

recent advances in phytochemistry

volume 25

Modern Phytochemical Methods

RECENT ADVANCES IN PHYTOCHEMISTRY

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volume 25

Modern Phytochemical Methods

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PREFACE

This volume contains reviews which are based on a symposium, given at the 30th meeting of The Phytochemical Society of North America, held at Laval University in Quebec City, Canada on August 11-15, 1990. During the past two decades, there have been major new developments in methods which can be applied toward the isolation, separation and structure determination of complex natural products. Therefore, the topic of this symposium, "Modern Phytochemical Methods", is a very timely one. The organizers of the symposium recognized that it would not be possible to cover in detail all new advances in phytochemical methodology. It was therefore decided to emphasize general reviews on recent developments of major separation techniques such as high performance liquid chromatography as well as supercritical fluid chromatography. In addition, advances in commonly used structure determination methods, mainly NMR and MS, are reviewed. Other topics include methodologies of micro-sampling for isolation and analysis of trichome constituents as well as recent breakthroughs on biosynthetic studies of monoterpenes using "enriched" basal cells of trichomes. The volume concludes with a review of quantitative structure-activity relationship (QSAR) studies of biologically active natural products.

In Chapter 1, K. Hostettmann and his colleagues give a general review of recent developments in the separation of natural products with major emphasis on preparative separations of biologically active plant constituents. The authors present a comparison of droplet countercurrent chromatography (DCCC) with the highly rapid and more versatile centrifugal partition chromatography (CPC). The wide range of applications of CPC in separations from milligram to gram quantities of substances of varying polarity, from very polar to nonpolar, provide a powerful addition to the arsenal of liquid-liquid chromatography. Besides representative examples from their own laboratory, the author provides a tabulation of recent literature reports on applications of CPC in the separation of a wide variety of different structural types in natural products.

Barron discusses developments in the prefractionation and semi-preparative separation of free and glycosidically bound volatile natural products

by HPLC. This new methodology is very useful in the analysis of volatiles in perfumes, aroma constituents and, in particular, of glycosidically bound "aroma precursors". The author summarizes in tabular form recent reports on the HPLC separation of mono- and sesquiterpenes, essential oils and aroma constituents as well as other plant-derived volatiles and related nonvolatile glycosidic precursors.

In Chapter 3, Davin, Lewis and Umezawa give an up-to-date account of enantioselective separations in phytochemistry. After a brief review, the authors outline various direct and indirect chromatographic methods by GLC and HPLC for the separation of enantiomeric mixtures. Enantioselective separations of natural and synthetic racemates on chiral stationary and/or mobile phases are discussed. Specific examples of separations of racemic terpenoids, lignans, isoflavonoids, catechins and a wide spectrum of other natural products provide convincing evidence that enantioselective separations will soon find routine applications in resolving stereochemical questions in structural as well as biosynthetic studies.

The ability to separate thermally labile and/or/nonvolatile compounds at ambient temperatures makes supercritical fluid chromatography (SFC) a highly valuable chromatographic complement to GLC and HPLC. Foley and Crow provide an extensive account of the use of SPC in the analysis of natural products. The authors provide detailed information of the methodology of SFC and discuss its application in the natural products field, which also includes supercritical fluid extractions.

Pidgeon and collaborators discuss their novel and potentially highly useful chromatography studies with immobilized artificial membranes (IAM). This new solid-phase membrane mimetics methodology shows considerable promise for the purification of membrane proteins. Other potential non-chromatographic applications of IAM include the correlation of drug-binding to IAM with drug transport through membranes.

Recent developments in mass spectrometry (MS) and their application in the structure determination of natural products are reviewed by Pyrek. After a general introduction to mass spectrometry, this extensive review presents applications of modern MS methods, including the well established GC-MS and the powerful new HPLC-MS separation-identification techniques. In addition, examples of field ionization (FI) and field desorption (FD) as well as secondary ion mass spectrometry (SIMS), fast atom bombardment (FAB), plasma desorption (PD), PD-MS and laser desorption techniques are presented. This comprehensive account closes with the use of isotopes in mass spectral studies. The presentation of Smith and coworkers provides specific examples of new

mass spectral methods with major emphasis on strategies for structure elucidation of natural products.

The last two decades have seen major developments in the application of nuclear magnetic resonance (NMR) techniques in structural as well as biosynthetic studies of natural products. The paper by Fischer, Vargas and Menelaou provides a review of commonly used 1D and 2D NMR techniques, which includes applications of the various polarization transfer experiments (DEPT, INEPT, INAPT, COLOC, etc.). The authors discuss representative examples (sesquiterpene lactones, flavonoids and polyacetylenes) from their own laboratory to demonstrate the strength and limitations of the various 1D and 2D ^1H NMR and ^{13}C NMR methods. The review concludes with a tabular summary of modern NMR methods in structural studies of a wide range of different types of natural products.

Spring discusses a simple, rapid and inexpensive trichome micro-sampling technique for the application of micromolecular natural products (sesquiterpene lactones) as markers in biochemical systematic studies in the Asteraceae. This technique, which permits qualitative and quantitative analyses by HPLC of trichome constituents, has considerable potential for the isolation of specific natural products from plant trichomes in sufficiently large amounts to be used for subsequent structural studies, by MS and NMR.

Gershenzon and colleagues of Croteau's laboratory present detailed new procedures for isolating secretory cells from glandular trichomes to be used for intact and cell-free preparations of monoterpenes in peppermint. Since their data clearly indicate that monoterpene biosynthesis is restricted to glandular trichomes, the described methodology will undoubtedly find extensive general applications in biosynthetic studies of other natural products.

The concluding chapter discusses quantitative correlations of biological activities of natural products in general and phototoxicity of natural and synthetic thiophenes, in particular. The authors provide a convincing case for the use of relatively simple models for quantitative structure-activity relationships (QSAR). Potential future applications include many areas of plant sciences, from plant physiology to chemical ecology.

Finally, we wish to thank Jeremy N. McNeil, Laval University, who arranged, with Murray Isman's help, the Quebec meeting. We also acknowledge the generous financial support of the National Sciences and Engineering Council of Canada.

February, 1991

N.H. Fischer
M.B. Isman
H.A. Stafford

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Chapter One

NEW DEVELOPMENTS IN THE SEPARATION OF NATURAL PRODUCTS

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INTRODUCTION

Over the last ten years, there has been a growing interest in drugs of plant origin. During this period, consumption of medicinal plants has almost doubled in industrialized countries of Western Europe where substances derived from higher plants constitute approximately 25% of prescribed medicines.¹

The isolation of pure biologically active constituents responsible for the properties of medicinal plants is essential for structure elucidation, structural

modifications and for studying the mode of action of drugs, their side-effects, toxicology, etc. For these investigations, quantities in excess of 1 g of the relevant compounds are generally required. It should be mentioned here that plants may contain hundreds of different constituents and that often a single compound (or a series of closely related substances) is responsible for the biological or pharmacological properties observed in crude extracts.

The process that leads from the plant to a pharmacologically active, pure constituent is very long and tedious, and requires a multidisciplinary approach involving botanists, pharmacognosists, chemists, pharmacologists and toxicologists. The latter can be divided into the following steps (see Fig. 1):²

- collection, proper botanical identification and drying of the plant material.
- preparation of appropriate extracts and preliminary chromatographic analysis by TLC and HPLC.
- biological and pharmacological screening of crude extracts.
- several consecutive steps of chromatographic separation, where each fraction has to be submitted to bioassays in order to follow the activity (activity-guided fractionation).
- verification of the purity of the isolated compounds.
- structure elucidation by chemical and physicochemical methods.
- partial or total synthesis.
- preparation of derivatives/analogs for the investigation of structure-activity relationships.
- large-scale isolation for further pharmacological and toxicological tests.

When screening for biologically active plant constituents, the selection of the plant species to be investigated is obviously a crucial factor for theulti-

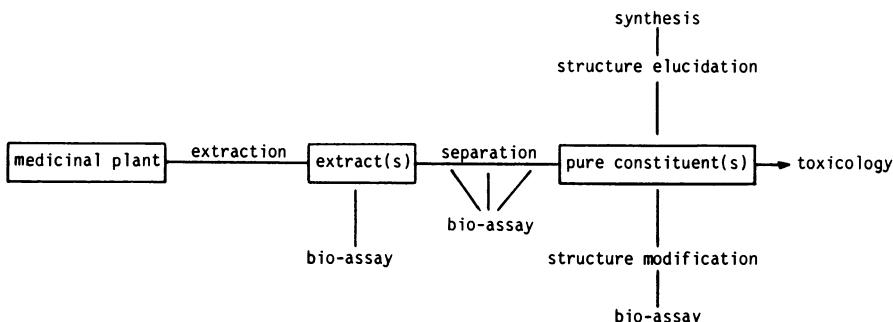


Fig. 1. Procedure for obtaining the active principles from plants.

mate success of the investigation. Besides random collection of plant material, targeted collection based on consideration of chemotaxonomic relationships and the exploitation of ethnomedical information is currently used. In particular, plants that have been used in traditional medicine are more likely to yield pharmacologically active compounds. However, the gathered information should be critically analyzed before undertaking a phytochemical investigation. For complex diseases, it is difficult for a healer in the village to make a correct diagnosis. Nevertheless, we believe that selection of plants based on information from traditional medicine is much more efficient than any random selection.

The discovery of promising extracts and the subsequent activity-guided isolation of constituents puts specific requirements on the bioassays to be used for that purpose. They have to be simple, rapid, reproducible, and inexpensive in order to be compatible with the large number of assays to be performed. In the crude extract, active principles are generally present at low concentration only. Inactive compounds interfering with the test system (tannins, etc.) may be present, and poor solubility under the test conditions is common. False negative or false positive results are therefore a much more serious problem than when dealing with pure compounds.

Most important of all during the whole isolation procedure is the judicious choice of chromatographic techniques for maximum yield of pure substances with a minimum of effort. Numerous chromatographic techniques have been developed recently for the preparative and semi-preparative separation of natural products.³ The past few years have seen an increasing interest in chromatography systems involving liquid-liquid partition (absence of solid stationary phase). Droplet countercurrent chromatography (DCCC) has found numerous applications⁴ but is limited by time considerations and by the choice of solvent systems. The newly emerging technique of centrifugal partition chromatography (CPC) has done much to overcome these limitations and provides a very useful addition to the methods available for the separation of different classes of compounds.⁵ CPC has been used for the separation of simple mixtures and complex plant extracts, containing both polar and non-polar constituents.

PREPARATIVE SEPARATION METHODS FOR PLANT CONSTITUENTS AND OTHER NATURAL PRODUCTS

Preparative scale separation is one of the most important operations which is carried out in a natural products laboratory. It is often tedious and time

consuming, especially when the mixture to be separated is complex. Over the past ten years, several new techniques, leading to an acceleration and simplification of complicated purification and separation steps, have emerged. However, there is no universal technique capable of solving all separation problems. All of them possess their advantages and their limitations, and best results are often obtained by the combination of two or more different methods.

The most important preparative separation techniques which have found application in the isolation and purification of natural products are listed in Table 1. A distinction has to be made between techniques using a solid stationary phase or a liquid fixed on an "inert" solid support, and all-liquid partition techniques. In the first category, column chromatography using solid stationary phases of different origins is very popular and used extensively. This technique has high load capacity, but the separation time is long and the resolution relatively poor. Several preparative systems have evolved that reduce the separation time. These include vacuum liquid chromatography⁶ and flash chromatography⁷ which give rapid separations with moderate resolution. Even in cases where high resolution is required, preliminary purification by these simple techniques allows for subsequent use of high-resolution separations without contamination of expensive high-performance liquid chromatography (HPLC) columns. Preparative HPLC is a more efficient method and is becoming very popular. For example, this method has been used by us for the isolation of pure compounds from crude medicinal plant extracts.⁸ The separation time was short and the resolution was high, but prepurification of the sample by one of the above methods prior to injection was necessary in order to avoid column contamination. The use of chemically bonded silica gel as the stationary phase in preparative liquid chromatography greatly increases the versatility of this technique. Numerous examples of separation of polar compounds using reversed-phase preparative HPLC have been reported.³

In many laboratories, preparative paper chromatography or preparative thin-layer chromatography (TLC) is still often used. The time-consuming recovery of the components from the stationary phase is avoided by a new and elegant centrifugally accelerated, radial, thin-layer chromatography instrument called the Chromatotron. In this system, the TLC plate (rotor) is not horizontal but inclined and thus allows a better collection of the eluate. Centrifugal chromatography can be achieved very fast with a small solvent consumption and may replace in numerous cases classical column chromatography and preparative TLC.^{3,9}

All the above mentioned techniques require a solid stationary phase or a liquid stationary phase fixed on an "inert" support. The presence of this solid

Table 1. Preparative separation methods for plant constituents

Paper chromatography
Preparative thin-layer chromatography
Centrifugal TLC
Open-column chromatography
Vacuum liquid chromatography
Pressure liquid chromatography
- Flash
- Low pressure LC
- Medium pressure LC
- High pressure LC (HPLC)
Liquid-Liquid chromatography
- Craig distribution
- Droplet countercurrent chromatography (DCCC)
- Rotation locular countercurrent chromatography (RLCC)
- Centrifugal partition chromatography (CPC)

packing material in a column, on a plate or on a disk may cause irreversible adsorption when dealing with very polar compounds or produce modification of the solute at the liquid-solid interface. In order to avoid complications arising from solid supports, various support-free liquid-liquid partition techniques have been developed.¹⁰ The process of liquid-liquid partition resembles simple extraction between two immiscible liquids in a separatory funnel. Separation results from the differing distribution of the various solutes between two phases as defined by the partition coefficient. Droplet countercurrent chromatography (DCCC) and centrifugal partition chromatography (CPC) have found numerous applications in the field of natural products isolation,¹¹ whereas rotation locular countercurrent chromatography^{12,13} (RLCC) is of limited interest.

DROPLET COUNTERCURRENT CHROMATOGRAPHY (DCCC)

Droplet countercurrent chromatography, developed by Tanimura *et al.*,¹⁴ is a simple all-liquid separation technique and possesses high efficiency. It is one of the earlier introduced liquid-liquid separation techniques which relies on

the partition of a solute between two immiscible solvents, the relative proportions of solute passing into each of the two phases being determined by the respective partition coefficients. Retention of stationary phase relies on gravity and the technique is slow. DCCC grew out of the observation that a light liquid phase with low wall surface affinity formed discrete droplets that rose through the heavy phase with visible evidence of very active interfacial motion. Under ideal conditions, each droplet could become a "plate" if kept more or less discrete throughout the system.¹²

A typical DCCC instrument consists of 200-600 vertically-arranged columns (20-60 cm in length) of narrow-bore silanized glass tubing (1.5 to 4 mm I.D.), interconnected in series by capillary Teflon tubes. The whole apparatus is first filled with the stationary phase of a biphasic solvent system before the sample is injected. The mobile phase is then pumped via the sample chamber into the first of the columns, forming a stream of droplets in the immiscible stationary phase. Depending on the choice of solvents for the mobile and stationary phases, these droplets are made either to ascend ("ascending mode") or descend ("descending mode") through the columns.¹⁵ As the mobile phase moves through the column in the form of droplets, turbulence promotes efficient partitioning of the solute between the two phases. Separation occurs according to the difference in the partition coefficients of the components of the sample.

Solvent systems which form two immiscible layers are usually suitable for DCCC. However, there are some limitations. The generation of droplets having suitable sizes and mobility is governed by factors such as the difference in specific gravities of the two liquid phases, the viscosity of solvents, the surface tension, the flow rate of the mobile phase, the diameter of the inlet tip, and the internal diameter of the column. In general, small-bore columns (less than 1.0 mm I.D.) produce flow plugs in which the entire contents of the tube are displaced. On the other hand, it should be noted that a large diameter of the tubes decreases the efficiency of separation. An internal column diameter of about 2 to 3 mm seems to be ideal for efficient separations. By using columns with 2 mm I.D., the choice of solvent systems is limited, as the formed droplets must have a smaller size than the internal diameter of the column. For successful DCCC separations, therefore, the choice of the two-phase solvent system is crucial. Ternary (or quaternary) systems should be used, such that the addition of a third (or fourth) component, miscible with the other components, diminishes the polarity difference between the two phases. The selectivity of the system is thus increased, allowing the separation of closely-related substances. Furthermore, the interfacial tension and viscosity of the system

should be diminished by the third component. Some basic solvent systems used in DCCC are listed in Table 2. The majority of DCCC separations involve polar compounds, especially glycosides, which are often difficult to purify. Chloroform-methanol-water systems of varying composition remain the most widely-used, in view of the good droplet formation and convenient viscosity of this combination.

As a means of selecting a suitable solvent system for DCCC separations, a thin-layer chromatographic (TLC) investigation of the mixture or extract may be made.¹⁵ Migration of the sample on silica gel plates with the water-saturated organic layer of a two-phase aqueous solvent system is carried out, and if the R_F values of the compounds to be separated are higher than about 0.50, the less polar layer is suitable for use as the mobile phase. For more polar solutes ($R_F < 0.50$), the more polar layer is used as the mobile phase. When the R_F values are in the range 0.40-0.60, the separation can be achieved by using either the more or the less polar layer as the mobile phase.

If the R_F values of the compounds to be separated are either too low ($R_F < 0.20-0.30$) or too high ($R_F > 0.70-0.80$), no elution will take place in a reasonable time and a different solvent system should be considered. However, the separation of a mixture with components of widely ranging polarities is possible if the organic phase is used first as a mobile phase to elute the less polar compounds. The polar components remaining on the column are pumped out and rechromatographed using the aqueous phase as the mobile phase for their separation.

Table 2. DCCC solvent systems

Basic binary system	Auxiliary solvent
<i>n</i> BuOH-H ₂ O	MeOH, EtOH, acetone, acetic acid, pyridine
CHCl ₃ -H ₂ O	MeOH, <i>n</i> PrOH, <i>i</i> PrOH
<i>n</i> C ₇ H ₁₆ -MeOH	chlorinated hydrocarbons, acetone
<i>n</i> C ₇ H ₁₆ -CH ₃ CN	chlorinated hydrocarbons, acetone

For non-aqueous solvent systems, TLC on silica gel is not suitable. Instead, chemically bonded phases such as RP-8 are employed.¹⁶ Although liquid chromatographic (LC) analysis of the distribution of a sample between two immiscible phases is also possible, TLC provides a very simple and rapid preliminary indication of the applicability of any given two-phase solvent combination of DCCC. An alternative method of selecting suitable solvents is to monitor the distribution of 5-10 mg of sample between 5-10 ml of each of the two phases constituting the solvent system.¹³ A system in which 15-25 % of the sample is distributed in one of the phases is chosen. The stationary phase employed is the one that contains the most sample.

Numerous examples of purification and separation of various classes of natural products from plant sources by DCCC have already been reported.^{4,11,15} These include saponins, flavonoids, anthraquinones, alkaloids, sugars and various terpenoids. DCCC gives highly reproducible separations and is easy to operate. It certainly seduces by its simplicity and separates various types of compounds on a preparative scale (quantities from milligram amounts up to several grams can be handled) with a relatively high resolution. It possesses, however, limitations arising from the facts that the efficiency of the method depends entirely upon droplet formation.¹⁵ Further-more, the separation time is relatively long. A separation, including the filling of the instrument with stationary phase, requires at least 12-15 hours, and can last several days. The newly emerging technique of centrifugal partition chroma-tography (CPC) has done much to overcome these limitations and provides a very useful addition to the methods available for the separation of natural products.

CENTRIFUGAL PARTITION CHROMATOGRAPHY (CPC)

Centrifugal partition chromatography (CPC) is a liquid-liquid chromatographic method employing two immiscible phases in which the liquid stationary phase is retained by a centrifugal force rather than by a solid support. Separation of a mixture is achieved by introducing the sample into the stationary phase and eluting with mobile phase. Those components with a partition coefficient in favour of the mobile phase will be eluted first. The technique was introduced in order to obtain separations analogous to stepwise liquid-liquid extraction but in a continuous mode.¹⁰ Centrifugal partition chromatography is rapidly proving to be an indispensable tool in the separation laboratory, and a number of preparative and analytical applications that illustrate the potential of the technique will be described here. Of particular interest is the separation of

bioactive components from crude extracts of plants, marine organisms and microbial fermentation media.

Instrumentation

There are two alternative designs of apparatus presently available:

- a) a system of cartridges arranged around the circumference of a centrifuge, as found in the instruments marketed by Sanki Engineering Ltd.
- b) a separation coil, composed normally of teflon, as found in the instruments developed by Y. Ito of the National Institutes of Health, Bethesda, Maryland, U.S.A.

Both approaches rely on the application of a centrifugal force to hold the stationary phase in the separation unit. In the cartridge system, method a), also known as centrifugal droplet countercurrent chromatography (CDCCC), each cartridge consists of numerous tiny channels in which the liquid-liquid partition process occurs. The principle of method b) can be explained by considering a static coiled column system. If the coil is filled with stationary phase of a biphasic solvent system and the other phase is pumped through the coil at a suitable speed, a point is reached whereby no further displacement of stationary phase occurs. The apparatus now contains approximately 50 % of each of the two phases and steady pumping-in of the mobile phase results in elution of the mobile phase alone. Both droplet countercurrent chromatography (DCCC) and rotation locular countercurrent chromatography (RLCC) are extensions of this gravity method. However, this basic system uses only 50 % of the efficient column space for actual mixing of the two phases. A more effective way of using the column space is to rotate the coil while eluting the mobile phase. A hydrodynamic equilibrium is rapidly established between the two phases and almost 100 % of the column space can be used for their mixing. Thus the interfacial area of the phases is dramatically increased.¹⁷ As there is no solid support, solute retention of injected samples depends only on the partition coefficients. The number of partition units in the column system can be augmented by increasing the number of coils, by reducing the internal diameter of the coiled column and by reducing the helical diameter. In the most recent development, known as "high-speed countercurrent chromatography", the mobile phase is pumped at high flow rates, while retaining the stationary phase in the coil by fast rotation speeds. Separation times are short and partition efficiency is high.¹⁸

The Sanki Centrifugal Partition Chromatographic System, Model LLN.

This instrument, manufactured by Sanki Engineering Ltd., Kyoto, Japan, was first described in 1982 by Murayama *et al.*,¹⁹ but it is only recently that its use has been fully exploited. It consists of a series of 12 cartridges arranged around the circumference of a centrifuge rotor (Fig. 2). Each cartridge contains 400 separation channels, the longitudinal axes of which are parallel to the direction of the centrifugal force (Fig. 3). The top of each channel is connected to the bottom of the next one. The cartridges, which contain a total of 250 ml of solvent, are connected by narrow-bore Teflon tubes.²⁰ It is not necessary to work with the maximum number of cartridges and they can very easily be removed (in pairs, to retain a proper centrifugal balance) until the required number remains. By this means, the speed of separation can be increased when high separation efficiencies are unnecessary.

The mechanism of solvent flow in the separation channels is shown in Figure 4. Spinning the centrifuge rotor allows retention of the stationary phase, enabling the mobile phase to be pumped through the system at high flow rates. As the mobile phase enters the separation channels, it forms a stream of droplets that traverses the stationary phase. The pressure in the cartridges increases linearly with the flow-rate and quadratically with the spin rate.²⁰ Rotary seals at the upper and lower axes of the centrifuge are necessary to allow passage of solvent under pressure into the apparatus. As the apparatus is enclosed in a constant temperature box, experiments can be performed with heating or cooling. For partial separations, recycling is possible.

The Multilayer Coil Planet Centrifugal Countercurrent Chromatograph.

The multilayer coil planet centrifuge, produced by P.C. Inc., Potomac, Maryland, U.S.A., is a technically simple instrument but very effective for liquid-liquid partition chromatography. Also known as the "multilayer coil separator-extractor", it consists of a single length of 2.6 mm (or 1.68 mm I.D.) PTFE tubing wrapped around a spool. The spool describes a planetary motion around a central axis and is counterbalanced by a counterweight²¹ (Fig. 5). The coiled column assemblies can readily be interchanged so that the scale of the separation is easy to alter. With a 2.6 mm I.D. tubing, there is a total capacity of 360 ml solvent. Rotation is normally performed at about 800 rpm, giving efficient and rapid separations with very little elution of the stationary phase.

The motion of the coil causes vigorous agitation of the two solvent phases, and a repetitive mixing and settling process, ideal for solute partitioning, occurs at over 13 times per second.²² However, the mechanism of hydrodynamic distribution of the solvent phases in the coil is not known. Retention of the stationary phase is strongly dependent on interfacial tension, density difference

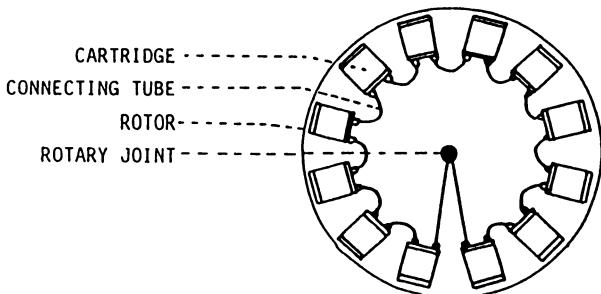


Fig. 2. Disposition of cartridges in the Sanki CPC instrument (Reprinted with permission from Marston *et al.*⁵).

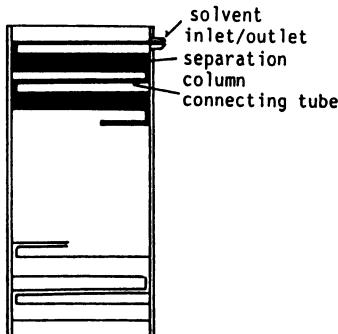


Fig. 3. Cross-section of a cartridge of the Sanki instrument (Reprinted with permission from Marston *et al.*⁵).

and viscosity of the two phases. The arrangement of the solvent exit and entry tubes is such that the chromatograph is seal-free, thus avoiding leakage and contamination problems. Backpressures tend to be low (2-6 bar) during elution, contrasting dramatically with the Sanki apparatus which is often operated at pressures approaching 50 bar.

The Multicoil Centrifugal Countercurrent Chromatograph. Very recently a family of innovative multilayer coil high-speed countercurrent chromatographs has been introduced by Pharma-Tech Research Corp., Baltimore, Maryland, U.S.A. One of these is a development of the multilayer coil planet centrifuge described above and consists of a pair of identical multilayer coils mounted symmetrically on each side of the rotary frame of the centrifuge.²³ This arrangement eliminates the problem of balancing the centrifuge system

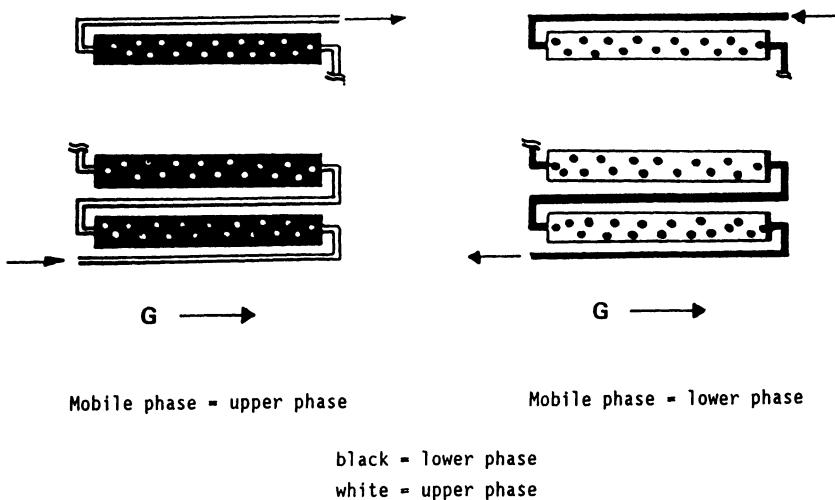


Fig. 4. Principle of operation of the Sanki CPC instrument (Reprinted with permission from Marston *et al.*⁵).

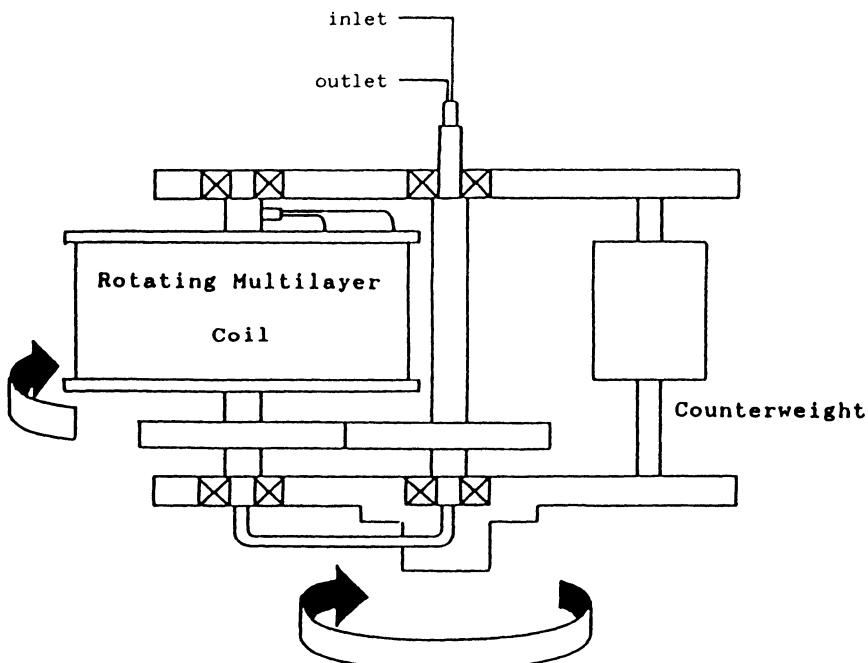


Fig. 5. Schematic diagram of the multilayer coil planet centrifuge (P.C. Inc.) (Reprinted with permission from Slacanin *et al.*²⁸).

with a counterweight. Each coiled column undergoes synchronous planetary motion in such a way that it revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity (ω). The two columns are equipped with a pair of flow tubes, arranged in such a fashion that they do not twist, enabling seal-free operation of the instrument. In contrast to the P.C. multilayer coil centrifuge, the Pharma-Tech variant accommodates the centrifuge in a vertical plane.

Pharma-Tech also markets a high-speed countercurrent chromatograph which holds three multilayer coils connected in series. With this instrument, the need for a counterweight is also superfluous and yet it is capable of seal-free operation. Increasing the number of coils allows an increase in column capacity and hence partition efficiency.²⁴ The actual volume of each column depends on the diameter and length of Teflon tubing used, while rotational speeds of up to 1500 rpm are possible.

Choice of Solvent Systems

The chromatographic literature contains numerous examples of solvent systems used in various countercurrent chromatographic separations^{3,10} and consultation of these references may give some leads as to possible systems useful for the separation in question. Alternatively, a classical chloroform-methanol-water system or a (less polar) *n*-hexane-ethyl acetate-methanol-water system can be chosen as the starting point and the proportions of the individual solvents changed until the required distribution of sample between the two phases is obtained. Chloroform-based solvent systems provide large density differences and relatively high interfacial tensions between the two solvent phases; they are consequently employed frequently for the separation of natural products by centrifugal partition chromatography. Because of their short settling times, chloroform-methanol-water systems normally produce satisfactory phase retention. One drawback, however, is that chloroform-methanol-water systems easily lead to overpressure problems with the Sanki apparatus. On the other hand, ethyl acetate-methanol-water solvent combinations are not always compatible with the multilayer coil planet centrifuge since the equilibrium state is difficult to reach and there is a continual leakage of stationary phase.

The method of TLC screening for solvent systems on silica gel plates used in droplet countercurrent chromatography¹⁵ can be applied to the search for suitable centrifugal partition chromatography systems, as long as some modifications are made. Thus, when using the organic layer of a two-phase aqueous solvent system as eluent for a TLC plate, the ideal R_F values of the sample for

chromatography on the Sanki apparatus should lie between 0.2 and 0.4. For the P.C. Inc. multilayer coil planet centrifuge, best results are obtained if the R_f values are a little lower. This method obviously gives only an approximate indication of the utility of a particular solvent system because TLC involves both partition and adsorption mechanisms, whereas the CPC instruments are based on purely liquid-liquid partition phenomena. Okuda et al.²⁵ have used TLC on cellulose plates for selection of solvent systems applicable to the separation of hydrolyzable tannins.

A knowledge of the partition coefficients of the solutes in question is of great benefit for the choice of suitable solvent systems. In fact, it is theoretically possible in CPC to predict the locations of eluted solute peaks once their partition coefficients are known. An analytical HPLC method has been described in order to determine the partition coefficients of the components of a mixture. In this method, the solutes are partitioned between two immiscible liquid phases and their respective concentrations are established by reversed-phase HPLC. The partition coefficient (K) of each component is calculated from the detector response following injection of a solution of the compounds before and after extraction with an immiscible solvent.²⁶ This procedure is of special relevance to natural product mixtures in which the identities of the individual components may not be known.

Applications

The separation of an artificial mixture of the flavanone hesperetin (1) and the flavonols kaempferol (2) and quercetin (3) with the same solvent system is shown in Fig. 6, in order to compare DCCC with the two CPC methods.^{27,28} Elution was by order of increasing polarity when the lower phase of the solvent system was used as the mobile phase. Whereas DCCC required more than 30 hours for complete separation, the two centrifugal chromatography methods took approximately 3 hours. Solvent consumption for DCCC and for the CPC methods was *ca.* 550 ml. When 12 cartridges instead of 6 cartridges were used in the Sanki instrument, resolution of the mixture was considerably improved but the separation time was 3.5 hours.

In liquid-liquid partition chromatography, composition and the ratio of stationary and mobile phase typically remain constant throughout the separation, and the proportion of the two phases depends on the particular solvent system chosen. When employing "isocratic" conditions, only compounds within a certain range of partition coefficients can be separated efficiently. A recent modification of the CPC instruments has improved their separation capability.

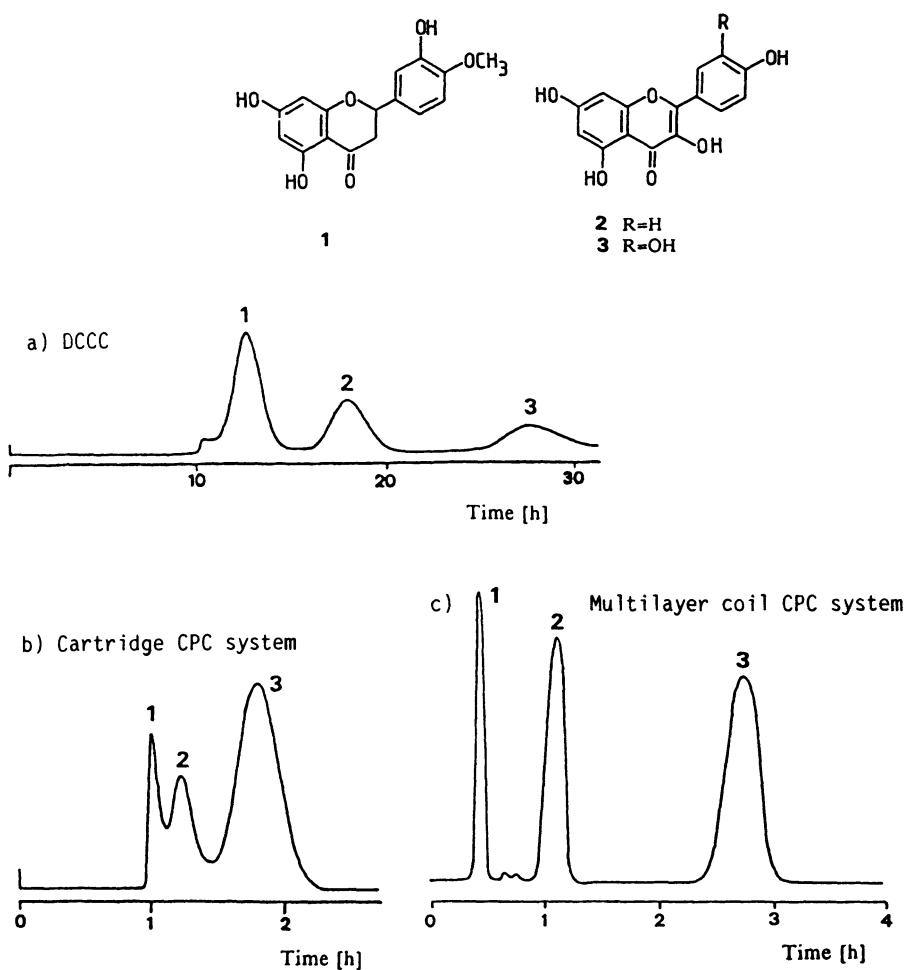


Fig. 6. Separation of hesperitin (1), kaempferol (2) and quercetin (3).

Solvent system: chloroform-methanol-water (33:40:27). Mobile phase: lower phase. Detection: 254 nm. a) Flow-rate: 48 ml/h. b) Flow-rate: 2 ml/min. Rotational speed: 600 rpm. 6 cartridges. c) Flow-rate: 3 ml/min. Rotational speed: 700 rpm. (Reprinted with permission from Marston *et al.*²⁷ and Slacanin *et al.*²⁸).

