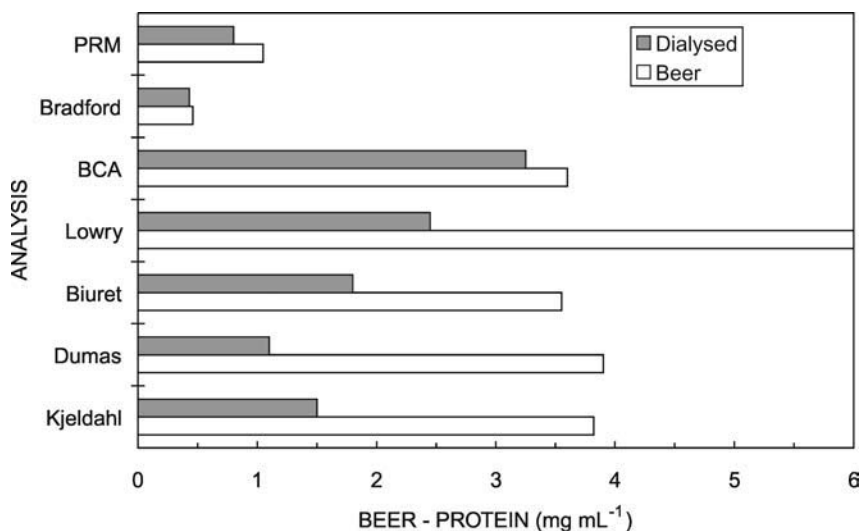


FIGURE 1 Apparent protein concentrations in stout beer as determined by seven methods. (Data from Ref. 7.)

TABLE 4 Barley Protein Analysis Using a Range of Techniques

Test method	Analysis time (min)	Regression line and correlation coefficient ^a	Standard error of analysis ^b
Biuret	10	$Y = 0.857 \text{ cP} + 1.942$ $R = 0.972$	0.336 and 0.2336
Dye binding ^c	15	—	—
Infrared	0.5–1.0	$Y = 1.060 \text{ cP} - 1.03$ $R = 0.96$	0.838–1.980
Alkaline distillation	90	$Y = 1.070 \text{ cP} - 0.670$	2.383 and 1.540

^acP, crude protein determined by Kjeldhal method ($N \times 6.25$); Y , response from the test method.

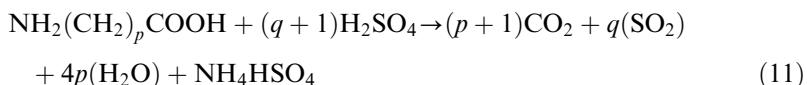
^bAssay standard error calculated from $\sum(Y_{\text{calc}} - Y_i)^2/(n)$.

^cNo information given.

Source: Ref. 8.

account of the Kjeldahl method can be found in the monograph by Bradstreet (12). The book is divided into five chapters: 1, introduction to the Kjeldahl method; 2, the Kjeldahl digestion; 3, digestion procedure (for fertilizers, leather, cereals, foods and proteins, coal and fuels); 4, the distillation and detection of ammonia. Chapter 5 is an extensive bibliography. A standardized Kjeldahl procedure appears in the International Standard ISO-1871 (13). Further descriptions are given by Gaspar (14) and Osborne (15).

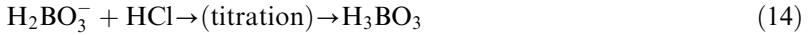
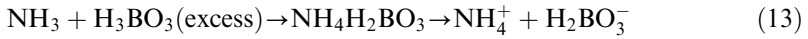
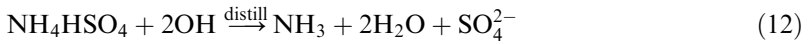
Initially, only sulfuric acid was used for sample digestion. Then solid potassium permanganate was added to facilitate sample oxidation. Mercuric oxide was introduced as a catalyst in 1885. During the acid digestion phase, the food sample is heated with concentrated sulfuric acid, which causes dehydration and charring. Above a sample decomposition temperature, carbon, sulfur, hydrogen, and nitrogen are converted to carbon dioxide, sulfur dioxide, water, and ammonium sulfate [Eq. (11)].



Digestion is complete when the mixture turns clear (light green color), usually after 20–30 minutes of heating. A further (after-boiling) period of heating is necessary to ensure quantitative recovery of nitrogen. Data from McKenzie and Wallace (cited in Ref. 14) show that adding X (mg) of potassium sulfate per mL of sulfuric acid increases its boiling point according to the relation $Y(^{\circ}\text{C}) = 55.8X + 331.2$. A maximum boiling point elevation of 130°C is achievable by adding 2 mg (potassium sulfate) per mL acid. A high boiling point reduces the sample digestion time. Sample digestion can also be facilitated by using a catalyst; the order of effectiveness for metal oxide catalysts is $\text{Hg} > \text{Se} > \text{Te} > \text{Ti} > \text{Mo} > \text{Fe} > \text{Cu} > \text{V} > \text{W} > \text{Ag}$ (16). A proprietary brand of Kjeldahl catalyst (Kjeltabs from Foss Electric Ltd.) comes as tablets. Each tablet contains 0.25 g of mercuric oxide and 5 g of potassium sulfate. A working selenium catalyst can be formulated with potassium sulfate (32 g), mercuric sulfate (5 g), and selenium powder (1 g). Chemical oxidants (hydrogen peroxide, perchloric acid, or chromic acid) can be added to the sulfuric acid to speed up sample digestion.

Ammonium sulfate is first neutralized with alkali to form ammonia. This is then distilled and trapped using 4% boric acid. Ammonium borate is then titrated with standard acid in the presence of a suitable indicator. Low-cost Quick-fit glassware is readily available for distillation and titration. Sophisticated semiautomatic distillation systems are also available. The

processes of neutralization, distillation, and titrimetric analyses are summarized as follows.



Suitable titration indicators include methyl orange, methyl red, Congo red, and Tashiro indicator (a 1:1 mixture of 0.2% methyl red solution and 0.1% methylene blue).

2.1. Nitrogen-to-Protein Conversion Factors

The Kjeldahl technique measures sample nitrogen (S_N) as ammonia. The value for S_N is later converted to crude protein (cP) by multiplying by a Kjeldahl factor, F_K .

$$\text{cP}(\%) = S_N F_K \quad (15)$$

The units for S_N are g-N 100 g⁻¹ (g-nitrogen released per 100 g of sample). The F_K (g-protein g⁻¹ N) is the amount of protein that produces a gram of nitrogen. F_K is also called the nitrogen-to-protein conversion factor. AOAC-recommended values for F_K for meat and other food are summarized by Benedict (17). Frequently, F_K is given a default value of 6.25 or 5.7 for animal and plant proteins, which are assumed to have an average N content of 16% and 17.5%, respectively. In fact, most proteins deviate significantly from these averages (18). F_K is also affected by the presence of NPN (e.g., adenine, ammonia, choline, betaine, guanidine, nucleic acid, urea, free amino acids). Soya beans have 3–10% NPN, which increases to about 30% for immature seeds. The amount of NPN also changes with growth conditions as well as with geographic factors. There is generally no correlation between NPN and protein content (19). No single F_K value applies to all food types. Ideally, F_K should be determined for each individual food type (Table 5).

