JOURNAL OF CHROMATOGRAPHY LIBRARY - volume 43

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FOREWORD

A text that provides a compilation of an active area of research should be authored by individuals who themselves have made significant contributions to that field. It is within this aegis that the present authors have edited this latest addition to the library of useful natural products publications.

In earlier publications in this series, two volumes have appeared which were the efforts of investigators who had made important contributions to antibiotic discoveries. These were "Chromatography of Antibiotics" in two editions, 1973 and 1978, and "Antibiotics; Isolation, Separation, and Purification", 1978. One of the authors, Gerald Wagman, has joined with Raymond Cooper to carry on this tradition of erudition.

Microbiology has continued its march into other prolific fields of exploration. Microorganisms display an impressive biosynthetic ingenuity, and have become a useful source of enzyme inhibitors, active peptides, and immunomodulators. This present volume has addressed these new developments. The organisms have not changed, but the techniques for the identification of their products have become more sensitive and selective. This volume, which compiles the experience of the experts in their fields, has allowed for the dissemination of these changing techniques. Since important bioactive compounds are also being found from terrestrial and marine sources, it is appropriate to expand the scope of this book to include them.

My perspective of the field is now viewed through a retired eye, but my vision is clear that this volume will be a valued addition to the continuing generations of microbial screeners and natural products chemists.

Marvin J. Weinstein, Ph.D.
Vice-President, Microbiology
Schering Corporation (Retired)

PREFACE

Progress in the discovery of natural products requires a multi-disciplinary approach involving the expertise of scientists in such fields as biology, botany, molecular biology, chemistry and pharmacology. Although any given project may be interdisciplinary in nature, the contributions of the natural products chemist to the isolation of a particular compound are unique. Using modern technology it has often been possible to purify and identify a new and potentially useful compound on a scale so small that several years ago was considered impossible. The advances in the biological, medicinal and pharmaceutical sciences have been maintained in no small part due to improvements in chromatographic methods and separation techniques. In the cases of isolation of small quantities of bioactive material, the challenge of structure determination has been complemented by the ever impressive sophistication of spectroscopic methods and instrumentation. The scope of this book is to encompass, in as much detail as possible, the most recent progress made in the isolation and separation of compounds of natural origin. This book is an outgrowth of the previous book in this field (Antibiotics, Eds. M. Weinstein and G. Wagman) but is expanded to cover entirely new material and other natural products. We have attempted this project by bringing together 14 diverse chapters aimed at presenting modern methods of isolating antibiotics, marine and plant derived substances, enzyme inhibitors and interferons. The chosen topics are organized to present modern methodology for separation of antimicrobials, antivirals and enzyme inhibitors from a variety of sources. These chapters cover families or groups of compounds that have gained prominence in the last ten years, e.g. avermectins, interferons.

The first two chapters are devoted to newer methodologies applied to purification of a variety of compounds. There is an extensive review on applications of counter-current chromatography. We are witnessing a renaissance in liquid chromatography due to some innovative technology, and a review of several different techniques is presented. The applications of the emerging HPLC - photodiode array technology are reviewed in Chapters 2 and 4. It should be noted in the previous volume that aminoglycosides, tetracyclines and macrolides were reviewed; in this volume HPLC methods development, detection and screening of these compounds are presented

reflecting the advancement and the present interest in these families of compounds within the pharmaceutical industry. Although affinity chromatography is not a new approach, a review of the first application to the separation of antibiotics directly from fermentation broth is given in Chapter 3. Using this approach, a large family of glycopeptides has been isolated.

There are several selected topics indicating the isolation of antimicrobials. These include the family of antitumor compounds known as the saframycins and a comprehensive review of a very important new class of antiparasitic agent, the avermectins. An important source for antimicrobials and other bioactive natural products has been from the sea and an update of isolation and purification of a variety of marine-derived compounds is given in Chapter 10. The prominence and importance of β-lactams in the field of antimicrobial chemistry cannot be underestimated. For this reason we have included three chapters indicating the major advances over the past 10 years. The monobactams and thienamycins are extremely unstable natural products produced in culture broth. The successful discovery and isolation of small quantities have led to their subsequent development as new β-lactam antibiotics in the market place.