The use of two solvent delivery pumps, one for the stationary and the second for the mobile phase, in conjunction with a four-way valve allows for the variation of the ratio of the two phases.²⁸ Simultaneous filling of the immobile coil or cartridges with both phases in the desired proportions before commencing rotation leads to a hydrodynamic equilibrium with the predetermined ratio once the system is rotating. Altering the proportion of the two phases during the separation run ("gradient" elution) is also possible, as illustrated by a separation achieved with the multilayer CPC instrument. By pumping simultaneously the lower phase with one HPLC pump and the upper phase of a two-phase system with the other HPLC pump, the proportions of phases in the coil change during a separation run. In the example shown (Fig. 7), the coil of the chromatograph was first filled with equivalent amounts of upper and lower phases of the solvent system chloroform-methanol-water (33:40:27). A mixture of flavonoids 1-3 was then injected. By pumping the upper phase through the apparatus at 4

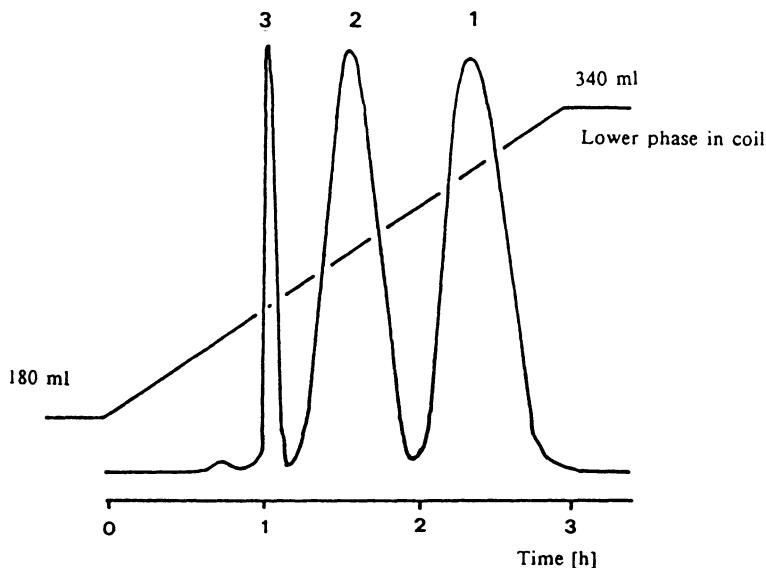


Fig. 7. Separation of flavonoids 1-3 with the multilayer coil planet centrifuge using "gradient" elution. Solvent system : chloroform-methanol-water (33:40:27). Detection: 254 nm. Mobile phase: upper phase (4ml/min) + lower phase (1 ml/min). Flow rate: 3 ml/min. Rotational speed: 700 rpm. (Reprinted with permission from Slacanin *et al.* 28).

ml/min and lower phase at 1 ml/min, the content of the lower phase in the coil increased from 180 to 340 ml over 3 hours. The separation of the three flavonoids was thus achieved in a tim 5 hours shorter than that shown in Figure 8a.

Both CPC systems are capable of "reversed-phase" operation: that is, referring to the cartridge system, at any point during the separation the flow direction can be reversed and elution commenced with stationary phase; in the coil system, phase reversal is achieved by switching the "head" and "tail" entry/exit tubes and eluting with the other phase. 27,28

This is illustrated with the separation of the previously used flavonoid mixture. While the separation of hesperetin (1), kaempferol (2) and quercetin (3) was achieved within 3 hours when using the lower phase as mobile phase (Fig. 6c), the separation took 8 hours if the upper phase was employed as the mobile phase (Fig. 8a). Starting the separation of flavonoids 1-3 with the upper phase as mobile phase and then changing to the lower phase after elution of quercetin (3) gave a dramatic increase in separation speed (Fig. 8b). This phase reversal during the separation (or "reversed-phase" operation) was achieved by activating a four-way valve between the solvent delivery system and the coil, without stopping operation of the instrument. These improvements introduce a greater degree of flexibility when establishing experimental parameters, and allow for the separation of a broader range of compounds within a single run.

The advantages of centrifugal partition chromatography over liquid-solid chromatography are illustrated by a practical example from our work on bioactive plant constituents. The shrub *Psorospermum febrifugum* (Guttiferae) is found in many parts of Africa and has numerous uses in traditional medicine - for the treatment of wounds and skin diseases and as a febrifuge. The light petroleum extract of the root bark contains a mixture of anthraquinone, anthrone and tetrahydroanthracene pigments, some of which have strong cell growth-inhibitory activities in a human colon carcinoma cell line 29. The extract and three of the pure lipophilic compounds exhibit marked *in vitro* antimalarial activity. Separation of the anthranoid constituents by flash chromatography and low-pressure reversed-phase liquid chromatography resulted in considerable material losses, due to irreversible adsorption on the sorbents. However, in a single CPC step (Sanki cartridge system), three pure compounds (4-6) and a mixture of two anthranoid pigments (7 plus a minor component) were obtained without loss of product (Fig. 9). A non-aqueous solvent system was employed for the separation. Increasing the number of cartridges permitted a better resolution of the peaks but required a longer separation time. With 12 cartridges, the separation could be scaled up to a 500 mg sample size.⁵

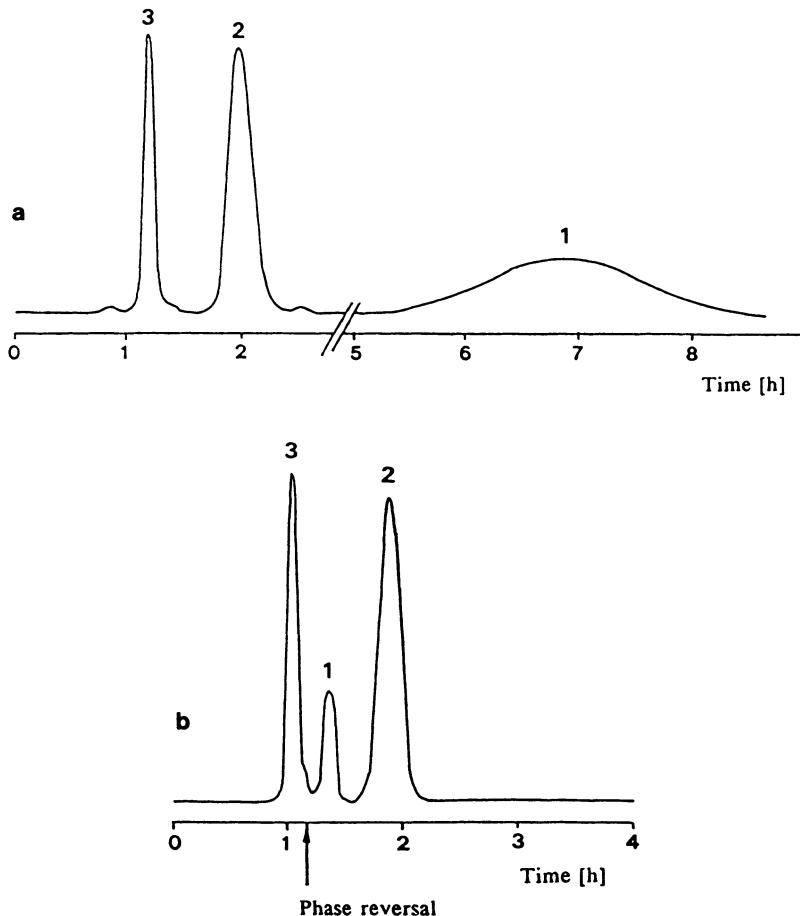


Fig. 8. Separation of hesperetin (1), kaempferol (2) and quercetin (3) with phase reversal, using the multilayer coil CPC system. Solvent system and detection as in Fig. 7. a) Mobile phase: upper phase. Flow rate: 3 ml/min. Rotational speed: 700 rpm. b) Mobile phase: upper phase to 70 min, then lower phase. Flow-rate: 3 ml/min. Rotational speed: 700 rpm. (Reprinted with permission from Slacanin *et al.* 28).

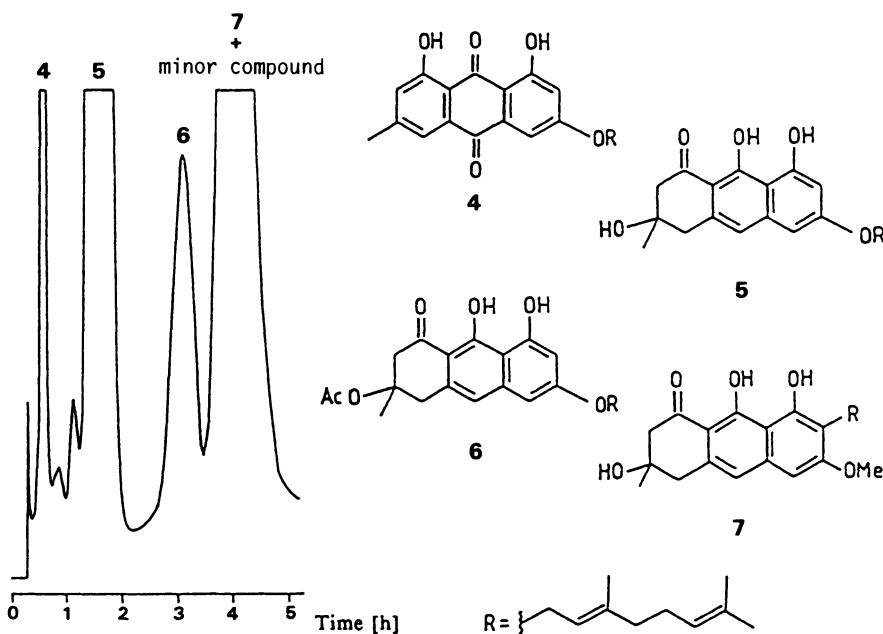


Fig. 9. CPC separation of a light petroleum extract of *Psorospermum febrifugum* root bark with the Sanki cartridge system. Solvent system: hexane-acetonitrile-methanol (40:25:10). Mobile phase: upper phase. Detection 254 nm. Rotational speed: 800 rpm. Flow-rate: 6.5 ml/min. Sample size: 500 mg. (Reprinted with permission from Marston *et al.*⁵).

Table 3. Selected applications of centrifugal partition chromatography in the isolation of natural products

Substances separated	Solvent system	Reference
Flavonoids	CHCl ₃ -MeOH-H ₂ O (4:3:2) CHCl ₃ -MeOH-H ₂ O (33:40:27)	30 27, 28
Flavonoid glycosides	EtOAc-94 % EtOH-H ₂ O (2:1:2) EtOAc- <i>n</i> BuOH-H ₂ O (2:1:2) EtOAc-H ₂ O→EtOAc- <i>i</i> BuOH-H ₂ O	27, 31 31 32
Chalcones	CHCl ₃ -MeOH-H ₂ O (7:13:8)	33
Tannins	<i>n</i> BuOH- <i>n</i> PrOH-H ₂ O (4:1:5) <i>n</i> BuOH- <i>n</i> PrOH-H ₂ O (2:1:3) <i>n</i> BuOH-0.1 M NaCl (1:1)	33 25, 34 35
Coumarins	<i>n</i> C ₆ H ₁₄ -EtOAc-MeOH-H ₂ O (18:42:30:30)	27, 36
Coumarin glycosides	CHCl ₃ -MeOH-H ₂ O (13:23:16)	37
Phenolic acids	<i>n</i> C ₆ H ₁₄ -EtOAc-MeOH-H ₂ O (18:42:30:30)	27, 36
Lignan glycosides	<i>n</i> C ₆ H ₁₄ -CH ₂ Cl ₂ -MeOH-H ₂ O (1:5:4:3)	38
Anthranooids	<i>n</i> C ₆ H ₁₄ -CH ₃ CN-MeOH (40:25:10)	27, 28
Cyclohexadienone derivatives	<i>n</i> C ₆ H ₁₄ -94 % EtOH-EtOAc-H ₂ O (83:67:33:17)	39
Saponins	CHCl ₃ -MeOH-H ₂ O (7:13:8)	27
Cardiac glycosides	CHCl ₃ -MeOH-HOAc-H ₂ O (5:3:1:3)	40
Retinals	Cyclohexane- <i>n</i> C ₅ H ₁₂ -CH ₃ CN-MeOH (500:200:500:11)	41
Carotenoids	CCl ₄ -MeOH-H ₂ O (5:4:1)	42
Sesquiterpenes	<i>i</i> C ₈ H ₁₈ -EtOAc-MeOH-H ₂ O (7:3:6:4)	43
Norditerpenes	CHCl ₃ -MeOH-H ₂ O (5:6:4)	44
Alkaloids	<i>n</i> BuOH-Me ₂ CO-H ₂ O (8:1:10) <i>n</i> BuOH-0.1 M NaCl (1:1) CCl ₄ -MeOH-H ₂ O (20:20:2) CHCl ₃ -0.07 M sodium phosphate (1:1)	45 46 47 48

A selection of examples of natural product separations by CPC is shown in Table 3. CPC is not only a preparative separation tool but also of practical use for the extraction of compounds from a large volume of solvent. In this case, solvent is employed as the mobile phase and a stationary phase is chosen which has a high affinity for the compound to be extracted. For a more detailed discussion of examples including separations of antibiotics and marine natural products, the reader is referred to recent review articles.^{5,11}

COMBINATION OF CHROMATOGRAPHIC METHODS FOR THE ISOLATION OF BIOLOGICALLY ACTIVE COMPOUNDS

Despite the efficacy of modern liquid partition chromatography, the isolation of active principles from a plant extract can only in a few cases be achieved by a single chromatographic step. Crude extracts typically consist of hundreds of primary and secondary metabolites. Especially when the compounds of interest are present in small concentrations, their isolation from the complex matrix usually requires two or more consecutive steps of chromatographic purification. A judicious combination of different chromatographic techniques is the key for rapid and efficient isolation. This will be illustrated by recent examples from our work on bioactive plant constituents.

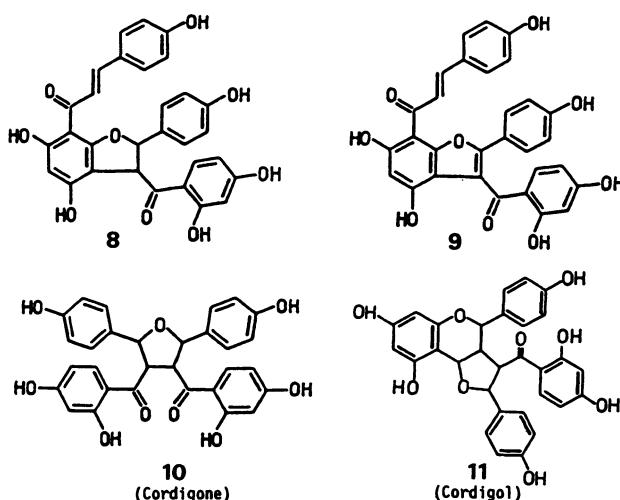


Fig. 10. Structures of phenols from *Cordia goetzei*.

Cordia goetzei (Boraginaceae) is a tree found in Tanzania and in other Southern and Central African countries. The roots are used in traditional medicine for the treatment of malaria and leprosy, while stem bark extracts help to heal sores. There is a bright yellow tissue layer between the grey outer stem bark and the reddish heartwood. Extraction of this layer with solvents of increasing polarity gave a methanolic extract with fungicidal activity. Bioassay-guided fractionation of the extract led to the isolation of four antifungal highly oxygenated polyphenols 8-11 (Fig. 10), two of which were previously undescribed compounds.⁴⁹

The first isolation was achieved by DCCC with the solvent system chloroform-methanol-water (43:27:20) in the descending mode, followed by low-pressure liquid chromatography on RP-8 with methanol-water (6:4). An improved separation scheme (Fig. 11) was employed for the isolation of additional quantities of these compounds, combining CPC with reversed phase chromatography on RP-18 (Fig. 12).

Species of the genus *Hypericum* (Guttiferae) are known to contain antibacterial and antifungal constituents. Of special interest is hyperforin, a prenylated phloroglucin antibiotic. During an expedition in Malawi, we collected leaves and twigs of *Hypericum revolutum*, a shrub native to South-East Africa growing at high altitude in open mountain grassland and at the margins of evergreen forest. The light petrol ether extract of the aerial parts showed antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum*. Activity-directed fractionation by various chromatographic techniques afforded

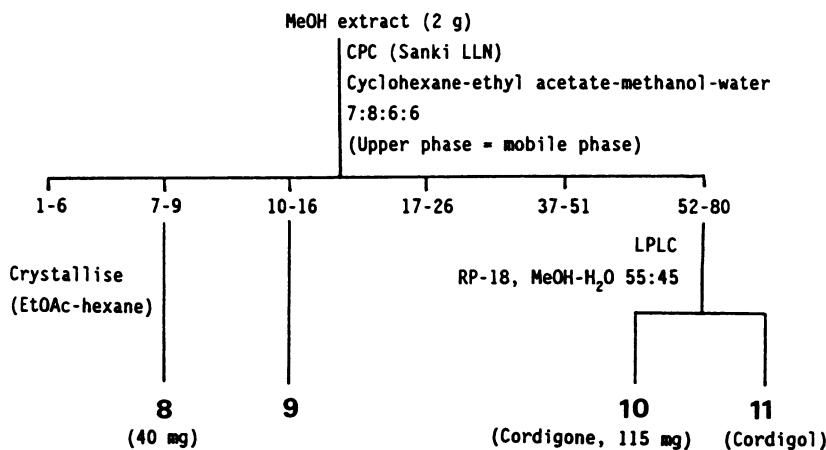


Fig. 11. Isolation of polyphenolic compounds from *Cordia goetzei* stem bark by CPC.

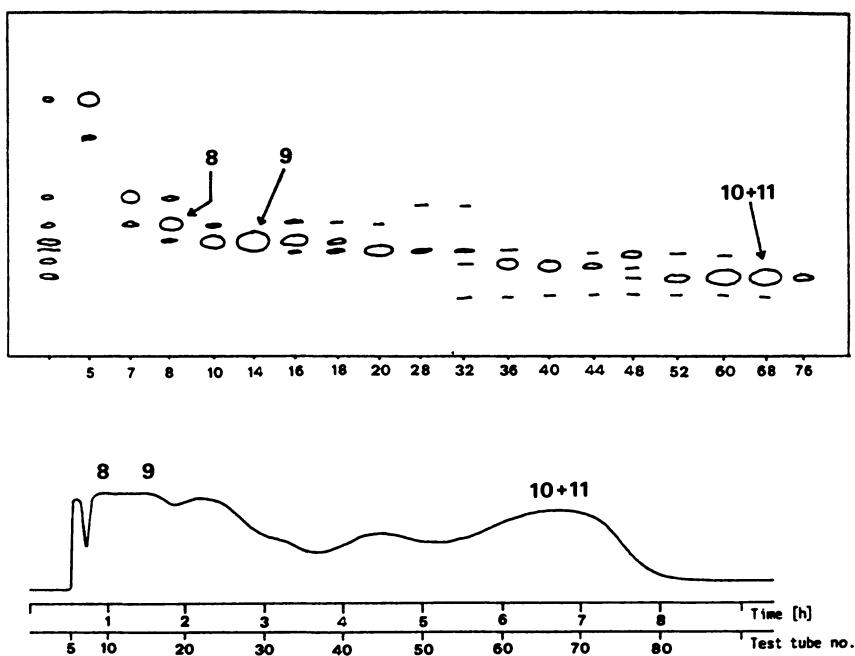


Fig. 12. Monitoring of the CPC separation of the *Cordia goetzei* extract. a) TLC monitoring. Stationary phase: silica gel. Mobile phase: chloroform-methanol-water (13:7:4), lower phase. Detection: 254 nm. b) monitoring by UV detector at 254 nm. Solvent system: cyclohexane-ethyl acetate-methanol-water (7:8:6:6). Mobile phase: upper phase. Flow-rate: 2.8 ml/min. Rotational speed: 700 rpm. Sample size: 2 g.

two novel chromenyl ketones.⁵⁰ The petroleum ether extract of the root bark of this plant displayed *in vitro* growth-inhibitory activity against the Co-115 human colon carcinoma cell line. Activity-guided fractionation of this extract by DCCC with light petroleum ether/94 % EtOH/AcOEt/H₂O (83:67:33:17), in the ascending mode yielded a crystalline mixture of two hyperforin derivatives. Compound 12 was subsequently separated from its higher homologue 13 (Fig. 13) by semi-preparative HPLC on RP-18 with MeOH-H₂O (83:17) (0.1 % AcOH added to the solvent).⁵¹ In this particular case, use of an acidic eluent proved to be indispensable, since the compounds were not retained on the column when using an eluent containing no acid. The unusual behaviour of the two hyperforin derivatives is undoubtedly linked to keto-enol tautomerism.

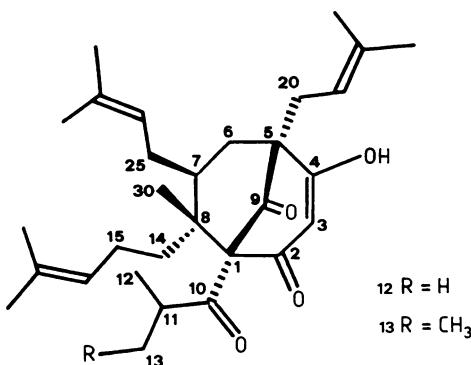


Fig. 13. Structures of new hyperforin derivatives from *Hypericum revolutum*.

The Far Eastern plant *Eleutherococcus senticosus* (*Acanthopanax senticosus*) (Araliaceae), commonly referred to as "Siberian ginseng", was introduced in the early 1960s in the Soviet Union as a substitute for ginseng root.⁵² This drug, used as a "tonic" or "adaptogen", (a preparation improving the adaptation and resistance of the organism to various external stress factors), is becoming increasingly popular in Western European countries. While a series of sterols, saponins and phenolic compounds have been isolated and identified, the rather non-specific pharmacological properties of the drug could not be attributed to certain members of these rather commonly encountered plant constituents. For the quality control of crude drug and phytopharmaceutical preparations, analytical methods are required which can assure the uniform composition of the final products. As the active principles of *E. senticosus* are unknown, the known constituents are considered as "marker" substances which can be used for the standardization of preparations containing *E. senticosus* extracts. In this context we became interested in developing an efficient isolation procedure for the phenolic constituents which are required as reference compounds for analytical purposes.

The structures of the isolated phenolic glycosides and the separation scheme are shown in Figures 14 and 15, respectively. A crude methanolic extract was first submitted to rapid column chromatography on silica gel. A water-containing eluent was employed in order to deactivate the stationary phase. Fractions I-III containing the compounds of interest were submitted to CPC with CHCl₃-MeOH-H₂O (7:13:8) using the lower phase as mobile phase. Final

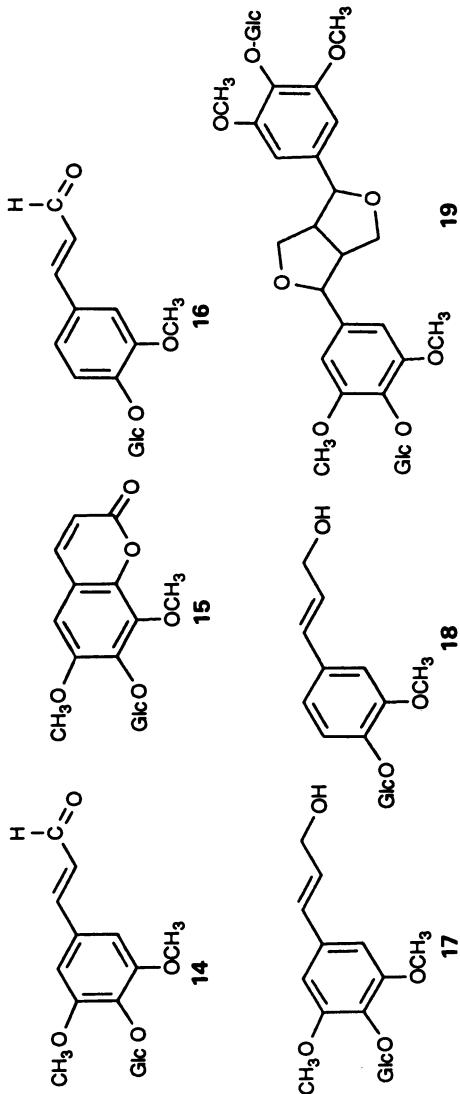
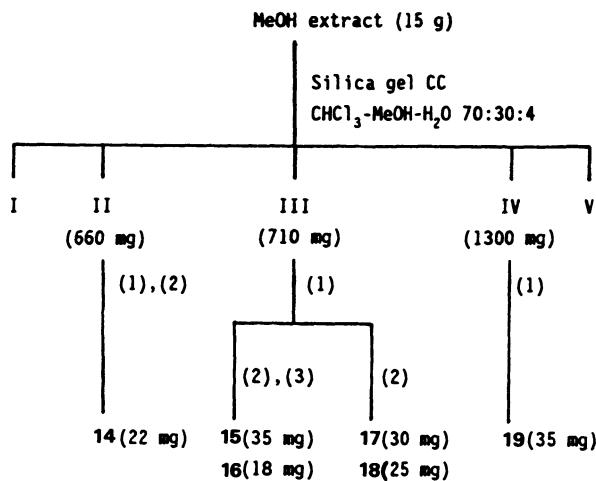


Fig. 14. Structures of phenolic glycosides isolated from *Elutherococcus senticosus* roots



(1) CPC, $\text{CHCl}_3\text{-MeOH}\text{-H}_2\text{O}$ 7:13:8 (mobile phase=lower phase)

(2) LPLC, RP-18, $\text{MeOH}\text{-H}_2\text{O}$

(3) HPLC semi-prep, RP-18, $\text{MeOH}\text{-H}_2\text{O}$ 15:85

Fig. 15. Isolation of constituents of *Eleutherococcus senticosus* roots.

purification of the eleutherosides **14-19** was achieved by low pressure or high pressure liquid chromatography on RP-18 with $\text{MeOH}\text{-H}_2\text{O}$ mixtures of varying proportions. The compounds were subsequently identified by standard spectroscopic methods such as sinapaldehyde-*O*- β -D-glucopyranoside (**14**), eleutheroside B₁ (**15**), coniferin aldehyde (**16**), eleutheroside B (syringin) (**17**), coniferin (**18**) and eleutheroside E (**19**).⁵³ Compounds **14** and **16** have not been reported previously from *E. senticosus*.

CONCLUSION

As the above-mentioned examples show, centrifugal partition chromatography has become an indispensable addition to the techniques available for the separation and purification of natural products. The wide range of applications, varying from apolar to very polar substances and from milligram to gram quantities, proves the versatility of this newly introduced variant of liquid-liquid chromatography. The rapidity of the method is one of the most attractive

attributes. Separation times are much shorter than DCCC; while DCCC may require 40 hours for a separation problem, the same sample can be separated in as little as 2 hours by centrifugal techniques. As no solid support is present, the phenomena of irreversible adsorption and contamination do not cause problems. These considerations are of special importance for the separation of crude extracts. The vast choice of two-phase solvent systems contributes greatly to the flexibility of CPC. This is in contrast to DCCC, which is restricted to solvent combinations that produce droplets in the columns. Several coil planet centrifuges and cartridge CPC systems are presently in production but most applications have been reported with the instruments described in this article. Further developments will include the refinement of existing instruments. Attempts are also being made to construct equipment which is capable of handling multigram to kilogram quantities of sample.

However, the isolation of natural products can often not be achieved by a single chromatographic technique. A judicious combination of chromatographic methods which display differing chromatographic selectivities is usually the solution for rapid and efficient isolation of the desired compounds. In this context, low-, medium- and high-pressure chromatography on chemically modified silica gel (RP-8, RP-18, DIOL, CN) have become increasingly important. Gel chromatography on Sephadex LH is a complementary and still highly useful technique. Future trends will be the introduction of polymer based solid supports for preparative chromatography and the development of CPC instruments capable of handling large quantities of sample.

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Chapter Two

RECENT ADVANCES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF VOLATILE NATURAL PRODUCTS

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INTRODUCTION

Gas chromatography (GC) is considered to be the method of choice in the analysis of volatile compounds, because of its high resolving power and the availability of universal detection using flame ionization (FID) detectors.

However, GC is mainly an analytical method and its application is limited when preparative isolation of volatile compounds is required. On the other hand, GC analysis of thermolabile and/or polar compounds is often difficult to achieve. Although these problems could be easily solved by the use of liquid chromatography, the latter technique has received limited application in the analysis of volatile constituents because of its poor resolution and the lack of a sensitive universal detector. In the past, the application of liquid chromatography in the analysis of volatiles has been mostly restricted to the prefractionation of crude extracts for GC analysis.

Compared to conventional open column liquid chromatography, high-performance liquid chromatography (HPLC) offers the advantages of shorter analysis time, as well as more reproducible injection and solvent gradient conditions. The applications of HPLC in the analysis of volatile compounds¹⁻⁷¹ can be placed into three major groups: i) the prefractionation of complex volatile mixtures prior to GC or GC-MS analysis; ii) the semi-preparative or preparative isolation of volatile compounds; and iii) the determination of specific constituents in complex mixtures of volatiles.

The optimization of conditions for the analysis and isolation of volatiles by HPLC is best performed on synthetic mixtures. Examples of such studies are listed in Table 1. Previous studies on the isolation and prefractionation of naturally occurring volatiles have been carried out mostly on essential oils (Table 2). Terpenoid analysis of essential oils and the isolation of essential oil constituents have been reviewed by Morin et al⁷² and Kubeczka^{73,74}, respectively. Using reversed phase HPLC on C₁₈ support, fractionation is carried out according to the polarity and the chain length of essential oil constituents³⁷. Monoterpene alcohols, monoterpene aldehydes, aliphatic ketones and aliphatic alcohols elute first, followed by esters of monoterpene alcohol and oxygenated sesquiterpenoids, monoterpene hydrocarbons, and finally sesquiterpene hydrocarbons. Using normal phase chromatography on silica gel, the pattern of elution is reversed, that is, hydrocarbons elute first, followed by aldehydes, ketones and esters, and finally terpene alcohols³⁶.

Quantitation of specific volatile constituents in complex mixtures is generally restricted to UV-absorbing or fluorescent compounds (Table 3). The high selectivity of these two types of detection, often considered to be a major limitation of HPLC in research of volatiles, turns out to be a major advantage when quantitation is needed. In HPLC, in fact, interference by other non UV-absorbing volatile constituents is rarely observed; this is generally not the case in GC unless extensive prefractionation of the extract is performed prior to GC

Table 1. Applications of HPLC in the analysis and the preparative isolation of synthetic mixtures of volatile constituents

Volatile compounds	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Carvone/Neral/ Geranial	Separation	Partisil 5 (silica)	Normal phase	Heptane/ MeCN	UV 242	9
Menthone/Carvone/ Neral/Geranial	Separation	Partisil 5 (silica)	Normal phase	Heptane/ EtOAc	UV 370 (DNP derivative)	9
Menthone/ isomenthone	Separation	μ -Porasil (silica)	Normal phase	Hexane/EtOAc	RI	14
	Semi-preparative isolation	Partisil 10 (silica)	Normal phase	Hexane/EtOAc	RI	14
Monoterpenes	Separation and semi-preparative isolation	Partisil 10 (silica)	Normal phase	CH ₂ Cl ₂ /EtOAc Hexane/EtOAc	RI	12

Table 1 continued

Volatile compounds	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Mono and sesquiterpenes	Separation	LiChrosorb RP-18	Reversed phase	Aq. MeOH	UV 220	75
		LiChrosorb Si-60 + 4.8 % H ₂ O	Partition	Pentane (-15°)	UV 220	75
Terpene hydrocarbons and oxygenated terpenes	Separation Preparative isolation	Silica gel	Normal phase	Pentane/Et ₂ O (-80 and +20°)	UV 220	38
Terpene alcohols	Separation	LiChroprep RP-18	Reversed phase	Aq. MeOH Aq. MeCN	UV 254	13
Terpene alcohols	Separation	Spherisorb ODS	Reversed phase	Aq. MeCN + Li perchlorate	Electrochem.	37
Farnesol isomers	Preparative isolation	μ-Porasil (silica)	Normal phase	Trimethylpentane /Et ₂ O	UV 210 RI	17

Table 1 continued

Volatile compounds	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Farnesol isomers	Preparative isolation	Styrene-divinyl-benzene copolymer	Gel filtration	MeOH Cyclohexane	RI UV	24
3-Vinyl-(4H)-1,2-dithiin	Preparative isolation	Spherisorb ODS	Reversed phase	MeOH/H ₂ O/ HCOOH	UV diode array	68
Model aroma compounds	Trace enrichment	Styrene-divinyl-benzene copolymer	Gel filtration	Aq. MeOH	UV 210 RI	48, 49
		Partisil ODS-3	Reversed phase	Aq. MeOH	UV 210 RI	48, 49

Table 2. Applications of HPLC in the analysis and the isolation of essential oil, perfume and aroma constituents

Volatile extract	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Valencia orange, tangerine, grapefruit oils	Prefractionation	Biobeads SX-2	Gel permeation	CHCl ₃		
		Permaphase ODS	Reversed phase	Aq. MeOH		
<i>Lindera</i> essential oil	Prefractionation	μ-Syrgeal	Gel permeation	Hex/ <i>iso</i> -PrOH CHCl ₃	RI/UV	1
		μ-Bondapak C ₁₈	Reversed phase	Aq. MeOH	Tetrahydrofuran	
Cocoa butter essence	Prefractionation	Partisil 10 (silica)	Normal phase	Pentane/Et ₂ O/ MeOH	UV 254/RI UV	4 7

Table 2 continued

Volatile extract	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Cocoa butter essence	Prefractionation	Partisil 10 PAC (Amino cyano)	Normal phase	Pentane/iso-PrOH		
		Spherisorb ODS	Reversed phase	Aq. MeOH	UV 254	7
Valeriana essential oil	Prefractionation	Silica (ROSL)	Normal phase	Hexane/MeOH	UV 220	30
Essential oils	Fractionation	Hypersil SAS (C1)	Reversed phase	Aq. MeOH	UV 260	9
		Spherasorb microbore	Exclusion	Heptane/EtOAc	UV	15
Cinnamon essential oil	Separation	Partisil-20 microbore	Normal phase	Heptane/EtOAc	UV	15
Bergamot oil						

Table 2 continued

Volatile extract	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Celery stems CH ₂ Cl ₂ extract	Separation of phthalides	Zorbax Sil	Normal phase	CH ₂ Cl ₂	UV 265	46
<i>Citrus</i> essential oil	Fractionation	LiChrosorb RP-8 or RP-18	Reversed phase	Aq. MeCN	UV 200, 210 or 254	21
<i>Ormenis</i> essential oil	Prefractionation	SI-100	Normal phase	Heptane/ <i>iso</i> -PrOH	UV 265	31
Lime oil	Prefractionation	Partisil PXS (silica)	Normal phase	Toluene/EtOAc CH ₂ Cl ₂ /EtOAc Hex/CH ₂ Cl ₂ /EtOAc	RI	36
Essential oils	Prefractionation	Nucleosil, LiChrosorb and Zorbax C ₁₈	Reversed phase	Aq. MeCN	UV 220	37

Table 2 continued

Volatile extract	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Quince fruit oil	Preparative isolation of monoterpenes	μ-Porasil (silica)	Normal phase	Hex/Et ₂ O	RI/UV	28
<i>Magnolia</i> bark	Separation of eudesmol isomers	Silica impregnated with silver nitrate	Normal phase	Hexane/EtOAc Hexane/Me ₂ CO Hexane/iso-PrOH	RI	18
Tolu balsam essential oil	Preparative isolation of sesquiterpene olefins	LiChrosorb Si-60 impregnated with silver perchlorate	Normal phase	Pentane/Et ₂ O	UV 220	42
<i>Amyris</i> oil	Preparative isolation of β-eudesmol	Microsorb silica	Normal phase	Pentane/MeOAc	RI	61
		Silica + 10% AgNO ₃	Normal phase	Petroleum spirit 40-60/MeOAc	RI	61

Table 2 continued

Volatile extract	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Lime oil	Preparative isolation of germacrene B	Silica (3 columns in tandem)	Normal phase	Hexane	RI	47
Umbelliferae spp rhizome hexane extracts	Preparative isolation of phthalides	LiChrosorb Si-60	Normal phase	Hexane/iso-PrOH	UV	40, 41
Jasmin concrete	Fractionation	Partisil 5 (silica)	Normal phase	Isooctane/EtOAc		
Perfumes	Fractionation before GC-MS of safrole	LiChrosorb RP-18	Reversed phase	Aq. MeOH	UV diode array	25
		Partisil M-9 10/50 PAC	Normal phase	Isooctane/CH ₂ Cl ₂	Fluorometric	29

Table 2 continued

Volatile extract	Application	Support	Type of Chromatography	Solvent	Detection	Ref.
Blackcurrant aroma compounds in food plant waste water	Trace enrichment	Styrene-divinylbenzene copolymer	Gel filtration	Aq. MeOH	UV 210 RI	50
Strawberry flavour extract	Prefractionation in view of chiral separation of lactones by LC-GC	Partisil ODS-3	Reversed phase	Aq. MeOH	UV 210 RI	50

Table 3. Applications of HPLC in the quantitation of specific volatile constituents

Compounds	Material	Chromatographic Support	Type of Chromatography	Solvent	Detection	Ref.
Vanillin	Vanilla essence	Microsorb C ₁₈	Reversed phase	H ₂ O/MeOH/ MeCN/AcOH	UV 275	60
Cinnamaldehyde and eugenol	<i>Cinnamon</i> oil	Corasil II (silica)	Normal phase	Cyclohexane/ EtOAc	UV 260	5
Eugenol	Pimento	ODS-Hypersil	Reversed phase	MeOH/phosphate buffer pH 7.0	UV 278 Electrochemical	32
Methyl anthranilate	Grape beverages	ODS/Sil-X-1	Reversed phase	MeCN/phosphate buffer pH 6.0	UV 217	6
β-Asarone	Calamus oil Alcoholic beverages	ODS/Sil-X	Reversed phase	Aq. MeOH	UV 254 Fluorometric	19
Carvacrol Thymol	<i>Origanum</i> <td>μ-Porasil</td> <td>Normal phase</td> <td>Hexane/CHCl₃</td> <td>UV 280</td> <td>20</td>	μ-Porasil	Normal phase	Hexane/CHCl ₃	UV 280	20

Table 3 continued

Compounds	Material	Support	Type of Chromatography	Solvent	Detection	Ref.
Carvacrol Thymol	<i>Thymus</i> essential oil	μ-Bondapak C ₁₈	Reversed phase	Aq. MeCN	UV 283	22
Safrole	Perfumes	Zorbax ODS	Reversed phase	Aq. MeOH	Fluorometric	29
β-Asarone/safrole isosafrole/anethole	Nutmeg/Sassafras/ Cinnamon/Anise oils	HS-RP 18	Reversed phase	Aq. MeCN	Fluorometric	43
Safrole Myristicin	Nutmeg & mace	LiChrosorb RP-8	Reversed phase	H ₂ O/MeCN/ MeOH/tetrahydrofuran	UV 282	51
Myristicin	Carrots	CN	Normal phase	Heptane/ tetrahydrofuran	UV	10
Menthol	Pharmaceutical product	μ-Bondapak C ₁₈	Reversed phase	Aq. MeOH + heptyl- <i>p</i> - aminobenzoate	Indirect UV detection 290	33

Table 3 continued

Compounds	Material	Support	Type of Chromatography	Solvent	Detection	Ref.
Furanol	Pineapple and grapefruit juices	Zorbax ODS	Reversed phase	MeOH/NaOAc buffer pH 4.0	UV 290	44
Pyrethrins	<i>Pyrethrum</i> extracts	Silica	Normal phase	Hexane/tetrahydrofuran	UV 229	52
Allicin and aliiin	Garlic	Spherisorb ODS-2	Reversed phase	Aq. MeOH/HCOOH MeOH/MeCN/H ₂ O MeOH/EtOAc/H ₂ O	UV diode array UV 254	67
Allicin	Garlic	ODS	Reversed phase	Aq. MeOH	UV 254	57
Ajoene and dithiins	Garlic	Spherisorb ODS	Reversed phase	MeCN/H ₂ O/MeOH	UV diode array UV 254	68

Table 3 continued

Compounds	Material	Support	Type of Chromatography	Solvent	Detection	Ref.
Ajoene and dithiins	Garlic	Spherisorb Si	Normal phase	Hexane/ <i>iso</i> -PrOH	UV diode	68
Aldehydes	Whisky	Develosil C ₈	Reversed phase	MeCN/phosphate buffer pH 7.0	Fluorometric NBD hydrazone derivatives	53
Aldehydes	Liquor	RP-18 microcolumn	Reversed phase	MeOH/phosphate buffer pH 9.5 NAD solution	Fluorometric Post column enzyme reaction	54

injection. Furthermore, when suitable chromophoric groups are present in the molecule, HPLC with UV detection may be more sensitive than GC-FID detection (Groel, J., Ravot, J., Barron, D., unpublished results). Fluorometric HPLC detection is even more sensitive than UV. Electrochemical detection has also been demonstrated to be more sensitive than UV detection in HPLC analysis of phenolic compounds³².

