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AMINO ACID ANALYSIS *by* GAS CHROMATOGRAPHY

Volume I

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Amino Acid Analysis by Gas Chromatography

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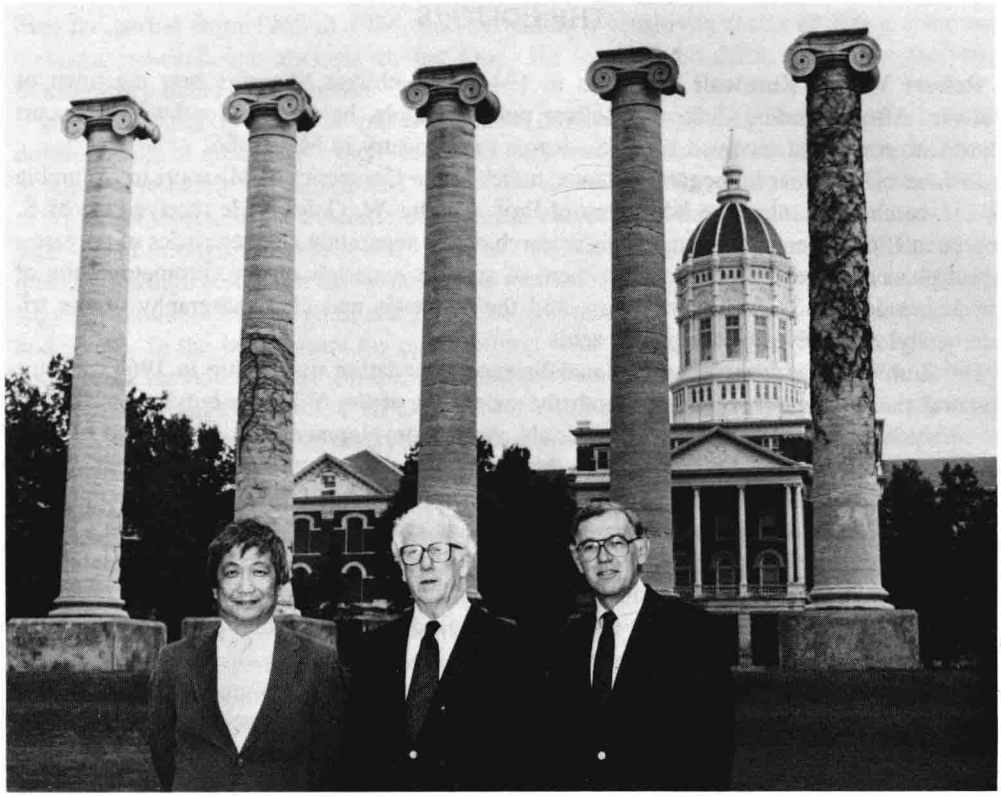

In the background the remaining columns of Academic Hall, first building of the University of Missouri, and Jesse Hall, administrative building for the University of Missouri-Columbia, presenting:

Dr. Charles W. Gehrke (center), Professor of Biochemistry and Director of the University of Missouri Interdisciplinary Chromatography Mass-Spectrometry Facility and Centennial President (1984) of the International Association of Official Analytical Chemists, Co-Principal Investigator on lunar analysis, Apollos 11 to 17, in search for life molecules.

Dr. Robert W. Zumwalt (right), Research Associate in Biochemistry and Co-Investigator on lunar analysis team of Apollos 11 to 17.

Mr. Kenneth C. T. Kuo (left), Senior Chromatographer and Research Chemist, and Co-Investigator on lunar analysis team of Apollos 11 to 17.

THE EDITORS

Robert Wayne Zumwalt was born in 1944 in southwest Missouri near the town of Bolivar. After attending Polk and Bolivar public schools, he entered Southwest Missouri State University and received the B.S. degree in chemistry in May 1966.

In June of that year he began graduate studies at the University of Missouri in Columbia as a research assistant in the laboratory of Prof. Charles W. Gehrke. He received the M.S. degree in 1968, after performing thesis research on the separation characteristics of polyester liquid phases, the effects of heat treatment of support materials on the chromatography of the amino acid *N*-TFA *n*-butyl esters, and the synthesis and chromatography of the trimethylsilyl derivatives of the amino acids.

Dr. Zumwalt was awarded a National Science Foundation traineeship in 1968, and his doctoral thesis research concerned both the extension of the *N*-TFA *n*-butyl ester method for analysis of complex biological materials and the development and application of high sensitivity methods for examination of the returned lunar samples for indigenous amino acids. He participated in the analysis of lunar samples returned by Apollo lunar missions for amino acids and extractable organic compounds at the NASA Ames Research Center, California, and the Laboratory for Chemical Evolution at the University of Maryland. He was co-inventor of a solvent venting system for gas chromatography (GC), and conducted the first derivatization and chromatographic studies using *bis*-(trimethylsilyl)trifluoroacetamide as a silylating agent for amino acids.

He, along with Dr. Gehrke and Mr. Kuo, reported the dual-column chromatographic system which allowed the quantitative analysis of the protein amino acids as the *N*-TFA *n*-butyl esters, and completed his thesis research with the development of ion-exchange purification procedures which allowed amino acid analysis of physiological fluids and other complex materials.