Today, directed screens for enzyme inhibitors is an integral part of a natural products program. Isolation and purification of these natural products from fermentation and plant sources are included. The chapter on enzyme inhibitors was written by Professor Hamao Umezawa shortly before his untimely death. Dr. Umezawa's son, Dr. Kazuo Umezawa, has kindly given of his time to put the manuscript in final form suitable for publication. We wish to express our sincere appreciation for his efforts in completing this work.

The chapter which follows shows that a relationship exists between plant-derived enzyme inhibitors and those derived from microorganisms. In discussing alkaloidal glycosidase inhibitors, this work reflects the interest of the plant chemist in the isolation of water soluble compounds. The final chapter is a futuristic essay indicating the challenges of the plant chemist to isolate minute amounts of natural products. By discovering these bioactive principles it may open the door to studying the fascinating biological properties which they possess.

We have chosen to include in this volume a chapter devoted to the isolation of interferons in recognition of the impact of molecular biology in the field of natural products and also in part as a reflection of the direction microbial products may take in the years to come.

We wish to thank Drs. Vincent Gullo, George Miller and Claude H. Nash of Schering Research for their encouragement and help in making this book possible. We also wish to acknowledge the excellent cooperation of Dawn Foster, for her assistance in the typing. Lastly, and most important, this volume would not have been possible without the superb cooperation we received from all of the chapter authors. It was a real pleasure to have worked with them and we appreciate their patience with us!

Gerald H. Wagman
Raymond Cooper

CONTENTS

Chapter 1
COUNTERCURRENT CHROMATOGRAPHY

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1. INTRODUCTION.

Countercurrent chromatography is a liquid-liquid partition chromatography technique employing two immiscible liquid phases. One phase is designated as the stationary phase and is retained as such by either gravitational or centrifugal forces while the second phase, the mobile phase, is passed through the stationary phase. Separation of the components of a mixture is achieved by introduction of the sample at one end of the stationary phase with the mobile phase. Each component is then retained by the stationary phase to a degree dependent on its partition coefficient for the two-phase system. Individual components are eluted from the other end of the stationary phase by the mobile phase. That component with the partition coefficient most in favor of the mobile phase will be eluted first.

As a separation technique, countercurrent chromatography has several inherent advantages. The number of two-phase systems available is virtually limitless and in order to achieve a separation between any two compounds all that is required is that a two-phase system be found in which the two compounds have significantly different partition coefficients. Loss of sample by irreversible absorption or chemical degradation, a common problem in chromatographic methods involving a solid support, is seldom experienced during countercurrent chromatography. Several of the more modern instruments give rapid chromatographies and, with the appropriate solvent systems, can provide the mildest possible conditions for a chromatographic separation.

Varieties of new instruments have recently been developed or are currently enjoying rapid advances and these offer the chemist high resolution separation techniques which may be tailored to any particular purification problem. Ito and Conway have written several detailed chapters on the design and theory behind the many different instruments designed by the former.[1-9] The reader is directed to these writings for greater appreciation of the historical development of current instruments and a more thorough understanding of the theoretical aspects. The latter is dealt with here only to that extent required for optimal utilization of the technique. Hostettmann and Hostettmann have provided many practical reviews with emphasis on the separation of plant products mainly with Droplet Countercurrent systems.[10-15] Elsewhere in this volume Mynderse *et al* give a detailed description of the use of the Centrifugal Countercurrent with Rotating Joint Seal for the separation and purification of tunichromes.

Although most of the examples discussed herein have been drawn from the antibiotic area, we have included examples from other areas where these have involved (i) the use of unusual solvent systems or (ii) compounds of uncommon polarity or (iii) where the number of published examples of use of a specific instrument was particularly limited. Anticipating that the chemist will find the tables most useful we have endeavored to include a wide a variety of solvent systems and to cover as many different classes of natural products as possible.