Applications of HPLC in perfume^{25,29,63} or aroma^{6,19,43,50,53,54,69} analyses are infrequent (Table 2). The use of HPLC in aroma and flavor studies has been reviewed by Kubeczka⁷⁵, Bitteur⁷⁶, and Rousseff⁷⁷. The possibilities and limitations of off-line HPLC-GC in flavor research have been described by Sandra et al⁷⁸. Chiral HPLC separation of optically active aroma constituents is receiving increasing attention^{58,59}. Finally, HPLC is of the method of choice in the analysis of polar, bound aroma constituents, the so called "aroma precursors". Two reverse-phase HPLC methods have been developed for the isolation and analysis of grape "aroma precursors"^{79,80}.

The lack of information on the behavior of aroma constituents on HPLC supports warrants further investigation. In fact, although prefractionation of crude aroma extracts can be performed by conventional column chromatography, it is a time-consuming method which cannot be applied for routine analyses of a large number of samples. On the other hand, trapping of GC fractions for proton NMR analysis⁸¹ is commonly used in aroma research. However, it requires excellent technical expertise and the quantities of an isolated products limit the acquisition of spectroscopic data to proton NMR.

In this chapter, recent developments in the HPLC prefractionation and the semi-preparative isolation of free and glycosidically bound forms of aroma constituents will be presented with emphasis on the use of silica and diol-bonded silica supports.

ADVANCES IN THE PREFRACTIONATION OF FREE AROMA EXTRACTS

Since silica gel has been successfully used in the pre-fractionation of aroma extracts by conventional liquid chromatography⁸²⁻⁸³, we chose first a silica HPLC support. Compared to conventional open column chromatography, however, we expected some differences in the elution profile of aroma compounds, especially in the recovery of the most polar aroma constituent, due to the high activity of HPLC silica supports. The method described by Palmer⁸³ uses deactivated silica gel. Deactivation of an HPLC silica column, although

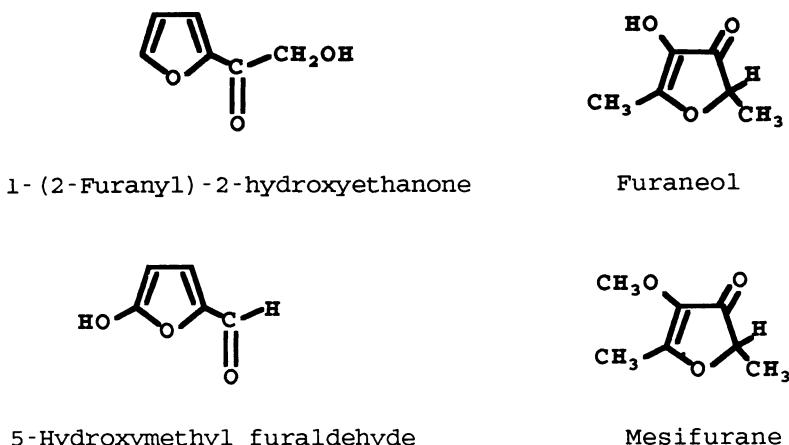


Fig 1. Structures of selected strawberry jam aroma constituents.

possible, is not recommended due to i) the risk of extensive swelling of the support leading to over-pressure problems; and ii) the difficulty in reactivating the silica gel.

For our separation studies, we chose a strawberry jam aroma extract because one of its important aroma constituents, furaneol (Fig. 1) is particularly unstable. This compound would be a good indicator of the recovery of unstable aroma constituents.

Prefractionation by Filtration on Silica Sep Pak® Cartridge

In order to quickly test the fractionation pattern on the HPLC silica support, the aroma extract was filtered through a small silica Sep Pak® cartridge and eluted with solvents of increasing polarity (pentane, dichloromethane, and diethyl ether). Since the pentane eluate was not significantly odorous, it was not further analysed. The dichloromethane and the ether fraction were subjected to GC-MS analyses after concentration (Table 4).

Methyl and ethyl hexanoates, 2-heptanone, 2-acetyl furan, and furfural were present in the dichloromethane extract. On the other hand, hydroxy esters (methyl 3-hydroxybutanoate), hydroxy ketones (3-hydroxy-2-butanone), and hydroxy aldehydes (5-hydroxymethyl furaldehyde), were eluted with ether. Unsaturated aldehydes such as 2-heptenal, 2,4-hepta- and decadienal, or 2,4,7-

Table 4. Sep Pak® fractionation of strawberry jam aroma extract on silica

Compound	CH ₂ Cl ₂ Extract	Et ₂ O Extract
KETONES		
2-Heptanone	+	
Acetyl furan	+	
3-Hydroxy-2-butanone		++
ALDEHYDES		
Furfural	++	
2-Heptenal	+	+++
2,4-Heptadienal	+	+++
2-Decenal		+++
2,4-Decadienal	+	+++
2,4,7-Decatrienal	+	++
Benzaldehyde		+
Vanillin	+	++
5-Hydroxymethyl furfural		+
ESTERS		
Methyl hexanoate	+	
Ethyl hexanoate	+	
Methyl 3-hydroxybutanoate		+
LACTONES		
γ-Octalactone	+	+
δ-Decalactone		+
γ-Dodecalactone	+	

Table 4 continued

Compound	CH ₂ Cl ₂ Extract	Et ₂ O Extract
ALCOHOLS		
1-Octanol		+
1-Dodecanol	+++	++
Linalool	+	
α -Terpineol	+	
<i>trans</i> -Nerolidol	+	
Benzyl alcohol	+	
ACIDS		
Butanoic		++
Isobutyric		+
2-Methyl butanoic	Trace	++++
Hexanoic	+	++++
Octanoic		++
Decanoic		+
Dodecanoic		++
Tetradecanoic		+++
Hexadecanoic (palmitic)	+++	+++
9-Octadecenoic	+++	+++
9,12-Octadecadienoic (linoleic)	+++	+++
Cinnamic		++++
MISCELLANEOUS		
Mesifurane		+++
Furaneol	-	-

Approximate relative amounts measured from GC peak heights:

+ : small; ++ : moderate; +++ : large; ++++ : very large

- : compound present in the crude extract which was not recovered after Sep Pak® filtration

decatrienol were more strongly retained on the silica support and, therefore, were present at higher concentrations in the ether extract. Of the aromatic aldehydes, benzaldehyde was found in the ether extract only, while vanillin was detected in both extracts, although at a higher concentration in the ether extract.

Monoterpene and sesquiterpene alcohols, as well as benzyl alcohol, were eluted with dichloromethane. The distribution of aliphatic alcohols between the two extracts was not distinct. The concentration of 1-dodecanol was much higher in dichloromethane, while 1-octanol was detected only in the ether extract. Similarly, lactones were partitioned between the dichloromethane and/or ether eluates, with no obvious structural basis.

Acids are very major constituents of the ether extract. However, the presence of large amounts of fatty acids, such as palmitic or linoleic acid, caused extensive saturation of the cartridge. Thus fatty acids were present at high concentration in the dichloromethane extract as well.

Among the important constituents of strawberry jam aroma⁸⁴ (Fig. 1), 2,5-dimethyl-4-methoxy-3-(2H)-furanone (mesifurane) was eluted with ether from the silica Sep Pak®. Its analog 2,5-dimethyl-4-hydroxy-3-(2H)-furanone (furaneol) could not be detected in either extract, even though it was present in the crude extract. On the other hand, although 5-hydroxymethyl furaldehyde was present in the ether extract, it was detected at low concentration, despite being one of the major constituents of the crude aroma extract.

Recovery of Polar Aroma Constituents by HPLC on LiChrosorb Si-60 Using Water-saturated Solvents

Injection of reference compounds on a Lichrosorb Si-60 analytical column using ether as eluent, confirmed the fact that furaneol remains irreversibly adsorbed on the silica support. This compound, however, was eluted with a satisfactory retention time (6 to 7 minutes), using water-saturated diethyl ether as the solvent. Under such conditions, mesifurane, vanillin, cinnamic acid, and 5-hydroxymethyl furaldehyde were eluted in less than 11 minutes (Figure 2). With the exception of the above-mentioned compounds, most of the other volatiles emerged at the solvent front (3 to 4.5 minutes after injection). Thus, it appeared that a one-step prefractionation of aroma extracts could not be achieved via HPLC on silica gel. The water-saturated diethyl ether solvent did not cause an irreversible deactivation of the support since standard chromatographic conditions were recovered by re-equilibrating the column with normal anhydrous solvents.

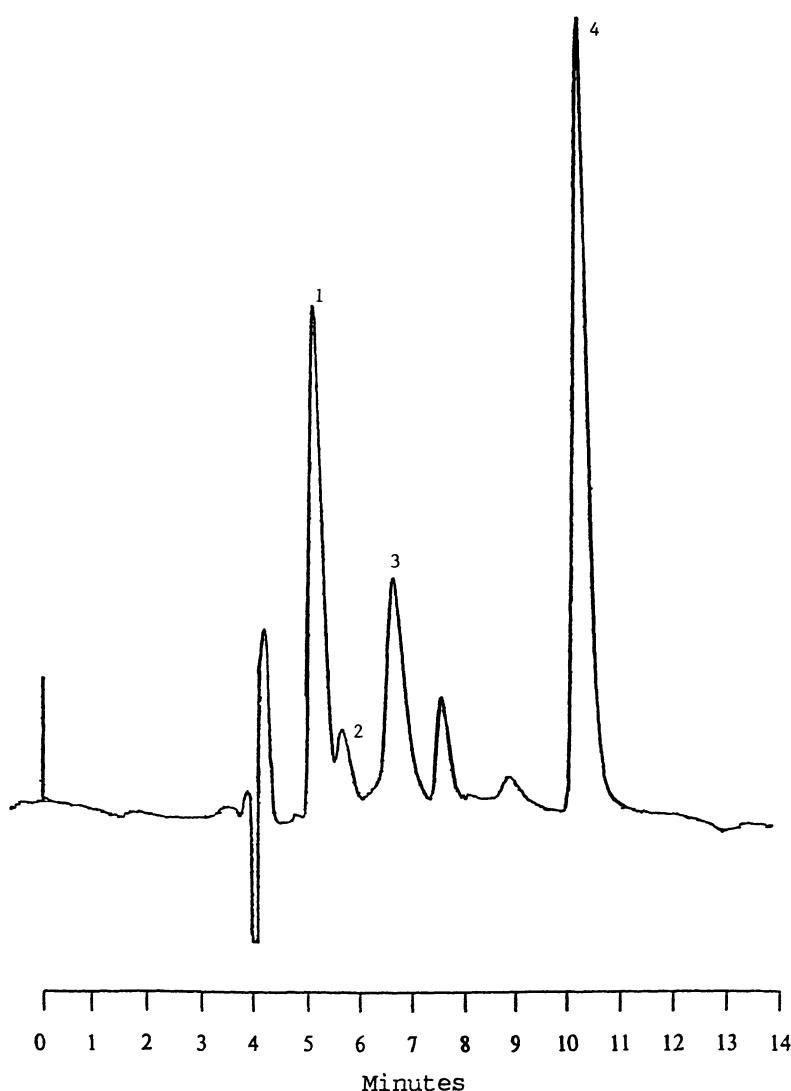


Fig. 2. HPLC of strawberry jam aroma extract on silica gel using water-saturated diethyl ether as solvent. Flow rate: 1 ml/min. Detection: UV 285 nm. 1: Cinnamic acid; 2: Mesifurane; 3: Vanillin and furaneol; 4: 5-Hydroxymethyl furaldehyde.

Prefractionation by HPLC on LiChrosorb diol

This support offers the advantage of being less acidic than silica gel and is very appropriate for the analysis of polar/unstable compounds which are irreversibly adsorbed on silica gel.

Before HPLC injection, the dichloromethane aroma extract was neutralized by partition with aqueous sodium bicarbonate and filtration on Sephadex LH-20⁸⁵. Fractionation of the extract was performed using a gradient of diethyl ether in pentane. Composition of the collected HPLC fractions was determined by GC-MS. The results are summarized in Table 5. The order of elution of compounds was similar to that observed on silica gel, in which hydrocarbons, esters, aldehydes and ketones were eluted first. Although unsaturated and aromatic carbonyl compounds were retained slightly longer than the saturated analogs, the separation between the two types of compounds was not as great as that observed on silica gel. However, as with silica gel, hydroxy esters/ketones were more strongly retained by the diol phase. Similar long retention was observed in the case of alcohols and lactones. Furaneol was successfully recovered from the diol support.

GC-MS of the crude aroma extract permitted the identification of more than 60 constituents. The total number of compounds identified, however, was increased to about 150 after GC-MS of the HPLC fractions, demonstrating the efficiency of the diol column as a prefractionation tool, while allowing the recovery of the more polar aroma constituents. A typical example is represented in Figure 3, where the mass spectra (GC-MS) of 1-(2-furanyl)-2-hydroxyethanone are compared before and after HPLC on the diol column.

ADVANCES IN THE SEMI-PREPARATIVE ISOLATION OF AROMA CONSTITUENTS

Whereas the diol support had distinct advantages over silica in prefractionation studies, complete purification of volatile compounds was rarely obtained by HPLC on diol columns because of the lower resolving power of the latter support. Thus, the first prefractionation on diol support usually needs to be followed by a final purification step on silica gel using water-saturated solvents.

Table 5. HPLC fractionation of a neutral strawberry jam aroma extract on LiChrosorb diol^a

Table 5 continued

Fraction number	5	6	7	8	9	10	11	12	13	14	15	17
LACTONES												
γ -Butyrolactone												—
γ -Valerolactone												—
γ -Lactones (Hexa-dodeca)												—
δ -Lactones (Hexa-dodeca)												—
ALCOHOLS												
Aliphatic, saturated												—
Aliphatic, unsaturated												—
Geraniol/Nerol												—
Linalool												—
<i>trans</i> -Nerolidol												—
Linalool oxides												—
Epoxilinalool												—
PHENOLS												
Eugenol												—

^aThe following conditions were used: Linear solvent gradient from pentane-Et₂O 9/1 to Et₂O in 30 min. Flow rate: 2 ml/min. Detection: UV 230 nm. Fractions collected every 2 min. after injection.

^bElution was checked by injection of the reference compound.

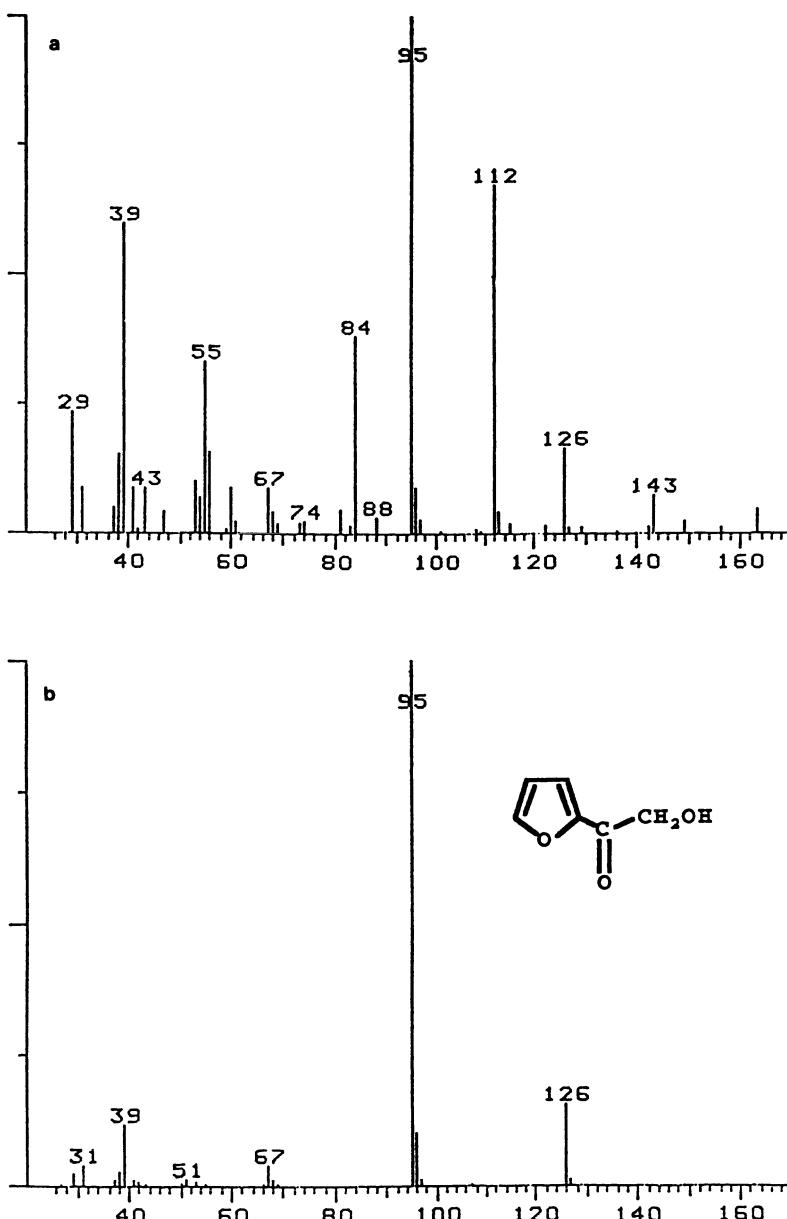


Fig. 3. GC-MS data for 1-(2-Furanyl)-2-hydroxyethanone. A: Injection of the crude dichloromethane aroma extract; B: Injection of the diol HPLC fraction N° 17.

Isolation of 1-(2-Furanyl)-2-hydroxyethanone from a Crude Strawberry Jam Dichloromethane Aroma Extract

Under the conditions used for capillary GC⁸⁴, 1-(2-furanyl)-2-hydroxyethanone coeluted with 3-hydroxy-2-pyranone, and consequently, its direct isolation by trapping of GC fractions was not possible.

Purification of the compound for NMR identification was accomplished by gel filtration of the dichloromethane aroma extract on Sephadex LH-20 (pentane:idichloromethane 1:1), followed by semi-preparative HPLC on diol using pentane-diethyl ether 9:1 as the solvent (Fig. 4), and finally by a second

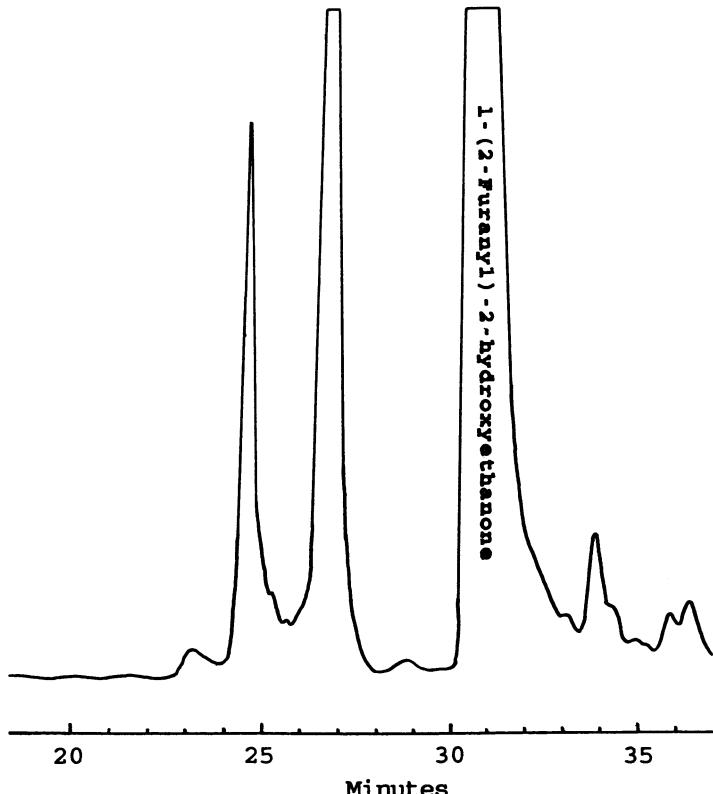


Fig. 4. Semi-preparative isolation of 1-(2-furanyl)-2-hydroxyethanone by HPLC on a diol column. Solvent: pentane-Et₂O 9/1. Flow rate: 2 ml/min. Detection: UV 280 nm.

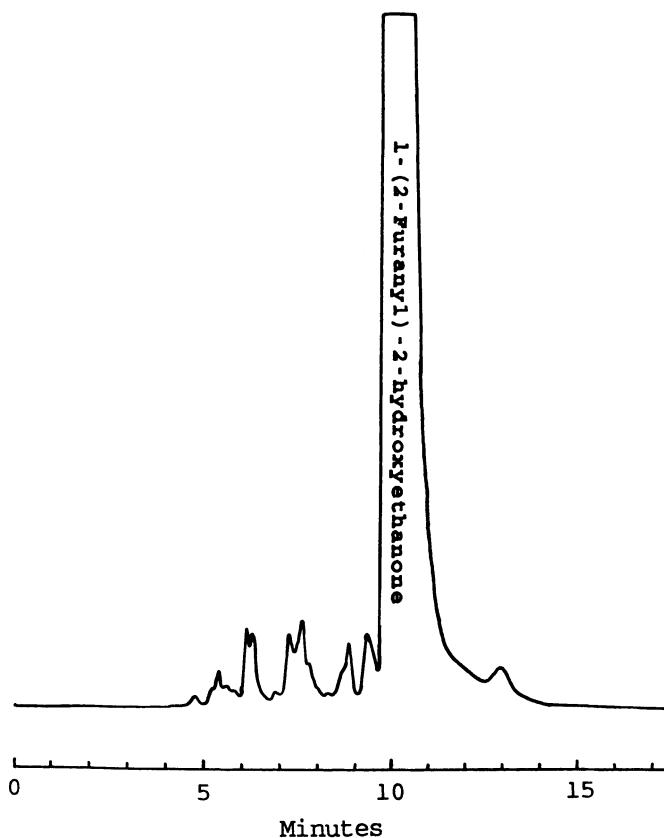


Fig. 5. Final purification of 1-(2-furanyl)-2-hydroxyethanone by semi-preparative HPLC on silica gel. Solvent: Water-saturated Et₂O. Flow rate: 2 ml/min. Detection: UV 280 nm.

semi-preparative HPLC on silica using water-saturated diethyl ether as the solvent (Fig. 5). The ¹H NMR spectrum of the isolated compound is shown in Figure 6.

Isolation of Unknown Sesquiterpene Alcohol Oxides from a Crude Dichloromethane Strawberry Jam Aroma Extract

Several unidentified compounds appeared in the GC as a group of three peaks with mass spectra similar to those published for nerolidol- and bisabolol oxides. A few significant differences from the published spectra, however, led us to undertake more complete identifications. A mixture of the three isomers was

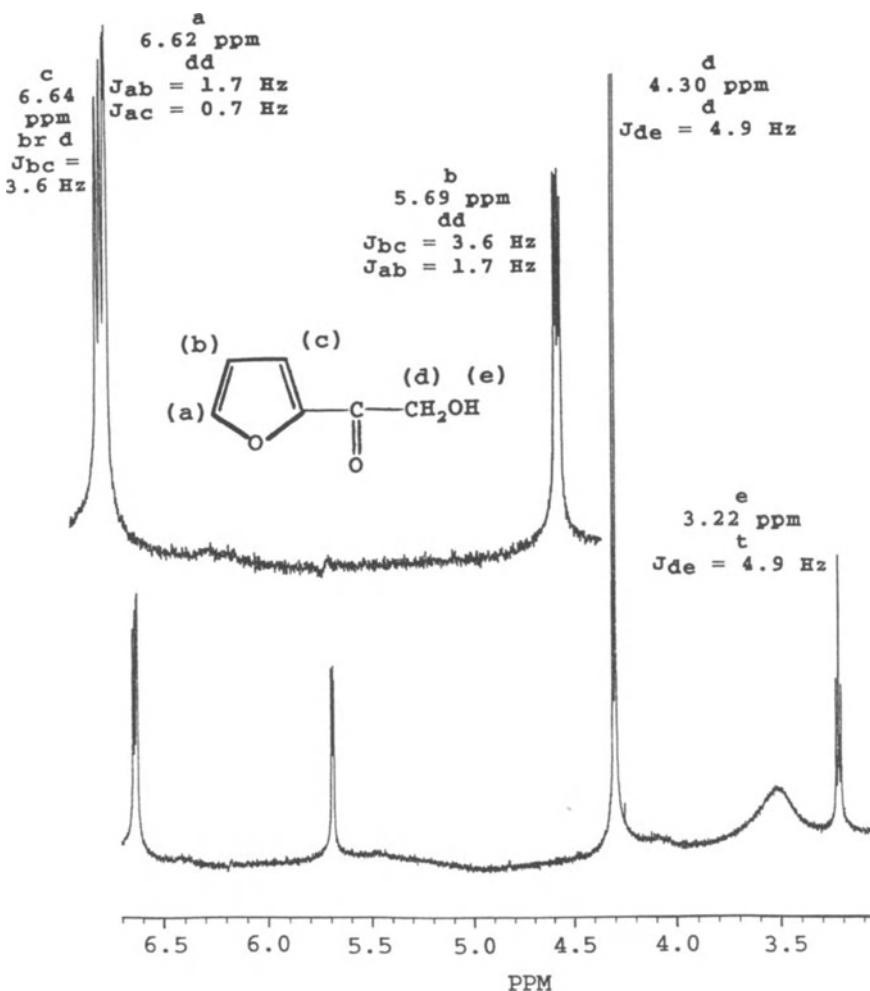


Fig. 6. ^1H NMR spectrum of the HPLC-isolated 1-(2-furanyl)-2-hydroxyethanone (400 MHz, C_6D_6 , δ ppm/TMS).

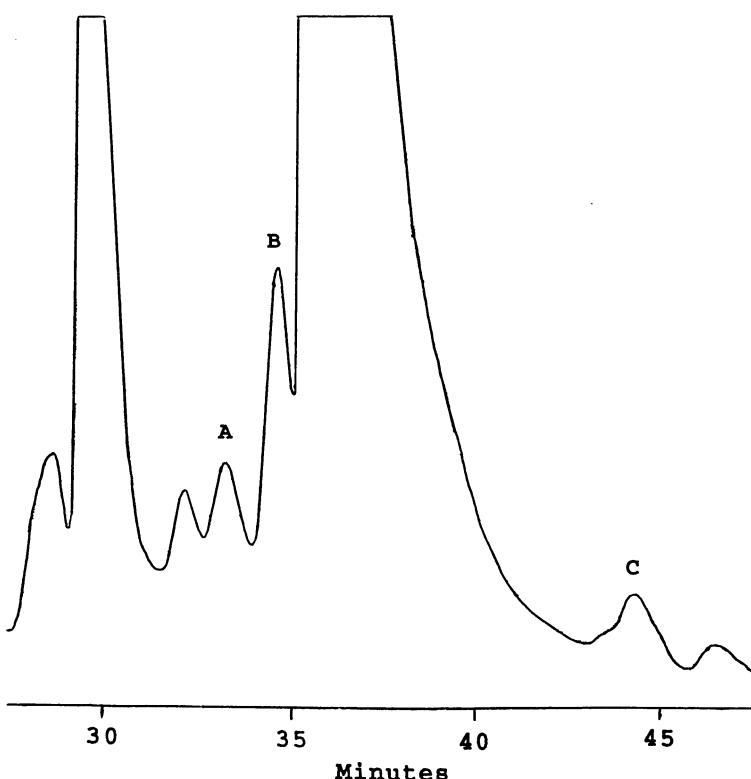


Fig. 7. Semi preparative isolation of unknown sesquiterpene alcohol oxides by HPLC on silica gel. Solvent: Pentane-Et₂O 8/2 (saturated with water). Flow rate: 2 ml/min. Detection: UV 210 nm. Peaks A, B, and C correspond to the three isomers.

first obtained by semi-preparative HPLC on a diol column (for conditions, see Table 5) and collected in fractions 4-7. Separation of the three isomers was subsequently achieved by semi-preparative HPLC on silica using water-saturated pentane-diethyl ether as the solvent (Fig. 7). Analysis of the ¹H NMR spectra of the isolated compounds (Fig. 8) led to the assignment of the structures as bisabolol oxides, rather than nerolidol oxides. Further confirmation of the structures was obtained after two of the three isomers were synthesized by 3-chloroperbenzoic acid (MCPBA) oxidation of α -bisabolol. A mixture of three isomers, however, was produced through the MCPBA oxidation of *trans*-nerolidol, followed by heating of the oxidation products at pH 3.0 (i.e. under similar conditions to those used during the preparation of the jam). This,

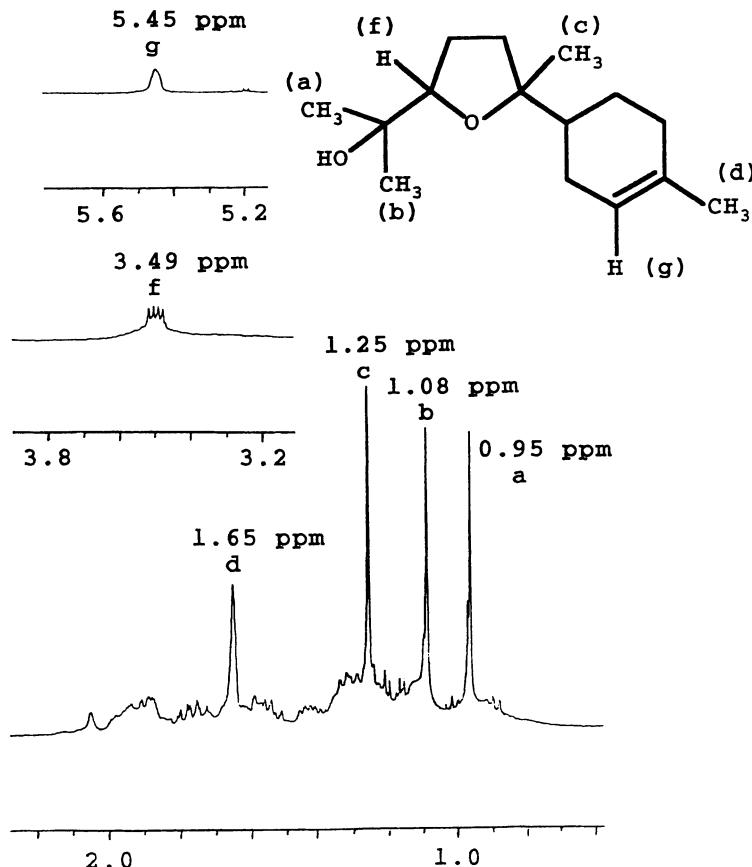


Fig. 8. ^1H NMR spectrum of isolated oxide A (400 MHz, C₆D₆, δ ppm/TMS).

together with the fact that, unlike α -bisabolol, *trans*-nerolidol is a major constituent of strawberry aroma, makes the latter compound the most probable precursor of the oxides in strawberry jam (Barron, D., Le Quéré, J.L., in preparation).

ADVANCES IN THE ISOLATION OF AROMA PRECURSORS

While 4-(4'-hydroxyphenyl)-butan-2-one (raspberry ketone; Fig. 9) is considered to be an important constituent of raspberry aroma, surprisingly, no

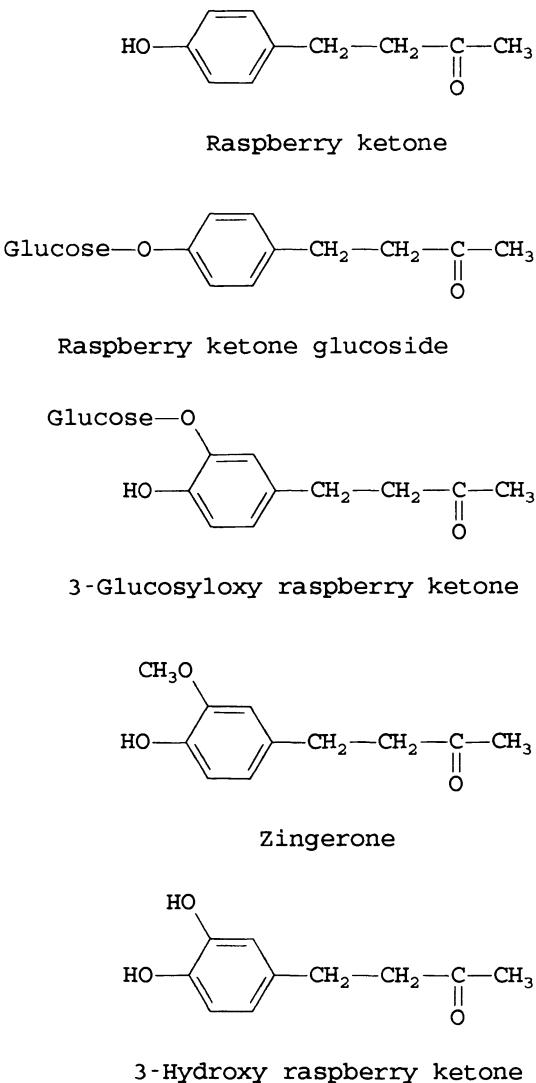


Fig. 9. Structures of a few raspberry fruit constituents.

Table 6. Reversed phase (RP-18) medium pressure liquid chromatographic separation of raspberry glycosidically bound aroma fraction

Elution solvent (H ₂ O-MeOH)	Released aglycone (enzymatic hydrolysis)
7/3	Raspberry ketone 3-Hydroxy raspberry ketone Zingerone
6/4	Benzyl alcohol
5/5	2-Phenylethanol 3-Oxo- α -ionol 4-Oxo- β -ionol 4-Hydroxy- β -ionone
3/7	Linalool
2/8	α -Ionol

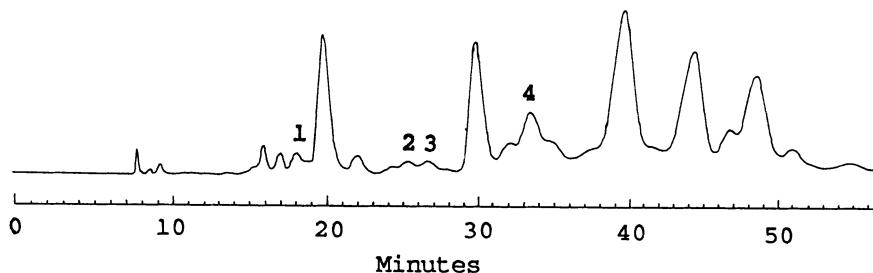


Fig. 10. Fractionation of the 30% aqueous MeOH RP-18 fraction by HPLC on diol support. Solvent: Isocratic Hexane-n-BuOH-MeOH-H₂O 50/40/9/1 for 15 min., increased to Hexane-n-BuOH-MeOH-H₂O 30/60/9/1 in a 25 min. gradient. Flow rate: 2 ml/min. Detection: UV 230 nm. 1. Benzyl alcohol conjugate; 2: Raspberry ketone- and 3-hydroxy raspberry ketone glucosides; 3. Zingerone conjugate; 4: Benzyl alcohol conjugate.