After receiving the Ph.D in 1972, Dr. Zumwalt's research efforts turned to the development of analytical methods for detecting and measuring biological markers of neoplastic disease. These studies included development of GC and HPLC techniques for measurement of methylated bases and nucleosides in patients physiological fluids. Dr. Zumwalt was associated with the College of Veterinary Medicine, University of Missouri, for 4 years, with the U.S. Fish and Wild Life Service's Columbia National Fisheries Research Laboratory for 1 year, then returned to the University of Missouri Department of Biochemistry in 1981. Dr. Zumwalt lectures in a graduate level course in Analytical Biochemistry-Chromatography at the University of Missouri-Columbia, and is an author of more than 50 scientific publications in the field of the development and application of quantitative chromatographic methods in biochemical research.

Kenneth Ching-Tien Kuo, was born in 1936 in China. He studied at Chun-Yen Institute of Science and Engineering, Taiwan, receiving a B.S. degree in Chemical Engineering in 1960. After fulfilling a military service obligation, he enrolled at the University of Houston. In 1963 he joined the Chevron Chemical Co. in Richmond, California, developing pesticide residue analytical methods and studying pesticide metabolism. Recognizing the power of gas-liquid chromatography (GLC) and the need of high resolution, sensitivity, and speed in the analysis of amino acids, he applied and was accepted as a member of the research team under Prof. Charles Gehrke at the University of Missouri-Columbia in 1968. He developed mixed phase columns for histidine, arginine, and cystine, which allow the dual column complete quantitation of protein amino acids in 30 min by GC. He along with Drs. Gehrke, Stalling, and Zumwalt, invented the Solvent-Vent Chromatographic System (U.S. Patent No. 3,881,892) which eliminates the sample solvent effect in GC analysis. This solvent-venting device was used in the search for amino acids in the returned Apollo lunar samples

over the period from 1969 to 1974, thus providing a sensitivity factor of 100 greater than classical ion-exchange analysis at that time. He received his M.S. degree in analytical biochemistry under Prof. Gehrke in 1970.

During the last 17 years he and Dr. Gehrke have dedicated their research efforts to the developments of quantitative high resolution chromatographic methods for biochemical and biomedical research. He participated in the NASA Apollo Returned Lunar Sample consortium of scientists searching for evidence of chemical evolution in the lunar samples from Apollo missions 11 through 17 (1969 to 1974). He has studied biomarkers for cancer, and developed quantitative high resolution chromatographic methods for polyamines, protein-bound neutral sugars, β-aminoisobutyric acid, and β-alanine; and modified ribonucleosides in human urine and serum. In the last 5 years his major efforts have been directed to the developing of a package of methods for the complete quantitative composition analysis of DNA, mRNA, and tRNA by high resolution HPLC. Through these methods, more than 70 major and modified ribonucleosides, 15 deoxynucleosides, and 9 mRNA cap structures can be identified and measured in nucleic acids or body fluids. He was an invited scientist by the Chinese Academy of Sciences in 1982 and lectured throughout China on the chromatography of nucleosides. He has contributed to over 50 scientific publications in analytical chemistry and biochemistry.

Charles William Gehrke was born in 1917 in New York City. He studied at the Ohio State University receiving a B.A. in 1939, a B.S. degree in education in 1941, and a M.S. degree in 1941. From 1941 to 1945 he was professor and chairman of the Department of Chemistry at Missouri Valley College, Marshall, Missouri, teaching chemistry and physics to selected Navy midshipmen from the destroyers, battleships, and aircraft carriers of World War II in the South Pacific. These young men then went back to the war theater as Deck and Flight Officers. In 1946, he returned to the Ohio State University as an instructor in agricultural biochemistry and received his Ph.D. degree in 1947. In 1949 he joined the College of Agriculture at the University of Missouri-Columbia, where at present he is professor of biochemistry and manager of the Experiment Station Chemical Laboratories and Director of the University Interdisciplinary Chromatography Mass-Spectrometry Facility. His duties also include those of State Chemist for the Missouri Fertilizer and Limestone Control Laws.

Prof. Gehrke is the author of over 250 scientific publications in analytical and biochemistry. His research interests include the development of quantitative, high-resolution GC and LC methods for amino acids, purines, pyrimidines, major and modified nucleosides in RNA, DNA, and methylated "CAP" structures in mRNA; fatty acids, and biological markers in the detection of cancer; characterization and interaction of proteins, chromatography of biologically important molecules, and automation of analytical methods for nitrogen, phosphorus, and potassium in fertilizers. Automated spectrophotometric methods have been developed for lysine, methionine, and cystine.