F_K can be calculated from amino acid data (18,20–24). Table 5 lists the 20 naturally occurring amino acids along with their formula weight, number of nitrogen atoms, percent nitrogen, and the value for F_K . For arginine, F_K is 3.11 (= 100/32.15). An idealized protein having all 20 amino acids in equal numbers has a nitrogen content of 14.73%. The F_K value is therefore 6.79 (100 g/14.73). Evaluating F_K from amino acid data (for

TABLE 5 Determination of the Kjeldahl Factor for Milk Protein Using Amino Acid Composition Data^a

1	2	3	4	5	6	7	8	9	10
Amino Acid	Formula weight (b_i)	N atoms	% N	Amino Acid F_K	AA composition (mg/g N)	AA-N (mg/g N)	AA (moles/g N)	Mole fraction (X_i)	$b_i X$
Arginine	174.2	4	32.15	3.11	234.0	75.2	0.001343	0.027082	4.71761
Histidine	155.2	3	27.06	3.70	188.0	50.9	0.001211	0.024422	3.79022
Asparagine	132.1	2	21.20	4.72	292	61.9	0.00221	0.044564	5.88694
Glutamine	146.1	2	19.16	5.22	783	150.1	0.005359	0.108048	15.7859
Lysine	146.2	2	19.15	5.22	487.0	93.3	0.003331	0.067157	9.81828
Glycine	75.1	1	18.64	5.36	128.0	23.9	0.001704	0.034362	2.58058
Alanine	89.1	1	15.71	6.36	219.0	34.4	0.002458	0.049553	4.4152
Tryptophan	204.2	2	13.71	7.29	90.0	12.3	0.000441	0.008886	1.81447
Serine	105.1	1	13.32	7.51	338.0	45.0	0.003216	0.064837	6.81433
Proline	115.1	1	12.16	8.22	571.0	69.5	0.004961	0.100016	11.5118
Valine	117.1	1	11.96	8.36	428.0	51.2	0.003655	0.073687	8.6288
Threonine	119.1	1	11.75	8.51	278.0	32.7	0.002334	0.047059	5.60469
Cystine	121.1	1	11.56	8.65	47.0	5.4	0.000388	0.007825	0.94756
Isoleucine	131.2	1	10.67	9.37	290.0	30.9	0.00221	0.044563	5.84662
Leucine	131.2	1	10.67	9.37	600.0	64.0	0.004573	0.092199	12.0964
Aspartic acid	133.1	1	10.52	9.51	214.0	22.5	0.001608	0.032415	4.3144
Glutamic acid	147.1	1	9.52	10.51	574.0	54.6	0.003902	0.078669	11.5723
Methionine	149.2	1	9.38	10.66	148.0	13.9	0.000992	0.019999	2.98379
Phenylalanine	165.2	1	8.47	11.80	341.0	28.9	0.002064	0.041615	6.87481
Tyrosine	181.2	1	7.73	12.94	297.0	22.9	0.001639	0.033045	5.98774
				Sum =	5669.7	943.5	0.04960	1.0	132.0
		Average =	14.73						
		$F_{K(1)} =$	6.79	$F_K =$	6.01				

^a $F_{K(1)}$ is determined from the average nitrogen content for all amino acids, i.e., 14.73%. $F_{K(2)}$ = value of whole protein or sum of amino acid nitrogen divided by sum of weight of nitrogen. $F_K = 5669.7/943.5$. Columns 8–10 contain data for calculating total protein content from quantitative amino acid analysis (Sec. 4).

skimmed milk) can be achieved in the following steps: (a) express each amino acid as mg per gram of total nitrogen (see column 6 of Table 5) and (b) calculate the mass of nitrogen derived from each amino acid (column 7 in Table 5). F_K is the weight of amino acids divided by the weight of amino acid nitrogen (AA-N).

$$F_K = \frac{\text{total weight of AA}}{\text{total weight of AA - N}} = \frac{5696.7}{943.5} = 6.01 \tag{16}$$

Some typical values for F_K are listed in Table 6 for a range of foods. The use of F_K values for quantitative amino acid analysis is discussed in Sec. 4.

TABLE 6 Nitrogen-Protein Conversion (F_K) Factors for Selected Food Protein Sources

Food product	F_K	Food product	F_K
Dairy products and egg		Roots and tuber	
Casein	6.15	Carrot	5.80
Milk	6.02	Beet	5.27
Cheese	6.13	Potato	5.18
Egg	5.73	Potato protein	5.94
Egg white solids	5.96		
Meat and fish products		Fruit	
Beef	5.72	Tomato	6.26
Chicken	5.82	Banana	5.32
Fish	5.82	Apple	5.72
Leafy vegetables		Microbial and fungal	
Lettuce	5.14	Yeast	5.78
Cabbage	5.30	Mushrooms	5.61
Cereals and legumes			
Wheat	5.71–5.75	Buckwheat	5.53
Rice	5.61–5.64	Oats	5.50
Corn	5.72	Millet	5.68
Sorghum	5.93	Mustard seed	5.40
Field pea	5.40	Rapeseed meal	5.53
Dry bean	5.44	Sunflower meal	5.36
Soya bean	5.69	Flax meal	5.41

Source: Data from Refs. 18 and 24.

2.2. Macro- and Micro-Kjeldahl Analysis

Kjeldahl digestion methods are discussed in this section. Illustrative examples are given to establish a pattern of work. Individual results are described in later parts of the chapter.

A. Grain and Cereals

Kaul and Sharma (25) analyzed a range of legume and cereal grains by micro-Kjeldahl analysis. About 200 mg of each sample was weighed into several 75-mL Kjeldahl digestion tubes. Concentrated sulfuric acid (3 mL), hydrogen peroxide (1.5 mL), and one Kjeltab tablet were added. The tubes were heated using a Tectator digestion block at 374°C for exactly 25 minutes and then allowed to cool. The contents of each tube were diluted to 75 mL and any ammonia produced quantified by colorimetric analysis (Sec. 3).

B. Potatoes

Mohyuddin and Mazza (26) analyzed proteins from 14 potato cultivars. Potato tubers were peeled, sliced, diced, and dried in a vacuum oven at 70°C and 48.8 mm Hg pressure. Each sample was milled and sieved through a 40-mesh sieve. Potato flour (100 mg) was added to each 100-mL Kjeldahl flask, followed by concentrated sulfuric acid (3 mL), hydrogen peroxide (30% solution; 1.5 mL), and commercial catalyst (500 mg; 10:0.7 w/w ratio of potassium sulfate and mercuric oxide). Heating for 45 minutes digested the samples. After cooling to room temperature, the contents of Kjeldahl flasks were diluted to 75 mL and then subjected to colorimetric analysis to determine ammonia.

C. Dried Milk Powder

Venter et al. (27) described a semimicro-Kjeldahl analysis for low-fat, medium-fat, and high-fat dried milk. About 200–250 mg of sample was mixed with 2.1 g of selenium catalyst and digested by heating with 10 mL of concentrated sulfuric acid. The digest was cooled and diluted to 100 mL with distilled water. Then 60 mL of 45% (w/v) NaOH solution was added and the liberated ammonia was distilled into 20 mL of 4% (w/v) boric acid solution. Titration was with 0.02 M HCl to an end point of pH 4.8. The results agreed well with the macro-Kjeldahl method [International IDF Standard (1962) No. 20].