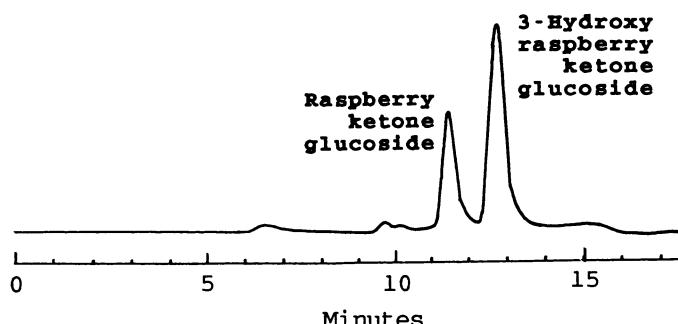


Fig. 11. Separation of raspberry ketone glucoside from 3-glucosyloxy raspberry ketone by semi-preparative HPLC on diol. Solvent: CH_2Cl_2 - $\text{MeOH}-\text{H}_2\text{O}$ 80/19/1. Flow rate: 2 ml/min. Detection: UV 280 nm.

glycosilated conjugate of raspberry ketone had been previously isolated from raspberry fruit. Enzymatic hydrolysis studies on raspberry fruit pulp, however, demonstrated that such a conjugate was present in raspberries⁸⁶. In order to isolate and identify this conjugate, a glycosidic fraction was obtained from raspberry fruit by standard methods.⁸⁷ The glycosidic extract was prefractionated by preparative medium pressure liquid chromatography on RP-18 using gradients of MeOH in H_2O as the solvent. Identification of the aroma precursors in each fraction was based on enzymatic hydrolysis of an aliquot, followed by ether extraction of the released aglycones and subsequent GC injection. The results of reverse-phase separations are given in Table 6. A raspberry ketone conjugate was eluted in 30 % aq. MeOH, together with 3-hydroxy raspberry ketone, and zingerone conjugates. Elimination of the zingerone conjugate was performed by semi-preparative HPLC on a diol column, using gradients of hexane/butanol/MeOH/ H_2O as the solvent (Fig. 10). Complete separation of the raspberry ketone conjugate from its 3-hydroxy analog was achieved by means of a second semi-preparative HPLC on a diol support, using dichloromethane/MeOH/ H_2O as the solvent (Fig. 11). Complete spectroscopic analysis (^1H and ^{13}C NMR, DCIMS) identified the two compounds as raspberry ketone glucoside, and 3-hydroxy raspberry ketone 3-glucoside, respectively⁸⁷.

CONCLUSION

High-performance liquid chromatography on diol-bonded silica is a very efficient tool for the separation of both free and glycosidically bound volatiles. For the prefractionation of aroma extracts, HPLC on diol bonded silica, has, over non-modified silica, the major advantage of allowing the recovery of all aroma constituents, including the more polar/unstable compounds. The weaker retention of compounds on the diol support implied lower resolution, and therefore less discrimination between the different classes of aroma constituents. This, however, did not affect the prefractionating potential of the diol column, as demonstrated by the considerable amount of additional volatiles which could be identified after diol HPLC. Therefore, HPLC on diol-bonded silica should be preferred in the future to HPLC on silica gel in the prefractionation of complex mixtures of volatiles.

When the semi-preparative isolation of a specific volatile constituent is required, individual fractions obtained from the diol column can be further purified by HPLC on silica gel. In fact, successful purification of a number of polar volatile compounds such as 1-(2-furanyl)-2-hydroxy ethanone was obtained on silica gel by using water-saturated ether solvents. Such solvents do not irreversibly deactivate the silica support.

Finally, we demonstrated the efficiency of the diol column in the purification of complex mixtures of glycosidically bound aroma constituents. Certainly, important developments via HPLC on diol support can be expected in the near future, especially in the normal phase chromatography of polyphenolic glycosides for which silica gel is rarely appropriate.

ACKNOWLEDGEMENTS

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Chapter Three

ENANTIOSELECTIVE SEPARATIONS IN PHYTOCHEMISTRY

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INTRODUCTION

Plant biochemical pathways produce a host of phytochemicals whose stereochemical configurations are determined by precise enzymatic transformations. As a result, most naturally-occurring organic compounds have a certain chirality (or handedness), e.g., sugars, proteins, nucleic acids, polysaccharides, etc.¹ This is true irrespective of whether the substances are cell wall polymers (e.g., cellulose) or are not (e.g., alkaloids, lignans, flavonoids, terpenoids, proanthocyanidins, etc.).

When one considers molecular chirality, it is noteworthy that some phytochemicals exist in only one enantiomeric form, whereas with others the optical rotation of the metabolite can vary between species. For instance, the sesquiterpenoid, abscisic acid, an important hormone in plant growth and development, seems to occur only as the (+)-*S*-enantiomer.² On the other hand, there are many examples where both antipodes are found, e.g., (+)-epipinoresinol from *Forsythia intermedia*³ and (-)-epipinoresinol from *Xanthoxylum ailanthoides*⁴ (see Fig. 1).

A striking feature about chiral recognition of molecules by living organisms, such as enzymes and receptors, is in the difference in the relative effect of each enantiomer in racemic mixtures of drugs, herbicides, pesticides, and growth factors. A few examples serve to illustrate this point: (a) (-)-abscisic acid has no comparable plant growth hormone properties when compared to its corresponding (+)-antipode;⁵ (b) the *S*-(-)-form of propranolol has β-adrenergic blocking activity, whereas the *R*-(+)-antipode has membrane stabilizing properties;⁶ and (c) although both thalidomide enantiomers are effective as sleep inducing drugs, only the *S*-(-)-form causes a marked teratogenic effect.⁷ For such reasons, there has been a growing trend towards obtaining medicinal compounds, such as herbicides, in the desired optically pure form.

Development of methodology for resolution of racemic mixtures began with the pioneering work of Pasteur in 1848 by fractional crystallization of (+)- and (-)-sodium ammonium tartrates. It has since been estimated that more than 7,000 racemates have been resolved by this means.⁸ This method, however, is tedious and often accompanied by poor recovery.⁹ Consequently, the last decade has witnessed a heavy emphasis towards methods development for the rapid chromatographic resolution of racemic mixtures, notably by high-performance liquid chromatography (HPLC) and, to a lesser extent, by gas liquid chromatography (GLC).

The purpose of this chapter is to demonstrate the usefulness of chiral separations in the plant sciences; the examples chosen do not represent an exhaust-

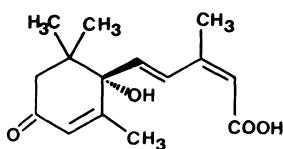
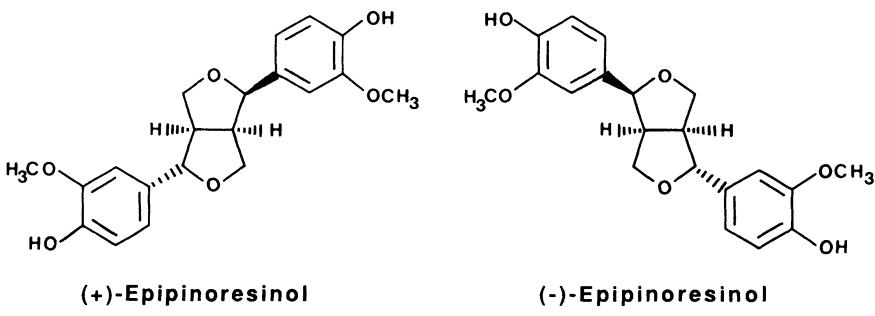
**(+)-S-Abscisic acid**

Fig. 1. (+) Abscisic acid and (+)- and (-)-epipinoresinol.

tive account, but instead serve to illustrate the breadth of applications available. These methods are of critical importance in certain phytochemical studies, where rapid and conclusive information about the optical purity of a particular metabolite is required, or when resolution of stereochemical questions in various biosynthetic pathways is needed, as in the case of particular lignans,¹⁰⁻¹² and terpenoids. This chapter begins by briefly reviewing the methodologies currently available for chiral separations. None are treated in detail, as the methods themselves have been adequately reviewed and/or described elsewhere.

CHROMATOGRAPHIC RESOLUTION OF RACEMIC COMPOUNDS

Attempts to chromatographically resolve racemic mixtures were described as early as 1904 by Willstätter, even though the resolution of (\pm)-atropine was not obtained following passage through either wool or silk.¹³ In

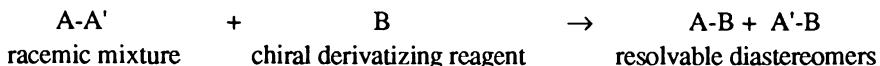
spite of several attempts thereafter, the resolution of *d,l-p*-phenylene-bisimino-camphor by differential adsorption chromatography, using lactose as a stationary phase, was not achieved until 1939 by Henderson and Rule.¹⁴ Later, Kotake *et al.* in Japan separated *d*- and *l*-forms of glutamic acid and tyrosine by paper chromatography using a chiral mobile phase (CMP).¹⁵ Interestingly, the order of elution of the *d*- and *l*-amino acids remained unchanged regardless whether the CMP was *d*- or *l*-methyl-(β -phenylisopropyl)-amine. It was concluded that the interactions with the chiral stationary phase, cellulose, were the most important factors affecting enantioselective separation. Chiral separations by (capillary) gas-liquid chromatography (GLC) followed in 1966, when Gil-Av *et al.* separated optical forms of (2-butanol esters of *N*-trifluoracetyl)-alanine, -valine, and -leucine.¹⁶ This was achieved using a pyrex capillary column coated with the chiral reagent, *N*-trifluoroacetyl-L-isoleucine lauryl ester. Over the last 15 years, new chiral reagents and high-resolution capillary columns have been introduced, thereby expanding the chromatographic resolution to a wider range of compounds. GLC has, however, the disadvantage of being restricted to volatile components and is, therefore, of limited usefulness in the enantioselective separation of many phytochemicals. For such reasons, chiral resolution by high-performance liquid chromatography (HPLC) has been the focus of much attention during the last decade, and over 40 different columns have been commercialized.¹⁷

Today, almost every laboratory is equipped with some sort of general purpose HPLC and GLC apparatus. When one considers enantioselective separations by either method, there are two ways by which this can be achieved: (a) indirectly, via derivatization of the racemic mixture with a chiral (i.e., optically active) reagent followed by chromatographic separation of the resulting diastereomers, and (b) directly, via reversible formation of transient diastereomeric complexes formed between each enantiomer and a "chiral environment".¹⁸ In the latter case, this chiral environment can be either the stationary phase (for HPLC or GLC), or the mobile phase (HPLC applications only). Both indirect and direct approaches to the optical resolution of racemic mixtures are described in greater detail below.

Indirect Method

Enantiomers of a given racemic mixture have identical physical and chemical properties, save for their behavior in plane polarized light, or in a chiral environment such as an enzyme active site.¹⁸ For these reasons, early attempts to resolve racemic mixtures directly using either normal (e.g., silica) or reversed-

phase (e.g., C₁₈) adsorption chromatography failed. To overcome this limitation, racemates were converted into diastereomeric forms by reaction with a pure enantiomer of a chiral derivatizing reagent. (See below for reaction scheme.) The resulting diastereomers, which have different physicochemical properties, could then be separated by conventional normal, or reversed phase, chromatography.



This technique has several limitations that substantially restrict its use when compared to more direct means of enantioselective analysis. First, the racemic compound must contain a functional group (e.g., -OH, -NH₂, -CO₂H) able to undergo derivatization with the chiral reagent. Second, the chiral reagent must be optically pure to prevent complication by formation of other diastereomers that could interfere with the separations. Third, the chemical derivatization procedure must not result in racemization of either the chiral derivatizing agent or the enantiomers undergoing derivatization; nor should there be different reaction rates with each enantiomeric form. Fourth, in some cases, each purified enantiomer is needed for subsequent studies; this requires its regeneration from the diastereomeric derivative previously formed.

Direct Method

The direct method is the most commonly used method for chromatographic resolution of racemic mixtures.¹⁸ With HPLC, separation of optical isomers can be obtained using either chiral stationary or mobile phases. Separations rely upon the "chiral discriminant" having different enantioselective affinities for individual enantiomers, with most "discriminants" being stationary phases. Chiral stationary phases include biopolymers, modified biopolymers, and synthetic polymers, either used as such or coated onto a matrix, e.g., silica. Chiral stationary phases can also be formed by linking small chiral molecules (either covalently or ionically) to a solid support. Less commonly, enantioselective mobile phases can be used for optical resolution of racemic mixtures. In the latter case, dynamic diastereomeric complexes are formed between each enantiomer and the chiral mobile phase, and separations occur because of stability differences of the complexes formed with each antipode. A number of enantioselective mobile phases, ranging from proteins to smaller molecules such as quinine, have been shown to be effective. With GLC, only chiral stationary

phases are used.¹⁹ The section below describes chiral phases (both stationary and mobile) currently available; these are discussed separately according to whether they are used for HPLC or GLC.

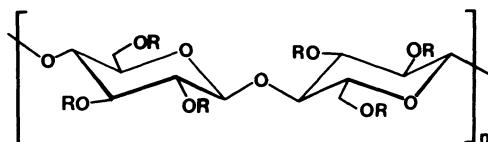
ENANTIOSELECTIVE SEPARATIONS BY HPLC AND GLC

HPLC Chiral Stationary Phases: Biopolymers, Modified Biopolymers, and Synthetic Polymers

Several biopolymers, and their derivatives, have been examined for their usefulness as chiral stationary phases. These include carbohydrates (cellulose, amylose, and related polysaccharides) and proteins (silk, wool, α_1 -acid glycoprotein, bovine serum albumin, ovomucoid).

Carbohydrates. Of the different polysaccharides studied for chiral recognition properties, most attention has been given to cellulose, although amylose, curdlan, chitosan, pullulan, mannan, xylan, dextran, and fructan have also been briefly evaluated. All have some enantioselective properties (e.g., cellulose has been used to separate the D- and L- forms of tryptophan²⁰), but their resolving powers are generally low and they find no commercial application as such¹⁷.

A different situation exists with modified biopolymers. Typically these include esters²¹ and carbamates^{17,22} which are most often used as coatings on macroporous silica gel and, less frequently, as the sole stationary phase. Of these biopolymers, modified cellulose (Fig. 2) has received the most attention,



R = H; Cellulose

R = CH₃CO; Cellulose triacetate

R = C₆H₅NHCO; Cellulose tris(phenylcarbamate)

Fig. 2. Cellulose and some of its derivatives used for enantioselective separations.

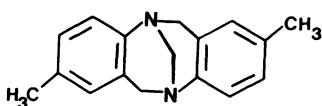


Fig. 3. Tröger's base.

with triacetate,^{17,23-31} tricinnamate^{17,31} and tribenzoate^{17,28,31,32} esters, tribenzyl ethers,³¹ and tris(phenyl carbamate),^{17,31,33-36} being evaluated. Interestingly, when cellulose triacetate (CTA), obtained by heterogeneous acetylation of microcrystalline α -cellulose,^{23,25} was used as a chiral stationary phase, it showed very different chiral recognition properties to that of a soluble cellulose triacetate polymer adsorbed onto macroporous silica gel.^{28,37} For example, with Tröger's base (Fig. 3), the order of elution of its enantiomers was reversed when separated on either microcrystalline CTA or CTA coated on silica, respectively. While the reasons for this difference in elution order are not well understood, it has been suggested that this may be due to differences in tertiary structure (i.e., cellulose I vs. cellulose II), or to soluble cellulose triacetate liquid crystals imparting some degree of order when coated onto macroporous silica.²³

Proteins. Like the native carbohydrate polymers, various proteins such as those from silk and wool have been evaluated for their chiral recognition properties, but show only low resolving power.¹⁷ However, when immobilized on silica gel, proteins can exhibit considerable usefulness as HPLC chiral stationary phases, e.g., with human plasma protein, α_1 -acid glycoprotein (Mw ~ 41,000),^{17,38-43} bovine serum albumin (Mw ~ 69,000),^{17,44-46} and the egg protein, ovomucoid (Mw ~ 28,000).^{17,47}

Synthetic polymers. The most commonly employed synthetic polymers for chiral separations are helical (+)-poly(triphenylmethyl methacrylate),^{17,31,48-51} poly(diphenyl-2-pyridylmethyl methacrylate)^{17,52,53} and, to a lesser extent, chiral polyacrylamides⁵⁴ and polyamides.^{25,55,56} (+)-Poly-(triphenylmethyl methacrylate) is obtained by polymerization of triphenylmethyl methacrylate in the presence of (-)-sparteine-butyllithium complex⁴⁸ and is the most widely used (Fig. 4). It is a (+)-helical isotactic polymer with one-handed helicity, and is normally coated onto preactivated macroporous silica gel. As with cellulose derivatives, (+)-poly(triphenylmethyl methacrylate) has also been evaluated as the sole stationary phase, but normally the silica-coated polymer is used since it has superior resolution capabilities.⁴⁹ When compared with poly(triphenylmethyl methacrylate), poly(diphenyl-2-pyridylmethyl methacrylate) has

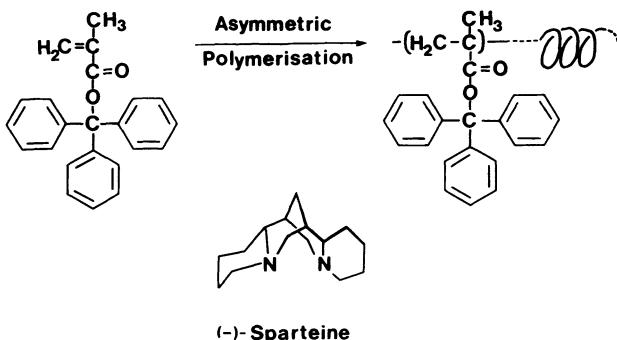


Fig. 4. Formation of (+)-poly(triphenylmethyl methacrylate).

poorer resolving power, but has a longer HPLC lifetime.¹⁷ Polyacrylamide and polyamide coatings onto macroporous silica gel are also used, since these polymers on their own are unsuitable as stationary phases for HPLC applications.²⁵ They have mostly been used for the enantioselective resolution of polar drugs.

HPLC Stationary Phases Containing Bonded (Monomeric or Oligomeric) Chiral Molecules

Quite a large number of chiral molecules have been attached, either covalently or ionically, to a solid achiral matrix. These enantioselective discriminators are conveniently subdivided into three main categories, with each relying upon a different principle of separation. They are ligand exchange,^{31,57-60} cavity,^{31,61-65} and so-called "brush" type^{31,66-73} phases.

In ligand exchange, an optically active molecule such as an amino acid (e.g., L-proline or L-valine) or another compound, e.g., L-(+)-tartaric acid, is typically attached to silica, normally via a covalently bonded organic "spacer" molecule. These chiral reagents are then complexed with a coordination metal salt (e.g., Cu²⁺), and enantioselective separations depend upon differences in the stability of the dynamic coordination complexes formed with each optical isomer. This technique has been used to resolve racemic mixtures of amino acids,^{31,59,60} amino alcohols,^{31,59} and catecholamines.⁶⁰

Cavity, or host-guest phases, include cyclodextrins^{31,61-64} and chiral crown ethers,⁶⁵ (see Fig. 5) covalently attached to a silica matrix, again normal-

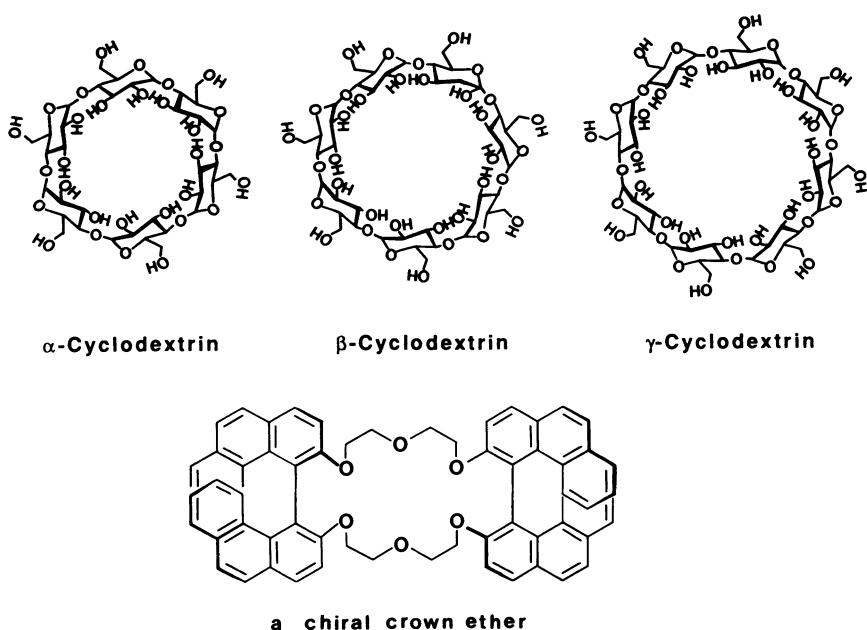


Fig. 5. Chiral discriminators based on cyclodextrins or crown ethers.

ly through some sort of organic "spacer" molecule. Cyclodextrins are obtained by action of the amylase of *Bacillus macerans*, *Klebsiella pneumonia*, or other types of bacillus on amylose, with the cyclodextrin oligomers resulting having from 6 to 12 glucose units bonded through $\alpha-(1,4)$ linkages with a cavity within.¹⁹ The three smallest homologs, α -cyclodextrin (cyclohexylamylose), β -cyclodextrin (cycloheptylamylose), and γ -cyclodextrin (cyclooctaamylose), are commercially supplied (Astec, ICT, Frankfurt, Germany) as Cyclobond III(α), II(γ), and I(β), with the I form (β -cyclodextrin) being the most commonly employed for HPLC applications.³¹ Separations rely upon the stability of dynamic interactions formed between pendant functional groups around the hydrophobic cavity and each enantiomer. Chiral crown ethers^{9,65} have also been covalently attached to silica gel and used to resolve amino acid racemic mixtures.

The last type of chiral stationary phase is often trivially referred to as a "brush" phase. In such cases, a bonded silica (e.g., octadecylsilane or amino-propyl silanized silica) is further modified by subsequent attachment of organic groups to the exterior of the silica network. There are a large number of different

types of such brush phases, e.g., Pirkle columns^{31,66-68}, which are amino-propyl silanized silica normally covalently attached to *N*-(3,5 dinitrobenzoyl) derivatives of amino acids via urea-amide like linkages, etc. With such columns, it is thought that the relative stability of the diastereomeric transition states formed with each enantiomer is dependent upon hydrogen-bonding, charge-transfer interactions, "dipole" stacking, steric effects, $\pi-\pi$ interactions, and the like.³¹ Other representative chiral stationary phases include (*R*)- and (*S*)-1-(α -naphthyl)ethylamine⁷⁰, (1*R*,3*R*)-*trans*-chrysanthemic acids⁶⁹ and L-valine⁷⁰ linked to LiChrosorb-NH₂ columns via D-phenylglycine⁶⁹ and "spacer" moieties, respectively. *Cinchona* alkaloids (e.g., quinine and quinidine) have also been used, with these being covalently attached to silica via reaction with γ -mercaptopropylsilanized silica.⁷¹⁻⁷³

HPLC Chiral Mobile Phases

In this case, a chiral selector is dissolved in the mobile phase and enantiomeric separations occur following passage through an achiral matrix (e.g., LiChrosorb-RP-18, etc.). A rather large number of chiral mobile phases have been evaluated and tested for their efficacy.⁷⁴⁻⁸² The principles of separation are as before, except that the enantioselective interactions now take place in solution rather than with the solid matrix, i.e., the chiral mobile phase can dynamically bind enantiomeric substrates giving diastereomeric ion pairs (via electrostatic interactions, hydrogen-bonding, or complexation with metal salts) or by inclusion chromatography (e.g., with α -or β -cyclodextrins). A number of chiral mobile phases use Cu²⁺ complexes of amino acids or derivatives and find application in the enantiomeric separations of both free amino acids⁷⁴ and their dansyl derivatives.⁷⁵⁻⁷⁸ Other chiral mobile phases include (*R,R*)-tartaric acid mono-*n*-octylamide complexed with Cu²⁺ or Ni²⁺ (for separation of amino acids),⁷⁹ (+)-10-camphorsulfonic acid, *Cinchona* alkaloids, albumins and (+)-di-*n*-butyltartrate (for separations of amino acids, amino alcohols, alkaloids, sulfonic, and carboxylic acids).⁸⁰ Finally, both α - and β -cyclodextrins have been successfully used as CMPs for the separation of chiral mandelic acid derivatives⁸¹ and barbiturates.⁸²

GLC Chiral Stationary Phases

The chiral stationary phases used for optical resolution can be grouped into three categories: amino acid, carbohydrate (notably cyclodextrin), and metal complex derived.

Amino acid derived phases. Since 1966,¹⁶ a variety of chiral stationary phases derived from amino acids have been developed. Most are wall-coated on capillary columns⁸³ and are usually classified into three groups: peptides, diamides, and ureides.

The peptide phases, (e.g., *N*-trifluoracetyl-L-isoleucine lauryl ester, *N*-trifluoroacetyl-L-valyl-L-leucine cyclohexyl ester) are suitable for the enantioselective separation of highly-volatile amino acid derivatives, mostly as their *N*-trifluoroacetyl (*N*-TFA) derivatives.⁸³

The diamide phases, when coupled to polysiloxanes, are used for chiral separation of enantiomers of both low and highly volatile *N*-TFA derivatives of amino acids,⁸⁴ as well as drug⁸³ (e.g., amphetamine, methamphetamine as their *N*-trifluoroacetyl-L-propyl chloride) derivatives. Modified diamide phases have been introduced for the resolution of enantiomers of hydroxy acids,⁸⁵ carbohydrates,⁸⁵ and carboxylic acids.⁸⁶

The ureide type stationary phase, strictly named carbonyl bis(amino acid esters) are used for the enantioselective resolution of amine derivatives.⁸³

Carbohydrate derived phases. With the development of high resolution capillary columns and the availability of many peralkylated cyclodextrins, new chiral stationary phases for GLC were introduced over the last three years.¹⁹ These columns again utilize the ability of cyclodextrins to form enantioselective complexes with guest molecules, and have been used for chiral chromatographic separations of various naturally-occurring substances (e.g., various pheromones and flavors) as well as pharmaceuticals.^{19,87-98} In many cases the enantiomers can be separated without the need for derivatization.

Metal complexes. In this case, chiral separations depend upon the formation of dynamic enantiospecific complexes formed between a chiral solute and an optically-active organometallic compound.⁹⁹ The mechanism for separation is analogous to that described for ligand exchange chromatography. In this way, enantioselective chromatography of racemic ethers, ketones, alcohols, and acetate esters have been obtained using manganese(II), cobalt(II), or nickel(II)bis[3-(heptafluorobutanoyl)-(1*R*)-camphorate] stationary phases.¹⁰⁰

CHIRAL COLUMN CHROMATOGRAPHIC APPLICATIONS IN PHYTOCHEMISTRY

In contrast to the situation for the HPLC or GLC chiral column separation of enantiomers of racemic synthetic drugs, herbicides and pesticides, the application of this methodology to phytochemistry has thus far been

minimal. This situation will undoubtedly change dramatically in the coming years. Of the limited number of chromatographic applications in phytochemistry, most have used modified cellulose (rather than other carbohydrate polymers), synthetic polymers, α - and β -cyclodextrins, chiral crown ethers, and various "brush-type" chiral phases. Described below are some examples of the excellent separations that can be achieved.

Terpenoids

During the last six years, a growing number of enantioselective column chromatographic applications, have been used in mono-, sesqui-, and tetraterpene (carotenoid) studies. For monoterpenes, this was apparently achieved only by chiral column GLC, using either indirect or direct means for racemate resolution. The indirect method, although infrequently used, requires initial formation of diastereomeric derivatives with subsequent separation by GLC. Diastereomeric derivatives were prepared by reaction of each racemic mixture, e.g., (+)- and (-)-forms of α -and β -pinene,¹⁰¹ (+)- and (-)-camphor and fenchol,¹⁰² with chiral reagents.

This approach, however, has been overshadowed by direct enantioselective chromatographic methods, which avoid the need for derivatization. Direct methods have been applied to the separation of both mono- and sesquiterpene optical isomers and have employed both GLC and HPLC techniques. GLC applications mainly use cyclodextrin-based columns and, less frequently, either metal complexes coordinated to a chiral reagent (so called Chirametal phases)¹⁰⁰ or chiral amide coatings (e.g., with XE-60-*S*-valine-*S*- α -phenylethylamide¹⁰³).

The cyclodextrin-based columns have proven to be of great utility in separating mono- and sesquiterpenes as well as other compounds, and these developments were recently thoroughly reviewed.¹⁹ For the monoterpenes, enantioselective separations were first achieved by coating celite⁸⁷⁻⁹⁰ or Chromosorb GLC⁹¹ columns with a solution of α -cyclodextrin in formamide. While these columns permitted the facile separation of various compounds (e.g., (+)- and (-)-forms of α - and β -pinenes,⁸⁷⁻⁹¹ *cis*- and *trans*-pinane,^{89,90} and Δ^3 -carene,⁹⁰) by host-guest complexation, they had short lifetimes due to bleeding of solvent, low theoretical plate numbers, and a limited temperature range (max. 70°C). Thus, their technical usefulness was severely limited.¹⁹

Subsequent generations of cyclodextrin columns are vastly improved. They are thermally stable and find widespread application in high-resolution capillary chromatography. This success was obtained by using the more stable

peralkylated forms of α -, β - and γ -cyclodextrins. Of these, the most common are permethylated cyclodextrin, i.e., heptakis (2,3,6-tri-*O*-methyl)- β -cyclodextrin, usually dissolved in OV-1701 (containing 5% cyanopropyl groups, 7% phenyl groups, and 88% methyl groups), or per-*n*-pentylated cyclodextrin, i.e., octakis (3-*O*-butanoyl-2,6-di-*O*-*n*-pentyl)- γ -cyclodextrin).¹⁹ These cyclodextrin derivatives are then coated on either pretreated glass or fused silica capillary columns to afford the chiral stationary phase.

Permethylated cyclodextrin columns have been used for enantioselective separations of various monoterpene hydrocarbons, e.g., α -pinene, limonene,^{92,93,97} β -pinene, camphene, α - and β -phellandrene,⁹³ as well as terpene ketones (e.g., pulegone) and alcohols (e.g., 3-menthanols).¹⁹ Interestingly, this technique has been applied not only for the separation of menthol enantiomers, but also for the diasteromers of neo- and iso-menthol⁹⁷ (Fig. 6). In a similar manner, the per-*n*-pentylated cyclodextrin chiral column (coated onto deactivated glass) was used for the separation of (+)- and (-)-forms of the terpenoid hydrocarbons: α -pinene, camphene, limonene, α -phellandrene,⁹⁵ *trans*- and *cis*-pinane,⁹⁴ the terpene ketones fenchone, menthone, isomenthone, camphor^{95,104} and piperitone, myrtenal,⁹⁵ as well as the alcohols β -citronellol,^{95,104} *trans*-pinocarveol, myrtenol, and linalool.⁹⁵

Monoterpene have also been separated using chirametal and chiral amino acid stationary phases. Chirametal columns use high-resolution glass or fused-silica columns, coated with a chiral reagent such as Ni(II)bis[3-(heptafluorobutanoyl)-(1*R*)-camphorate], and have been successful in resolving (+)- and (-)-forms of menthone, isomenthol, and isomenthone.¹⁰⁰ Columns using the chiral amide coating, XE-60-*S*-valine-*S*- α -phenylethylamide, were used to resolve various terpene alcohols, e.g., (+)- and (-)-menthol, terpinen-4-ol, etc., as their isopropyl urethane derivatives.¹⁰³

Sesquiterpenes have also been resolved by both chiral GLC and HPLC. For GLC, the enantioselective separation of the optical forms of α -copaene (from plant sources such as hops, Bartlett pear leaves, and myrrh oil) and δ -elemene (from ginger oil) was reported using 10% heptakis (2,3,6-tri-*O*-methyl)- β -cyclodextrin in OV-1701.⁹³ HPLC methodology has been applied recently, particularly to abscisic acid (ABA) and its various derivatives. Initially, attempts to directly resolve (+)- and (-)-ABA by HPLC were unsuccessful, and time-consuming derivatizations were necessary, e.g., by formation of their methyl 1',4' *cis*-diols and subsequent enantioselective chromatography on Pirkle Type A and Pirkle covalent *R*-phenylglycine columns in series,¹⁰⁵ or by conversion to (\pm)-abscisic acid methyl esters and chiral separation on a Chiralcel OD column (cellulose tris-3,5-dimethylphenylcarbamate coated on silica, Daicel, Japan).¹⁰⁶

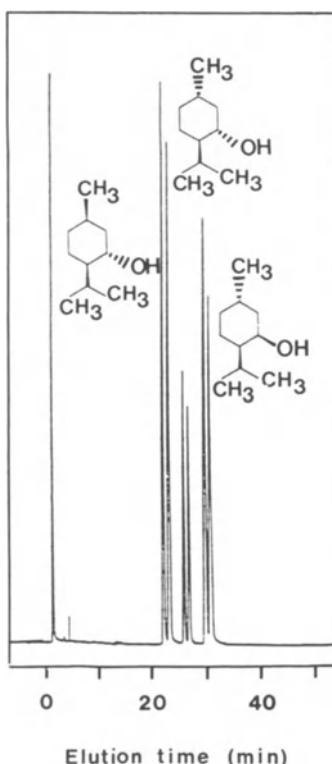


Fig. 6. Enantioselective chromatographic resolution of racemic isomenthol, menthol, and neomenthol. Column: fused silica, 25 m x 0.25 mm coated with heptakis (2,3,6-O-trimethyl-) β -cyclodextrin in OV-1701 ($c=0.07$ m); $T = 85^\circ\text{C}$; $p = 1.0$ bar H_2 . (Redrawn from reference 97.)

More recently, ABA enantiomers were resolved directly using α_1 -acid glycoprotein,⁵ cellulose tris(3,5-dimethylphenylcarbamate)¹⁰⁷ (see Fig. 7) and ovomucoid^{108,109} columns, which were all individually coated onto macroporous silica gel. Although excellent separations were achieved in each case, those carried out on α_1 -acid glycoprotein or ovomucoid columns were noted to be very pH dependent.^{5,108,109} Interestingly, the ovomucoid column was used not only for abscisic acid, but also for the enantiomeric chromatography of its metabolites, (\pm)-phaseic acid and (\pm)-1',4' *cis*- and *trans*-diols.¹⁰⁹

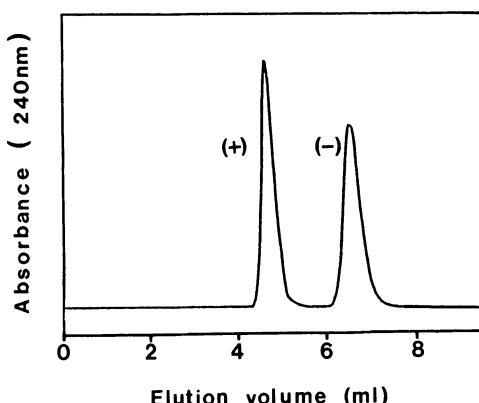


Fig. 7. Resolution of (\pm)abscisic acids on a cellulose tris(3,5-dimethylphenylcarbamate) column with hexane:2-propanol:TFA (80:20:1) as mobile phase, flow rate: 0.5 ml min^{-1} . (Redrawn from reference 107.)

Chiral separations of carotenoid-derived flavor compounds have been obtained using chiral GLC, e.g., (i) α -damascone and theaspirane diastereomers (metabolites of the oxidative degradation of carotenoids, and constituents of black tea aroma) were resolved on a permethylated β -cyclodextrin stationary phase (see Fig. 8),¹¹⁰ and (ii) dihydroactinidiolide, a flavor compound from apricots (obtained by photooxidation of β -carotenes¹¹¹), was separated into (+)- and (-) forms on a per-*n*-pentylated- β -cyclodextrin phase.¹¹² One last application of chiral column separations within the terpenoids is worthy of mention, even though it is not plant related. This involves the facile separation of 3-hydroxy-4-oxocarotenoids, which widely occur in aquatic animals. These separations were carried out by HPLC using a Sumipax OA-2000 column (Sumitomo Chemicals, Japan).¹¹³

Flavonoids: Flavanones, Procyanidins and Isoflavonoids

Flavonoids are a diverse group of metabolites having several key functions: the pigmentation of flowers and fruits,¹¹⁴ phytotoxins against pathogens,¹¹⁵ insects,¹¹⁶ fish, etc. This class of compounds is typified by the characteristic flavonoid skeleton, which is found in nature not as the unsubstituted flavanone skeleton shown, but with oxygen-containing substituents as in naringenin (Fig. 9). An added complexity is the stereochemistry of the pendant

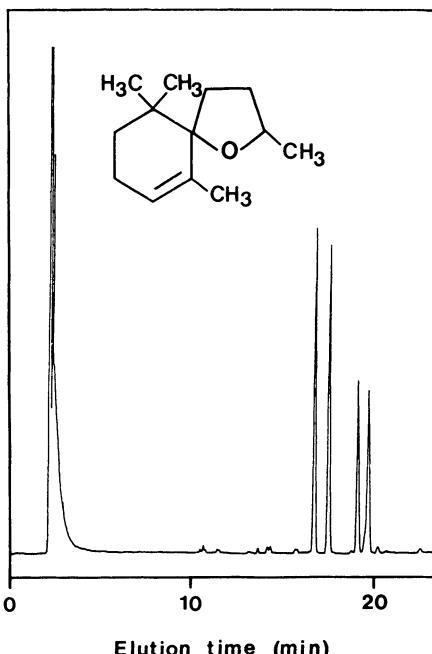


Fig. 8. Chiral separation of (+)- and (-)-forms of theaspirane diastereoisomers on a 38 m x 0.23 mm i.d. open-tubular glass column, coated with 10% heptakis (2,3,6-tri-*O*-methyl)- β -cyclodextrin in OV-1701-vi; carrier gas H₂; inlet pressure 70 kPa, program: 120°C isothermal. (Redrawn from reference 110.)

aryl ring at C-2 and, often, oxygen-bearing substituents at C-3. This has made the chiral separation of such compounds challenging.