Prof. Gehrke has been an invited scientist to lecture on GLC of amino acids in Japan, China, and at many universities and institutes in the U.S. and Europe. He participated as a co-principal investigator in the analysis of lunar samples returned by Apollo flights 11, 12, 14, 15, 16, and 17 for amino acids and extractable organic compounds with Prof. Cyril Ponnamperuma, University of Maryland, and with a consortium of scientists at the NASA Ames Research Center, California. In 1971, he received the annual Association of Official Analytical Chemists' (AOAC) Harvey W. Wiley Award in Analytical Chemistry and was the recipient of the Senior Faculty Member Award, UMC College of Agriculture, in 1973. In August 1974, he was invited to the Soviet Academy of Sciences to make the summary presentation on organic substances in lunar fines to the Oparin International Symposium on the "Origin of Life." In 1975, he was selected as a member of the American Chemical

Society Charter Review Board for Chemical Abstracts. As an invited teacher under the sponsorship of five Central American governments, he taught chromatographic analysis of amino acids at the Central American Research Institute for Industry in Guatemala, 1975.

He was elected to Who's Who in Missouri Education, 1975, and recipient of the Faculty-Alumni Gold Medal Award and was the recipient of the prestigious Kenneth A. Spencer Award from the Kansas City Section of the American Chemical Society for meritorious achievement in agricultural and food chemistry, 1979 to 1980. He received the Tswett "Chromatography Memorial Medal" from the Scientific Council on Chromatography, Academy of Sciences of the USSR, Moscow, 1978, and Sigma Xi Senior Research Award by the University of Missouri-Columbia Chapter, 1980. He has been an invited speaker on "Modified Nucleosides and Cancer" in Freiburg, West Germany, 1982, and gave presentations as an invited scientist throughout Japan, mainland China, Taiwan, Philippines, and Hong Kong, 1982. He was selected to the Board of Directors and Editorial Board of the AOAC from 1979 to 1980, President-Elect of the AOAC international organization, 1982 to 1983; and was honored by the election as the centennial President, 1983 to 1984. He developed "Libraries of Instruments" interdisciplinary research programs on strengthening research in American universities. Dr. Gehrke is founder and chairman of the Board of Directors, Analytical Biochemistry Laboratories, Inc., 1968 to present, a private corporation of 150 scientists, engineers, biologists, and chemists specializing in chromatographic instrumentation, and addressing problems worldwide to the environment.

Over 60 masters and doctoral students have received their advanced degrees in analytical biochemistry under the direction of Prof. Gehrke. In addition to his extensive contributions to amino acid analysis by GC, Dr. Gehrke and colleagues have pioneered in the development of sensitive, high-resolution, quantitative HPLC methods for over 80 major and modified nucleosides in RNA, DNA, mRNA, then applied their methods in collaborative research with scientists in molecular biology across the world. Prof. Ernest Borek at the International Symposium on Cancer Markers, Freiburg, West Germany, in 1982, stated that Prof. Gehrke's chromatographic methods are being used successfully by more than half of the scientists in attendance at these meetings.

In 1986, Dr. Gehrke was the recipient of the American Chemical Society's Midwest Award for outstanding research in analytical biochemistry. The Smithsonian Institution has requested the gas chromatograph which Gehrke and colleagues modified and used to analyze the Apollo lunar samples for display in the National Aeronautics Air and Space Museum.

PREFACE

The central role of proteins (French *protéine,* "primary substance [of the body]" from Greek, *prōtos,* first) and their building blocks, the amino acids, in biology has evoked intense and continued interest in protein and amino acid chemistry by scientists representing a wide spectrum of disciplines. The array of substances subjected to examination for their amino acid content is therefore extraordinarily broad, ranging from meteorites and lunar samples to newly synthesized or isolated peptides and proteins. Over the past 4 decades, chromatographic techniques have emerged as the dominant means of amino acid determination, and milestones of that development are apparent. Partition chromatography of the *N*-acyl amino acids was performed by Nobel Laureates A. J. P. Martin and R. L. M. Synge (1941), working in the laboratories of the Wool Industries Research Association in Leeds, England. They addressed the problem of analyzing the amino acids in protein hydrolysates and laid the theoretical foundation on which liquid-liquid chromatography is based. Nobel Laureates Stanford Moore and William Stein, along with D. H. Spackman at the Rockefeller University, New York, pioneered the elegant automated ion-exchange amino acid analysis (1958) which has had a profound impact on amino acid and protein research. In 1952, Nobel Laureates A. T. James and A. J. P. Martin described the fundamental parameters of gas-liquid chromatography (GLC), including a theory of its operation in terms of the theoretical plate concept, and laid the foundation for further development of the technique.

The earliest gas chromatographic (GC) method for analysis of amino acids was described by Hunter et al. in 1956, and involved separation of the aldehydes which resulted after decarboxylation and deamination with ninhydrin. In 1958, Bayer reported the GC separation of the *N*-trifluoroacetyl (*N*-TFA) methyl esters, and *N*-acylated amino acid esters have subsequently emerged as by far the most widely used class of derivatives. In the 1960s, Charles W. Gehrke and his doctoral students William Lamkin and David Stalling at the University of Missouri-Columbia, laid the foundations that resulted in the synthesis of the reference standard derivatives and in establishing the organic reaction and chromatographic separation conditions for the first quantitative amino acid analysis by GLC of the 20 *N*-TFA *n*-butyl esters. Their research on GLC methods for amino acids led to intensive research in more than 100 laboratories across the world directed to studies on the merit of different derivatives, chromatographic columns, detectors, and applications to research in medicine, agriculture, and the environment.