D. Beer Protein

Concentrated sulfuric acid (2 mL) was added to 50 mL of beer (bitter, lager, or stout) and the mixture was heated until nearly dry (7). Kjeldahl catalyst (10 g) and more sulfuric acid (20 mL) were added, followed by further heating for 25 minutes. After cooling for 2 hours, water (250 mL) was added and the Kjeldahl flask was connected to a condenser with one end immersed in a 2% boric acid solution (200 mL). Bromocresol green was used as indicator. Sodium hydroxide (10 M, 70 mL) was added, followed by heating until the distillate tested neutral. The borate solution was later titrated with 0.1 N HCl. The nitrogen content in beer was calculated from the relation

$$\%N = 14V_a/W_d \tag{17}$$

where V_a (mL) is the volume of HCl required to neutralize ammonia and W_d (g) is the dry weight of beer. Table 7 summarizes characteristics of the Kjeldahl method used in the brewing and allied industries (28).

TABLE 7 Macro-Kjeldahl Procedures Currently in Use in the Brewing and Allied Industries

Parameter	Comment ^a
Instrumentation ^b	Kjel-Tec 1030 (Auto), Kjel-Tec 1007/ 1002*; Kjel-Foss 16120 [†]
Sample weight	0.92 ± 0.23 g (barley or malt) range 0.5–1.5 g, 14 ± 8.4 mL (beer) range 5–25 mL
Catalyst (weight range) ^c	K ₂ SO ₄ + CuSO ₄ + TiO ₂ ; 3.5–15.8 g
Volume of conc. H ₂ SO ₄	16 ± 4.3 mL, range 10–25 mL
Digestion temperature ^d	411 ± 14°C, range 380–430°C
Digestion time ^e	75 min or 9.6 min
End-point detection	Mainly colorimetric

^aAverage values unless otherwise stated.
^bLocal suppliers *Tectator Ltd. and Perstop Ltd., both of Bristol, UK, † Foss Electric (UK) Ltd., The Chantry, Bishopthorpe, York, UK.
^cA large amount of a single catalyst or a smaller quantity of a combination catalysts was used. HgO was used in 2 of the 25 laboratories.
^dDigestion temperatures were not reported for seven laboratories using a manual Kjeldahl technique.
^eDigestion time plus after-boiling time. The digestion time is 9.6 min when H₂O₂ is used as a prooxidant.
Source: Summarized from Ref. 28.

2.3. Automated Kjeldahl Analysis

Kjeldahl analysis has undergone three forms of automation. The Kjel-Foss[®] instrument mechanizes the entire micro-Kjeldahl procedure (digestion, neutralization, distillation, and titration). The Kjel-Tec[®] technique uses a digestion block in conjunction with apparatus for automated distillation and titrimetric analysis. The final form of automation is the Technicon AutoAnalyzer Instrument[®], which uses continuous flow analysis (CFA).

A. The Kjel-Foss Instrument

The Kjel-Foss instrument (N. Foss Electric Ltd., Hillerød, Denmark) performs the entire Kjeldahl procedure automatically (29–31). Automation reduces the analysis time from 3 hours to 6 minutes. The first analysis is completed in 12 minutes and succeeding analyses every 3 minutes. The sample throughput is 120–160 analyses per day. The Kjel-Foss instrument requires a reliable supply of electricity and tap water for installation and adequate drains and ventilation. A fume cupboard is not essential. Accuracy, precision, and economics of the Kjel-Foss method were compared with those of the manual Kjeldahl method, neutron activation analysis, proton activation analysis, combustion analysis, and the Kjel-Tech method (32). Results of the Kjel-Foss and manual Kjeldahl methods were highly correlated.

Fish meal was analyzed using the Kjel-Foss instrument by Bjarno (33). Seven collaborators compared the efficiency of antimony versus mercuric oxide as catalyst. After modifying the Kjel-Foss procedure slightly with higher acid settings, the differences in recovery and repeatability of the two procedures were $< \pm 1\%$. Using mercuric oxide catalyst poses environmental concerns if the effluent from the Kjel-Foss instrument is to be disposed of through the sewers. McGill (34) compared the Kjel-Foss method with an improved AOAC Kjeldahl method for meat and meat products. Over 80 analyses were performed with low (25%) fat, high (40%) fat, and dry sausage (50% fat). As shown in Fig. 2 and Table 8, the two techniques were highly correlated ($Y = 0.9904X + 0.1797$; $R^2 = 0.9896$). There was no systematic error in the Kjel-Foss technique over the range of protein concentrations examined by McGill. This work validated the Kjel-Foss instrument for meat product analysis.

Suhre et al. (35) also evaluated the Kjel-Foss instrument for meat analysis using the AOAC Kjeldahl method as reference. Twenty-three different laboratories analyzed six meat products having 10–30% crude protein. Eight laboratories used the automated Kjel-Foss instrument, five used the official AOAC method, and eleven used a block digester with steam

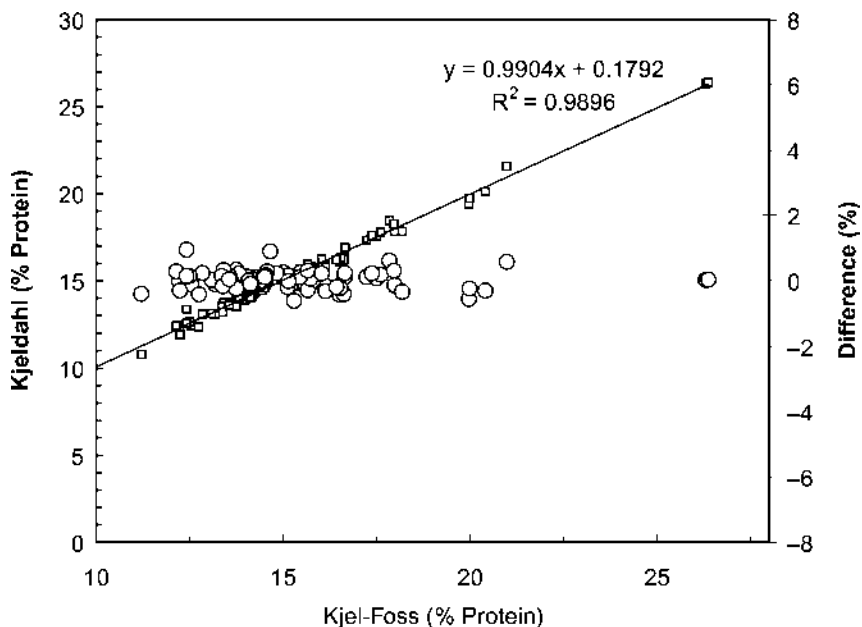


FIGURE 2 Calibration graph for the Kjel-Foss automated method for protein determinations. (Data from Table 1 of Ref. 34.)

distillation. Recommendations from this work led to the block digester–steam distillation method being awarded “first action” status.