Initial work in resolving flavonoids was carried out with the unsubstituted flavanone that only has one chiral center at C-2 (Fig. 9). Enantiomeric separation was achieved using cellulose triphenylcarbamate supported on silica gel³³ (Chiralcel OC, Daicel, Japan). However, this column was unable to separate flavanones containing more than two hydroxyl groups, a serious limitation since naturally-occurring compounds contain numerous hydroxyl groups, e.g., naringenin, hesperetin, eriodictyol, homoeriodictyol, pinocembrin, and isosakuranetin. This difficulty was overcome by using microcrystalline cellulose triacetate as the stationary phase.¹¹⁷ As can be seen from the example naringenin in Figure 10, chiral resolution was achieved in each case and those

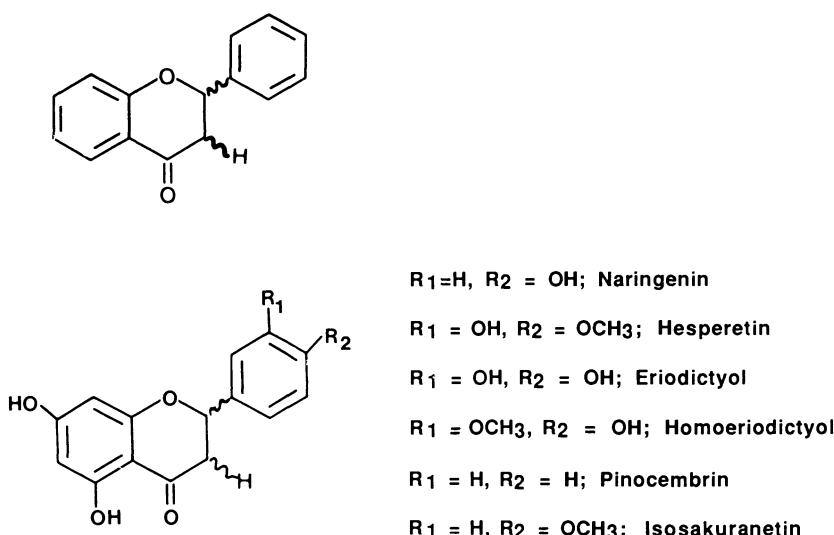


Fig. 9. The flavanone skeleton and various naturally occurring flavanones.

isomers having a 2S- configuration were eluted first. For purposes of comparison, CTA adsorbed onto macroporous silica was also evaluated but gave poorer results; e.g., naringenin was incompletely resolved and the chiral recognition pattern was reversed for Tröger's base as previously observed. This is consistent with other observations that reprecipitated CTA has different enantioselective properties.

In a similar manner to the separations described above using cellulose triphenylcarbamate on silica, enantioselectivity has also been reported for the resolution of the flavan-3-ols, (+)- and (-)-catechin and epicatechin, respectively¹¹⁸ (Fig. 11). In this case, these flavan-3-ols were obtained by chemical degradation of procyanidins from the pith of sago palm (*Metroxylon sagus* Rottb.).

Pterocarpans such as (+)-pisatin in *Pisum sativum* are naturally occurring isoflavonoids with phytoalexin properties. A number of racemic pterocarpans, obtained synthetically, have been separated into their (+)- and (-)-antipodes¹¹⁹ on a poly(triphenylmethyl methacrylate) coated silica gel column (Chiralpak OT, Daicel, Japan). In all examples shown, the dextrorotatory enantiomers were eluted first.

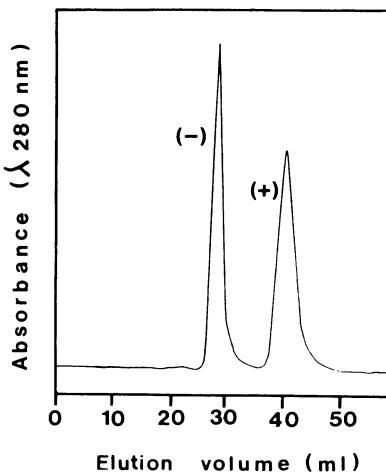
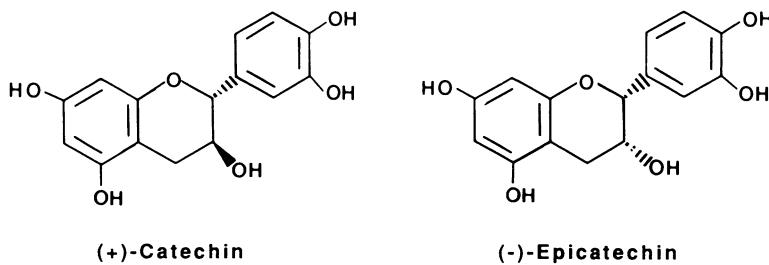


Fig. 10. Enantioselective separation of racemic naringenins. Column: 250 x 10 mm, stainless steel, packed with 10 μm microcrystalline cellulose triacetate (Merck, Darmstadt, F.R.G.), mobile phase: methanol, flow rate: 1 ml min^{-1} . (Redrawn from reference 117.)



(+)-Catechin

(-)-Epicatechin

Fig. 11. Flavan-3-ols, (+)-catechin and (-)-epicatechin.

Enantioselective separations have also been achieved with the rotenoids. These are a group of naturally occurring isoflavonoids found mainly in the Papilionatae division of the Leguminosae; the best known is $(-)(6aS, 12aS, 5'R)$ -rotenone (see Fig. 12) which is found in the genera *Derris*, *Lonchocarpus*, and a number of other species.¹²⁰ Rotenone is used as an insecticide and fish poison. Rotenoids readily undergo oxidation to afford the corresponding 12a-hydroxy analogues. Further, in mildly alkaline media, diastereomers can be formed by epimerization (see Fig. 12 for examples).

The enantioselective chromatographic resolution of rotenone and related rotenoids (isorotenone, elliptone, and deguelin), as well as their 12a-hydroxy analogues (rotenolone, isorotenolone, hydroxyelliptone, tephrosine, and α -toxicarol), has been investigated using three different chiral phases: (+) poly(triphenylmethyl methacrylate) (Chiraldak OT, Daicel), Pirkle-type columns (covalently or ionically bonded (*R*)-*N*-3,5-dinitrobenzoylphenylglycine adsorbed onto silica), and β -cyclodextrin bonded silica.^{121,122} The separations were found to be hydroxyl substitution, oxidation level and column dependent.

(\pm)-Rotenones, isorotenones, elliptones, and deguelins were readily resolved on the poly(triphenylmethyl methacrylate column); separations were explained on the basis that both nonpolar interactions and the presence of aromatic groups were important for chiral recognition.¹²³ As an example, Figure 13A shows the separation of 6a*S*, 12a*S*-(peak 1) and 6a*R*, 12a*R*-(peak 2) isorotenones. However, this column was not suitable for chiral resolution of rotenolone and isorotenolone, which differ only by hydroxylation at position 12a.

(\pm)-Rotenones, isorotenones, deguelins, and their corresponding 12a-hydroxy analogues can also be resolved on β -cyclodextrin stationary phases. Interestingly, the elution order is reversed when compared to that previously observed for the poly-(triphenylmethyl methacrylate) column separation (e.g., see Fig. 13B). Another difference with the β -cyclodextrin bonded phase is that neither elliptone nor its 12a-hydroxy analogue could be resolved. Thus, the presence of the isopropyl group on the E ring seems necessary for enantioselectivity with this chiral stationary phase.

Lastly, the Pirkle columns were found to be useful for the chiral separation of all 12a-hydroxy analogues except for α -toxicarol. They did not, however, resolve rotenone, isorotenone, elliptone or deguelin. Enantioselectivity has been rationalized as being dependent upon specific polar interactions (hydrogen acceptor-donor complexation) between the 12a-hydroxy group and the stationary phase. Note, though, that no satisfactory explanation for the failure to resolve α -toxicarol was given.

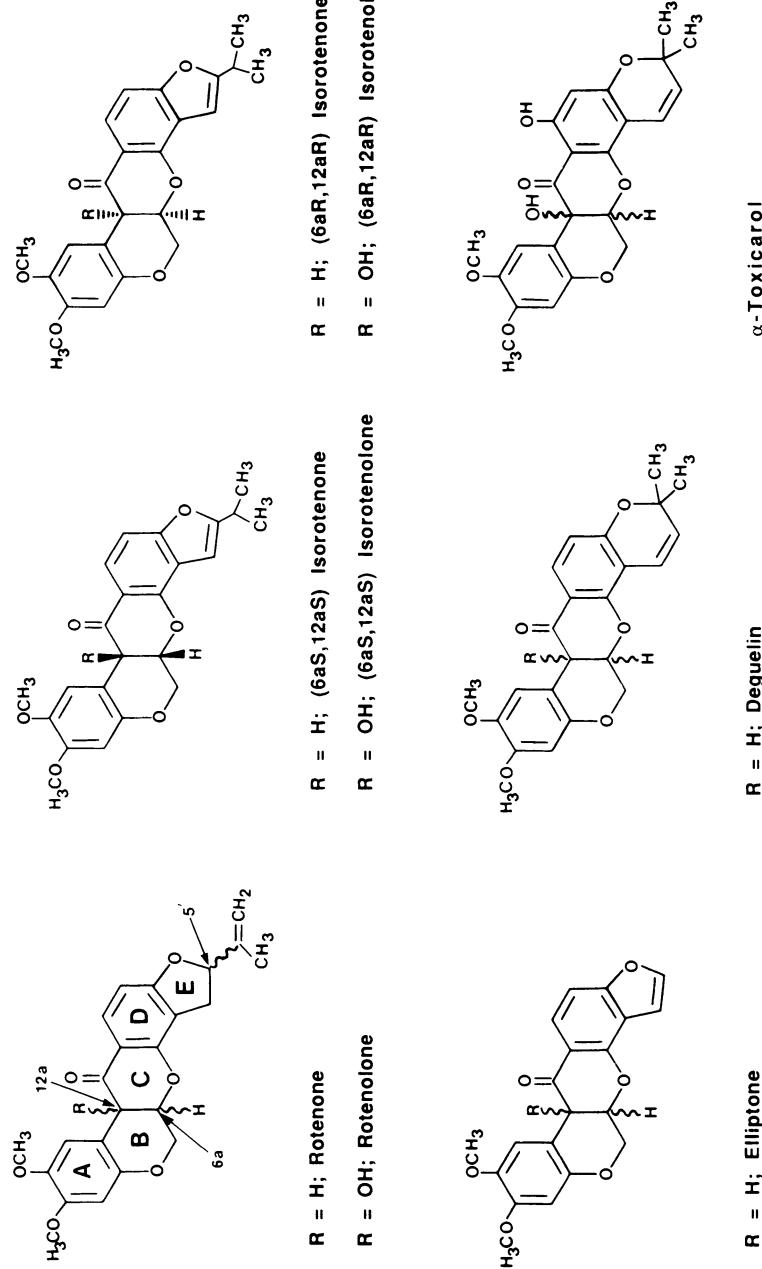


Fig. 12. Some naturally occurring rotenoids.

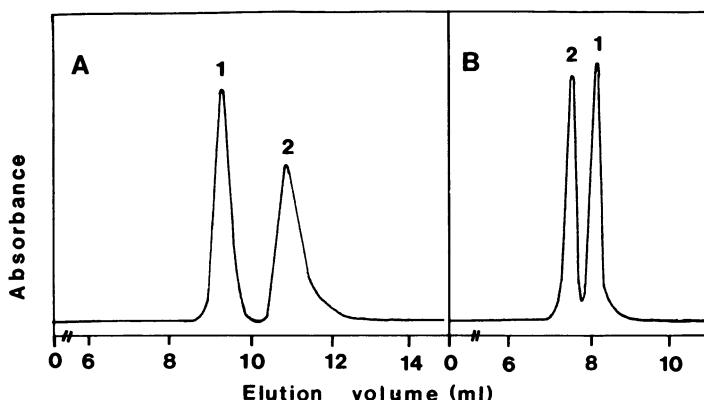


Fig. 13. Enantioseparation of isorotenone antipodes: (A) Chiral phase: helical (+)-poly(triphenylmethyl methacrylate)-silica (Daicel, New York, NY, USA), mobile phase: methanol, flow rate: 0.5 ml min^{-1} ; (B) Chiral phase: β -cyclodextrin bonded silica (Advanced Separations Technologies, Whippany, N.J., USA), mobile phase: $\text{CH}_3\text{CN:H}_2\text{O}$ (30:70), flow rate: 0.5 ml min^{-1} . Peaks: 1 = (6aS, 12aS) isorotenone, 2 = (6aR, 12aR) isorotenone. (Redrawn from reference 122.)

Truxinic Acids

Recently, the formation of truxillic acids in the cell walls of vascular plants, e.g., 4',4'-didehydroxytruxillic acid found in *Lolium multiflorum* has received much attention.¹²⁴ This is because their formation may have some importance in the cross-linking of cell-wall polysaccharides. The corresponding truxinic acids have not yet been found. Nevertheless, truxinic acids, such as the synthetic racemate *S*-truxinic diamide, are optically active and have been resolved using a Pirkle chiral stationary phase derived from *cis*-3-(1,1-dimethylethyl)-4-phenyl-2-azetidinone.¹²⁵

Lignans

Lignans are another widespread group of naturally-occurring phytochemicals of great structural diversity¹²⁶ which are normally found as dimeric phenylpropanoids. Many have interesting phytotoxic or pharmacological

properties.¹²⁷ Until recently, almost nothing was known about the biochemical pathways to this important class of compounds. However, in 1990, significant progress was made in this area with our study on the biogenesis of the *Forsythia* lignans, secoisolariciresinol and matairesinol (Fig. 14). These investigations were critically dependent upon enantioselective separations. Thus, we found that the coupling of two coniferyl alcohol units in *F. intermedia*¹¹ was stereochemically controlled, affording only (-)-secoisolariciresinol and not its (+)-enantiomer.¹⁰ Further, (-)-matairesinol formation occurred via the stereoselective dehydrogenation of (-)-, and not (+)-secoisolariciresinol. This was established both at the cell-free level and *in vivo*. These are the first two examples of enzymatic conversion leading to optically-active lignans and their derivatives *in vitro*. As an example of a typical enantioselective separation, Figure 15 shows the chiral resolution of synthetic (+)- and (-)-secoisolariciresinols. Other racemic lignans have also been resolved, e.g., the enantiomers of kadsurenone,¹²⁸ a neo-

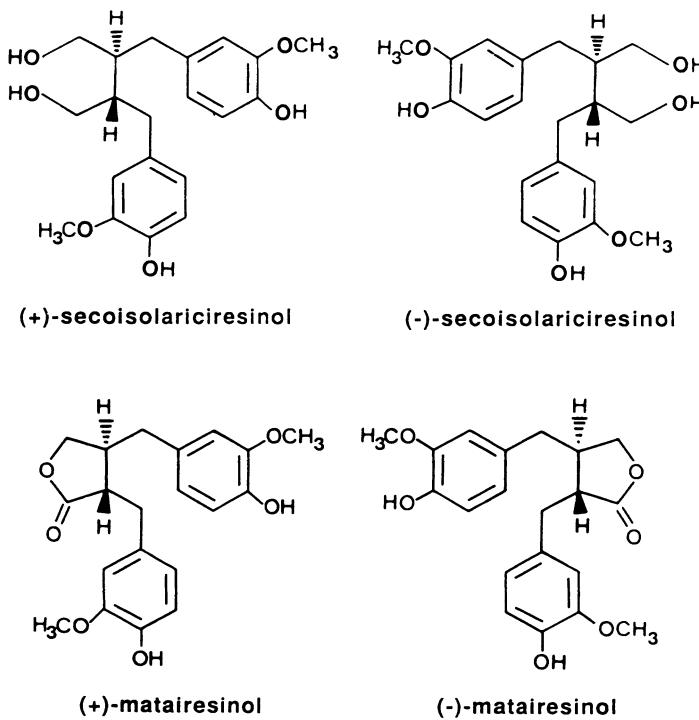


Fig. 14. Racemic lignans, secoisolariciresinol and matairesinol.

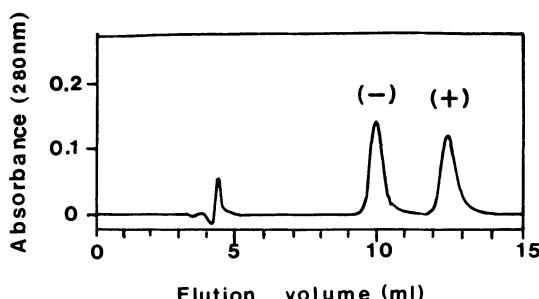


Fig. 15. Enantioselective separation of (+)- and (-)-secoisolariciresinols.

Column: Cellulose tris(3,5-dimethylphenylcarbamate) coated on silica (Chiralcel OD, Daicel, Japan), mobile phase: hexanes: EtOH (70:30) flow rate: 0.5 ml min⁻¹.

lignan, on poly(methyl methacrylate) coated silica (Chiralpak, Daicel, Japan), (\pm)-syringaresinol¹²⁹ and (\pm)-todolactol A¹³⁰ on cellulose triphenylcarbamate supported on silica gel (Chiralcel OC, Daicel, Japan).

Alkaloids

Like the lignans and terpenoids, alkaloids represent an important class of natural products. As before, the enantiomeric form can vary with the plant source from which a particular alkaloid is isolated. Chiral separations of alkaloids, normally obtained synthetically as racemic mixtures, have mainly used cyclodextrin-bonded silica columns, although α_1 -acid glycoprotein columns have also been employed. For example, Armstrong *et al.*⁶¹ separated the diastereomeric pairs of the cardioactive and antimalarial *Cinchona* alkaloids, quinidine-quinine and cinchonidine-cinchonine on a β -cyclodextrin stationary phase. The pharmaceutical drugs, *dl*-scopolamine (isolated from *Hyoscyamus niger* and other related shrubs), *dl*-hyoscyamine (formed by racemization of *l*-hyoscyamine during its extraction from *Atropa belladonna* and *Datura stramonium*) and *dl*-cocaine were resolved using two β -cyclodextrin bonded phase columns in series.¹³¹ In each case, the naturally occurring *l*-antipode was eluted last. Interestingly, the tobacco alkaloid, nicotine, obtained in racemic form by total synthesis, has been difficult to resolve directly, e.g., enantioselective separations required conversion to analogues such as *N'*-(2,2,2-trifluoro-

ethyl)nornicotine, *N*⁺-benzylornicotine, etc., with subsequent enantioselective chromatography using one or two β -cyclodextrin columns.¹³² Nicotine can, however, be directly resolved on an achiral C₁ microcolumn (1m x 250 μ m) with a saturated solution of β -cyclodextrin in acetonitrile:water (20:80) as mobile phase¹³³ (Fig. 16). Enantioselective separations of atropine, homatropine, cocaine, and ephedrine (from *Ephedra* species) have also been carried out with α_1 -acid glycoprotein columns.³⁹

Miscellaneous Chiral Separations

Pantothenic acid is a precursor of the biologically important coenzyme A with only the D-(*R*)-isomer being active. Synthetic pantolactone, calcium pantothenate, and panthenol when converted into pantoic acid, can be resolved into their corresponding enantiomeric forms via a ligand-exchange chiral stationary phase (MCI gel CRS 10W, Mitsubishi, Kasei Kogyo, Tokyo, Japan) with a copper sulfate solution (2 mM) containing CH₃CN (10%) as mobile phase.¹³⁴ These racemic compounds, when derivatized as either 3,5 dinitrophenyl carbamates or 3,5 dinitrobenzoyl esters, can be separated on a chiral polyacrylic stationary phase. This was achieved with pantothenyl alcohol, as its 3,5-dinitrobenzoylester,¹³⁴ pantolactone and calcium pantothenate, as their 3,5-dinitrophenyl carbamates.

2-Hydroxy fatty acids occur widely in biological tissues as constituents of unusual seed oils, as well as in sphingolipids and wool wax.¹³⁵ These racemates have been separated into their respective antipodal forms as their 3,5-

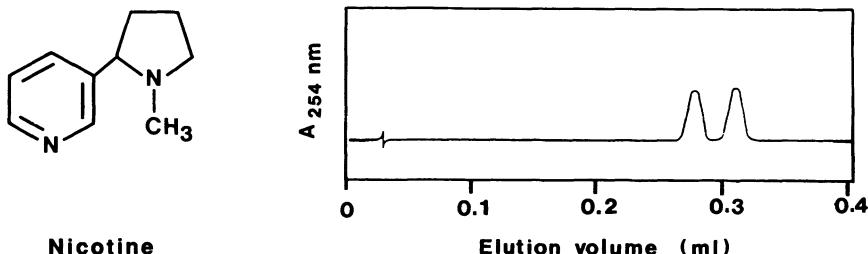


Fig. 16. Separation of (+) and (-) nicotine antipodes. The microcolumn was packed with achiral C₁ packing material. The mobile phase was a saturated solution of β -cyclodextrin in acetonitrile: water (20:80). The flow rate was 1.3 μ l min⁻¹ at a pressure of 230 atm. (Redrawn from reference 133).

dinitrophenylurethane derivatives.¹³⁶ A base line separation of 2-hydroxy-4-methylpentanoic and 2-hydroxyoctanoic acids was obtained using a capillary column packed with *N*-(S)-(4-chlorophenyl)-isovaleroyl-D-phenylglycine, ionically bonded to γ -aminopropyl silanized silica with *n*-hexane:1,2-dichloroethane:ethanol (20:5:1) as the mobile phase.

The separation of racemic amino acids by high-performance liquid chromatography is mainly based on ligand exchange chromatography. In such cases, the chiral ligands, chelated to a divalent metal ion, are either linked to the stationary phase or are present in the mobile phase.⁷⁹ When a chiral mobile phase is used, the stationary support is typically a reversed phase column. Generally, amino acids and their derivatives^{74,75,77,78,137} are used as the chiral chelating selector, although derivatives of tartaric acid have also been utilized.^{60,79} Copper (II) is most widely used as the metal ion for chelation.

Chiral stationary phases with bovine serum albumin covalently bound⁴⁵ or adsorbed⁴⁶ to silica have also been used to resolve alanine and threonine as their phthalimido derivatives,⁴⁵ alanine and serine as their *N*-benzenesulfonyl derivatives⁴⁵ and tryptophan.⁴⁶ Finally, chiral crown ether-coated octadecylsilyl silica has been used to resolve common amino acids,⁶⁵ such as alanine, leucine, phenylalanine, methionine, etc., with the L-enantiomers being first eluted.

CONCLUSION

The preceding sections served to illustrate some of the rapid enantioselective separations that can be attained today in phytochemistry. The development of such methods for the chiral resolution of optically active natural products is a welcome addition to the tools of the modern phytochemist. As time progresses, these methods will find routine application in helping to resolve outstanding stereochemical biogenetic questions at both the whole plant and cell-free levels. This is because stereoselectivity (or the lack of it) of various enzymatic transformations can now be quickly followed. Another application is in rapidly determining the enantiomeric purity of numerous plant-derived natural products. This may also be of significance in other areas where enantiomeric purity of a given phytochemical is important, as in foodstuffs, beverages or medicines. Indeed, since this technique permits the facile determination of the enantiomeric purity of a given compound of interest, this may provide a considerable disincentive to those wishing to either substitute, or dilute natural material, with synthetic racemic mixtures.

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Chapter Four

SUPERCRITICAL FLUID CHROMATOGRAPHY FOR THE ANALYSIS OF NATURAL PRODUCTS

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INTRODUCTION

As with many branches of science, advances in the field of natural products research have always depended largely on the analytical methods available. The separation, identification, structure elucidation, and quantitation of components in phytochemical samples are all essential steps to the study of natural products chemistry. As a result, many of the advances in this field parallel the improvement of analytical methodologies.

Chemical separations are the cornerstone of modern analytical chemistry, primarily because methods for chemical analysis are rarely, if ever, specific. Consequently, the separation of the analyte from potential interferences is an important step in most analytical procedures, qualitative or quantitative. Two of the most widely used and most powerful methods of performing analytical separations are chromatography and electrophoresis, techniques that have applications in virtually all branches of science. The appearance of chromatographic applications has been explosive over the last four decades, resulting not only from the development of several new types of chromatographic techniques but also from the growing need by scientists for better methods of characterizing complex mixtures. More recently, chromatography has also become quite popular for preparative separations, particularly high performance liquid chromatography (HPLC). The tremendous impact of chromatography on science is attested by the 1952 Nobel prize that was awarded to A.J.P. Martin and R.L.M. Synge for their discoveries in this field, and twelve other Nobel prizes awarded between 1937 and 1972 that were based upon research in which chromatography played a vital role.¹

The purpose of the present work is to introduce an emerging method of analytical separation and quantitation, supercritical fluid chromatography (SFC), to the natural products community. A brief discussion of the theory and methodology is provided, followed by a thorough review of applications to natural products. Recent results from our own laboratory are also included. Some familiarity with more conventional separation modes, gas chromatography

(GC) and liquid chromatography (LC), is assumed. Although we shall provide citations throughout this chapter, the reader is also alerted to the hundreds of additional references provided by recent critical reviews,²⁻⁴ books,⁵⁻⁸ and other non-critical reviews.⁹⁻¹¹

DEFINITION AND RATIONALE FOR SUPERCritical FLUID CHROMATOGRAPHY

Supercritical fluid chromatography (SFC) is an instrumental chromatographic method similar to liquid and gas chromatography, except that it employs a supercritical fluid as the mobile phase. Although SFC was first employed by Klesper et al. in 1962 for the separation of nickel porphyrins using supercritical chlorofluoromethanes as mobile phases,¹² it was not until some major technological improvements occurred in the early 1980's that its use became significant. SFC is currently growing steadily and rapidly, and today it is recognized as a necessity in many analytical laboratories.

A supercritical fluid can be defined as any substance for which both the pressure and temperature are above their critical values, and the substance can no longer be described as a liquid or a gas. This supercritical region is illustrated in the upper right quadrant (region A) of the phase diagram for carbon dioxide (Fig. 1), the substance most commonly employed for supercritical fluid-based separations.

The attractiveness of supercritical fluids as mobile phases for chromatography can be realized by examination of Table 1. Since supercritical fluids possess properties that are typically intermediate between gases and liquids, we can exploit their high solvating power (relative to GC), yet retain high solute diffusivities (relative to HPLC). For example, gas chromatography (GC) cannot be used for the analysis of thermally labile or involatile compounds, which constitute the vast majority (over 75 %) of all chemical compounds. On the other hand, liquid chromatography (LC) in theory is applicable to all types of compounds, but as currently practiced has a fairly low peak capacity (number of resolvable peaks) relative to GC and the lack of a sensitive, easy-to-use detector for compounds without a chromophore (cf. flame based detectors in GC).

With SFC, the densities of supercritical fluids are sufficient to dissolve many compounds at near-ambient temperatures (usually 32-100°C). Moreover, the solvating power of the fluid can be varied continuously by varying the pressure (density) and temperature. Mobile phase viscosity remains low enough

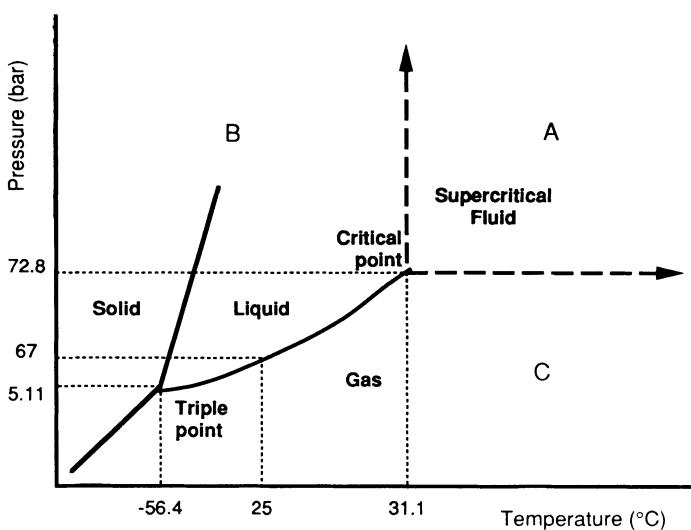


Fig. 1. Phase diagram for carbon dioxide. Although region A is the only true supercritical region (by definition), it should be noted that a phase transition will not occur if either (but not both) the temperature or pressure is reduced below its critical value, i.e., in going from region A to regions B or C. Nevertheless, region A is generally distinguished from regions B and C since a phase transition could occur if the temperature or pressure was lowered, respectively, in the latter regions.

Table 1. Physical properties of typical gases, liquids, and supercritical fluids used in chromatography^a

Property	Gas (helium)	Supercritical fluid	Liquid (water)
Density (g/mL)	0.001	0.3-0.9	1.0
Viscosity (poise)	10^{-4}	10^{-3}	10^{-2}
Solute diffusion coefficient (cm ² /sec)	0.01-1	10^{-3}	10^{-5}

a. Data obtained from reference 5.

to permit the use of long, open tubular (capillary) columns with minimal pressure drop, which results in very high efficiencies and peak capacities. Alternatively, packed columns can be employed as in HPLC, but with a much higher pressure drop per unit length. Finally, although lower than in gases, the diffusivities of solutes in supercritical fluids significantly exceed those in liquids, and the resulting optimal range of inner diameter for capillary columns is larger for supercritical fluids (15-35 μm vs 5 μm for liquids) and more easily interfaced to the injector and detector. With packed columns, the superior mass transfer characteristics (higher diffusivities) also allow the same efficiency to be achieved at higher linear velocities than in LC, and thus faster analyses with equivalent resolution. Because many natural products are involatile or lack a good chromophore, SFC is a very promising technique for these compounds. As noted in the review section following the discussion on methodology, numerous applications have already been reported.

METHODOLOGY

Supercritical Fluids

To be useful as a supercritical fluid in chromatography, a substance must have a relatively low critical temperature and pressure. Table 2 lists critical and related properties of a number of substances that have been used as supercritical fluids in chromatography.

Carbon dioxide has been the mobile phase of choice for SFC, primarily because of its relatively mild critical parameters, low cost, chemical inertness, and detector compatibility,¹³ including the flame ionization detector (FID) used widely in GC and the UV detector in LC. However, the utility of carbon dioxide as a mobile phase is somewhat limited because of its nonpolarity,¹⁴ and many polar compounds appear to be insoluble in it. The elution of polar compounds is difficult and the peak shapes for these polar compounds are usually poor. This latter difficulty may be due to active sites on the stationary phase rather than any inherent deficiency in the mobile phase itself.

Supercritical pentane has also been used as a nonpolar mobile phase, but almost exclusively with capillary columns in order to minimize the mobile phase volume and thus the risks associated with flammable gases. Nitrous oxide has recently been shown to be of considerable value for amines,¹⁵ and sulfur hexafluoride has been shown to provide excellent selectivity for group-type separations of hydrocarbons,¹⁶ although it requires the use of a gold-plated FID.

Ammonia has been suggested as an alternative to carbon dioxide for polar compounds, but it tends to be fairly reactive with various high-pressure seals on commercial equipment, as well as a potential environmental hazard. The problems of seal degradation (leaks) are compounded by the fact that ammonia can then react with carbon dioxide to form the insoluble salt, ammonium carbonate, which could potentially plug the entire system.

Xenon has the advantage of being very inert and completely transparent to infrared radiation, making it ideal for use with SFC/FT-IR. Unfortunately it is very expensive and is not a good solvent for polar compounds. The various freons listed near the bottom of Table 2 have been employed less frequently, although they show promise with polar compounds. Chlorodifluoromethane has been reported to be somewhat corrosive with respect to the flame ionization detector,¹⁷ but was much more effective in eluting various phenolic compounds than carbon dioxide.

In addition to the pure supercritical fluids listed in Table 2, much research has been performed on the use of modifiers with supercritical fluids.

Table 2. Critical and Related Parameters of Selected Pure Fluids^a

Substance	Symbol	T _c (°C)	P _c (atm)	ρ _c (g/mL)	p400 atm
carbon dioxide	CO ₂	31.2	72.9	0.46	0.96
pentane	n-C ₅ H ₁₂	196.6	48.3	0.20	0.51
nitrous oxide	N ₂ O	36.5	71.7	0.45	0.94
sulfur hexafluoride	SF ₆	45.5	37.1	0.74	1.61
ammonia	NH ₃	132.5	111.5	0.24	0.40
xenon	Xe	16.6	58.4	1.10	2.30
dichloromethane ^b	CCl ₂ H ₂	NA ^c	60	NA	NA
trifluoromethane	CHF ₃	25.9	46.9	0.52	NA
chlorodifluoromethane ^d	CHClF ₂	96.0	48.4	0.525	1.12
dichlorodifluoromethane	CCl ₂ F ₂	111.8	40.7	0.56	1.12

^a Data obtained from Perry's Chemical Engineering Handbook, and reference 5.

^b Fujimoto et al., *J. Chromatogr. Sci.*, 1989, 27, 325-328.

^c NA = not available.

^d Reference 17.

That is, rather than switching to a completely different supercritical fluid for the mobile phase, a small percentage of a secondary solvent can be added to modify the mobile phase while (hopefully) maintaining the mild critical parameters of the primary fluid. Through the use of modifiers, one can increase the fluid's dielectric constant, introduce hydrogen bonding, or alter mass transfer characteristics and the solvent viscosity.¹⁸ Modifiers allow the chemical tailoring of the mobile phase to meet a specific chromatographic need.

Modifiers have been observed to increase solvent strength, enhance selectivity, and improve peak shape and column efficiency;¹⁹ improvements in peak shape and efficiency are often due to the deactivation (covering up) of active sites present on some types of stationary phases. The following modifiers have proven to be useful in one way or another with carbon dioxide:^{5,20} various alcohols including methanol, ethanol, isopropanol, and hexanol; acetonitrile, tetrahydrofuran, 1,4-dioxane, water, formic acid, ion-pairing agents, dimethyl sulfoxide, dichloromethane, and N,N-dimethylacetamide, as well as others.

For pure supercritical fluids, Giddings has reported a classification based on the solubility parameter.²¹ Note that in contrast to liquids, the mobile phase strength of a supercritical fluid varies with density, as shown in Table 3. With modified mobile phases, it is somewhat more difficult to estimate their polarity. Polarity can be measured with solvatochromic probes,²² but the results may sometimes be misleading due to specific probe-fluid interactions.

Instrumentation

A schematic diagram of an SFC instrument is shown in Figure 2. With the exception of the restrictor at the end of the column which is required to maintain the pressure needed for the supercritical state, the components are basically the same as in GC and LC. As always, a high purity mobile phase is required, and SFC-grade fluids are available from a number of sources.^{23,24} Generally it is easier to deliver the supercritical fluid in the liquid state (cf. gaseous state) with a high pressure syringe pump or alternatively, a reciprocating piston pump. By cooling the pump heads with a circulating refrigerated bath, a more reliable solvent fill and delivery is achieved. With carbon dioxide and other nonpolar fluids, an adsorbent trap is generally installed prior to the pump to eliminate irreproducible amounts of polar compounds that could significantly influence the separation. When mobile phase composition programming is required, the output of two independent pumps containing different fluids can be changed with time to produce the desired gradient.

Table 3. Solubility parameters of selected compounds ^a

Compound	δ (cal/mL) ^{1/2}	Compound	δ (cal/mL) ^{1/2}
Carbon dioxide		toluene	8.9
0.1 (g/mL)	0.9	benzene	9.2
0.3	2.6	1-propanol	12.0
0.5	4.3	2-propanol	12.4
0.7	6.0	methanol	14.5
0.9	7.7	formamide	19.2
n-heptane	8.0	water	23.4

^a Data from Giddings et al., [Science] 4: 67 (1968); Tijssen et al., J. Chromatogr. 122: 185 (1976); and Karger et al., J. Chromatogr. 125: 71 (1976).

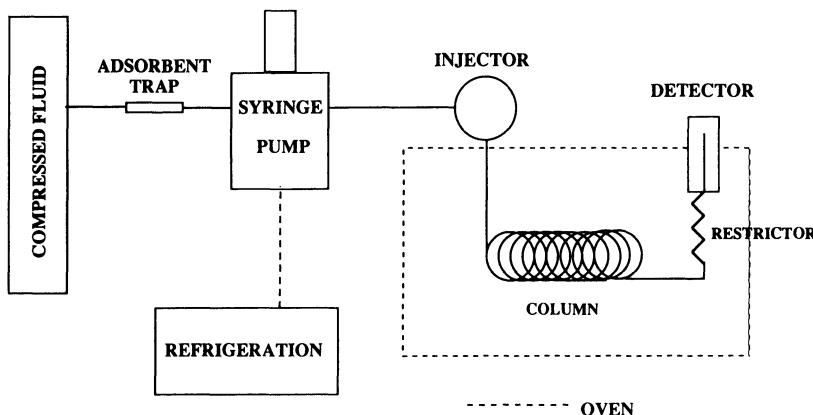


Fig. 2. Schematic diagram of typical instrumentation for supercritical fluid chromatography (SFC). With the exception of the restrictor which is required to maintain the pressure needed for the supercritical state, the components are basically the same as in gas and liquid chromatography: mobile phase, pump, injector, column, and detector.

Sample Introduction

Two types of injectors are frequently employed. For packed column SFC, a standard six port rotary valve with an external sample loop of 1-10 μL has proven to be quite reliable. For SFC with capillary columns, a similar rotary valve with an internal "loop" of 0.2 to 0.5 μL is typically employed. Frequently the rotor is pneumatically actuated in very rapid fashion to allow only a small fraction of sample to be introduced; this is done to avoid column overload. Alternatively, the flow from the injector is split off in the same fashion as in GC. A disadvantage of the latter mode is potential sample discrimination.

Another powerful means of sample introduction is a direct on-line extraction apparatus. A small amount of solid (or liquid) sample can be placed in the extraction vessel, followed by the introduction of the supercritical fluid. The material can be extracted at a specific temperature and pressure for a certain amount of time, and then transferred to the analytical column for analysis. This mode of sample introduction is described in more detail in a later section devoted to supercritical extractions.