Numerous reports of GLC techniques for amino acid determination began to emerge in the late 1960s, mainly spurred by the offer of improved resolution and speed of analysis as compared to the ion-exchange techniques of the day. Advances in GC detectors, column, materials, and quantitative derivatization methods during the 1960s and 1970s encouraged further research; and continued improvements in all phases of GC instrumentation and column technology into the 1980s have enhanced the capabilities of GC for amino acid analysis. Development of the GC methodology was followed by interfaced GC/mass spectrometric (MS) analysis and characterization of unknowns and analysis of amino acids enantiomers, and then by the more recent reversed-phase liquid chromatography approaches. This story on accomplishments continues with the excellent contributions of the pioneering scientists in the 20 chapters of this three volume treatise. Analytical and chromatographic strategies for separating, identifying, and quantitating amino acids in the array of matrices has been varied, dictated by both the methodology available and the demands presented by the specific analytical problem whether in a research setting or for compositional information. Improved ion-exchange, gas-liquid, and reversed-phase chromatographic techniques continue to evolve to meet the ever-increasing demands for better resolution, sensitivity, speed, and versatility.

The methodology of choice depends on the analytical requirements at hand. For a protein chemist involved in structural analysis of a particular protein, the analytical demands are

not the same as for a nutritional chemist involved in determining the nutritional quality of foods and feeds, the clinical chemist engaged in determining amino acids in physiological fluids to aid in diagnosis of disease, or the biogeochemist interested in the extent of racemization of amino acids in fossils.

The complexity and diversity of the sample matrices that are encountered requires a methodology providing high resolution, selectivity, and a wide linear response range of 10^6. GC/FID (flame ionization detection) is the method of choice and in these situations GC will provide more reliable data. The inherent strengths of GC methods (resolution, sensitivity, versatility, cost) to a wide range of amino acid analytical problems and applications are shown in these volumes.

In biomedical research, the problem is a general one, the need of new techniques and their application to solve old problems, and to probe new ideas of approach to solve intractable new problems. Whatever the disease or biochemical research objective, a research tool is required that will provide a reliable measurement of the molecules under study. GLC of amino acids in all of its ramifications provides the research scientist with powerful new tools and approaches to help in obtaining the needed answers to advance science.

The chromatographies and separation science, are a major "bridge" or "common denominator" as analytical methods in biological sciences research. The importance of research and new methods of measurement to the advancement of our society and the developing world depends upon expanding and new knowledge from every source for continued growth. Problems in nutrition, pollution, drugs, environment, and biotechnology are now being solved by chromatography and interfaced MS in weeks and months, where formerly years of study were involved. The genius of Mikhail Tswett, the father of chromatography, of the early 1900s has had a profound impact to this point in history. To illustrate the significance of Tswett's work, 56 world-leading chromatographers payed tribute to his accomplishments by contributing chapters to *75 Years of Chromatography — A Historical Dialogue*, which was published in 1979 to commemorate the 75th anniversary of Tswett's invention of chromatography. That volume, edited by L. S. Ettre and A. Zlatkis, provides a unique historical perspective as it relates developments and applications of chromatography by scientists from disciplines that range from petroleum chemistry to medicine. His accomplishments promises to open even wider doors as Chemistry and Biology are brought more closely together to more effectively serve mankind.

In 1954, Prof. William Albrecht, Chairman of Agronomy and Soils, at the University of Missouri, expressed the great need and asked me (CWG) to develop a more reliable method for the quantitative measurement of amino acids than the bacterial turbidity method used at that time. This was the challenge and start of my work on new methods by GC for amino acids. Our accomplishments have been most rewarding.

Our goals, at Missouri, in the Experiment Station Chemical Laboratories, have been the development of automated analytical, and chromatographic methods as "research tools" useful for advancing investigation in biochemistry, agriculture, space sciences, and medicine.

Charles W. Gehrke
Kenneth C. Kuo
Robert W. Zumwalt
Columbia, Missouri

A DEDICATION AND THANKS

I humbly dedicate this three-volume treatise on amino acid analysis by gas-liquid chromatography to my beloved son, Dr. Charles W. Gehrke, Jr., a Navy aerospace surgeon whose life was so early and tragically taken in the line of duty on March 1, 1982, at Pensacola, Florida, flight station. Charles was also my graduate student, colleague, closest friend, and an accomplished analytical biochemist. He truly understood the deep meaning and relationships of chemistry and medicine and enjoyed life to its fullest.

I further dedicate these three volumes to my 60 master of science and doctoral students, postdoctorals, colleagues, and visiting scientists, many of whom contributed significantly in the research to a number of chapters of the three volumes. Their efforts have been exemplary and their contributions to analytical biochemistry were meritorious.

Lastly, but not least, my special appreciation to my wife, Virginia, who so graciously supported and encouraged me during these past 3 years during the development and completion of this work.

To my son, Dr. Jon C. Gehrke, M.D. and daughter, Susan G. Gehrke, J.D. for their love and special understanding.

Charles W. Gehrke

ACKNOWLEDGMENTS

Our sincere thanks are extended to the many accomplished scientists from around the world who have graciously and diligently presented their research findings in their contributed chapters. Their efforts have allowed these volumes to present an international, and a comprehensive perspective of the field of gas chromatographic amino acid analysis. In this fast-developing science and technology, their research findings have, and will, play important roles in the advancement of science in many disciplines.