B. Automated Kjeldahl Continuous Flow Analysis

The Technicon AutoAnalyzer has two reaction modules (36). The first module digests water-dispersible samples. The digest is then pumped to a second module (AutoAnalyzer Sampler II). Colorigenic reagents are added in quick succession before the flow stream passes to a delay coil to allow color formation. Ammonia is detected using Berthelot’s reaction or the ninhydrin assay (Sec. 3). The AutoAnalyzer was applied for protein determinations in plant material (37), feedstuffs (38), grain flour (39), instant breakfasts, meat analogues (40), meat products (41), and over 40 assorted canned and processed foods (42,43). In general AutoAnalyzer results agreed with micro-Kjeldahl analysis.

The AutoAnalyzer digestion unit is heated in two stages at 380–400°C and 300–320°C. To achieve efficient digestion, the ratio of acid to sample is

TABLE 8 A Comparison of the Automated Kjel-Foss and Approved Kjeldahl Method for Protein Analysis in Sausage Samples

Sausage type	Kjel-Foss (% protein)	Kjeldahl (% protein)
Low-fat sausages		
Bologna	14.64	14.54
Polish	14.42	14.43
Krakow	16.76	16.60
Liver	16.64	16.74
Hungarian	16.01	16.02
Medium-fat sausages		
Breakfast	14.40	14.52
Italian	14.77	14.80
Pizza	17.42	17.33
Pork	14.09	14.21
Dry sausages		
Pepperoni	14.33	14.41
Summer	18.87	18.86

Source: Ref. 34.

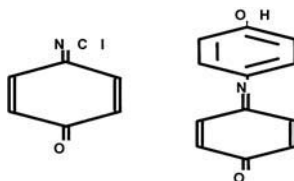
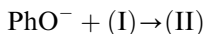
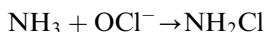
higher than normal for batch digestion. A superheated layer of acid forms, which facilitates sample digestion (44). Later tests showed that the recovery of nitrogen from refractory materials (arginine, creatine, or nicotinic acid) was only 70%. Davidson, et al. (45) concluded that the AutoAnalyzer digestion module was not reliable if an accuracy of 1% was desired for Kjeldahl analysis. Over 70 different animal feeds (corn grain, wheat, barley, rice, alfalfa, mixed feeds, feed concentrates) were analyzed using the AutoAnalyzer digestion module. The recovery of nitrogen was 88–90% (46). In contrast, using a Technicon block digester followed by AutoAnalyzer Sampler II led to 100% recovery of nitrogen from cattle supplement, swine ration, pig starter, and poultry ration (47). Suitable catalysts include copper sulfate and oxides of mercury, selenium, or titanium. Ammonia was detected using alkaline phenol reagent (Sec. 3.1). Quantitative recovery of nitrogen was also demonstrated by Kaul and Sharma (25), who used a Tectator[®] heating block to digest 23 assorted strains of rice and 15 other cereal-legume mixtures. The electrically heated block digests 40 samples per hour under controlled temperature conditions. Samples were then transferred to the AutoAnalyzer Sampler II for ammonia detection using the alkaline phenol reagent.

3. COLORIMETRIC ANALYSIS OF KJELDAHL NITROGEN

Colorimetric analysis simplifies Kjeldahl analysis and increases the sample throughput. Other benefits include increased sensitivity and a greater potential for automation. Reagents for colorimetric Kjeldahl-N analyses include (a) alkali-phenol reagent (APR), also called indophenol reagent; (b) ninhydrin (indanetrione hydrate) reagent; (c) Nessler's reagent; and (d) acetylacetone formaldehyde reagent. These colorimetric techniques are reviewed next.

3.1. Alkali-Phenol Reagent (Indophenol) Method

The alkali-phenol reagent is frequently used for the Technicon AutoAnalyzer. Under alkaline conditions, ammonia, sodium hypochlorite, and phenol react to form a blue product. Berthelot first reported this reaction in 1859. The principles of the APR assay have been reviewed (48–51) although the underlying reactions remain uncertain. Ammonia probably reacts with hypochlorite to form chloramine (NH_2Cl). This reacts with phenol to form *N*-chloro-*p*-hydroxybenzoquinone monoimine or quinochloroamine (I).



(I) Quinochloroamine and (II) Indophenol

Alternatively, ammonia may first react with phenol to form *p*-aminophenol, which is then oxidized by hypochlorite to form (I). Irrespective of how (I) forms, it reacts with 1 mole of phenol to form indophenol (II). The yellow compound ionizes under strongly alkaline conditions ($\text{p}K_a$ 13.4) giving a blue anion ($\Delta\epsilon = 1 - 2 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$). Substituted phenols (*o*-chlorophenol, *m*-cresol, and guaiacol) undergo the indophenol reaction. However, *para*-substituted phenols and some *meta*-derivatives do not react. Color intensity and the rate of color formation increase in the presence of manganese (+2) ions or acetone. Sodium nitroprusside ($10\text{--}40 \text{ mg L}^{-1}$) is another catalyst.

A simple APR assay suitable for detecting ≤ 3 ppm ammonia is described in Ref. 48 (Fig. 3). Indophenol formation is pH and temperature dependent. The linear dynamic range for ammonia was 0.3–3 ppm with a sensitivity of 0.3284 (absorbance units/1 ppm NH_3). The assay precision (for 1 ppm NH_3) was $\pm 3\%$. Although performed with boric acid as the background medium, the simple APR assay is probably not suitable for

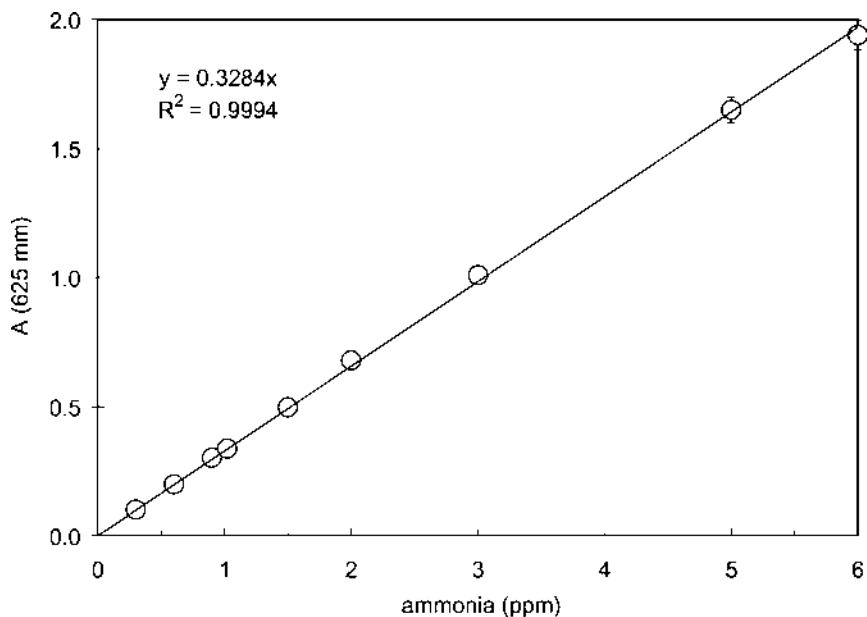


FIGURE 3 Calibration graph for ammonia determination using the alkali-phenol reagent assay. (Drawn from results in Ref. 48.)

Kjeldahl-N determination. Copper, zinc, and iron salts were found to act as interferences.