Columns

Both capillary and packed columns have been used successfully in SFC, although the debate over which was better in terms of separating power, quantitative reproducibility, and compatibility with polar analytes was formerly a source of great controversy. Not unexpectedly, SFC capillary columns are similar to the fused silica columns used in GC, except that their inner diameters are reduced from 200 to 250 μm down to 100 to 50 μm (or less) to reflect the reduced mass transfer (lower diffusion coefficients) in a supercritical fluid compared to a gas (Table 1), and the stationary phase is either crosslinked or bonded to the fused silica to avoid dissolution by the strongly solvating supercritical fluid mobile phase. Packed SFC columns used in the past were virtually identical to the bonded-phase HPLC columns, although usage of shorter, smaller inner diameter columns (0.5-2 mm i.d.) or packed capillary columns is now somewhat favored to make the flow rate more compatible with various detectors (FID, MS, etc.). Whereas packed columns at present nearly always provide better separation per unit time,²⁵ the much greater permeability (lower pressure drop per unit length) of capillary columns allows much greater lengths (1-10 m vs 5-10 cm for packed columns) to be employed and correspondingly greater total efficiency (peak capacity) to be achieved; this is sometimes desirable for complex

samples. However, one of the greatest limiting factors for packed column use in SFC has been the active sites present in conventional HPLC-type bonded-phase columns. This results in poor peak shape for polar compounds, and in many cases (basic compounds) solutes become irreversibly adsorbed and do not elute at all. The use of modifiers is frequently very beneficial in this regard, providing coverage of these active sites; and recently, better methods of deactivation and coating of porous silica particles for packed-columns have renewed hope in the successful use of these columns for polar and basic compounds.^{26,27}

It is beyond the scope of this review to exhaustively compare and contrast the features of packed and capillary columns; detailed treatments may be found elsewhere.^{25,28,29} It suffices to say that a plethora of commercially available columns exists, and the complementary nature of the two types of columns is now widely appreciated. Although new types of columns will no doubt continue to be introduced, at present much research is being performed for the purpose of improving the existing ones. For packed columns this means reducing the number of active sites while for capillaries it is reducing the inner diameter to a value that is closer to the theoretical optimum for mass transfer; the latter will unfortunately require further improvements in sample introduction and detection before it can be exploited completely.

Detectors

One of the nice features of SFC (depending on the mobile phase selected) is the potential ability to use on-line virtually any of the detectors employed in LC (before decompression) or GC (after decompression). Although the flame ionization (FID) and absorption (UV-vis) detectors, which provide little if any structural information, have seen the most widespread use until now, applications utilizing mass spectrometers (MS) or FT-IR spectrometers³⁰ continue to increase, along with those performed with element specific detectors. Detectors that have been employed to date (along with common abbreviations) are as follows: flame ionization (FID), absorption (UV-vis), Fourier-transform infrared spectrometer (FT-IR), mass spectrometer (MS), atomic emission (AE), inductively coupled plasma (ICP), nitrogen and phosphorous thermionic (NPD), chemiluminescence, flame photometric (FPD), electron capture (ECD), photoionization, light scattering, and nuclear magnetic resonance (NMR).

Restrictors

As noted earlier, this is both a unique and important component of

SFC. The restrictor serves to maintain the supercritical state of the fluid as well as to control the linear velocity of the mobile phase. It is across the restrictor that the supercritical fluid decompresses into a gaseous phase. In those instances where detection is performed after decompression (FID, MS, etc.), the restrictor design can be very important in terms of analyte precipitation within the restrictor or aggregation prior to detection. A variety of designs have been reported, including the linear, frit, integral, tapered, and the sheath flow restrictor.^{31,32} The linear restrictor is not recommended for general use except on the waste side of the sample splitter (if employed) or where detection is performed prior to decompression. Except for the sheath flow design, all restrictors are commercially available and are based on the principle of uncompensated flow constriction. At a given viscosity of the supercritical fluid [determined by its pressure (density) and temperature], the linear velocity or mass flow rate is determined by the amount of flow constriction provided by the restrictor, which is a function of its length and size of its orifice(s),³³ neither of which can be varied after the restrictor is made.

Although quite workable, the present commercially available restrictors do not permit as much control over linear velocity/mass flow rate as is desirable. This type of restriction necessitates a change of the restrictor whenever a different linear velocity is desired under the same condition or to maintain the same mass flow rate when conditions such as pressure or temperature are changed. In addition, column plugging and/or analyte precipitation/aggregation during decompression is sometimes a problem, although this can be alleviated to a degree by judicious regulation of the restrictor temperature.

SEPARATION THEORY AND OPERATION

Like all conventional chromatographic techniques (GC, LC, etc.), resolution in SFC is determined by the product of three terms—efficiency, selectivity, and retention—as shown in Equation 1:

$$R_s = \frac{\sqrt{N}}{4} \frac{\alpha-1}{\alpha} \frac{k'}{1+k'} \quad [1]$$

where N is the number of theoretical plates, α is the selectivity, and k' is the retention (capacity) factor. Each of the three terms in the fundamental resolution equation³⁴ can be optimized to improve the separation. Retention (k') and selectivity (α) should first be optimized via changes in the density or compo-

sition of the mobile phase, the temperature, and the gradients, if any, associated with these variables. Optimization of these two chromatographic terms is clearly the first step, since it will indicate if the current mobile phase/stationary phase combination is adequate for the separation being considered. The efficiency of a column, N , is determined by its length and the nature of the stationary phase, including column diameter or particle size. Given the square root dependency of resolution on N , large changes in parameters controlling N (e.g., column length or linear velocity) will result in only moderate changes in resolution, and thus should only be considered if changes in selectivity and retention do not suffice.

Whereas temperature and mobile phase composition are the primary variables used to modulate retention on a given column in GC and LC, respectively, in SFC it is feasible to employ several variables in combination to control retention--pressure (density), temperature, and composition—although each is often employed individually. From thermodynamic and other considerations, density has been shown to be a more fundamental variable than pressure; for those supercritical fluids for which density can be predicted from pressure via an accurate equation of state (e.g., Peng-Robinson), it is possible to attain a precise density simply by controlling the pressure and temperature. The variation of density with pressure at constant temperature is frequently nonlinear near the critical temperature of the fluid and gradually approaches linearity as the temperature is increased, as the curves in Figure 3 clearly illustrate for carbon dioxide.

Although the variation in retention is usually sufficient with changes in density, greater variation is usually possible via changes in the mobile phase composition. For the separation of homologous or oligomeric compounds with pure supercritical fluids, an asymptotic density gradient has been shown to be superior to a linear density or pressure gradient.³⁵ Even better results are possible if a temperature gradient is superimposed onto the density gradient. By including a temperature gradient, the reduction in column efficiency due to reduced mass transfer (reduced solute diffusion coefficients) that occurs with an increase in density is not as large.³⁶ The decreased efficiency that occurs with density programming in SFC is a minor disadvantage compared to temperature or mobile phase programming in GC and LC, respectively.

For nonhomologous or non-oligomeric compounds, optimization is much less predictable and is generally done on a trial and error basis. Results from a systematic, multi-parameter approach that we are developing in our laboratory will be summarized later.

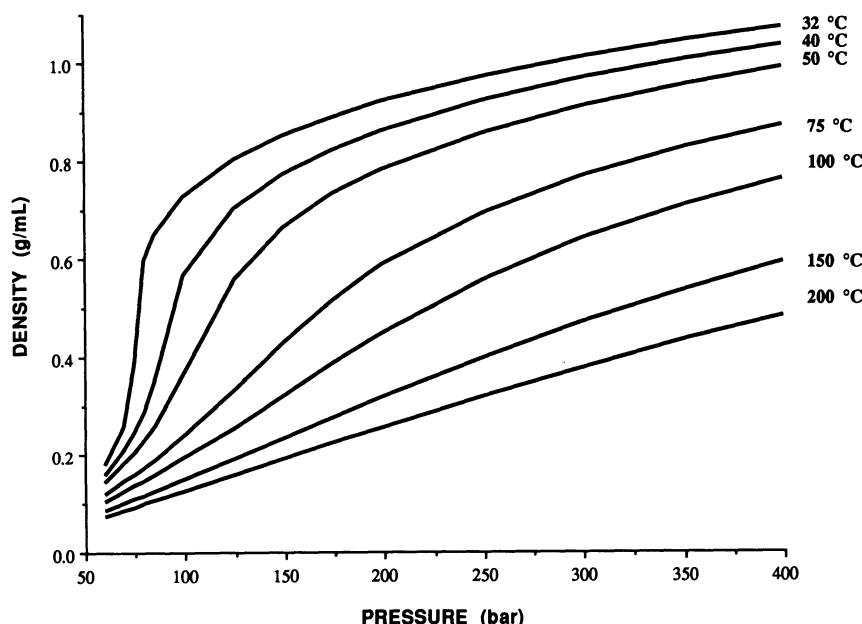


Fig. 3. Dependence of carbon dioxide density on pressure at several temperatures above the critical temperature (31.1 °C).

GENERAL APPLICATIONS

Historically, SFC has found its most widespread use in the chemical and petroleum industries for the separation and quantitation of a broad range of samples. Such samples typically have nonpolar to moderately polar constituents, with an upper molecular weight limit ranging from 2000 to 10,000 daltons. The classes of compounds successfully separated by SFC include high molecular weight petroleum hydrocarbons, surfactants, polystyrenes, polyethylenes, polymer additives, paints, siloxanes, adhesives, pesticides, waxes, fatty acids, alcohols, amines, amides, etc. They are frequently not amenable to GC or LC for reasons discussed earlier. SFC has also been useful for performing simulated distillations.³⁷

A representative SFC separation is shown in Figure 4. The sample was an isomeric mixture of C₁₀ aliphatic alcohols that had been ethoxylated to produce an oligomeric surfactant. The mobile phase was pure carbon dioxide and the stationary phase was a crosslinked polymethylbiphenyl siloxane (30%

biphenyl) capillary column. A density gradient was employed to achieve near baseline resolution of almost fifty oligomers; detection was by flame ionization. Details are given in the caption.

Although the above separation took over an hour because of the long capillary required for the necessary efficiency and peak capacity, it is feasible to perform simpler separations using packed columns or much shorter capillary columns. Shown in Figure 5 is a good example of the fast separations possible with the latter. Sample components are, in order of elution, hexane (solvent), acetophenone, propiophenone, bicyclohexyl, biphenyl, undecylbenzene, and benzophenone. The wide solvent peak shown here was greatly reduced in subsequent runs by replacing the original sample splitter with one that has a much lower internal volume; this illustrates the sensitivity of capillary SFC to extracolumn band-broadening.

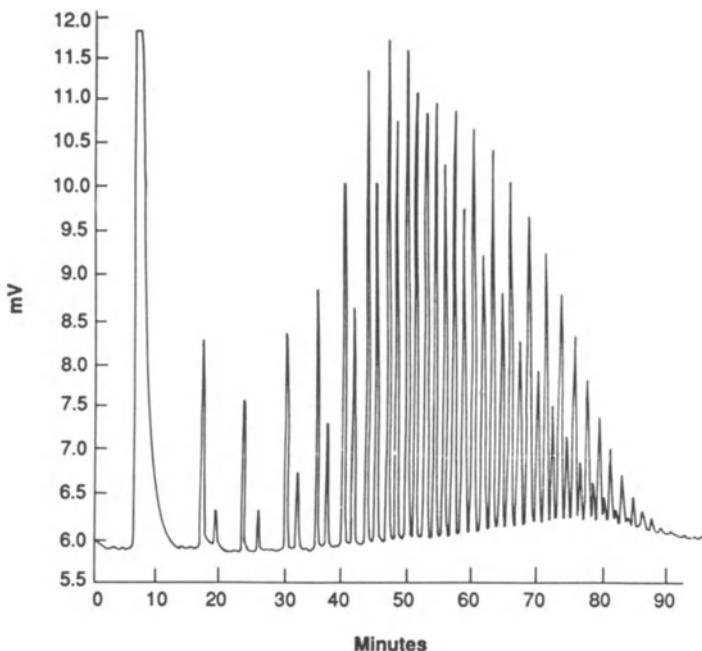


Fig. 4. SFC separation of ethoxylated alcohol (nonionic surfactant) using carbon dioxide and a 3 meter, 50 μm i.d. 30% biphenyl-polymethyl siloxane capillary column. Conditions: injection duration: 0.25 s; oven temperature: 80°C; density program: 0.1 g/mL for 15 min, then increased by 0.012 (g/mL)/min to 0.5 g/mL, then by 0.008 (g/mL)/min to 0.83 g/mL.

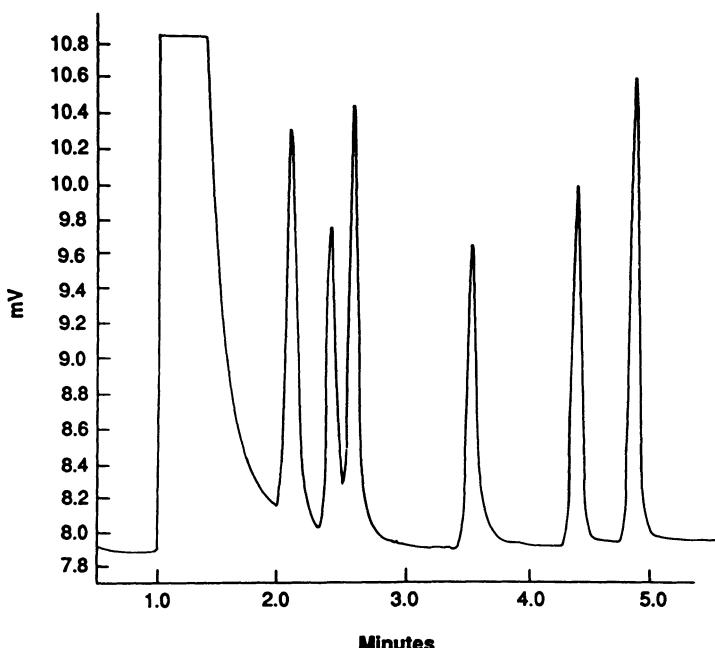


Fig. 5. Rapid capillary SFC separation of simple mixture using carbon dioxide and a 0.7 meter 30% biphenyl-polymethyl siloxane capillary column. Sample components: hexane (solvent), acetophenone, propiophenone, bicyclohexyl, biphenyl, undecylbenzene, and benzophenone. Conditions: injection duration: 0.25 s; linear velocity: 1.5 cm/s; oven temperature: 120°C; density program: 0.075 g/mL for 4 min, then increased by 0.0422 (g/mL)/min to a final density of 0.8 g/mL.

Similar separations are possible with packed column SFC. As described earlier, somewhat faster separations can typically be achieved, although the peak capacity is generally lower than the capillary separation shown in Figure 4. Hundreds of additional examples of both packed and capillary SFC applications may be found in the references cited earlier.^{2-8,10,11}

REVIEW OF NATURAL PRODUCTS APPLICATIONS

Very few reports of natural products applications appeared until the mid-to late-1980's, although in retrospect it seems clear from the successful

separations of industrial fatty acids, alcohols, etc. that SFC would be applicable to many classes of natural products. Indeed, the capability provided by SFC using a carbon dioxide mobile phase and an FID detector to separate and detect low levels of complex mixtures of nonchromophoric compounds at temperatures mild enough to avoid thermal decomposition or rearrangement has finally begun to become widely appreciated. Unless otherwise stated in the review of applications that follows, it is understood that the mobile phase was pure carbon dioxide.

Fatty Acids and Lipids

Several separations of fatty acids and lipids have been reported. Görner and Perrut³⁸ demonstrated the feasibility of separating unsaturated fatty acid methyl esters on a (polar) silica column. In contrast, Nomura et al.³⁹ successfully separated free fatty acids, their methyl esters, and other lipid materials on a nonpolar octadecyl-derivatized silica gel column (fewer active sites). Finally, Geiser et al.²⁰ compared the separation of lauric, myristic, palmitic, stearic, and arachidic acids on seven different stationary phases using dry or water-saturated carbon dioxide and found that the latter provided superior resolution and peak shape with all seven columns. According to the authors, the primary benefit of water appears to be the masking of undesirable retention sites (probably silanols) from the more polar compounds. Holzer and co-workers analyzed lipids obtained from a hydrothermal vent methanogen and associated vent sediment.⁴⁰ Neutral lipids of the thermophilic methanogen consisted of: (1) straight chain alkanes (*n*-C₂₂ to *n*-C₃₆), with *n*-C₂₄, *n*-C₂₈, *n*-C₃₂, and *n*-C₃₆ predominating; and (2) C₂₅, C₃₀, and C₃₅-isoprenoids and hydroisoprenoids, with the squalene (C₃₀) series being the most abundant (95.6%). Polar lipids of the thermophilic methanogen consisted of diphytanyl glycerol diether (61.6%), macrocyclic glycerol diether (15.3%), dibiphytanyl diglycerol tetraether (11.8%), and an unidentified component (11.4%).

Sugars / Carbohydrates

Following derivatization via trimethylsilation, Chester and Innis⁴¹ achieved excellent resolution of glucose oligomers from 2 to 18 units on a polymethylsiloxane (DB-1) capillary column with FID detection. Kuei and co-workers⁴² analyzed glucose polymers and three classes of glycosphingolipids after permethylating them, using a polymethylphenylsiloxane (5% phenyl) capillary column, a programmed density ramp, and a flame ionization detector.

Herbreteau et al. described an analysis of sugars performed on polar silica-based packed columns using a methanol-modified carbon dioxide mobile phase and a light-scattering detector.⁴³ The selectivity was considerably different from that found in HPLC. By using a methanol composition gradient, mono-, di-, and trisaccharides could be eluted in the same analysis without a substantial baseline shift.

Steroids

A number of different types of steroids have been examined with SFC. Raynor et al.⁴⁴ and Morgan et al.⁴⁵ described preliminary results of separation of ecdysteroids using carbon dioxide and a variety of polar and nonpolar silica-based packed columns. These groups later collaborated for the analysis of phytoecdysteroids from *Silene nutans* and *S. otites* by means of SFC and SFC/MS.⁴⁶ Shah and co-workers⁴⁷ reported the normal-phase HPLC and SFC separations of steroids with FT-IR spectrometric detection on a range of cyanopropyl columns. The order of elution of the steroids in both SFC and HPLC was related to the number of hydroxy groups present in the steroid molecule. David and Novotny used a phosphorus-selective detector following derivatization of the steroids with dimethylthiophosphinic chloride (using 4-dimethylaminopyridine as the catalyst) to analyze low levels of steroids in plasma and in urine (androsterone, estradiol, and estriol).⁴⁸

Alkaloids

Holzer and co-workers⁴⁹ exploited the greater efficiency of capillary SFC for the separation of pyrrolizidine alkaloids (PAs). Complete separation of the PAs of the retronecine and otonecine family was achieved with pressure-programming. The mild operating conditions prevented thermal decomposition of 8 PAs in a sample from *Senecio anonymus*. Balsevich and co-workers⁵⁰ used SFC/UV or SFC/MS (thermospray interface) for the separation in less than 8 minutes of 40-60 indole alkaloids from leaves of *Catharanthus roseus*; the high quality EI mass spectra obtained permitted the identification of several alkaloids.

Janicot and co-workers⁵¹ studied the optimization of the separation of six opium alkaloids (narcotine, papaverine, thebaine, ethylmorphine, codeine, and morphine) using silica and aminopropylsilica packed columns and mobile phases consisting of pure or modified carbon dioxide. The aminopropylsilica packed columns gave a faster separation, supposedly due to insufficient retention which could be increased somewhat by the addition of an aminated modifier to

the methanol-modified carbon dioxide. Longer analysis times (≈ 10 min.) and better resolution were obtained with the silica column and a complex mobile phase of carbon dioxide-methanol-methylamine-water.

Terpenes

Morin et al. reported a method for the separation and identification via on-line SFC/FT-IR of polycyclic aromatic hydrocarbons or sesquiterpene hydrocarbons.⁵² Later they separated and identified several sesquiterpene hydrocarbons obtained from copaiba balsam oil and ylang-ylang oil using silica as the stationary phase.⁵³ The low temperature employed (40°C) preserved structural information for these compounds, which included α -copaene, trans- α -bergamotene, β -caryophyllene, β -bisabolene, and humulene from copaiba balsam oil and β -caryophyllene, α -copaene, germacrene D and α -farnesene from ylang-ylang oil. The relatively high densities employed (0.6-0.9 g/mL) slightly obscured some of the anticipated spectral features. We have utilized SFC for the separation of a synthetic mixture of sesquiterpene lactones. Although this class of natural products is typically more polar than the sesquiterpene hydrocarbons, at least some of the lactones can be separated with pure carbon dioxide as a mobile phase.⁵⁴ For those compounds which show limited solubility in the pure carbon dioxide, a small amount of a polar modifier, such as formic acid, can be added to enhance their solubility in the mobile phase (vide infra).

Miscellaneous

Calvey and co-workers⁵⁵ described the separation of peracetylated aldononitrile derivatives and by-products of monosaccharides by SFC/FT-IR using a cyanopropyl column. Berry et al.⁵⁶ used silica and amino-bonded silica columns and carbon dioxide modified with methanol or methoxyethanol to effect separations of mixtures of xanthines, carbamates, sulfonamides, steroids, and ergot alkaloids in synthetic and natural samples, including an extract from *Claviceps purpurea*. Later and co-workers⁵⁷ demonstrated the feasibility of drug screening by SFC with a polymethylsiloxane capillary column and FID detection. Results for the analysis of steroids, antibiotics, and drugs of abuse such as cannabinoids in synthetic mixtures are given, as well as in human and equine urine. Roach et al.⁵⁸ reported an analysis of trichothecenes utilizing capillary SFC/negative ion chemical ionization (CI)-MS. A capillary SFC/MS interface incorporating a heated frit restrictor is described. The trichothecene mycotoxins, T-2 toxin, deoxynivalenol, and roridin A, were used to evaluate the

effect of restrictor temperature and carbon dioxide mobile phase on the negative ion CI spectra of these compounds under electron capture, proton abstraction, and chloride attachment conditions. When the restrictor temperature did not exceed the oven temperature by more than 100°C, sample transfer into the mass spectrometer was retarded, but neither temperature nor the carbon dioxide mobile phase significantly affected negative ion CI conditions. Yamauchi and Saito⁵⁹ described the fractionation of lemon-peel oil by semi-preparative SFC using a silica gel (10-20 µm) packed-column. Hydrocarbons (including terpenes), alcohols and aldehydes, esters, and other compounds could be separated by class.

RESEARCH PERFORMED IN OUR OWN LABORATORY

Systematic Optimization of Separations

The application of a systematic, multi-parameter approach for the optimization of SFC separations has yet to be reported. Until now, researchers have focused on only one or, at most, two experimental variables at a time, and have chiefly used trial-and-error as their optimization "strategy". Unfortunately, several variables in SFC, including temperature, the type of stationary phase, and the polarity, density (or pressure), and modifier content of the mobile phase, as well as gradients of temperature, density (pressure), and composition have been identified as significant. Moreover, it is clear from chemometric principles that any procedure which does not consider all the significant variables simultaneously will seldom, if ever, locate the true optimum conditions.

We have therefore begun to examine the optimization of SFC systematically using the sequential simplex optimization algorithm. The sequential simplex method is a multivariate optimization procedure that uses a geometrical figure called a simplex to move throughout the response surface in search of the optimum set of experimental conditions.⁶⁰ The simplex has been successfully used in various forms of chromatography, particularly HPLC⁶¹⁻⁶³ and GC.⁶⁴⁻⁶⁷ The simplex method is illustrated in Figure 6. The number of initial experiments conducted is one more than the number of parameters (temperature, gradient rate, etc.) to be simultaneously optimized. In Figure 6, where two parameters are optimized, the initial simplex is a triangle, with vertices labeled 1, 2 and 3. Once the initial simplex is established, the vertex with the lowest value is rejected (vertex 1), and a new point is found (vertex 4) by reflecting the simplex in the direction away from the rejected vertex. In this way the simplex proceeds toward the optimum set of conditions (vertex 8).

Figure 6 also illustrates the failure of the conventional univariate approach in finding the optimum. With this approach, all variables but one are held constant while the remaining variable is changed until an optimum response is found. The process is repeated until all variables have been "optimized." In Figure 6, the temperature is held constant at some arbitrary value while the density is varied (points A thru F). When the optimum density at that arbitrary temperature is located (point E), the temperature is then varied while holding the density constant at its "optimum" value (points G thru I) until an optimum temperature is found (point H). Point H corresponds to the best response found by the univariate approach, but it is obviously not the true optimum (vertex 8).

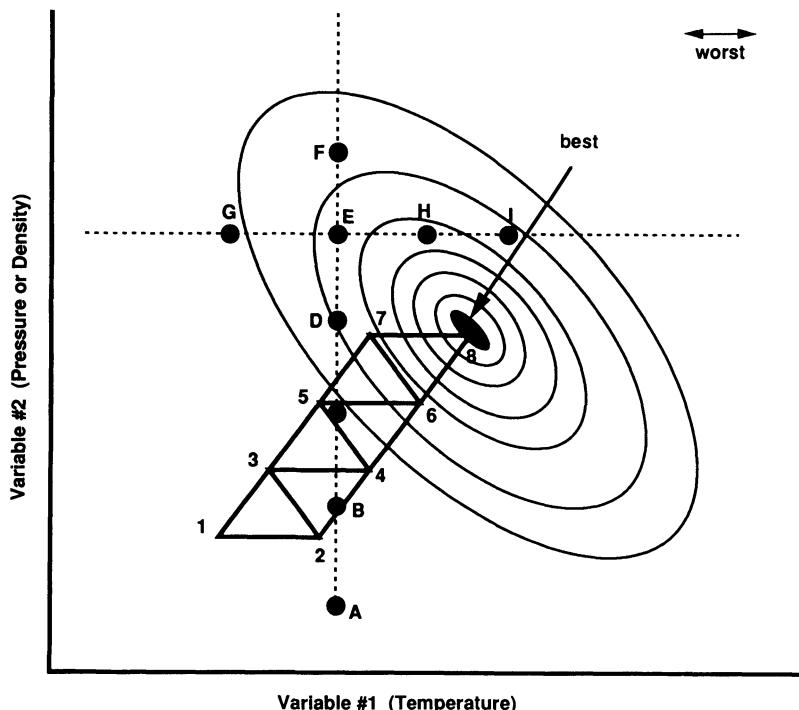


Fig. 6. Comparison of simplex and univariate optimization of a simple response surface with two interdependent parameters (density and temperature). Whereas the simplex finds the true optimum (vertex 8) after only 8 experiments, the conventional univariate procedure never locates the true optimum and instead incorrectly identifies point H as the optimum after 9 experiments.

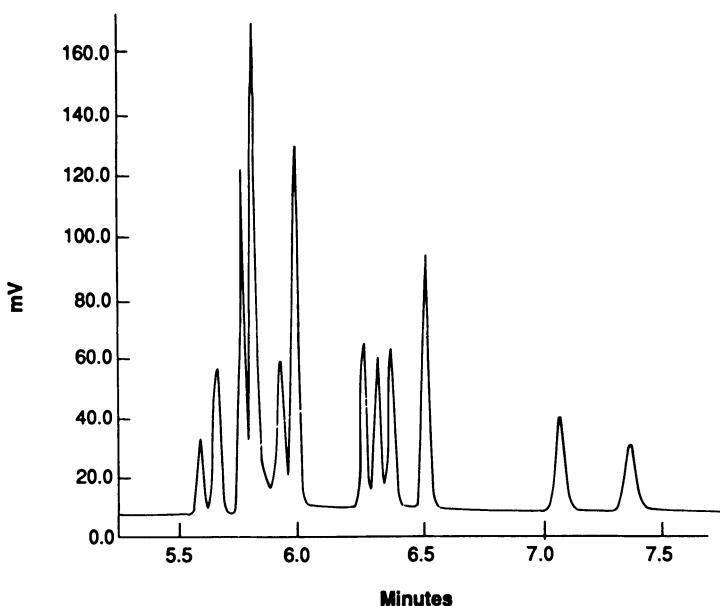


Fig. 7. Four parameter simplex optimization of the separation of a 12-component mixture. Variables: density, density gradient, temperature, and temperature gradient. Conditions: oven temperature: 90°C, increased by 21°C/min during the run; density: 0.23 g/mL, increased by 0.16 (g/mL)/min. Sample components: naphthalene, n-octadecane ($n\text{-C}_{18}\text{H}_{38}$), quinoline, isoquinoline, n-eicosane ($n\text{-C}_{20}\text{H}_{42}$), undecylbenzene, benzophenone, 2'-aceto-naphthone, diphenylamine, o-diethylphthalate, N-phenyl-1-naphthylamine, phenanthrene quinone.

While the univariate approach could be repeated at other initial temperatures in the hope of finding the true optimum, the probability of success is low. Moreover, it took more experiments to find a false optimum with the univariate procedure than to find the true optimum in the first attempt with the simplex approach (9 vs 8). Further details on the simplex algorithm, as well as other chemometric techniques of optimization, are available elsewhere.^{60,68-72}

To our knowledge, we are the only research group to exploit systematic procedures such as the simplex in SFC. Our initial efforts at adapting the simplex method for SFC optimization have been successful,⁵⁴ and we are well on our way to completing a follow-up study. We plan to continue to investigate the simplex and other systematic, multi-parameter algorithms (e.g., optiplex,

simulated annealing, window diagrams) that have been successfully applied to other chromatographic techniques.

The systematic, multi-parameter optimization strategies described above are in principle applicable to any type of sample. At present we are using our strategies to optimize the SFC separation of various classes of natural products and other compounds for which gas and liquid chromatographic methods have proven to be inadequate. These compounds include phenolic soil phytotoxins, leaf alkaloids, and sesquiterpene lactones. We have already reported the optimization of the separation of a synthetic mixture of three sesquiterpene lactones, glaucolide A, burrodin, and psilostachyin A.⁵⁴

More recently, we have been testing a four-parameter simplex approach (density, density gradient, temperature, temperature gradient) on samples that contain both volatile and nonvolatile analytes and therefore give complex changes in elution order as experimental conditions are changed. The success of the simplex approach in optimizing the separation of such samples is illustrated in Figure 7 for a 12-component sample containing alkanes, amines, polyaromatic hydrocarbons, ketones, esters, etc. Most notably, the optimization process provided an increase in the number of peaks resolved from 10 to 12. To obtain baseline resolution for all components, either a longer column or a slower flow rate can be employed as described previously.⁵⁴

Development of New Supercritical Fluid Mobile Phases to Facilitate the Solubilization of Polar Molecules

One of the principal limitations of SFC is the inability of the supercritical fluid to solubilize many classes of polar compounds, such as organic acids and carbohydrates. Carbon dioxide has been the dominant mobile phase of choice for SFC, mostly because of its relatively mild critical parameters, low cost, chemical inertness, and detector compatibility⁷³. However, this fluid has a limited usefulness as a mobile phase because of its nonpolarity⁷⁴. The elution of polar compounds is frequently very difficult and their peak shapes are often poor. The selectivity obtained in conjunction with nonpolar stationary phases is also usually poor, since both mobile and stationary phase are of similar polarity. This is especially unfortunate since nonpolar stationary phases are more stable, more resistant to oxidation, generally more efficient, and have higher operating temperature limits than polar phases.

As part of an effort to utilize the unique capabilities of supercritical fluids with a much wider range of compounds, including biological ones, many alternative pure and modified supercritical fluids have been investigated.

However, to date none have been found that provide improved solubility for polar and ionic compounds while simultaneously satisfying the other criteria (FID compatibility, etc.) noted above that are responsible for the popularity of pure carbon dioxide.

We are presently investigating the modification of carbon dioxide with small amounts of highly oxidized, polar compounds that are invisible to the flame ionization detector, in contrast to traditional organic modifiers discussed earlier such as methanol, isopropanol, 1,4-dioxane, etc. Such "invisible" compounds include formic acid and formamide, and at present we have chosen to focus on the former, using a concentration of about 0.3 % (w/w) in CO₂. Our results to date are very encouraging. The addition of formic acid to carbon dioxide drastically reduced the retention and significantly altered the selectivity for several polar compounds without disturbing the separation of nonpolar analytes (e.g., n-alkanes) to any significant degree. Thermodynamic studies revealed greater solute-fluid interactions for the polar analytes as well as a more ordered solvation environment, presumably as a result of clustering of formic acid around the polar solutes. The larger heats of solvation in the formic acid modified mobile phase and the large reduction in the capacity factor indicates an increased solubility of the polar compounds. Table 4 shows the reduced retention of polar compounds when a small amount of formic acid is added to pure carbon dioxide. Just 0.3 % (w/w) of formic acid in CO₂ results in an average decrease in retention of about 50 %. There are many potential applications of SFC with formic acid modified-CO₂, including the sesquiterpene lactones and other polar natural products, sugars and other carbohydrates, as well as many other classes of polar compounds.

SUPERCritical FLUID EXTRACTIONS

Although an in-depth discussion is beyond the scope of this work, we would be remiss if we did not at least mention extractions, a very important application of supercritical fluids. Generally speaking, extractions and other sample preparation techniques lag far behind most other analytical procedures. Sample preparation still requires hours in most cases, and is usually the limiting step in an analysis. This is particularly true in the analysis and characterization of natural products. Soxhlet devices have improved extractions over batch techniques, but such devices are still very time-consuming (several hours to over a day) and can only utilize pure solvents with relatively low boiling points. Moreover, the relatively high temperature required for the Soxhlet or similar

Table 4. Comparison of the capacity factors obtained with mobile phases of pure carbon dioxide and formic-acid modified carbon dioxide^a at a temperature of 100°C

solute ^b	density g/mL	k' pure	k' modified	% change
VA	0.1	0.501	0.288	-42
n-C ₁₆ H ₃₄	0.2	0.515	0.505	-1.9
n-C ₁₈ H ₃₈	0.2	0.889	0.928	+4.2
AcNap	0.2	1.562	1.016	-35
mBrBzA	0.4	0.401	0.185	-54
pNPAA	0.4	0.804	0.384	-52
oBBzA	0.4	1.622	0.668	-59
mBrBzA	0.5	0.185	0.080	-57
pNPAA	0.5	0.352	0.141	-60
oBBzA	0.5	0.638	0.224	-65

^a 0.3% (w/w) formic acid in carbon dioxide.

^b VA = valeric acid; AcNap = acetonaphthone;

mBrBzA = m-bromobenzoic acid;

pNPAA = p-nitrophenylacetic acid;

oBBzA = o-benzoylbenzoic acid.

extraction is incompatible with many natural products that are thermally unstable. Some extractions could be made more selective by using mixed solvents systems such as ethanol-water/hexane, but the mixed systems nearly always require crude batch techniques. In addition to the initial extraction, subsequent purification steps must be performed before any analysis can be made. These procedures can be quite involved, depending of the compound(s) of interest. Each extraction step within the purification procedure must be performed exhaustively to obtain high yields since single-step recoveries are not always very high.

With the advent of supercritical fluid extraction (SFE), sample preparation can be reduced to several minutes and higher recovery percentages can be obtained. This technique has been repeatedly shown to be vastly superior to conventional extraction methods, yet the advantages of SFE remain largely unappreciated in many areas of science which would clearly benefit from this technique. Moreover, SFE is easily coupled to GC and SFC, permitting extraction and analysis to be performed in the same step. Following a brief description of the apparatus typically used for on-line SFE/GC or SFE/SFC, a review of natural products applications is provided.

Apparatus

A schematic diagram of the instrumentation typically employed in SFE/SFC is shown in Figure 8. With the pump valve in position 1, the supercritical fluid passes through the extraction cell at a predetermined temperature and pressure (density). The extraction can be performed in a static mode or a dynamic mode. In the static extraction, the material is extracted for a certain length of time before it is allowed to pass on to the focusing trap. When a dynamic extraction is performed (Fig. 8), the supercritical fluid is continuously passed over the sample matrix in the cell carrying away with it any extractable material. When the supercritical fluid and analyte reaches the trap, the fluid is vented (losing its solvating power at the lower temperature and pressure) and the extracted material precipitates inside the glass-lined cryofocusing trap. After a certain length of time, the pump valve is switched to position 2, and the trap is also heated (by removing cooling carbon dioxide). This allows the extracted material to be moved onto the analytical column for separation and analysis to be completed.

In Figure 8, the 8-port valve is shown in the vent mode. This mode is commonly employed to remove any interfering components from the sample. For example, if the material is known to be soluble at a given density, some interfering material in the matrix could be extracted at conditions just under that density, and this portion could be vented to waste (through ports 1 and 2 in Fig. 8). After this interfering material is removed, the valve can be switched to extract position (through ports 2 and 3) and the density raised to just above the required density to extract the material of interest. In this fashion, we can perform a partially selective extraction of the sample; this is yet another important advantage of SFE over other extraction techniques.

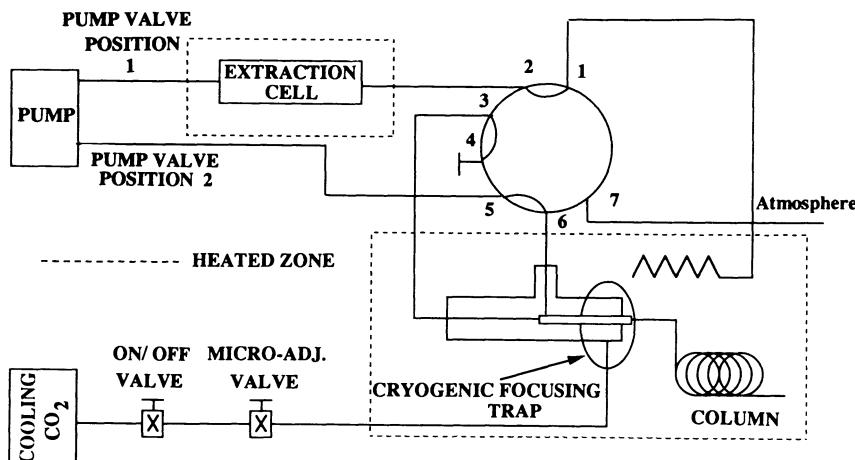


Fig. 8. Schematic diagram of an on-line supercritical fluid extraction (SFE) apparatus. See text for discussion.

Natural Product Applications

Except as noted, pure supercritical carbon dioxide was used as the extraction solvent in the brief review of applications that follows. Also, the majority of SFE natural product applications have been performed off-line rather than on-line, despite the numerous advantages of the latter.

Imanishi et al. reported the supercritical fluid extraction of fish oil;⁷⁴ vapor-liquid equilibrium data for a carbon dioxide/fish oil system were measured at 313-353 K and 20-35 MPa. The solubility of fish oil increased as pressure increased and temperature decreased (i.e., as density increased). On the other hand, the selectivity in supercritical carbon dioxide of lipids and triglycerides which come from fish oil increases as pressure decreases and temperature increases. The fractional separation of triglycerides is possible with supercritical carbon dioxide.