We thank Nancy Rice, Jennifer Welch, Lori Sampsel, and Wylonda Walters Jones for their preparation of finished manuscripts which constitute several chapters of these volumes, and our thanks also to the editors of CRC Press, Inc., for their help in the publication of these volumes.

GENERAL INTRODUCTION

As a result of investigations to answer a wide variety of scientific questions, development of chromatographic techniques for determination of amino acids has advanced continuously since A. J. P. Martin and R. L. M. Synge described partition column chromatography and its application to amino acids in 1941. This wide-ranging interest has had two effects which are apparent in the literature on this subject. First, nearly every form of chromatography has been brought to bear, at one time or another, on problems dealing with the measurement of amino acids. Second, this broad interest has resulted in chromatographic methods for determining amino acids in a broad spectrum of sample types from artificial sweeteners to meteorites.

We undertook this three-volume work to provide the scientific community with information on the development of new research tools and an opportunity to explore the application of packed column and capillary column gas chromatography (GC) to amino acid analysis in a number of different research areas. Packed columns possess certain advantages which include ease of preparation and operation, larger sample capacity, and reduced cost. Capillary columns on the other hand offer increased resolution, selectivity and sensitivity, while fused-silica bonded-phase columns are especially inert to reactive sample components.

VOLUME I — INTRODUCTION

Chapter 1

The author-editors begin with a chapter directed to the critical first step in obtaining accurate measurement of the amino acid content of proteinaceous substances; sample preparation and protein hydrolysis. The main sources of variance between the amino acid content of the HCl hydrolysate and the protein from which it was derived are discussed, and results are presented from studies which compared high-temperature, short-time hydrolysis (145°—4 hr) with the more traditional (110°—24 hr) procedure for a diverse set of sample types. The results demonstrate the feasibility of the higher temperature, shorter hydrolysis time for many applications. The results of a prehydrolysis oxidation for analysis of cystine as cysteic acid and methionine as methionine sulfone for the diverse samples were evaluated, and amino acid values extrapolated from multiple hydrolysis times at 145° were compared with multiple hydrolysis at 110°, illustrating a means for obtaining values for amino acids such as isoleucine, valine, threonine, and serine which are more accurate than those from a single hydrolysate, and more rapid than the typical 110°-multiple hydrolysate technique.

We also evaluated interlaboratory variations in sample preparation, which revealed that although differences resulting from hydrolysate preparation by two different laboratories can be minimized, those variations are greater than the chromatographic or analytical variability. These studies further demonstrate that use of glass tubes with Teflon®-lined screw caps as hydrolysis vessels compares favorably with sealed glass ampules and that 145°—4 hr hydrolysis with these tubes, after careful exclusion of air, is a rapid, precise, and practical method for protein hydrolysis. The importance of careful sample preparation to the achievement of accurate data is stressed in Chapter 1, which also provides both a review of HCl hydrolysis of proteins and practical information on preparation of hydrolysates.

Chapter 2

The GC analysis of amino acids as the *N*-trifluoroacetyl (*N*-TFA) *n*-butyl esters is the subject of Chapter 2. This established method, principally developed in the author-editors' laboratories at the University of Missouri-Columbia, provides an effective and reliable means of amino acid determination that is applicable to a very wide range of analytical needs. Prof. Gehrke, graduate students, and colleagues during the period from 1962 to 1975 established the fundamentals of quantitative derivatization, conditions of chromatographic separation, and defined the interactions of the amino acid derivatives with the stationary and support phases. Their studies and continued refinements since 1975 have resulted in a precise and accurate, reliable, and straightforward method for amino acid measurement.

The chapter begins with an extensive review of the applications of GC to amino acid analysis of a wide range of matrices, from pine needle extracts to erythrocytes. The *Experimental* section provides a thorough description of the quantitative analytical procedures developed by the authors, including preparation of the ethylene glycol adipate (EGA) and silicone mixed phase chromatographic columns. The EGA column which is used to separate and quantitate all the protein amino acids except histidine, arginine, and cystine is composed of 0.65 w/w% stabilized grade EGA on 80/100 mesh acid-washed Chromosorb® W, 1.5 m × 4 mm I.D. glass. For quantitation of histidine, arginine, and cystine the silicone mixed phase of 1.0 w/w% OV-7 and 0.75 w/w% SP-2401 on 100/120 mesh Gas-Chrom® Q (1.5 m × 4 mm I.D. glass) performs extremely well. They also describe the preparation and use of ion-exchange resins for sample cleanup, and complete sample derivatization to the *N*-TFA *n*-butyl esters. The amino acids are esterified by reaction with *n*-butanol · 3 *N* HCl for 15 min at 100°C, the excess *n*-butanol · 3 *N* HCl removed under vacuum at 60°C, any remaining moisture removed azeotropically with dichloromethane, then the amino acid esters are trifluoroacylated by reaction with trifluoroacetic anhydride (TFAA) at 150°C — 5 min

in the presence of dichloromethane as solvent. Immediately following the *Experimental* section are valuable comments on various parts of the method which provide guidance to the use of the entire technique, from sample preparation to chromatography to quantitation.