Tetlow and Wilson (49) added ethylenediaminetetraacetic acid (EDTA) to APR to reduce metal ion interference. Temperature control was also improved using a thermostated water bath. An outline protocol is described below.

Method 1

Analysis of ammonia using the APR assay (48,49).

Reagents

1. Phenol (crystalline, $\geq 85\%$ pure)
2. Sodium hydroxide solution (5 N)
3. Sodium hypochlorite solution (or commercial bleach)
4. Ammonium chloride solid
5. EDTA (6% w/v)
6. Acetone

Procedure

Preparation of alkali-phenol reagent. Place 62.5 g of solid phenol in a 500-mL beaker and add 135 mL of sodium hydroxide (5 N) slowly with stirring. Caution: Use an ice bath to avoid excessive heat buildup. Add 12 mL of acetone and make up the volume to 500 mL with deionized water.

Sodium hypochlorite (1% w/v available chloride). Prepare by diluting commercially available bleach.

Ammonium chloride standards (1000 ppm NH_3 and 100 ppm NH_3).

Dissolve 314.1 mg of solid NH_4Cl in 100 mL of water and then dilute 10-fold. Prepare a working standard solution (0.5 ppm NH_3) daily.

The APR assay sequence. Place 1 mL of sample (or standard) in a test tube. Add EDTA solution (100 μL) with gentle shaking. Next, add APR (1 mL) and hypochlorite (0.5 mL) in quick succession, mixing after each addition. Finally, add 2.5 mL of water and incubate at 25°C for 60 minutes. Take A_{625} readings for samples. Prepare a reagent blank as described next.

Reagent blank (reverse addition method). First, mix hypochlorite (0.5 mL) and APR (1 mL) solutions and allow to react for 5–10 minutes. Next add EDTA (100 μL) followed by 3.4 mL of water (or the designated blank solution).

When reverse addition is used, hypochlorite reacts with phenol first. Traces of NH_3 present in the blank are not detected (48,49). Reverse addition is useful where ammonia-free water is not available for sample preparation. After optimization, the linear dynamic range for ammonia analysis was 50–500 ppb. Assay sensitivity was 200% greater than the results shown in Fig. 2. Color formation with 50–800 ppb NH_3 was virtually complete after 15 minutes at 14–30°C. Temperature variations had little effect on the reaction. Thermostating at 25°C for 60 minutes improved the precision.

Addition of acetone to APR increased the response to ammonia by 10-fold. The color yield with 500 ppb ammonia declined by 2.65%, 4.8%, and 6.8% for 4.5-hour-, 1-day- or 5-day-old APR. Addition of EDTA prevented interference from 100 ppb copper. The intervals between addition of various reagents must not exceed 1 minute to ensure optimum precision. Comparing results for normal and reverse addition provides a means for detecting very small amounts of NH_3 in samples—such as water. Otherwise, ammonia-free water is needed for preparing reagents and blanks. The calibration curve for the APR assay is described by the equation $A_{625} = 0.7120X$, where X is the concentration of nitrogen (ppm). The analytical sensitivity was 0.7120 (absorbance units) for 1 ppm ammonia. The SD for the reagent blank was ± 0.0005 ppm. These values lead to an expected LLD for ammonia of 1.6

TABLE 9 The Comparative Costs of Manual APR Assay and Other Techniques^a

Technique	Capital cost ^b	Running cost per year ^c	Analysis per year	Cost per analysis ^d
Manual APR method	6000 (1)	5200 (1)	8000	0.72 (1)
Micro-Kjeldahl	12000 (2)	7000 (1.3)	2000	4.1 (5.4)
AutoAnalyzer APR test	81,000 (13.5)	17,000 (3.3)	32,000	0.78 (1)

^aCosts are given in deutsche marks. At current exchange rate 2.8 DM = \$1.4 = £1. All methods employ a digestion unit costing DM6000.

^bCapital costs for the micro-Kjeldahl method include the cost of a distillation unit and an autotitrator.

^cThe running cost includes DM5000 for miscellaneous chemicals.

^dCalculated for a 10-year period as capital cost/10 + running cost/no. of samples. Ratios of costs are given in parentheses for each column.

ppb. Assuming a default F_K of 6.25, the LLD for protein is 10 ppb. The APR assay is widely used in conjunction with the AutoAnalyzer. The composition of the APR used in CFA is pretty much the same as described earlier (45,52).

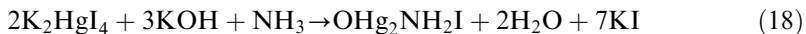
Kaul and Sharma (25) describe a rare attempt to deploy a manual Kjeldahl-APR assay for protein analysis. They used a Tectator heating block for micro-Kjeldahl digestion of grain. Sample nitrogen was then analyzed by the APR assay. The analytical performance was similar to results obtained with the AutoAnalyzer-APR assay or the conventional micro-Kjeldahl analysis. From Table 9, the capital cost for the manual Kjeldahl-APR assay was 2 times lower than for the micro-Kjeldahl and 13.5 times lower than for the AutoAnalyzer method. Running costs were also lowest for the manual APR assay.

For laboratories handling 40 or more analyses per day, it may be worth investing in an automated technique. The manual Kjeldahl-APR analysis was advantageous for small laboratories lacking the wherewithal to purchase an AutoAnalyzer. Mohyuddin and Mazza (53) used the manual Kjeldahl-APR assay to analyze potatoes (see Sec. II.B.2). The mean protein content for 14 potato cultivars was 10.65 (± 1.23)% by the manual APR assay and 10.53 (± 1.13)% using the AutoAnalyzer.

3.2. Nessler's Reagent

Ammonia reacts with alkaline potassium iodo-mercurate II (Nessler's reagent) to form a colloidal complex ($\lambda_{\max} = 430\text{--}460$). The linear range

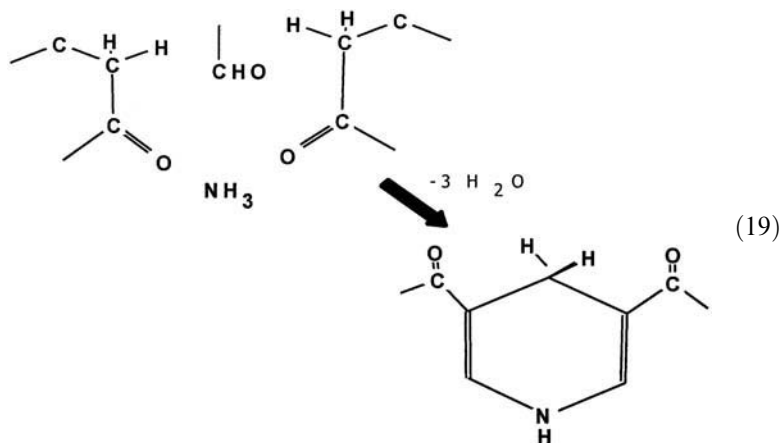
for analysis extends to 75 mg (ammonia) ml⁻¹. A possible reaction scheme for Nesslerization is



Hach et al. (54) developed a commercial Nesslerization reagent for use in Kjeldahl analysis. A sulfuric acid-digested sample (0.4 mL) is diluted with 24.6 mL of 0.01% (w/w) polyvinyl alcohol (PVA) solution. One ml of Nessler's reagent is added and the sample is agitated mechanically before absorbance measurements are recorded at 430 nm. As the product of Nesslerization is colloidal in nature, spectrophotometric analysis is sensitive to the degree of sample agitation. PVA acts as a colloidal stabilizer and improves the precision of the Nessler method.