1.1 Historical Development.

Countercurrent chromatography was originally developed in the early 1950's with much of the pioneering work done by Lyman Craig. This work is well documented [16] and culminated in the

design and commercialization of multi-tube glass countercurrent distribution instruments which were commonly referred to as "Craig machines". The high resolution capabilities inherent in the method surpassed anything available from other separation techniques at the time. The instruments, however, were large, cumbersome, and fragile and required dedicated venting systems to remove organic solvent vapors. Moreover a typical separation, including set-up and instrument cleaning would encompass a week's work. None-the-less, the technique was used to achieve difficult, or otherwise impossible separations. Gross [17] for example, separated individual components of the Gramicidin complex, a family of pentadecapeptide antibiotics the individual members of which in some instances differed by a single residue change of valine to isoleucine.

With the advent of high resolution solid-phase support chromatography systems, especially HPLC, that allowed for rapid and convenient separations, countercurrent distribution fell into relative disuse. The design and commercialization, within the last ten years, however, of several new continuous-flow countercurrent chromatography instruments has provided a resurgence of interest in this separation methodology.

1.2 Basic Theory.

For the Craig machine, where each cell is a discrete unit and equilibrium distribution is achieved between each transfer, the mathematics of distribution of a component of a given partition coefficient for a set number of transfers is relatively simple. Some examples of these are shown in Figure 1-1. Given the volumes of upper and lower phase in each cell and the partition coefficient of a particular component of a mixture, the number of transfers required to elute that component can be readily calculated. These calculations assume that the partition coefficient of one

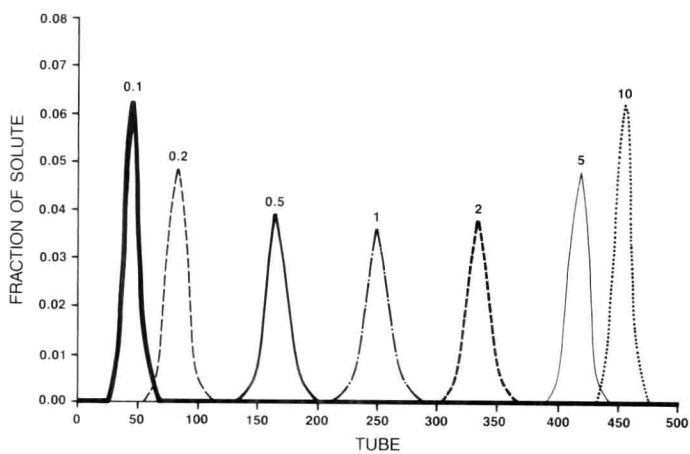


Figure 1·1 Theoretical distribution curves of solutes of various Partition Coefficients from 0.1 to 10 in the tubes of a Craig machine after 500 transfers with equal volumes of each phase

component in the two-phase system is not significantly affected by the other components of the analyte mixture and in practice this seems to be the case. For continuous-flow countercurrent chromatography the number of unknown variables makes a rigorous mathematical analysis virtually impossible, however, extrapolation from a distribution analysis modified by the results of experience is the most common *modus operandi*.

In principle, all that is required to separate two components is a two-phase system in which the two components have different partition coefficients. The number of transfers needed in a Craig machine to obtain baseline separation is a function of ß, the ratio of the partition coefficients. Craig **16** calculated the number of transfers required to give 99.73% pure compounds from a mixture of equal parts of two components for various ß values. For a ß value of 1·5 this degree of purity required 872 transfers whereas if the ß value was increased to 10 only 24 transfers gave the same separation.

Compounds with partition coefficients favoring the mobile phase will be eluted from the system as relatively sharp bands however as the countercurrent chromatography continues, the bands broaden. Figure 1-2 shows the theoretical distribution of compounds with partition coefficients of 8, 1 and 0·1 after the number of transfers required to bring the peak concentrations of these bands to tube 25 of a Craig machine. For the partition coefficient (K) of 8, this required only 28 transfers, for K=1, 50 transfers were required and for K=0.1, 275 transfers were needed.