Of particular value in this chapter is the comparison of GLC and IEC results of hydrolysates of diverse matrices. This extensive comparison of an array of sample types showed that the values obtained by the two techniques were generally in close agreement.

GLC and IEC analyses of multiple hydrolysates were performed to evaluate the reproducibility of hydrolysate preparation and to compare GLC and IEC analyses of the same hydrolysates. The total amino acids found in the same hydrolysates were essentially identical by both GLC vs. IEC. As the sets of three hydrolysates were prepared at the same time under identical conditions, it might be expected that differences between the GLC and IEC analyses of the same hydrolysates would be greater than the differences between identically prepared hydrolysates. However, slight differences in the amounts of certain amino acids present in the different hydrolysates can be observed, emphasizing that variations do arise due to the hydrolysis itself, even under preparation conditions most conducive to reproducibility.

As the sulfur-containing amino acids are of particular interest in nutrition, cystine and methionine analyses are discussed in detail. The quantitative determination of amino acids in addition to cystine and methionine in preoxidized hydrolysates by IEC is described, and a rapid oxidation-hydrolysis procedure is presented which allows accurate analysis of cystine, methionine, lysine, and nine other amino acids in feedstuffs and other biological matrices.

One of the authors of Chapter 2 (FEK) has used the *N*-TFA *n*-butyl ester method for more than 17 years on a routine basis in a commercial laboratory (Analytical Biochemistry Laboratories, Columbia, Mo.) and his observations on the analysis of an extremely wide range of sample types over this time span are presented in a special section of this chapter. *Experiences of a Commercial Laboratory* provides valuable practical information into amino acid analysis by GC.

The analysis of amino acids as the *N*-TFA *n*-butyl esters is an established technique that offers much to scientists concerned with the determination of amino acids. The method offers excellent precision, accuracy, selectivity, and is an economical complementary technique to the elegant Stein-Moore ion-exchange method.

Chapter 3

In this chapter, we provide both a detailed account and historical perspective of our development of GC amino acid analysis, and describe the solution of problems encountered as the methods evolved. The *N*-TFA *n*-butyl ester and trimethylsilyl (TMS) derivatives are discussed, including reaction conditions, chromatographic separations, mass spectrometric (MS) identification of both classes of derivatives, interactions of the arginine, histidine, and cystine derivatives with liquid phase and support materials, and application of the methods.

The acylation of arginine posed a problem in early studies, and the successful solution of this particular problem paved the way to a high-temperature acylation procedure which is now widely used with numerous acylating reagents. Likewise, esterification of the amino acids was investigated in detail, resulting in a direct esterification procedure which quickly and reproducibly converts the amino acids to the *n*-butyl esters. This approach has also been widely used to form various amino acid esters.

Chapter 3 describes the development of chromatographic columns for separation of the *N*-TFA *n*-butyl esters, from early efforts to obtain a single column separation of the protein amino acids to the successful development of a dual column system which has been used on a routine basis for some 15 years for quantitative amino acid analysis.

The early development of GLC analysis of iodine- and sulfur-containing amino acids as the TMS derivatives is described, with the finding that bis(trimethylsilyl)-trifluoroacetamide

(BSTFA), a silylating reagent which we invented, is an effective silylating reagent for forming amino acid derivatives.

Our studies on the derivatization of the protein amino acids with our new reagent, BSTFA, is described in which the amino acids are converted to volatile derivatives in a single reaction step. Although certain amino acids tend to form multiple derivatives which contain varying numbers of TMS groups, high-temperature, long reaction time derivatization permits quantitative analysis of the amino acids as the TMS derivatives. Our studies on the GLC separation of the TMS amino acids resulted in the development of a 6 m column of 10% OV-11 on Supelcoport® for separation of the TMS derivatives.

The development of a chromatographic column system for the *N*-TFA *n*-butyl esters came about from the realization that the derivatives of arginine, histidine, and cystine were not reproducibly eluted from columns with polyester liquid phases, although this type column was excellent for analysis of the other protein amino acids. We developed a siloxane mixed phase column specifically for these 3 amino acids, with the developed system being an EGA for 17 amino acids and the mixed phase column for these remaining 3. Studies on derivative interactions with liquid phases and support materials confirmed that the polar liquid phase EGA was primarily responsible for the destruction of the histidine derivative, and that the derivatives of arginine, histidine, and cystine are all subject to complex temperature-dependent interactions with EGA and nondeactivated support material.

Our invention of a GC solvent venting system is also described in Chapter 3. The venting system allows injection of 100 $\mu\ell$ or more, but prevents the solvent and excess acylating reagent from traversing the column while allowing quantitative transport of the amino acid derivatives through the column to the detector. This system was invented as a direct result of our involvement in the search for amino acids in the returned lunar samples, for which we wished to increase the volume of sample injected in order to search for part-per-billion amounts of amino acids. At that same time in the lunar studies we also conducted a survey of potential sources of low-level amino acid contamination which is also described in Chapter 3.