3.3. Acetylacetone-Formaldehyde Reagent

The acetylacetone-formaldehyde assay is based on the Hantzsch reaction for the synthesis of pyridine (55). Prediluted digest is reacted with a mixture of acetylacetone and formaldehyde in the presence of sodium acetate. The yellow product (3,5-diacetyl-1,4-dihydropyridine) is measured at 410 nm ($\Delta\epsilon = 1.4 \times 10^3 \text{ Lmol}^{-1}\text{cm}^{-1}$). The color-forming reaction is shown in Eq. (19).



Acetylacetone-formaldehyde reagent was used for the analysis of medicinal agents such as paracetamol, sulfanilamide, and chloropramide. The potential for colorimetric Kjeldahl analysis is obvious.

Method 2

Determination of ammonia using acetylacetone-formaldehyde reagent (55).

Reagents

Acetylacetone-formaldehyde reagent. Place 15 mL of formaldehyde (37% w/v) and acetylacetone (7.8 mL) into a 100-mL flask. Make up to 100 mL with distilled water.

Sodium acetate (2M). Dissolve sodium acetate (82 g) in 1 L of distilled water.

Procedure

Add prediluted Kjeldahl digest (< 2 mL; 25–100 $\mu\text{g N}$) to a 25-mL conical flask followed by sodium acetate solution (3 mL) and acetylacetone-formaldehyde reagent (3 mL). Incubate the mixture at 97.8°C for 15 minutes and cool to room temperature.

Bring the total volume to 25 mL and record A_{412} values using a 1-cm cuvette.

The linear dynamic range for the preceding assay was 0.5–6.0 $\mu\text{g N}$ (per final reaction mixture). The calibration graph was described by $A_{412} = 9.8 \times 10^{-2}X + 4.2 \times 10^{-3}$ ($R^2 = 0.9999$), where X is the amount of nitrogen present in the final (25-mL) reaction mixture. The new method shows levels of accuracy and precision equal to those of the micro-Kjeldahl method.

3.4. Ninhydrin (Indanetrione Hydrate) Assay

Ninhydrin* reacts with amino acids (Fig. 4) in two stages: (a) the amino acid is oxidized to aldehyde and ammonia while ninhydrin is converted to hydrindantin and (b) hydrindantin and 1 mole of ninhydrin react with ammonia to form Ruhemann's purple (56,57). For ammonia determination an added reducing agent is necessary to convert ninhydrin to hydrindantin. Ninhydrin solution is available commercially. Results from the ninhydrin-Kjeldahl assay agree closely with those from Kjeldahl analysis (56–58). The linear dynamic range for colorimetric Kjeldahl assay depends on the extent of sample dilution just before ninhydrin analysis. A 2-mL standard ammonium sulfate solution containing 5.6 μg (or 2.8 ppm) reacted with 2 mL of ninhydrin solution yields an A_{570} reading of 0.805. Interference was noted for concentrations of selenium above 86 $\mu\text{g mL}^{-1}$ (prediluted digest). No interferences were observed with Fe, Zn, Pb, Cu, Ca, Ba, Al, Mg, Co, or

* Ninhydrin is often encountered in detective novels as a reagent for fingerprint analysis.

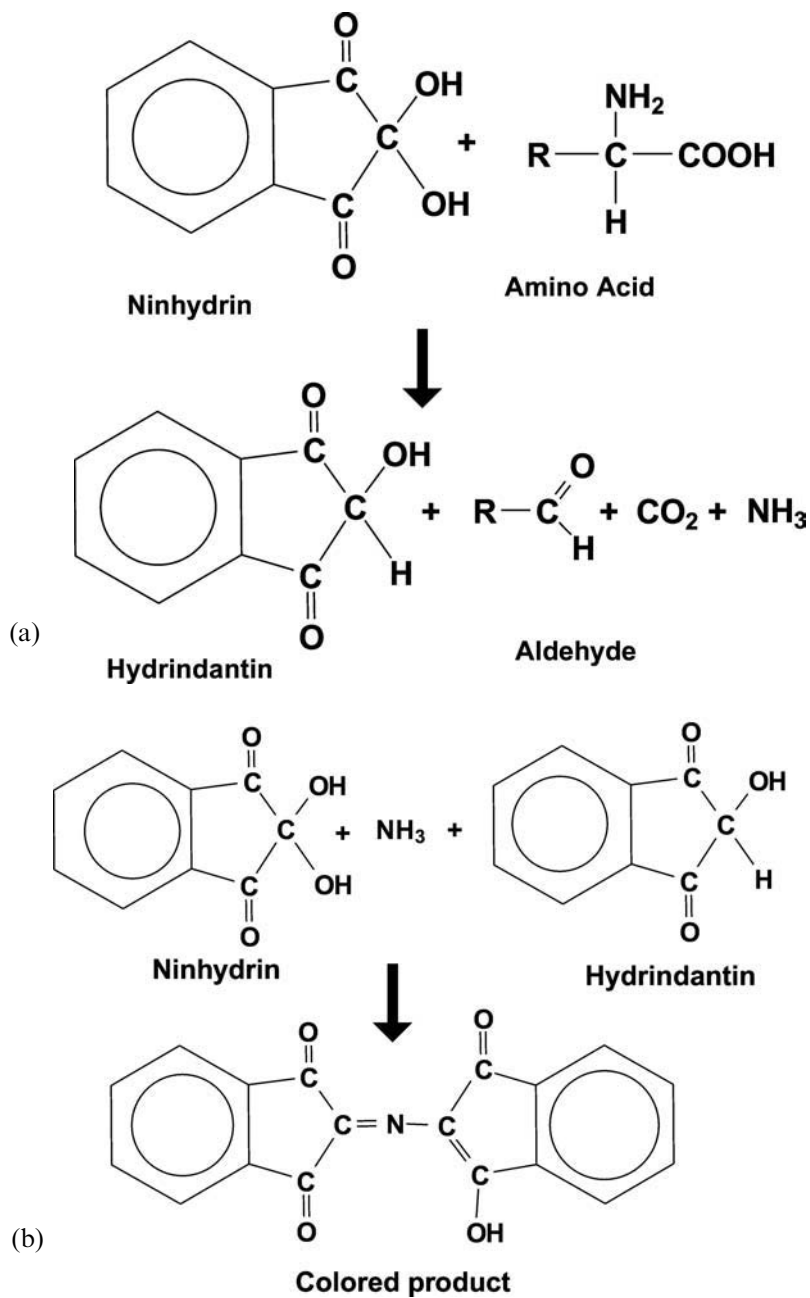


FIGURE 4 Reaction scheme between amino acids and ninhydrin reagent.