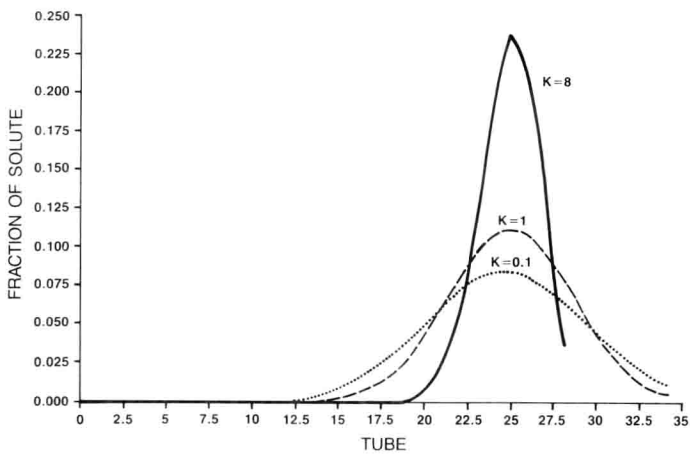


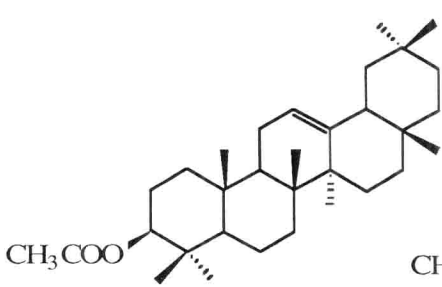
Figure 1·2 Theoretical distribution curves of solutes of various Partition Coefficients after the number of transfers of equal phase volumes required to bring the peak concentration to tube 25

1.3 Scope and Limitations

The spreading of a band can be avoided only if one component favors the stationary phase very strongly and no attempt is made to elute the sample from the column with mobile phase. Then the sample is recovered from the stationary phase at the end of a chromatography thus involving

fewer transfers. Under these conditions countercurrent techniques have been used basically as means of efficiently extracting away impurities, although it can be used to achieve a classical chromatographic separation. For example, benzanthrins A and B were separated and each recovered from the stationary phase after CPC chromatography.[18]

For compounds of intermediate polarity, as is the case for many bioactive secondary metabolites, there exists, in the literature, a wide variety of two-phase solvent systems that will give partition coefficients in a useful range (see Tables in section 5). However for very polar or very non-polar compounds the choice of solvent systems can be quite limited and we have endeavored to include all such systems in the accompanying tables. The capacity of a particular system is dependent on the solubility of the analyte components in each phase . Problems arising from poor solubility are most common with solvents at either end of the polarity spectrum. None-the-less, fine separations can be achieved at these extremes. For example, the triterpenes ß-amyrin acetate **1** and taraxasterol acetate **2** differ only in the positions of a double bond and a methyl attachment and



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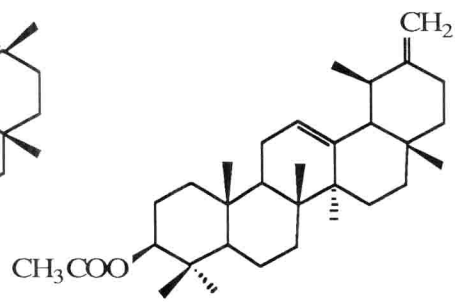

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might be regarded as a chromatographic challenge under any circumstance. However they were separated on the Ito coil planet centrifuge with a system of hexane-acetonitrile-methanol-ethyl acetate (5:5:4:2).[19]

Solubility problems can often arise in the form of a "self inflicted wound" where the investigator has deliberately chosen poor solvents such as carbon tetrachloride or n-heptane as components of the organic phase in order to force the partition coefficients of the analytes more in favor of the aqueous phase.

Continuous-flow countercurrent chromatography is most commonly used as a small scale preparative separation tool, but it does have other applications. It has also been used for extraction where a compound was present in a large volume of a solvent that could be used as the mobile phase of a two-phase system in which the compound partitioned strongly in favor of the other (stationary) phase. An example of this use is the extraction of the metabolites of daunorubicin and adriamycin from urine (the mobile phase) by buffer-saturated n-BuOH (the