The power of GC/MS for identifying amino acids in complex matrices prompted us to obtain and publish the electron-impact mass spectra of the *N*-TFA *n*-butyl ester and TMS derivatives of the amino acids. We obtained mass spectra of 48 *N*-TFA *n*-butyl amino acids and 46 TMS amino acid derivatives, and Chapter 3 summarizes the major characteristics of their spectra.

Chapter 3 ends with a summary which points out that the foundation of successful amino acid analysis by GC is composed of two elements: (1) reproducible and quantitative conversion of amino acids to suitable derivatives and (2) separation and quantitative elution of the derivatives by the chromatographic column. This chapter provides the reader with valuable background information into the development and successful use of GC for amino acid analysis.

Chapter 4

S. L. MacKenzie of the Plant Biotechnology Institute, NRC, Canada, a leader in the successful development of the *N*-heptafluorobutyryl (*N*-HFB) isobutyl ester derivatives, describes the rationale leading to his extensive work, and he presents in detail the derivatization, separation and applications of this derivative in Chapter 4. His chapter contains a section entitled *Important Comments,* pointing out that derivatization is the most crucial factor in reproducible analysis of amino acids by GLC. His observations in that section will be particularly helpful to the analyst interested in utilizing the *N*-HFB isobutyl ester method. MacKenzie then discusses the analysis of protein hydrolysates and free amino acids, reviews the analysis of nonprotein amino acids and presents interesting applications of the analysis of free amino acids, such as studies of the free amino acid pool of starfish tissue, and

whether sulfur amino acids are involved in the mercury resistance of certain fungi. Chapter 4 concludes with an informative section on electron impact and chemical ionization mass spectral analysis of the *N*-HFB amino acid isobutyl esters.

Chapter 5

Noting that there are some 50 diseases known to be due to anomalies of amino acid metabolism, J. Desgres and P. Padieu of the National Center for Mass Spectrometry and the Laboratory of Medical Biochemistry at the University of Dijon, France, describe the development of the N-HFB isobutyl derivatives for the clinical analysis of amino acids, and the adaptation of the method to the routine practice of a clinical biochemistry laboratory in Chapter 5. The experimental protocol is described in detail, followed by application of the procedure for analysis of normal and pathologic physiological fluids including phenylketonuria, maple syrup disease, idiopathic glycinemia, cystathionase deficiency, cystathionase synthetase deficiency, and renal absorption disorders. Desgres and Padieu have routinely used their method for more than 6 years in a clinical biology laboratory, and conclude that GLC is perfectly suited for the daily analysis of more than 30 amino acids; they also point out the importance of GC/MS in elucidating metabolic disorders.

Chapter 6

I. Moodie of the National Research Institute for Nutritional Diseases and the Metabolic Unit, Tygerberg Hospital, South Africa, describes in Chapter 6 his development of an efficient GLC method specifically for routinely producing accurate determination of protein amino acids in fishery products. Moodie presents a thorough account of his comprehensive studies, describing his choice of suitable derivative and columns, modification of derivative preparation, and sample preparation techniques for packed column analysis of the *N*-HFB isobutyl esters. He describes his chromatographic procedure in detail, then reports the use of the procedure to statistically study the effects of sampling and hydrolysis on the precision of analysis. Moodie finds that a major contribution to reduced precision arises during sampling and hydrolysis. Moodie has extensively studied glass capillary columns for amino acid analysis and describes column preparation and use, column longevity, and comparisons of packed and capillary columns. His chapter serves as an excellent example of the practical utilization of amino acid analysis in nutrition by GLC.

VOLUME II — INTRODUCTION

Chapter 1

Development of the chiral diamide phases for resolution of amino acid enantiomers is the subject of Chapter 1 by E. Gil-Av, R. Charles, and S.-C. Chang of the Weizmann Institute of Science, Israel.

Pioneers in research on the separation of enantiomers, Gil-Av et al. discuss the evolution of optically active phases from the α-amino acid derivative phases (e.g., *N*-TFA-L-Ile-lauroyl ester) to dipeptide phases (e.g., *N*-TFA-L-Val-L-Val-*O*-cyclohexyl) to the still more efficient and versatile diamide phases of the formula $R_1CONHCH(R_2)CONHR_3$. Gil-Av et al. describe the synthesis and purification of the diamide phases, and the determination of their optical purity.

The influence of structural features of the diamides on resolution and thermal stability is discussed in detail. In these extensive studies, the structures of R_1, R_2, and R_3 in the above formula were varied with R_1 and R_3 representing various *n*-alkyl, branched alkyl, and alicyclic groups, and R_2 representing various aliphatic and aromatic groups. Chain lengthening of R_1 and R_3 groups produced the desired increase in thermal stability, yielding phases operable at 200°C and above.

Gil-Av then follows with an elegant discussion of the mechanism of resolution of amino acid enantiomers with the diamide phases, using models based on the consideration of conformations and modes of hydrogen bonding of simple diamides.