Choi and co-workers⁷⁵ employed SFE for rapid and efficient (good recoveries) sample preparation in the TLC characterization of lipids from algae *Scenedesmus obliquus*. Neutral lipids consisted primarily of diglycerides, triglycerides, hydrocarbons, free sterols, and sterol esters; glycolipids were mostly monogalactosyl diglyceride, digalactosyl diglyceride, esterified sterol glycoside, and sterol glycoside; and the main constituents of phospholipids were

phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine. The predominant fatty acids were found to be 16:0, 16:1, 16:2, 16:3, 16:4, 18:1, 18:2 and 18:3 (α).

Ndiomu and Simpson⁷⁶ presented some examples of SFE for the extraction of caffeine and quinine from various plant materials and of morphine from serum. Results are compared with those obtained by liquid extraction with methanol and THF, Soxhlet extractions, and solid-phase extractions. Sugiyama and Saito⁷⁷ employed SFE off-line for the GC-MS analysis of lemon peel oil; the results compared favorably with those samples that were cold-pressed.

McDonald et al.⁷⁸ used supercritical methanol or acetone (360°C, 28 MPa) to extract chemicals from forest products such as southern pine and Douglas-fir bark. Schaeffer et al.⁷⁹ employed supercritical carbon dioxide-ethanol mixtures to extract and separate monocrotaline from the seeds of *Crotalaria spectabilis* using a cation-exchange resin. Miyachi and co-workers⁸⁰ used methanol, ethanol, or water modified supercritical carbon dioxide to extract coumarins, lignans, and prenylflavonoids from plants including *Fraxinus japonica*, *F. mandshurica* var. *japonica*, *Forsythia suspensa*, and *F. viridisima*.

A pilot-scale SFE system was described by Temelli et al.⁸¹ to evaluate the extraction and separation of aroma and flavor compounds from citrus essential oils. Conditions were optimized to facilitate extraction of the terpenes, leaving behind the oxygenated (flavor) compounds.

Finally, the pioneering research of Hawthorne and co-workers demonstrated that SFE can be coupled on-line with capillary GC and GC/MS to achieve rapid extraction and analysis (< 1 hour) of a variety of samples, including flavor components from food products⁸² and flavors and fragrances from spices, chewing gum, orange peel, spruce needles, and cedar wood.⁸³

CONCLUSION

Supercritical fluid chromatography (SFC) is an analytical method of separation and quantitation that, although introduced in 1962, has only recently come of age due to a variety of technological improvements. The ability to separate thermally labile or nonvolatile, high molecular weight compounds under mild conditions (near ambient temperatures) along with the capability to detect these compounds at low levels even if they have no chromophores makes SFC an important chromatographic complement to gas and high performance liquid chromatography. The potential of SFC for the analysis of natural products is very high, as evidenced by the survey of successful applications in this report.

In particular, the successful on-line coupling of SFC with mass spectroscopy and/or Fourier transform infrared spectroscopy (FT-IR) has important implications to natural products characterization and analysis; indeed, we expect that many modes of SFC will eventually become everyday tools for the phytochemist.

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Chapter Five

IMMOBILIZED ARTIFICIAL MEMBRANE CHROMATOGRAPHY

Initial Studies Using Monomyristoylphosphatidylcholine as a Detergent for Solubilizing and Purifying Membrane Proteins

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INTRODUCTION

Immobilized artificial membranes (IAM) are solid-phase-membrane-mimetics.¹ Synthesis of IAM particles entails bonding cell membrane lipid

molecules to solid surfaces at high molecular surface densities.¹⁻⁴ Thus IAM surfaces are intended to mimic the lipid environment of cell membranes and consequently the initial applications of IAM particles relate to endogenous solute—membrane interactions. Non-chromatographic applications of IAM include reconstitution of phospholipase D (unpublished observation), and the correlation of drug-binding to IAM with drug-transport through human skin.⁵ Chromatographic applications include the purification of cytochrome P-450 (submitted for publication and also refs.^{6,7}), and the purification of other membrane proteins.⁸ Although both non-chromatographic and chromatographic applications are evolving, the most useful application (s) of IAM will be for the purification of membrane proteins.

Two IAM bonded phases containing only immobilized phosphatidylcholine were used for this work. One bonded phase, denoted as IAM.PC, contains a near-monolayer of phosphatidylcholine (PC) covalently linked to silica propylamine.¹ After PC bonding, residual unreacted propylamines create a chemically basic sub-surface approximately 15 Å below the IAM.PC surface. These unreacted propylamines significantly increase, sometimes by a factor of ten, the retention time of acidic compounds (C. Pidgeon, unpublished observation). For this reason, the unreacted sub-surface propylamines were endcapped with methylglycolate via amide bond formation at Regis Chemical Company (Morton Grove, IL). This second IAM.PC bonded phase is denoted IAM.PC.MG, whereby MG merely indicates endcapping with methylglycolate; this sub-surface is neutral. The chromatographic results obtained to date indicate that IAM.PC, or other IAM chromatographic surfaces, have the potential of becoming one of the critical chromatographic steps in the purification of any membrane protein. Although it is unlikely that only one chromatographic step will be sufficient for the purification of a given membrane protein, we believe IAM surfaces will provide significant chromatographic advantages over conventional methods, particularly as the methodology matures. Currently only IAM.PC and IAM.PC.MG are commercially available and this limited number of IAM bonded phases is significantly inhibiting the development of technology because selective binding of membrane proteins to IAM surfaces will depend on the immobilized lipid molecule; consequently our lab is synthesizing several other IAM bonded phases. Preliminary results using IAM bonded phases containing carboxyl groups at the immobilized membrane interface, i.e., IAM.FA and IAM.PC.acidic, are also presented. These bonded phases were synthesized in our laboratory.^{1,2}

The specific objective of this report is to demonstrate that non-chromatographic batch-binding studies of membrane proteins to IAM.PC

particles are useful in identifying irreversible binding of membrane proteins to the IAM.PC surface prior to injecting the proteins onto IAM high performance liquid chromatography (HPLC) columns. Batch-binding studies can identify if the membrane protein under study has no-affinity, weak-affinity, or high-affinity (i.e., irreversible binding) to the confluent layer of immobilized lecithins that form the IAM.PC surface. Thus batch-binding studies provide guidelines for using IAM columns with regard to the solubilized membrane sample. Membrane preparations containing proteins that irreversibly bind to IAM particles should not be injected onto IAM columns.

CHEMICALS, SOLVENTS, SOLUTIONS, IAM COLUMNS

All chemicals and solvents were analytical grade or better and purchased from standard suppliers. Chromatography packing material, for batch-binding studies of membrane proteins and IAM.PC particles, were synthesized in our laboratory with established procedures.^{1,2} However, for these experiments, the IAM.PC particles were 5 μ in size containing 300 Å pores, and obtained from the same synthetic batch. Based on FTIR analysis of the particles, the lecithin loading gave the expected approximate-monolayer surface coverage.² Three types of IAM surfaces containing different immobilized lipids on each surface were also used for this study; IAM.PC, IAM.FA, and IAM.PC.acidic. IAM.FA and IAM.PC.acidic were synthesized in our laboratory using established procedures,^{1,2} and contain carboxyl groups at the immobilized membrane interface. Each of these IAM surfaces were evaluated in both batch-binding studies and HPLC studies. However, only the results from IAM.PC are shown because the work on the other surfaces is in progress and will be reported in subsequent publications. Commercially available IAM.PC and IAM.PC.MG chromatography columns (Regis Chemical Company) were used for this work and had the following specifications: 12 μ , 300 Å, and 4.6 mm x 15 cm. A Rainin Dual pump HPLC system interfaced with a Macintosh computer for gradient control and data processing was used.^{1,3} Mobile phase buffer conditions are given in the appropriate figure legend. Gel electrophoresis under denaturing conditions using 10 to 12 % acrylamide gels was performed according to the procedure of Laemmle.^{9,10} All detergents were used as received except monomyristoylphosphatidylcholine (lysoMPC). LysoMPC was purchased from Avanti Polar Lipids (Birmingham, AL). Sodium dodecylsulfate (SDS) was purchased from BIORAD and Triton X 100 from Aldrich Chemical company. Lubrol and 3-[*(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate*

(CHAPS) were purchased from Sigma Chemical Company. The BCA protein assay kit (Pierce Chemical Co.) was used in both the 'standard' and 'enhanced' protocols. The effect of lysoMPC and Tris (2-amino-2-hydroxymethyl,1,3-propamediol) on both of these protocols was carefully evaluated and is described below.

DETERGENTS FOR IAM CHROMATOGRAPHY

The binding of both soluble proteins and membrane proteins to IAM.PC and IAM.PC.MG is sufficiently strong that organic solvents or detergents are needed for protein elution. We use detergents instead of organic solvents to purify membrane proteins because of the tendency of organic solvents to denature proteins. Currently no guidelines for choosing a detergent for IAM chromatography exist. We are attempting to establish detergent guidelines by identifying non-denaturing detergent conditions that elute membrane proteins from IAM columns.

It is well known that single chain PC analogs, denoted as lysolecithins, disrupt cell membranes. Lysolecithins are micelle forming lipids that destroy endogenous cell membranes via detergent solubilization. We exploited this membrane solubilization effect of lysolecithins and evaluated the ability of lysoMPC to (i) solubilize membrane proteins, and (ii) to elute membrane proteins from IAM.PC. LysoMPC is a single chain analog of the double chain PC analog bonded to silica. Thus lysoMPC is a *detergent* with a similar alkyl chain length and an identical polar headgroup as the lecithin ligand immobilized on IAM.PC bonded phases. Consequently, lysoMPC can function as an 'affinity-detergent' to displace membrane proteins adsorbed to the immobilized phospholipid monolayer comprising IAM.PC. We define an affinity-detergent as a mobile phase lysolecithin that competes with immobilized-lecithin for the binding of membrane proteins.

PURIFICATION, SYNTHESIS, AND PHYSICO-CHEMICAL PROPERTIES OF lysoMPC

Purification of lysoMPC

The HPLC purification of lysoMPC was as follows. Three grams of lysoMPC, dissolved in 23 ml chloroform/methanol (90:10) was injected onto a

200 Å, 20 µ, silica semi-prep-column, 15 mm x 21.1 cm in size (Regis Chemical Company) that had been pre-equilibrated with chloroform. Sample injection was through the HPLC priming valve in front of the pump using a 50 ml glass syringe. The silica column was washed with 300 ml of chloroform, 200 ml of chloroform/methanol (9:1), and 200 ml of chloroform/methanol (4:1). After application of this wash-gradient, the mobile phase was changed to 200 ml of chloroform/methanol (7:3), and fraction collection was initiated (8 ml/fraction). The final mobile phase solvent system was 700 ml of chloroform/methanol (3:2). Phosphate positive fractions were pooled to obtain 'pure-lysoMPC'. Phosphate positive fractions were identified using silica gel TLC plates developed in chloroform/methanol/water (65/25/4) and sprayed with Phospray (Supelco Inc., Bellefonte, PA).

Synthesis of lysoMPC

Synthetic lysoMPC was purchased from Avanti Polar lipids. LysoMPC was synthesized by diacylating glycerophosphocholine (GPC) with myristoyl-anhydride, followed by phospholipase A2 cleavage of the fatty acid in the glycerol sn-2 position. To reduce the cost of lysoMPC, GPC was diacylated with myristic acid that was 98 % pure. However, the contaminates of myristic acid are merely saturated fatty acids ± 2 methylene groups in length. Although these impurities would be unacceptable for biophysical studies of lipids, for detergent solubilization the impurities are insignificant. This synthetic route reduced the cost by 50% and currently lysoMPC can be purchased for \$45/g which does not prohibit using lysoMPC as a detergent to both solubilize and elute membrane proteins during IAM chromatography. The critical micelle concentration (CMC) of lysoMPC is 100 times less than the CMC of CHAPS (which costs ~\$12/g). Thus the cost of using lysoMPC is about four times greater than the cost of using CHAPS unless detergent concentrations near the CMC are used whereby lysoMPC becomes the less expensive detergent.

Physico-chemical and Chromatographic Properties of lysoMPC

Lysophospholipids cooperatively bind to several membrane proteins¹¹⁻¹³ and discrete interaction sites exist in proteins for anionic and neutral phospholipid detergents (e.g., myelin basic protein¹⁴). The CMC of lysolecithins (and also other lysolipids) can be determined by both detergent dialysis^{11,15,16} and ³¹P NMR,¹⁶; however, the sensitivity of ³¹P NMR

permits using this technique for determining CMC values only above ~100 μM . The CMC of lysoMPC is approximately 55-70 μM (~0.0026 to 0.0032 % W/V) or approximately 3 mg/100 ml.¹⁶ The CMC of lysoMPC is 100 times less than the CMC of CHAPS (CMC ~ 6.5 mM). The CMC of zwitterionic phospholipids, including lysoMPC, varies little with ionic strength ($I \leq 0.5$) and lysolipids have well defined CMC aggregation numbers. The average lysoMPC micelle size contains approximately 103 ± 3 molecules and a molecular weight ~45,000 daltons.¹⁷ The molecular weight or size of lysoMPC micelles containing solubilized protein depends on the lipid-micelle-protein interactions. For example, myelin basic protein binds two phospholipid micelles per polypeptide.¹¹

Several advantages exist for developing lysolipids as detergents for solubilizing membrane proteins and using lysolipids to elute membrane proteins from IAM bonded phases. The advantages are: (i) lysolipids are non-absorbing, chemically defined, and easily synthesized; (ii) synthetic routes are available to make lysolipids cost-effective-detergents; (iii) lysolipids have well defined CMC values; (iv) the interactions of lysolipids with membrane proteins are biologically relevant, and reports characterizing protein-lipid interactions are abundant (e.g., 11-14); (v) lysolecithins do not interfere with protein determination by standard methods;¹⁷ and most importantly, (vi) lysolipids may function as affinity-detergents for IAM chromatography. In addition, lysolipids can be removed from the protein sample by dialysis.

Chromatography of IAM phases should always characterize the detergent elution profile obtained without injecting protein. An example of a typical blank-run elution profile using a lysoMPC gradient is shown in Figure 1a. The weak absorbing peaks in this chromatogram (peaks A, B, C, D, E) suggested impurities in lysoMPC. Consequently, lysoMPC was purified by semi-preparative scale HPLC and the same blank-run repeated, as shown in Figure 1b. Purification removed peaks A, B, and C, which we attribute to detergent impurities. We also note that the large asymmetric peak in Figure 1b (eluting after 150-160 minutes) and the small double peak (eluting between 60-75 minutes) routinely occur with lysoMPC gradients. These variable, low absorbing non-protein-peaks routinely occur as the column is equilibrated to either higher or lower detergent concentrations and also as the column ages from multiple sample injections. The shape and intensity of these peaks vary. Although these peaks are potentially misleading, the peak intensities are low and they occur in a defined region of the chromatogram. However, based on the data in Figure 1, when small protein amounts are loaded on IAM columns, we suspect that low absorbing peaks elute as the detergent gradient changes. The

protein-composition of these peaks must be verified by BCA protein assay and gel electrophoresis. The main conclusion from data presented in Figure 1 is that regardless of purity, lysoMPC gradients cause non-protein peaks in the HPLC chromatogram. Under isocratic conditions, these peaks never occur if the column has reached equilibrium.

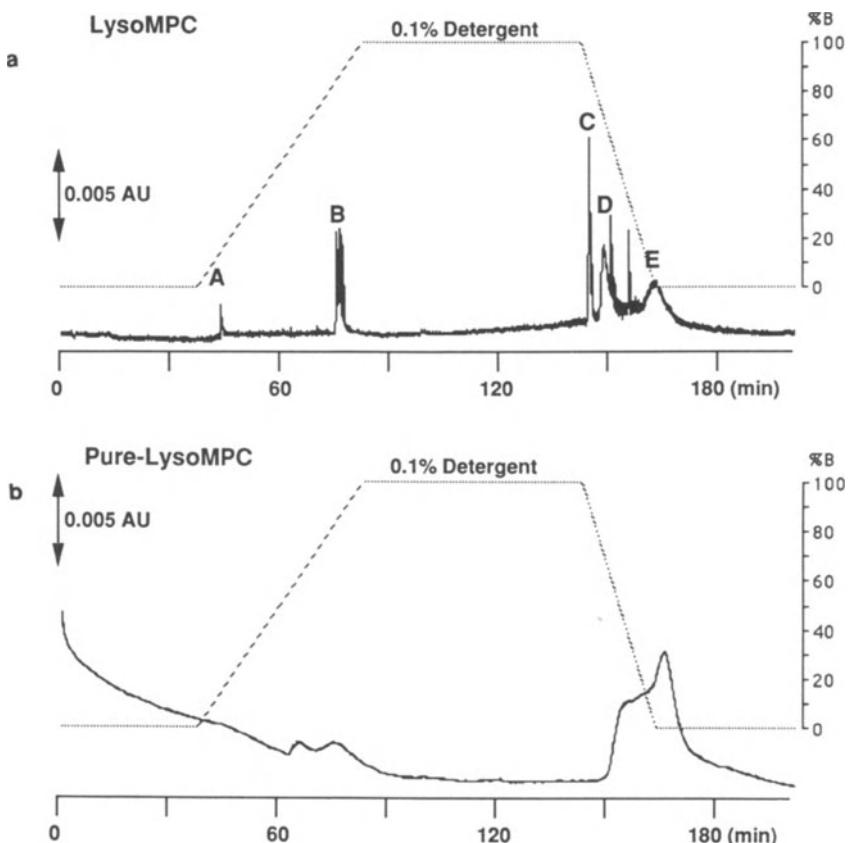


Figure 1. HPLC chromatograms demonstrating impurities in lysoMPC. (a) lysoMPC was utilized as received from the supplier. (b) lysoMPC purified by HPLC was utilized. Both chromatograms were obtained on the same new IAM.PC column, 4.6 mm X 15 cm, detection at 280 nm, flow 0.75 ml/min. Buffer A contained 0.003% lysoMPC, 62.5 mM Tris, pH 7.026 with 1 M HCl. Buffer B was identical to buffer A except that lysoMPC was 0.1%.(w/v).

During method development we routinely inject small amounts (< 50 µg) of protein on IAM columns and proteins eluting from the IAM column are usually diluted. Consequently, we frequently need to quantitate 2-3 µg of protein in 100 µl of HPLC eluent. Although many buffers and detergents are reported not to interfere with the BCA assay, we have found significant interference from these components when low amounts of protein are being quantitated.

The 'standard' BCA protocol calls for incubating samples with reagents for 30 minutes at 37° and this protocol quantitates 1 to 50 µg-protein. The 'enhanced' BCA protocol calls for incubating samples for 30 minutes at 60° and this protocol quantitates 1 to 25 µg-protein. LysoMPC does not significantly affect protein determination by the 'standard'-BCA assay (Figure 2) or the 'enhanced'-BCA assay (Figure 3) but HPLC purified lysoMPC elicits ~50 % less background absorbance in these assays (inserts in Figs. 2 and 3). Both purified and non-purified lysoMPC background absorbance values in the BCA assay are equivalent to less than 1-2 µg/ml of bovine serum albumin.

Although lysoMPC does not interfere with the 'enhanced' BCA method, Tris buffer significantly affects this protocol, but the background absorbance is saturable at approximately 50 mM Tris (Fig. 4). This variable background absorbance is troublesome because the sample volume chosen for BCA assay is not constant, e.g., 100 µl. The sample volume depends on the amount of protein in the HPLC eluent and varies from 10 to 200 µl. Since both BCA assay protocols dilute all samples to a final volume of 200 µl, varying the HPLC eluent-sample-volume leaves different amounts of Tris in the sample during protein determination. Thus the Tris concentration and detergent concentration vary during sample handling. To avoid the background absorbance of Tris we routinely add enough Tris to a final concentration of ~50 mM in both the blank and sample during the enhanced BCA protocol. This eliminates the changing background caused by different Tris concentrations. Unfortunately, the effect of lysoMPC is not saturable (Figs. 2 and 3) and consequently we can not add a fixed amount of lysoMPC to BCA samples to eliminate background absorbance due to this detergent. Nevertheless, we routinely quantitate 2 µg protein in 200 µl HPLC eluents using the enhanced BCA protocol by adding Tris and using the appropriate detergent background.

LysoMPC occasionally generates an insoluble precipitate in the 'enhanced' BCA assay protocol. This precipitate forms slowly and if protein absorbance values are not measured within 30-45 minutes of sample preparation, then very high background absorbances cause the BCA assay to be highly inaccurate. However, BCA sample-protein-solutions containing the precipitate can be diluted with 1 volume of chloroform and the aqueous phase can be used

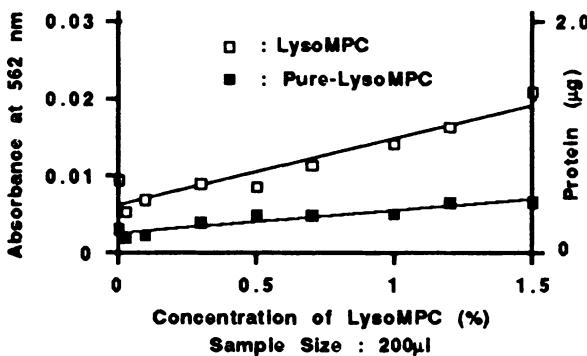
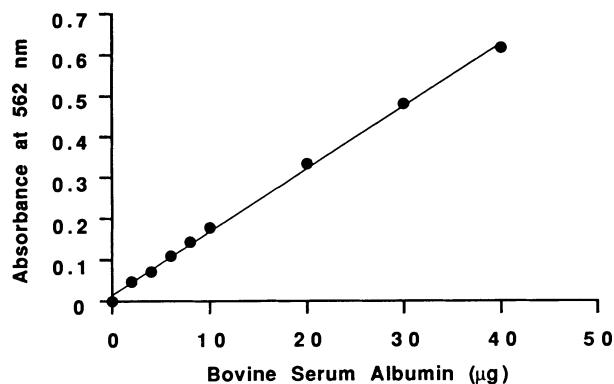


Figure 2. (top graph) Standard curve for bovine serum albumin in the 'standard' BCA protein assay; (bottom graph) LysomPC effect on standard BCA protein assay. Absorbance values from 'standard' BCA assay in the presence of either lysomPC or HPLC-pure lysomPC. Purifying lysomPC decreased the background absorbance ~50%. All data were obtained on 100 μl sample volumes containing 62.5 mM Tris pH 7, diluted to 200 μl with water before BCA assay.

directly for quantitating protein content. Treating the sample-protein-solution with chloroform does not change the slope of the standard curve using bovine serum albumin or membrane proteins obtained from red cell ghosts (not shown). The insoluble precipitate is most likely hydrolyzed fatty acid from lysomPC caused by heating (60° C, 0.5 hr) the alkaline (pH 11) reagent-solution required in the enhanced-BCA assay.

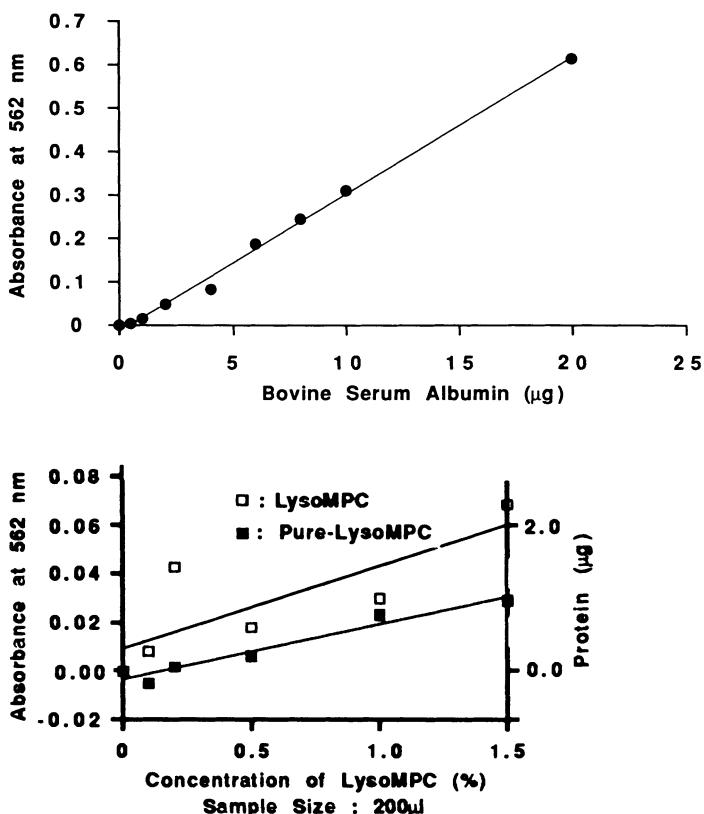


Figure 3. (top graph) Standard curve for bovine serum albumin in the 'enhanced' BCA assay; (bottom graph) LysoMPC's effect on 'enhanced' BCA protein assay. It shows that lysoMPC exhibits low absorbance values equivalent to ~1 mg-protein (for 0.1% lysoMPC) to 2 mg-protein (for 1.5% lysoMPC), depending on the concentration of lysoMPC. All data were obtained on 100 ml sample volumes containing 62.5 mM Tris pH 7 that was diluted to 200 ml with water before BCA assay.

Extensive evaluation by Avanti Polar Lipids (Birmingham, AL, personal communication from Walt Shaw) has shown that lysoMPC does not contain any detectable protein (i.e., phospholipase A2 from the synthesis of lysoMPC). Nevertheless, because the lysoMPC background absorbance in the BCA assay is low (≤ 0.06 absorbance units for the 'enhanced' BCA assay), and

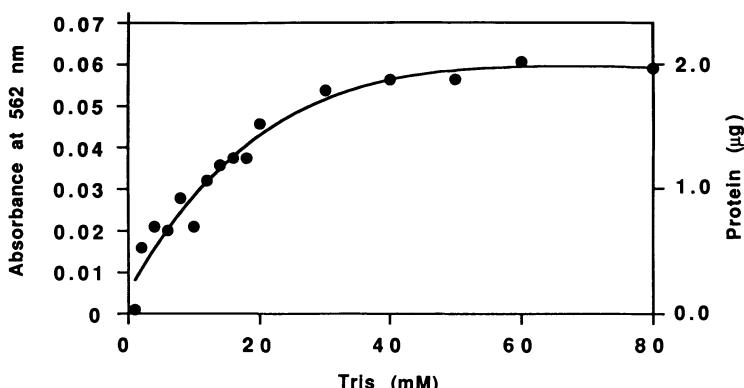


Figure 4. Effect of Tris buffer on the enhanced BCA assay protocol. All data were obtained on a sample volume of 200 μ l; therefore, the Tris concentration reflects the molar concentration in a typical sample subjected to protein-analysis in the enhanced BCA protocol. A water background was used - the protein standard curve is shown in Figure 3.

the impurity A_{280} is ~ 0.005 in the HPLC chromatograms (Fig. 1), we do not routinely purify lysoMPC during IAM chromatography.

MEMBRANE PROTEIN SOLUBILIZATION

Membrane proteins must be extracted and solubilized prior to injection on IAM columns. We are evaluating if lysoMPC can function as both (i) a general-use detergent to solubilize membranes and (ii) a detergent to elute membrane proteins during IAM chromatography. To our knowledge, only one report is available in which a lysolecithin was used both to solubilize a membrane (i.e., a mitochondrial membrane) and elute membrane-proteins during chromatography.¹⁸ However, the solubilized mitochondrial membrane proteins were eluted from a hydroxyapatite/celite column using 1-palmitoyl-sn-3-glycerophosphocholine (a C18 lysolecithin) with poor results; the mitochondrial membrane proteins quantitatively eluted in the column void volume.

For this work, the model membrane proteins solubilized with lysoMPC were obtained from human red blood cell membranes. Red blood cell membranes contain well characterized peripheral and integral membrane proteins and we believe the solubilization and chromatographic results can be extended to membrane proteins derived from other sources that include bacteria, plants, and viruses. Red cell membrane proteins were chosen as model compounds because

the protein-protein and protein-lipid interactions are well established (for an excellent review, see ref. 17). In addition, red cell membranes are the best understood cellular membrane system, yet they are far less complex than non-erythroid cells; thus membrane proteins from red cells are excellent model proteins for experimentally characterizing IAM surfaces.

Solubilization of Red Cell Membrane Proteins

Three membrane preparations were used for this work; red cell membrane ghosts (denoted as only ghosts), KI-stripped-inside out vesicles (KI-IOV's), and pH 11 stripped IOV's (IOV's^{pH11}). Ghosts,¹⁹ KI-IOV's,²⁰ and IOV's^{pH11} (ref. 21) were prepared as described. Ghosts contain both peripheral and integral membrane proteins and consist of the plasma membrane proper. KI-IOV's contain predominantly integral membrane proteins and are largely depleted of peripheral membrane proteins. Both KI-IOV's and IOV's^{pH11} are prepared from ghosts, but the extent of peripheral membrane protein depletion in KI-IOV's and IOV's^{pH11} varies. IOV's^{pH11} are essentially devoid of peripheral proteins and approximately ~3/4 of the residual protein is band 3 (Fig. 5); the remaining proteins are primarily glycophorins. For KI-IOV's, small quantities of peripheral membrane proteins (spectrin, actin, ankyrin, and bands 4.1, 4.2 and 7) are present.

Anionic SDS and zwitterionic CHAPS are reported to be good detergents for solubilizing ghosts; however, SDS denatures ghost membrane proteins whereas CHAPS does not.²² We compared zwitterionic lysoMPC to both SDS and CHAPS regarding the solubilization of ghost proteins. Both 1.8 % SDS and 1.35% lysoMPC completely solubilized ghost membrane proteins, whereas 1.8% CHAPS does not solubilize the sample (Fig. 5, lanes 1-3 non-boiled samples). This is in contrast to the report that CHAPS is a good detergent to solubilize ghost proteins.²² We note that the standard SDS-PAGE procedure recommends brief (1-2 minutes) sample-boiling to assure protein denaturation.²³ Brief boiling unfolds (denatures) proteins and maximizes SDS adsorption to the solubilized proteins. Maximum SDS adsorption permits the negatively charged SDS molecule to dominate the protein surface charge, which is needed for SDS-PAGE gel electrophoresis.

A few reports are available suggesting that membrane proteins will aggregate if the samples are boiled in preparation for SDS-PAGE.²¹ Figure 5 (lanes 4-6) demonstrates that solubilized ghosts also elicits substantial protein aggregation if the samples were boiled. This is evident by the depletion of band 3 (compare lane 1 vs lane 4; also compare lane 2 vs lane 7) and other membrane

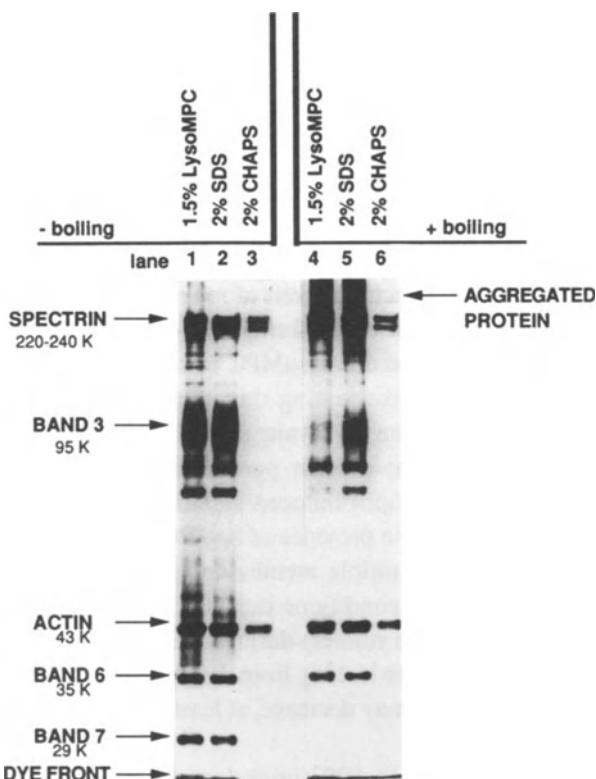


Figure 5. Inability of CHAPS to solubilize red cell membrane ghosts. Ghosts (0.2 ml, ~1.5 mg-protein/ml) were diluted with 1.8 ml of non-buffered detergents (1.5% lysoMPC, 2% SDS, 2% CHAPS) and incubated for 1 hour at 4°C. Prior to sample preparation for gel electrophoresis, samples were centrifuged at 35,000 g x 30 minutes to remove insolubles. Sample boiling in preparation for gel electrophoresis with and without sample boiling is shown. Lanes 1-3 are non-boiled samples and lanes 4-6 are boiled samples.

proteins from the solubilized sample. In addition high molecular weight dark protein bands in the gel above spectrin (MW ~ 240 KD) are indicative of protein aggregation. We have found that sample boiling is unnecessary and causes gels with several diffuse high molecular weight bands.

Aggregation is not apparent when ghosts were solubilized with CHAPS primarily because CHAPS does not solubilize ghost proteins, i.e., very little membrane proteins were in the supernatant (Fig. 5, lanes 3 and 6) and therefore no aggregation was detectable. Formation of insoluble protein aggregates has been reported with charged detergents^{24,25} including charged lysolipids¹² and SDS,¹² whereas neutral zwitterionic lysolipid detergents have not induced protein aggregation under the conditions tested.¹² Although we have not quantified red cell membrane protein aggregation in the presence of lysoMPC, we have repeatedly observed that gel electrophoresis of red cell membrane proteins shows less aggregation (i.e., less high molecular weight diffuse bands in SDS-PAGE gels) when the proteins are stored in lysoMPC compared to CHAPS or SDS. A few reports are available demonstrating that egg lecithin (i.e., double chain phosphocholine) stabilized the rat brain sodium channel during detergent solubilization and subsequent protein purification.^{26,27} However 10 mM calcium was needed for the lipid induced stabilization. In addition, DEAE sephadex chromatography in the presence of egg-PC permitted a 2-fold increase in protein recovery of this multiple membrane spanning protein. Systematic studies that identify solution conditions that stabilize membrane proteins (as found for egg-PC in the above studies) during detergent-solubilization, protein chromatography, and storage are lacking from the literature. However, the above results suggest that lysolipids may decrease, at least partially, the aggregation of membrane proteins.

Coomassie staining is 100 times less sensitive than silver staining,^{28,29} and consequently aggregated proteins are difficult to detect using Coomassie staining. For example, Figure 6 shows that Coomassie could not identify aggregated proteins in ghost samples boiled in preparation for SDS-PAGE whereas silver staining easily detected the aggregates. Using bovine serum albumin we find that Coomassie staining can detect approximately 1 μ g of protein, whereas silver staining easily detects 0.1 μ g of protein. Merril has provided an excellent review of gel-staining techniques.³⁰

PROTEIN BINDING TO IAM PARTICLES AND IAM CHROMATOGRAPHY COLUMNS

Complex biological samples contain several proteins and we have found several apparently-solubilized membrane protein samples to irreversibly bind to IAM.PC and IAM.PC.MG (unpublished observations). For this reason we typically perform binding studies of the solubilized-membrane sample with IAM

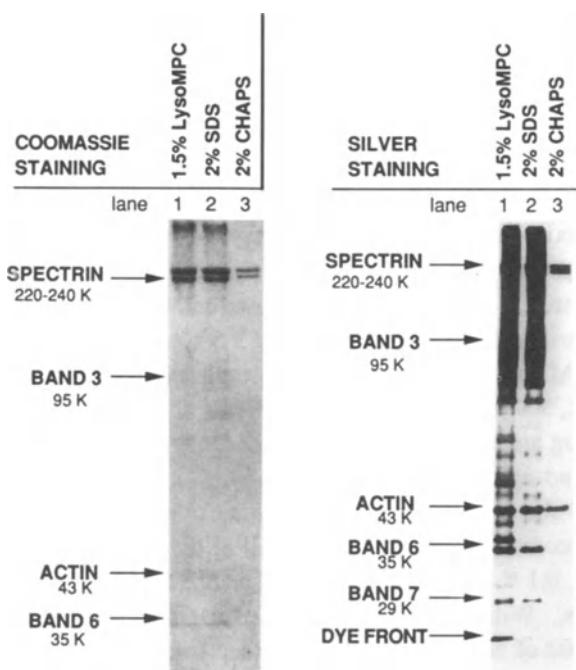


Figure 6. Coomassie staining vs silver staining of aggregated ghost proteins. Ghost protein samples were aggregated by boiling 2 minutes, as is routinely done for sample preparation for SDS-PAGE. Samples were solubilized as described in Figure 5. Silver staining was performed according to Merril,³⁰ while Coomassie staining was performed according to Laemmle.⁹ The detergent lanes have equal amounts of protein, e.g., lane 1 and lane 4 have equal protein loading.

particles, i.e., IAM particles not packed in HPLC columns. We assume proteins that irreversibly bind to IAM particles will also irreversibly bind to IAM HPLC columns; we do not assume that reversible binding of membrane proteins to IAM particles indicates reversible binding to IAM HPLC columns. The latter assumption reflects the differences in the protein adsorption-desorption process between IAM batch binding studies and elution of proteins from IAM HPLC columns. HPLC columns may concentrate proteins during the chromatographic step; protein concentration may result in aggregation, denaturation, and irreversible binding to the IAM column regardless of whether reversible binding occurs during a batch-binding experiment. These assumptions are currently

under investigation in our laboratory and an example correlating protein binding to IAM particles with protein binding to IAM HPLC columns is given below.

Protein binding studies to IAM particles are performed in microfilterfuge tubes containing a 0.5 ml capacity insert with a 0.2 μ filter at the base of the insert. Dry IAM powder (50 mg) is placed on the insert-filter and 250 μ l of the solubilized membrane preparation (~100-200 μ g protein) is applied with gentle mixing by a closed end capillary tube until the IAM particles are completely suspended.