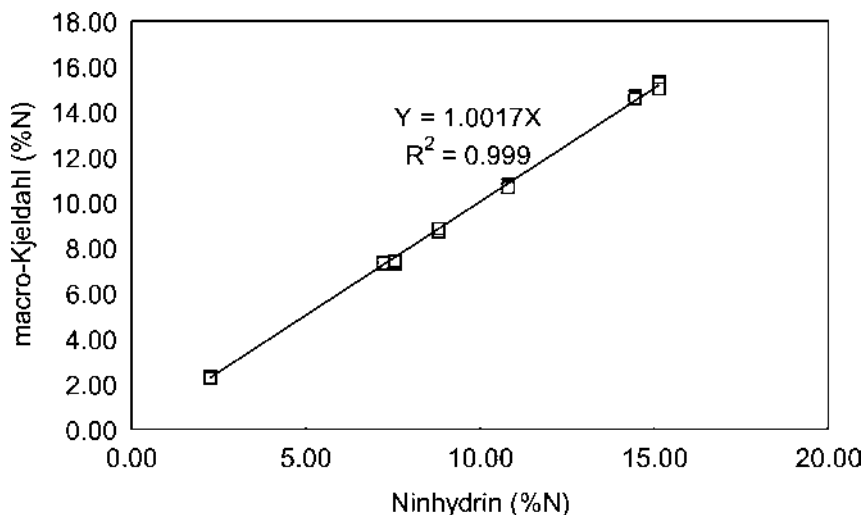


FIGURE 5 Colorimetric ninhydrin analysis of Kjeldahl nitrogen calibrated against the conventional macro-Kjeldahl analysis. (Drawn from data in Ref. 58.)

Ni at concentrations of $50 \mu\text{g mL}^{-1}$ or from Hg at $30 \mu\text{g mL}^{-1}$. The overall impression is that the ninhydrin assay is resistant to metal ions.

Quinn et al. (58) analyzed rapeseed flour, rapeseed concentrate, soybean concentrate, and bovine serum albumin using the ninhydrin assay in conjunction with an AutoAnalyzer (Fig. 5). The precision of analysis was 1.40–1.76%. A manual Kjeldahl-ninhydrin assay has not been reported recently. This seems a pity. Compared with other colorimetric methods, the ninhydrin assay is more resistant to interferences from metal catalysts. The color is also formed at a more easily buffered pH between 4.9 and 5.4.

4. QUANTITATIVE AMINO ACID ANALYSIS

The following steps are involved in quantitative amino acid analysis: (a) hydrolyze a sample of food using concentrated hydrochloric acid, (b) determine the amino acid profile, (c) calculate the *concentration* of each amino acid in the sample, and (d) calculate the weight of each amino acid.

Quantitative amino acid analysis is reportedly one of the most reliable methods for protein quantitation (59–61).

4.1. Principles of Quantitative Amino Acid Analysis

Crude protein (cP) is expressed by Eq. (20), where C_i (mole) is the amount of each amino acid in the sample and b_i (g mole⁻¹), is the formula weight for each amino acid.

$$\text{cP} = \sum_{i=1}^{20} C_i b_i \quad (20)$$

However, amino acid profiles are reported in terms of mole fraction of each amino acid (X_i):

$$X_i = C_i / C_{\text{net}} \quad (21)$$

where C_{net} is the net concentration of amino acids found in the sample. Substituting $C_i = C_{\text{net}} X_i$ in Eq. (20),

$$\text{cP} = C_{\text{net}} \sum_{i=1}^{20} (b_i X_i) \quad (22)$$

The term $\sum(b_i X_i)$ is called the mean residue weight, W (g mole⁻¹). This is the average formula weight for all amino acids in the sample adjusted for their frequency.*

$$W(\text{g mole}^{-1}) = \sum (b_i X_i) \quad (23)$$

and

$$\text{cP} = WC_{\text{net}} \quad \text{or} \quad \text{cP} = FC_{\text{net}} \quad (24)$$

In Eq. (24), F is the mean residue weight. Usually, W (g mole⁻¹) is adjusted to take into account two routine errors in amino acid analysis: (a) many colorimetric reagents for amino acids do not react with proline and (b) tryptophan is destroyed during acid hydrolysis of proteins. Proline and tryptophan are usually determined by separate experiments. After correcting for such errors, one gets the conversion factor, F (g mole⁻¹):

$$F = \frac{\sum_{i=1}^{18} (b_i C_i)}{C_{\text{net}} - C_{\text{Pro}} - C_{\text{Trp}}} \quad \text{or} \quad F = \frac{\sum_{i=1}^{18} (b_i X_i)}{1 - X_{\text{Pro}} - X_{\text{Trp}}} \quad (25)$$

where X_{Pro} and X_{Trp} are the mole fractions of proline and tryptophan. For a

* Horstmann called this parameter the weight equivalent (WE).

range of meat products, F (g mole⁻¹) was 10–20% larger than W [Eq. (23)]. Calculations of F (g mole⁻¹) appear in last three columns of Table 5. Typical values for F (g mole⁻¹) are given in Table 10. Values for F range from 100 to 125 g mole⁻¹ for most proteins. Most protein sources are now routinely analyzed for amino acid scores during nutritional evaluations (Chapter 14). This yields all the information necessary for total protein estimation. Zarkadas and co-workers in Canada are strong exponents of quantitative amino acid analysis (Table 11).

4.2. Quantitation of Specific Proteins

Meat collagen was determined by measuring 5-hydroxylysine (5OH-Lys). This amino acid is found in collagen and no other meat protein. The concentration of collagen (P_j) was calculated from a modified Eq. (24) (64,65);

$$P_j = C_i \frac{1000W_j}{n_jM_i} \tag{26}$$

TABLE 10 Corrected Mean Residue Weights (F) for Selected Food Protein Sources

Protein source	F (g mole ⁻¹)
Barley flour	129.84
Soya bean flour	119.82
Pea flour	118.85
Fish meal	112.70
Beef sausage	107.06–109.01
Pig skin (rind)	94.02
Bone meal	104.21
Soya bean protein	
Flour	114.43
Isolate	114.48
Concentrate	115.69
Wheat	
Flour	113.13–116.00
Gluten	108.4–108.52
Egg white solids	118.43
Potato protein	108.52
Milk (nonfat) powder	112.98

Source: Data calculated or taken from references in Table 11.

TABLE 11 Protein Determination by Quantitative Amino Acid Analysis

Sample/comments	Reference
NASA—Skylab meals	Heidelbaugh et al. (59)
Mushroom total protein	Weaver et al. (62), Braaksma and Schaap (63)
Porcine muscle and connective tissue proteins (myosin, actin, elastin, and collagen)	Zarkadas et al. (64), Zarkadas et al. (65)
Actin, myosin, and collagen in composite meat products; mixed meat sausages, bologna, frankfurters, sausages, hamburgers	Karatzas and Zarkadas (66)
Additives and ingredients for meat products including soybean, wheat products, potato protein	Zarkadas et al. (67)
Porcine skin (rind)	Nguyen et al. (68)
Chicken meat and connective tissue	Karatzas and Zarkadas (69)
Apple flower buds	Khanizadeh et al. (70)
Bone isolates	Zarkadas et al. (71)
New soybean cultivars	Zarkadas et al. (72)

where C_i is the concentration of 5OH-Lys in the meat hydrolysate, W_j is mean residue weight from the amino acid profile for collagen (averaged for the different collagen types), n_j is the number of 5OH-Lys residues per 1000 residues in collagen, and M_i is the formula weight for 5OH-Lys. Typically, $W_j = 91.01 \text{ g mole}^{-1}$, $N_i = 10$ residues, $M_j = 145.18$, and consequently

$$\text{Amount of collagen} = 62.75[5\text{OH-Lys}] \quad (27)$$

Similarly, the concentration of N^{ϵ} -methylhistidine and 4-hydroxyproline was the basis for assessing the amount of myofibrillar protein and connective tissue (collagen and elastin) in meat. These methods are satisfactory for meat and meat products. They may have questionable validity for composite foods. Plant foods may contain 4-hydroxyproline-rich glycoproteins (extensins, lectins, salt-extractable glycoproteins). For example, alfalfa protein and potato protein contain significant levels of 4OH-Pro.