The effect of *N*-acyl and ester groups on chromatographic separation is described, with the conclusion that isopropyl esters are the best compromise with regard to volatility, resolution, and ease of preparation. Differences among the *N*-trifluoroacetyl (*N*-TFA), *N*-pentafluoropropionyl (*N*-PFP), and *N*-heptafluorobutyryl (*N*-HFB) groups were reported to be small, but perhaps could be exploited to overcome problems of peak overlap of amino acid derivatives emerging very close to each other. Gil-Av also presents information on the influence of the side chain attached to the asymmetric carbon of the amino acids on resolution.

This chapter provides an excellent description of enantiomeric analyses with diamide phases, with discussions of analyses of samples without and with peak overlap, and the use of phases with opposite configuration for identification purposes. In conclusion, Gil-Av et al. compare GLC and HPLC for resolution of amino acid enantiomers, and notes the advantages of the GLC techniques.

Chapter 2

The separation of amino acid enantiomers using chiral polysiloxane liquid phases and quantitative analysis by the novel approach of "enantiomeric labeling" is presented by E. Bayer, G. Nicholson, and H. Frank of the University of Tübingen, West Germany. The synthesis of a novel type of chiral stationary phase was accomplished by Bayer et al. which possesses sufficiently high thermal stability to enable GC separation of the enantiomers of all the protein amino acids within about 30 min. The stationary phase, subsequently named Chirasil-Val®, is a polysiloxane functionalized with carboxy alkyl groups to which valine-*t*-butylamide residues are coupled through and amide linkage. Bayer et al. describe the criteria which the stationary phase must fulfill, and discuss the steps taken to synthesize the stationary phase to meet those requirements. They also describe the preparation of capillary columns with Chirasil-Val® stationary phases and GC conditions needed to optimize the separations.

Bayer et al. studied the effects of the acyl and ester groups on the "enantiomeric resolution" and relative retention times of each amino acid on Chirasil-Val®, and concluded that for complete resolution of all protein amino acids, a number of acyl ester combinations are possible, notably the *N*-PFP *n*-propyl esters and isopropyl esters, and the *N*-TFA *n*-propyl esters.

The concept of the use of D-amino acids as internal standards for the quantitative analysis of the L-amino acids is first and elegantly described in Chapter 2. Bayer et al. point out the prior to analyses of amino acids in biological fluids, a procedure comprised of several steps is usually required to separate the amino acids from high molecular weight material, carbohydrate derivatives, fatty acids, oligopeptides, biogenic amines, etc., and that recovery of the amino acids during this process must be either quantitative or of a constant and known percentage. Also, variations can occur during derivatization and in the response of the detector, and discriminatory effects in the injector can arise when using the split technique of sample injection.

By using the optical antipodes as internal standards, not only are volumetric losses during work-up, derivatization and GC compensated, but also differences in chemical behavior such as recovery from ion-exchange columns during cleanup, yield of derivatization, and stability of the derivatives. With ''enantiomeric labeling'', the internal standard suffers exactly the same fate as its optical antipode. Bayer et al. illustrate the use of ''enantiomeric labeling'' for analyses of blood serum and gelatin, and present the mathematical expressions for determining the concentration of each amino acid in samples. Sensitivity, precision, and accuracy of the enantiomeric labeling technique are discussed, with accuracy of determination of synthetic samples surpassing ion-exchange chromatography (IEC). Thus GC analysis of amino acids by ''enantiomeric labeling'' is a powerful technique for a wide variety of applications. When many analyses are required Bayer also has introduced a new automated derivatization robot.

Chapter 3

The analysis of amino acid racemization and the separation of amino acid enantiomers is presented by H. Frank of the University of Tübingen, West Germany. The author presents examples of enantiomers of free amino acids and amino acid analogues which exhibit different biological properties. A tragic illustration is the glutamic acid derivative thalidomide, a sedative drug used in the 1950s which induced malformations of newborns. The L-enantiomer of a metabolite is teratogenic, the D-antipode is not.

The importance of stereochemical analysis of amino acids for studies on the biochemical, pharmacological, or toxicological properties of anantiomers is described, as well as its utilization in structure elucidation, peptide synthesis, racemization monitoring, and pharmaceutical research.

Frank discusses the assessment of racemization in peptide synthesis by separation of dipeptide diastereomers, detailing factors which give rise to racemization during peptide synthesis and describing how racemization can be suppressed. Then follows a comprehensive discussion of mechanisms involved in amino acid racemization, including factors such as the chemical state (free amino acid, derivative, or in peptide linkage) temperature and pH.

The chromatographic measurement of the D- and L-enantiomers as diastereomers and directly as enantiomers is discussed and compared, including factors which introduce error into the analysis. The use of GC/chemical ionization-mass spectrometry (CI-MS) in racemization studies is described in which deuterium/hydrogen exchange during hydrolysis is monitored by GC/CI-MS to ascertain the extent of incorporation of deuterium into each enantiomer.

Frank presents a thorough discussion of the mechanisms involved in hydrogen/deuterium exchange, which is a powerful tool for determination of true enantiomer ratios in synthetic peptides and proteins. Detailed analysis of racemization mechanisms is possible by this procedure, and in many cases it represents the sole possibility to discriminate between racemate generated during hydrolysis and the amount originally present.

Deuterium/hydrogen exchange and GC/MS allow measurement of much lower amounts of D-enantiomers in proteins than GC alone. Using only GC, a lower limit of measurement