4.3. Examples and Relation to Kjeldahl Method

Quantitative amino acid analysis is arguably one of the most accurate methods for food protein quantitation. One source of error is the high concentration of free amino acids in some foods. The protein content in nine strains of *Agaricus* was 28% (± 3.4)% by quantitative amino acid analysis (62). The results were poorly correlated ($R = 0.4$) with Kjeldahl results (22.4% protein per dry weight basis). A more recent analysis of freeze-dried mushroom powder led to estimates of 7.0% protein by dry weight (63). In the later study, mushroom powder was extracted with 0.5 M NaOH and precipitating with trichloroacetic acid (TCA) before analysis. This removed large amounts of TCA-soluble NPN associated with mushrooms. Of the total NaOH-soluble nitrogen extracted from mushrooms, 20% was protein, 60% was urea or ammonia, and 20% was free amino acids. Food samples are now routinely extracted with organic solvents to remove NPN before quantitative amino acid analysis (Table 11). Advocates for quantitative amino acid analysis point to its compatibility with plant proteins. There is no interference from phenols, tannins, and lignin. By contrast, the Kjeldahl method is unsuitable for plant tissues regardless of the conversion factor used (73).

5. COMBUSTION NITROGEN ANALYZERS

The Dumas assay predates Kjeldahl analysis by 50 years (Table 1). The former technique was invented by Jean Baptiste Dumas. Early applications include the analysis of plant materials (74,75), meat (76), casein, whole powdered milk, soybeans, and maize flour (77). The first-generation instruments for the Dumas method were not user friendly. The volume of nitrogen gas produced by combustion was determined with a manometer. The advent of easy-to-use and highly accurate combustion nitrogen analyzers (CNAs) rekindled interest in the Dumas method.

CNAs from various manufacturers work on the same principle. The sample is dropped into a 950–1050°C furnace, purged free of atmospheric gas, and filled with pure (99+%) oxygen. Complete sample combustion leads to CO₂, water, SO₂, NO₂, and N₂. The product gases are cooled and a portion is passed through tubing packed with hot lead chromate, copper, sodium hydroxide (solid), or phosphorus pentoxide to remove SO₂, O₂, CO₂, and water, respectively. The NO₂ is then reduced to N₂ and measured with a thermal conductivity detector (TCD). Sample protein content is calculated by taking into account the mass of sample injected, the

proportion of the combustion gases analyzed, and the nitrogen-protein conversion factor (F_K). The calculations are now automated.

5.1. Collaborative Studies and Approved Status for CNAs

CNAs were calibrated with the Kjeldahl method. Interlaboratory studies appearing after 1987 are listed in Table 12. Such trials led to CNAs receiving approved status from the AOAC (Association of Official Analytical Chemists), AOCS (American Oil Chemists' Society), ASBC (American Society of Brewing Chemists), AFI (American Feed Industry), BRFI-International (Brewing Research Foundation-International), IOB (Institute of Brewing), and EBC (European Brewing Convention).

The Canadian Grain Commission and U.S. Department of Agriculture (USDA) Federal Grain Inspection Services (FGIS) approved CNAs in

TABLE 12 Food Protein Analysis Using the Dumas Combustion Method

Sample ^a	Reference
Animal feedstuffs, fertilizers	Sweeney and Rexroad (78), Schmitter and Rhihs (79), Sweeney (80), Sachen and Thiex (81), Tate (82)
Beer	ASBC (83), Johnson and Johansson (84,85)
Brewing grains—barley, malt, rice	ASBC (86), Buckee (28,87), Krotz et al. (88), Johansson (89), Angelino et al. (90)
Cereal grains—wheat, barley, corn, sorghum	Bicsak (91), Bicsak (92), Williams et al. (93)
Dairy products—skimmed, powdered milk etc. chocolate milkshake, cheeses, etc.	Wiles and Gray (94), Wiles et al. (95), Simonne et al. (96,97)
Fruit—guava, peaches, plum	Simonne et al. (96,97), Huang et al. (98)
Infant food	Bellemonte et al. (99)
Meat and meat products, fish (raw, fish in oil, tuna)	King-Brink and Sebranek (100), Simonne et al. (96,97), Buschmann and Westphal (101)
Oilseeds (soybean, canola, sunflower, corn)	Bicsak (91), Duan and DeClercq (102), Berner and Brown (103)
Potatoes	Young et al. (104)
Vegetables—cabbages, broccoli, ketchup, tomato	Simonne et al. (96,97)

^aApproximate sample classification; classes contain the other foodstuffs.

TABLE 13 Advantages of the Dumas Method

-
1. Greater ease of operation
 2. Higher operator safety owing to the nonrequirement for hazardous chemicals
 3. The absence of wetchemistry
 4. Reduced time of analysis
 5. Higher performance characteristics (greater accuracy, repeatability)
 6. Absence of waste disposal concerns (Table 14)
 7. Simple instrument installation without a requirement for specialized ventilation
 8. Low cost per analysis
-

1994 and 1996, respectively (91–93). Trials for the combustion method usually follow guidelines described by Youden and Sleiner (105):

1. The number of laboratories ranges from 7 to 12. Studies involving as few as three laboratories have been reported.
2. All studies compare CNAs with Kjeldahl analysis.
3. Interlaboratory studies focus on a single food group. Therefore, CNAs tend to receive approval for one food group at a time (Table 12).
4. Trials usually test a “generic combustion method” and are independent of the choice of instruments.

Minimum performance guidelines for CNA instruments include (a) a furnace temperature of 950°C, (b) a separation system for trapping CO₂ and water, (c) a thermal conductivity detector for nitrogen, (d) sufficient accuracy to produce results within + 0.15% of the mean (% nitrogen) results for 10 successive measurements using a standard compound, and (e) sufficient precision to produce a relative standard deviation of 0.01%. The LECO FP-428 analyzer was used by about 80% of the laboratories involved in collaborative trials. The Foss-Heraeus Macro-N analyzer, Carlo Erba NA-5000, and Perkin Elmer PE2410 also feature. The LECO FP-2000 combustion analyzer appears in the latest trials.

5.2. Advantages of the Combustion Method

The modern CNA has advantages over the Kjeldahl method (Table 13). There is greater speed of analysis and greater operator safety stemming from the nonuse of aggressive chemicals. The estimated cost for analysis is \$0.37–\$0.50 per sample with the LECO FP-2000 protein analyzer (LECO Corporation, Saint Joseph, MI) compared with \$1.0 per test for the Kjeldahl method (106–109).