The insert is then placed in an unused microfuge tube and subjected to low speed centrifugation for approximately 3-5 seconds (filtrate 1). The insert containing IAM particles and adsorbed proteins is transferred to an unused microfuge tube, 500 μ l of non-detergent-buffer is added and the sample is mixed well again using a closed end capillary as described above. The suspension is then centrifuged again (filtrate 2). This wash-mix-centrifuge procedure is repeated until the weakly adsorbed proteins wash off the IAM particles. Although unnecessary, it is convenient if all of the filtrates from a given wash condition (e.g., 0.1 % detergent) are pooled as one fraction and subjected to gel electrophoresis. With unknown solubilized samples we recommend 3 non-detergent washes of 500 μ l volume as a pooled fraction for gel electrophoresis. After washing the IAM particles with non-detergent buffer, the particles are sequentially washed with increasing concentrations of detergent using the same conditions.

In the above binding assay, the centrifugation time needed to quantitatively force the wash-filtrate through the IAM particles, without drying the IAM particles, depends on protein concentration and detergent concentration (manuscript in preparation). Because particle drying may (i) denature proteins, (ii) irreversibly adsorb proteins to IAM particles, or (iii) cause protein aggregation, we do not let the IAM powder become dry by excessive centrifugation. Long centrifugation times are thus avoided when using microfilterfuge 'inserts' to collect the particles during the IAM.PC binding study.

Figures 7a and 8a are typical results of the IAM.PC binding assays using red cell membrane preparations. Figure 7a shows that solubilized-ghost-proteins quantitatively and irreversibly bound to IAM.PC particles in the binding assay. For this experiment the wash solutions from a given wash-condition were not pooled and the individual fractions were subjected to gel electrophoresis. As shown in lanes 1-3, the first non-detergent wash filtrates contain no detectable protein (which was confirmed by BCA assay). No protein in these washes indicates that the ghost proteins were quantitatively bound to IAM.PC. However, three 500 μ l washes with 0.1 % lysoMPC (lanes 4-6), then three 500

μl washes of 0.6 % lysoMPC (lanes 7-9), then three 500 μl washes of 1.5 % lysoMPC (lanes 10-12) did not elute any of the red cell membrane proteins, which indicates that the bound proteins had irreversibly adsorbed to the packing material. Figure 7b shows the chromatogram obtained from injecting solubilized-ghost-protein onto IAM.PC HPLC columns. Virtually no proteins were eluted from the column based on both gel electrophoresis and the BCA protein assay. Using a new IAM.PC column, approximately 10 injections (300- μg -protein/injection) were required before solubilized-ghost-proteins were quantitatively eluted from IAM.PC (not shown). We have extensively evaluated IAM.PC and IAM.PC.MG with both ghosts and KI-IOV's and we find similar results as shown in Figure 7a. We speculate that the solubilized membrane proteins in KI-IOV's and ghost proteins retain significant protein-protein interactions that inhibit the elution of single components from IAM.PC. Regardless of mechanism, membrane proteins in KI-IOV's and ghost proteins irreversibly bind to IAM.PC particles; these proteins also irreversibly bind to new IAM.PC columns. Solution conditions necessary to elute membrane proteins found in ghosts and IOV's are under investigation in our laboratory.

In contrast to the irreversible protein binding to HPLC IAM.PC columns of solubilized-ghost-proteins and solubilized KI-IOV proteins, HPLC IAM.PC columns do not irreversibly bind membrane proteins in IOVsPH11; this correlated with the batch-binding studies (Fig. 8). Gel electrophoresis of samples shown in Figure 8 demonstrates that; (i) no proteins were detected in the initial wash buffer (lanes 1-3) indicating the membrane proteins adsorbed to the IAM surface; (ii) no proteins were detected in the 0.1 % lysoMPC wash (lanes 4-6); however, (iii) virtually all of the non-band 3 proteins were eluted during the first 0.6 % wash (lanes 7-9). Band 3 or other membrane proteins were not eluted by increasing the detergent concentration to 1.5% lysoMPC (lanes 10-12). When this binding study was repeated whereby the 0.6 % lysoMPC wash condition was omitted, the non-band 3 membrane proteins were eluted in 1.5 % lysoMPC. Thus the elution of non-band 3 membrane proteins adsorbed to IAM.PC is not a bell shaped curve in which there is an optimum detergent concentration for elution. Several other proteins exhibit a bell-shaped detergent-solubility curve whereby high detergent concentrations result in less protein solubility. For lysoMPC this does not appear to be the case, since 1.5 % lysoMPC would not have eluted the non-band 3 membrane proteins as efficiently as 0.6 % lysoMPC. Figure 8 indicates that the protein adsorption-desorption process observed in the batch-binding study correlates with the ability to elute membrane proteins from new IAM HPLC columns.

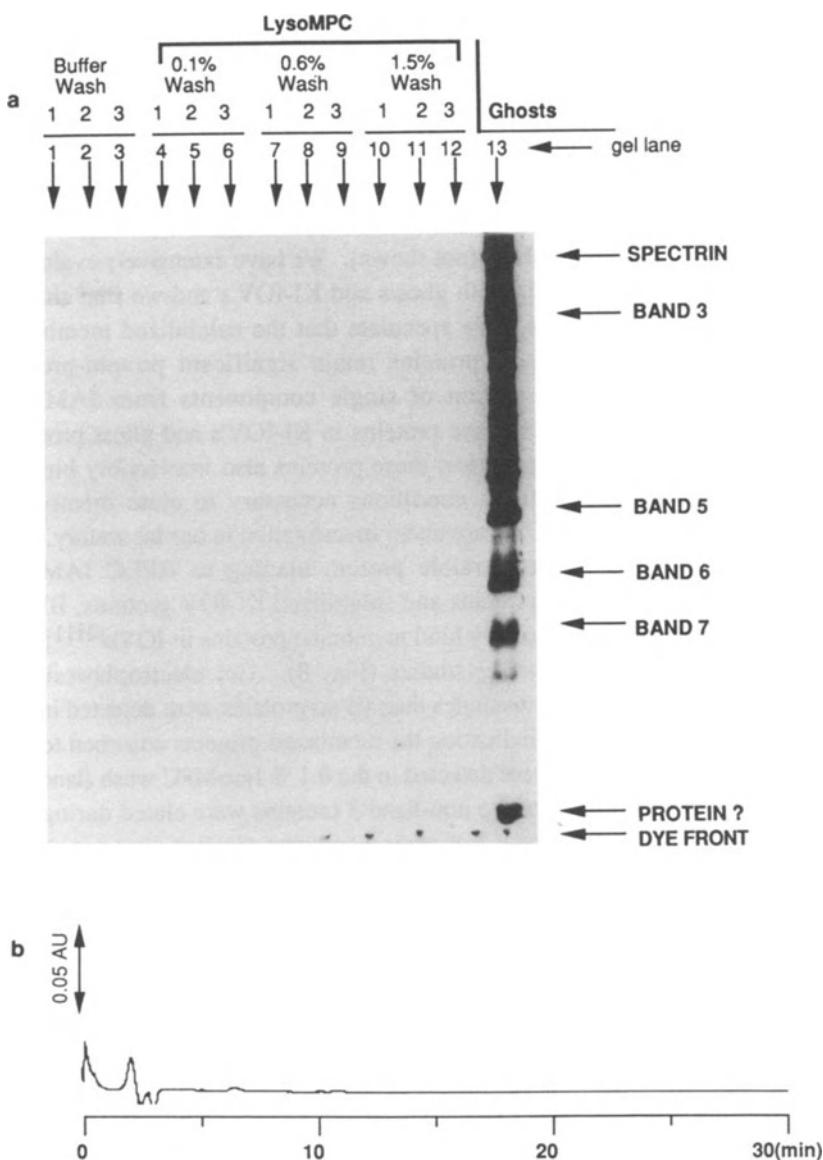
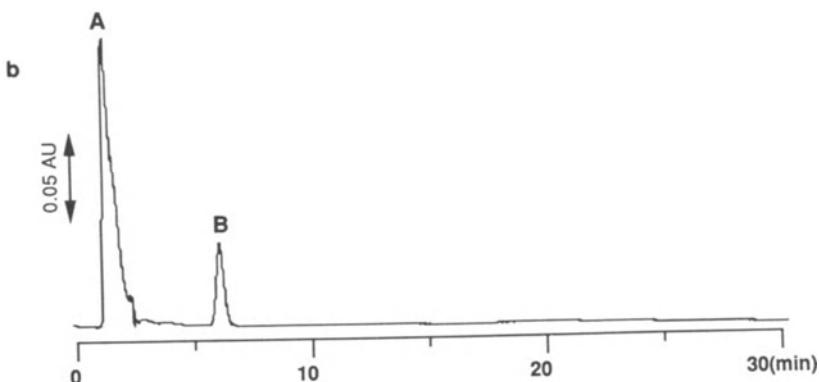
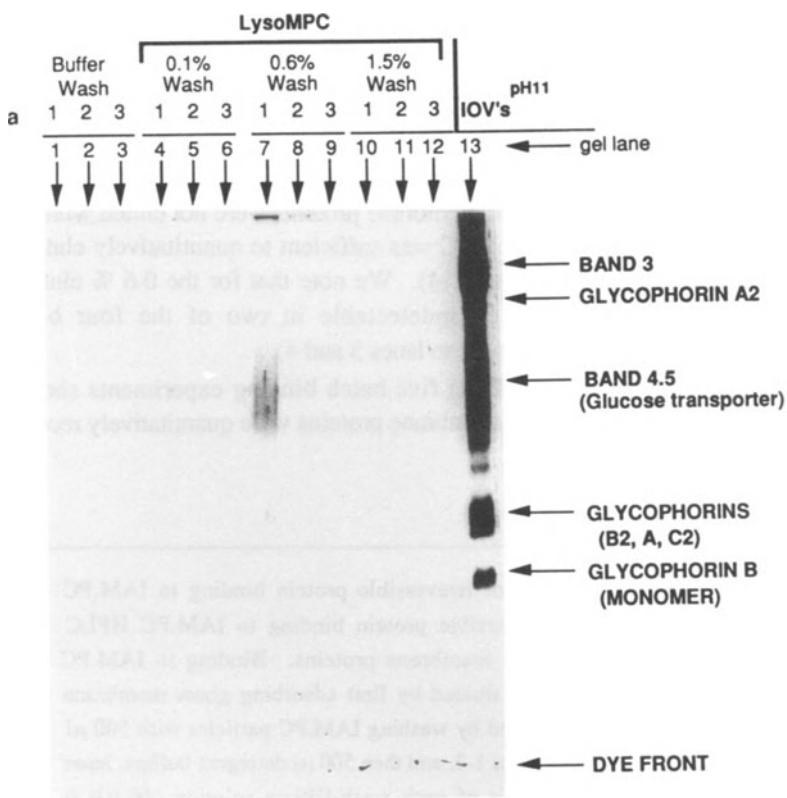


Figure 9 shows the results of 4 additional batch-binding experiments under identical experimental conditions used to obtain the binding data shown in Figure 8a. For these experiments fresh unused IAM.PC particles were used and wash-filtrates were pooled. Wash-filtrates were pooled because it was previously established by gel electrophoresis that only the 0.6 % lysoMPC wash-filtrate fractions contained protein. These 4 binding experiments gave virtually identical results as the first binding experiment: (i) the proteins were not eluted with 0.1 % lysoMPC (lane 7); and (ii) the membrane proteins were not eluted with 1.5 % (lane 6) because the 0.6 % lysoMPC was sufficient to quantitatively elute non-band 3 membrane proteins (lanes 1-4). We note that for the 0.6 % elution of membrane proteins, band 3 is undetectable in two of the four binding experiments (compare lanes 1 and 2 to lanes 3 and 4).

From BCA analysis of the five batch binding experiments shown in Figures 8 and 9, the non-band 3 membrane proteins were quantitatively recovered

Fig. 7. (a) Correlation of irreversible protein binding to IAM.PC particles (7a) with irreversible protein binding to IAM.PC HPLC columns (7b) with ghost membrane proteins. Binding to IAM.PC particles (50 mg) was evaluated by first adsorbing ghost membrane proteins (200 µg), followed by washing IAM.PC particles with 500 µl non-detergent buffers, lanes 1-3, and then 500 µl detergent buffers, lanes 4-12. Gel electrophoresis of each wash-filtrate solution (40 µl) is shown. The non-detergent buffer (lane 1-3) contained 62.5 mM Tris pH 7. Detergent buffers were prepared from this buffer by adding increasing amounts of lysoMPC. The gels were heavily stained in an attempt to determine if any proteins were washed off the IAM particles during the batch binding study. Because the gels were heavily stained, the control lane (lane 13) is diffuse. When this study was repeated 5-8 times on fresh IAM particles, small amounts of spectrin, actin and other peripheral membrane proteins appeared in small amounts on the gels. (b) For isocratic IAM.PC chromatography (7b) a new column (4.8 x 15 cm) was used, detection was at 280 nm, and the flow rate was 0.75 ml/min, and the mobile phase was 1.5% lysoMPC buffered with 62.5 mM Tris pH 7. Gel electrophoresis of the peak in 7a did not exhibit any protein and based on the BCA protein assay, the peak in 7a indicated that < 3% of the injected proteins were recovered in this peak.



from the particles, whereas band 3, which is about 3/4 of the IOVs^{pH11} membrane preparation, was quantitatively removed from the IOVs^{pH11}. Thus the IAM.PC binding study can also be considered as a batch extraction when particular proteins irreversibly bind as found for Band 3. We have characterized the binding isotherm of IOVs^{pH11} regarding the mg of protein that can be loaded on to IAM.PC before band 3 begins to elute. We find that 150 µg-protein/50 mg-IAM.PC is optimum for retaining band 3, yet eluting the other membrane proteins (manuscript in preparation). In other words, we can group fractionate in one step, all of the glycophorins, and non-Band 3 membrane proteins from IOVs^{pH11} membrane preparations. The correlation between batch-IAM binding studies with protein elution from IAM HPLC columns shown in Figures 7 and 8 demonstrates unambiguously that sample preparation is a significant variable in IAM chromatography. Both of the membrane preparations contain similar proteins yet one membrane preparation was more demonstrate that the IAM microfilterfuge binding assay can be used to screen or identify initial solubilization and elution conditions for IAM HPLC chromatog-

Figure 8. Correlation of reversible protein binding to IAM.PC particles (8a) in the batch-binding assay with reversible protein binding to IAM.PC HPLC columns (8b) with IOVs^{pH11} membrane proteins.

(a) Binding to IAM.PC particles (50 mg silica containing 2.5 mg of immobilized lecithin) was evaluated by first adsorbing membrane proteins (200 µg) to IAM.PC particles, followed by washing IAM.PC particles (500 µl each wash) with non-detergent buffers (lanes 1-3) and then detergent buffers, lanes 4-12. Gel electrophoresis of each wash-filtrate solution (40 µl) is shown. The non-detergent buffer (lanes 1-3) contained 62.5 mM Tris pH 7. Detergent buffers were prepared from this buffer by adding increasing amounts of lysoMPC. The gels were over-stained to detect the small amount of protein washing off the IAM.PC particles at 0.6%. The control lane (lane 13) has ~10 µg protein.

(b) For isocratic IAM.PC chromatography, a new column (4.8 x 15 cm) was used, detection was at 280 nm, the flow rate was 0.75 ml/min, and the mobile phase was 1.5% lysoMPC buffered with 0.06 M Tris pH 7. Gel electrophoresis of the major peak in 8a demonstrated that all of the non-Band 3 injected proteins were eluted from the column and BCA assay demonstrated that 20-30% of the injected proteins were recovered in this peak.

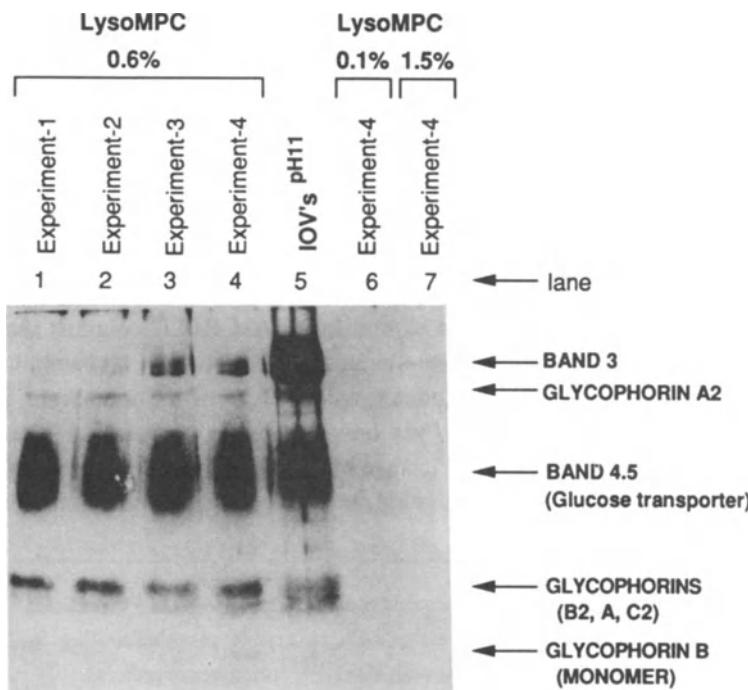


Figure 9. Reproducibility of the IAM.PC-IOVs^{pH11} batch-binding assay. Four separate batch-binding experiments were performed with new IAM.PC particles for each experiment. The binding experiments were performed exactly as described in Figure 8a except the filtrate fractions for a given wash condition were pooled and subjected to gel electrophoresis. Lanes 1-4 (~6-8 µg-protein/lane) are the 0.6% lysoMPC wash-filtrates from the four binding experiments. Lane 5 is the IOVs^{pH11} sample (6 µg-protein/lane) applied to the particles. Lane 6 is 0.1% lysoMPC and lane 7 is 1.5% lysoMPC, both from binding experiment 4. Based on the BCA assay no protein was detected in the 0.1 and 1.5 % wash-filtrates and this is evident by the lack of proteins in lane 6 and lane 7. Glycophorin A2 is the weak band below Band 3; this protein and the other glycophorins quantitatively eluted at 0.6% lysoMPC.

raphy. We have injected solubilized ghost-proteins, KI-IOVs and IOVs^{pH11} onto IAM.PC acidic and IAM.FA columns and have found that protein recovery from these columns includes all of the individual proteins in the membrane preparation (based on gel electrophoresis studies of the eluted proteins and is an unpublished observation). Although each type of injected protein was recovered, the extent of recovery varied. In addition, batch-binding assays involving adsorption of solubilized IOVs^{pH11} to IAM.PC.acidic and IAM.FA showed that all of the IOV's^{pH11} proteins did not irreversibly bind to these acidic IAM particles; this included band 3. Although membrane proteins from IOV's^{pH11} were eluted from IAM.PC columns (Fig. 8), protein separation or selectivity was not apparent (i.e., gel electrophoresis of the peak did not show selective protein elution across the peak). However, using IAM.FA and IAM.PC acidic, column selectivity was apparent; this work is in progress. These initial observations indicate that column selectivity will depend on the IAM surface. We have recently found that the elution of red cell membrane proteins from IAM surfaces, with lysoMPC as the mobile phase detergent, depends significantly on the amount of protein loaded onto the IAM surface. This is in contrast to the purification of cytochrome P-450, which uses lubrol as the mobile phase detergent. Purification of cytochrome P-450 can be achieved on analytical IAM.PC columns with sample loading of > 8 mg of total protein.

CONCLUSION

Lysomyristoylphosphatidylcholine efficiently solubilizes red cell membrane proteins. Lysomyristoylphosphatidylcholine partially inhibited the aggregation of red-cell membrane proteins during storage and sample handling. Binding studies of proteins to IAM particles are useful in choosing detergents for solubilizing and eluting membrane proteins from IAM HPLC columns. Band 3 irreversibly binds to IAM.PC particles unless the particles are overloaded. IAM HPLC column selectivity of membrane proteins depends on sample preparation, the IAM surface, and the mobile phase detergent.

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Chapter Six

MASS SPECTROMETRY IN THE CHEMISTRY OF NATURAL PRODUCTS

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INTRODUCTION

Mass spectrometry (MS) is one of the very first spectral methods dating back to the beginning of this century (1913, J.J. Thomson, parabola spectrograph; 1918, A.J. Dempster, spectrometer, and 1919, F.W. Aston, spectrograph).¹ It measures mass, one of the two basic properties of matter, as the mass-to-charge-ratio of ions, (m/z in atomic mass units, scale relative to ^{12}C) while other spectral methods usually measure frequency, either absorbed or emitted. Thus, in a sense, the mass spectrometer is an extension of the balance, the principal tool of a chemical laboratory. Sensitivity of mass spectral measurements approaches single ion detection and the accuracy of mass determination may be in order of ppm. The quest for this precise information characterizes all three major phases of the application and development of mass spectrometry: detection of isotopes, "inorganic phase", followed by extensive analysis of molecules of relatively low molecular weight, "organic phase", and the most recent "biological phase" in which ionization and analysis of ions derived from a much wider variety of polar and large organic molecules becomes possible. Immense developments of instrumentation, increasing analytical applications, and an ever increasing range of compounds accessible to measurement do not change the heart of mass spectrometry and the simplicity of the fundamental information provided.

This review attempts to address the development of mass spectral methods and to illustrate their application to analysis and identification of low molecular weight natural products. For the sake of clarity, most examples used represent two lines of investigation, one concerning terpenoids from *Compositae*, another bile acids from marsupials. Literature included in this review is selective and the reader ought to consult other sources for fundamental and current references.^{1,2}

Low molecular weight natural compounds represent a relatively small group of limited structural variability reflecting the existence of established metabolic pathways. Even classes represented by numerous varieties of basic skeletons by no means exhaust the possibilities of isomerism. An interesting calculation, recently performed,³ demonstrated that for a simple case of a branched $\text{C}_{167}\text{H}_{336}$ hydrocarbon the number of the structurally possible isomers reaches a value greater than the number of particles in the whole Universe. Not only in view of this comparison, but also when compared to the prolific synthetic effort of chemists, the number of small molecules in nature is surprisingly low. This makes the endeavor of chemists involved in the exhaustive isolation and structural identification of so called "secondary metabolites"

fully justified, apart from any utilitarian goal. The complete recognition of structures of a predominant number of natural compounds is fully realizable with currently available spectral methodology. This knowledge is of paramount importance to our understanding of life on Earth, especially the interdependence of species and their mutual biochemical evolution. This structural knowledge also delineates the subsequent endeavor in elucidation of biochemical reactions leading to these compounds.⁴ Its understanding is also more straightforward, less convoluted, and less dependent on apparently attractive but certainly largely oversimplified models explaining events on a macromolecular scale. In the eyes of some, however, this enduring effort of natural product chemists is devoid of both sophistication and purpose.

MASS SPECTROMETERS

Analysis of ions according to their mass-to-charge ratio (m/z) can be accomplished by different combinations of static, slowly changing, and radio-frequency modulated electric and magnetic fields. This measurement requires high vacuum for the unrestricted motion of ions, i.e. the mean free path of an ion must be much longer than dimensions of the mass spectrometer.

Mass spectrometers are composed of three major sections which perform in succession (a) ion formation, (b) ion analysis, and (c) ion detection. Their functions require a complex system of supporting vacuum and electronic devices. Almost all newly built mass spectrometers are controlled by a dedicated microprocessor performing verification of the status, scanning, and acquisition, as well as permitting easy access to data. Magnetic sector and quadrupole mass spectrometers, the two most commonly used, utilize the ion source separate from the section performing ion analysis. This is also the case for time of flight mass spectrometers. Ion cyclotron and ion trap instruments, however, may operate with either an external source or utilize the same volume for ion formation and analysis. Magnetic instruments certainly represent the main route of the development of mass spectrometry with ever increasing perfection in their construction. The following discussion starts with their description.

Magnetic Sector Instruments

Ions of mass "m" and charge "z" are expelled from the ion source by the accelerating potential "V", between the source and the first entrance slit. This allows their passage through subsequent regions with velocity "v", directly

calculated from the ion kinetic energy "k", and reach the magnetic analyzer:

$$v = \sqrt{2*V*(z/m)} \quad [1]$$

$$k = z*V = m*v^2/2. \quad [2]$$

In the perpendicular magnetic field "B" ions with velocity "v" are deflected with the radius "R", unique for each "m/z" value and meaning that ions are deflected in a way proportional to the intensity of the magnetic field "B" but inversely proportional to the square root of m/z:

$$R = (m/z) * (v/B) = (1/B) * \sqrt{2*V*m/z} \quad [3]$$

$$(m/z) = (1/2*V) * (R*B)^2 \quad [4]$$

Thus, if ions of m/z=18 are directed to the exit slit, ions with m/z=180 will require a 3.16 times stronger field, and ions m/z=1800 a ten times stronger field. Conversely, if the magnetic field is kept constant, the accelerating voltage ought to be lowered 10 and 100 fold, respectively, in order to direct these ions to the exit slit. Scanning usually follows the first approach, keeping a constant or possibly a high accelerating voltage, because sensitivity and resolution are proportional to this voltage. Although the dispersion of a magnetic analyzer, similar to other devices, can be calculated, mass assignments are performed by direct comparison with standard compounds providing precise mass scale and calibration. The resolving power of a mass spectrometer ($RP=M/\Delta M$) is the measure of separation of close ions with the average mass "M" differing by " ΔM ". It is usually defined for the "10 % valley" between peaks of an equal size. The magnetic field exerts the "direction focusing" of ions of the same m/z and aberrations in this focusing result, amongst others, from the dispersion of the ion beam. As a result, the resolving power of a magnetic instrument is determined by its overall geometry and width of both slits (S_1 and S_2): $RP=R/(S_1+S_2+A)$, where factor "A" corrects for such aberrations.

These factors determine that the construction parameters of a magnetic mass spectrometer suitable for high mass analysis requires a strong magnetic field and a large deflection radius. The introduction of the 60° sector magnet, i.e. with the deflection angle smaller than the originally used 180° magnets has been the first important step in this direction (1940, by A.O.C. Nier). Instruments of a manageable size can now be built with no need to incorporate exceedingly big magnets, which would have been required even for ions approaching 1000 amu. Thus, mass spectrometers with a mass range of 10,000 amu at 8 kV accelerating potential may have a 60° magnet with weight below 1 ton.

Magnetic properties of the solid magnet core require relatively long times between scans, or may even completely preclude fast, repetitive scanning of the whole mass spectrum. The situation of the high resolution magnetic analyzer built today, however, is different. Large laminated magnet cores permit a fast scan of even 0.1 sec per decade (e.g. 500 to 50 amu) with a short interscan time of about 0.2 sec., retaining full advantage of the mass range and resolving power. Alternatively, the construction of magnetic instruments based on electromagnets with no core permits even faster scanning.

Magnetic deflection of ions, as described above, assumes an equal kinetic energy for all ions expelled from the ion source, but this is not the case. Ions of the same m/z differ in velocity due to different thermal energies and finite dimensions of the volume in which ionization takes place. Increased acceleration potential lowers the relative effect of such contributions. The maximal resolving power achievable with a single magnetic sector may even reach several thousands and is obtained by narrowing both slits, at the considerable sacrifice of ion transmission and thus also sensitivity. The incorporation of an electric sector is beneficial in correcting this problem. It can be placed either before or after the magnetic analyzer giving EB (normal geometry) and BE (reverse geometry) doubly focusing instruments, respectively. Ions with kinetic energy resulting from acceleration by potential "V" [2] are deflected in a circular electric field "E" of a radius "P". Curvature of this deflection follows equation [5] from which "P" can be calculated:

$$z^*E = m^*v^2/P \quad [5]$$

$$P = 2^*V/E \quad [6]$$

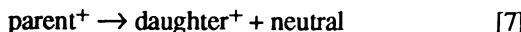
Notably, this radius is independent from m/z . Only ions of a selected velocity are deflected with the radius equal to that of the electric sector and are able to reach either the magnetic sector or exit slit. Slower and faster ions are not able to negotiate this turn and reach the next stage. Double focusing, i.e. in the direction (B-sector) and velocity (E-sector) significantly increases resolving power and transmission. With well-designed overall geometry and shape of the magnetic and electrostatic poles, and by correcting aberrations in ion focusing, resolving power can approach 100,000.

Accurate measurement of m/z requires high resolving power, but even at medium resolution of several thousands useful measurements can be performed for monoisotopic ions by "peak-matching". By varying the accelerating voltage (and electric sector voltage on double sector instruments) the ion of a precisely known mass (usually a fluorocarbon as their accurate mass is always below the

nominal mass) and the unknown ion are alternately brought to focus. From the linear dependence mentioned above, the accurate mass can be calculated from the ratio of respective accelerating voltages and the known mass of a standard. With two standard ions, one on each side of the unknown, even better accuracy of this determination can be obtained. This type of measurement for the mixture with a standard under high resolution conditions followed by the interpolation, now conveniently performed by suitable computer software, produces the accurate mass of all ions present in the spectrum.

Mass spectrometers measuring isotope ratios require high ion transmission and extreme accuracy of ion current measurement. Trace isotopes may be measured in multiple ion analyzers, one separating and measuring the major isotope, the second producing the final separation of the residual amount of the major isotope and measuring the minor isotope. Very high enrichment factors for trace isotopes can be obtained this way.

Focusing conditions are met for stable ions formed in the ion source and reaching the detector slit. If, however, ions decompose during the time of flight, products of such unimolecular reactions, called metastable ions, may either not reach the detector or may be partly focused at an apparent mass different from the true one. This is because part of the original kinetic energy is partitioned to another neutral or ionic product. Thus, the decomposition:



occurring between the ion source and the magnetic sector, a first field free region of a single sector magnetic instrument, produces metastable ions focused at the apparent value of m/z: .

$$(m/z)^* = (m/z)_d^2 / (m/z)_p \quad [8]$$

This relation is also fulfilled for decompositions taking place between E- and B-sectors, a second field free region, of the normal geometry, EB instruments. In contrast to stable ions, focused as sharp peaks with the full resolving power, peaks are broad and their shape and precise position reflects energetics of the specific decomposition process, due to metastable ions.

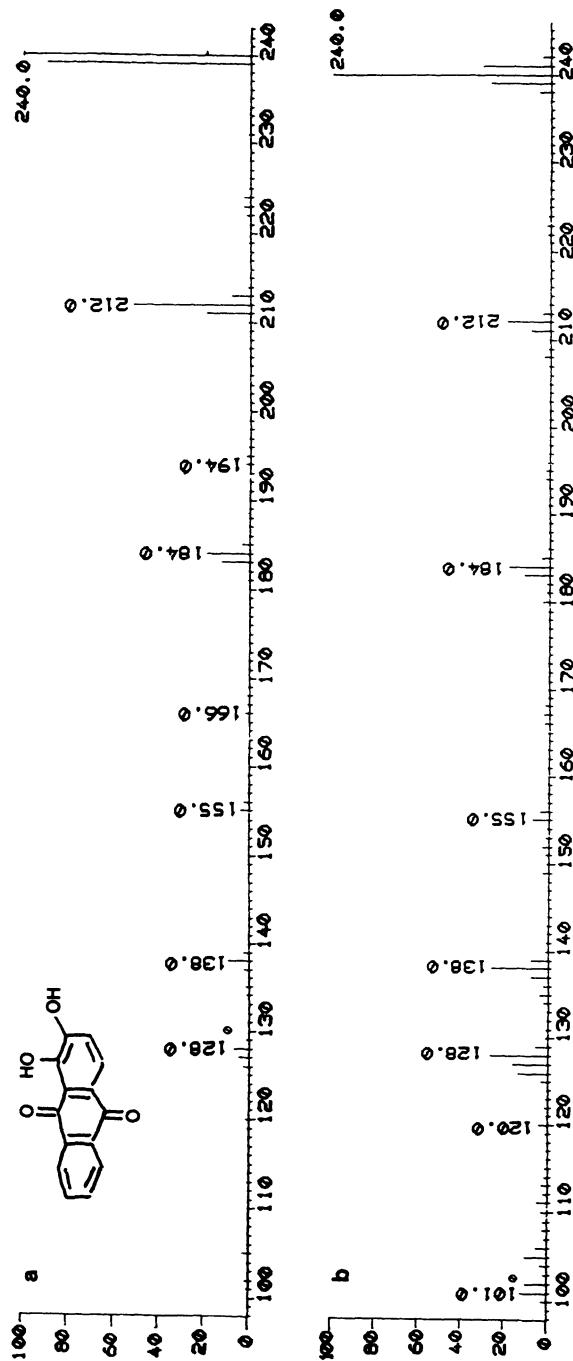
For decompositions in the first field free region of an EB instrument, the lower kinetic energy of metastable ions precludes their transmission through the E-sector. These metastable ions, however, can be "refocused" with the proportional increase of accelerating voltage. In such "high voltage scan" all "normal" ions are eliminated from the spectrum and parents of a selected daughter

ion can be identified. Alternatively, by adjusting the E-sector voltage, while keeping constant acceleration voltage, selected parent ions are able to pass this analyzer and enter the magnetic analyzer tuned to the daughter ion. The original energy of the parent ion is partitioned between products proportionally to their mass, thus the independent scan of the E-sector on the reversed geometry BE instrument, with the B-sector selecting parent, permits one to obtain a low resolution spectrum of products (known as mass analyzed ion kinetic energy spectroscopy, MIKES; or direct analysis of daughter ions, DADI).

Still another way of metastable ion detection involves both E- and B-fields varied in concert, B^2/E and B/E linked-scans, which permits detection of either parent or daughter ions. Ions related by "the constant neutral loss" can be identified in spectra by using mathematically more complex linked-scan. These three latest methods, preferably performed with a precise computer calibration, produce relatively well resolved spectra. The daughter ion spectrum (B/E linked-scan) obtained from the molecular ion of alizarin (m/z 240) shows a series of ions due to eliminations of one to four molecules of carbon monoxide, in addition to the loss of a hydrogen radical and water molecule (Fig. 1).

The detection of metastable ions is limited to unimolecular reactions occurring in a small window of the decomposition rate constant (usually between 10^{-4} and 10^{-6} sec^{-1}) determined by length of field free regions ($\sim m$) and ion velocity ($\sim 10^5 \text{ m/sec}$). Introduction of a special region in which ions interact with a neutral gas enhances such decompositions (collision induced, or activated decomposition, CID, CAD). The gas collision cell is usually placed in the first field free region of double sector instruments. Other methods of inducing dissociation are also possible.⁵

Metastable ions are fundamentally important for the assignment of the sequence of fragmentation reactions and their kinetic and energetic parameters. Consequently, better ways of generation of this vital information are being searched for. One possibility is to use multistage instruments separately tuned to the parent and daughter ions, and many such MS/MS combinations, especially of sector and quadrupole instruments, have been constructed.⁶ MS/MS, apart from supplying direct information on fragmentation processes, produces an analytical tool of multiple advantages. The first stage mass spectrometer serves as a separatory device in which ions, including molecular ions, can be selected from the spectrum of even a complex mixture. Subsequently, their decomposition facilitated by CID can be followed in the next stage. This further improves the already high sensitivity and selectivity of mass spectral detection and is especially powerful for the study of relatively stable ions resulting from "soft-ionization".



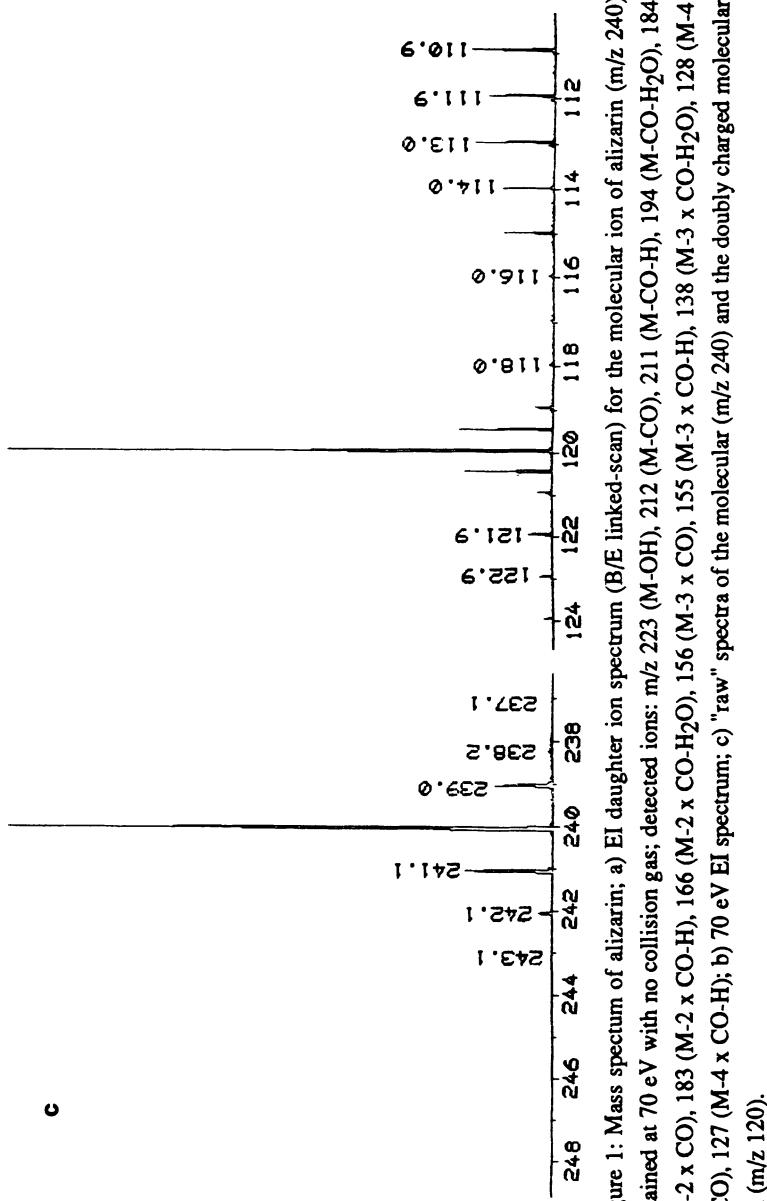


Figure 1: Mass spectrum of alizarin; a) EI daughter ion spectrum (B/E linked-scan) for the molecular ion of alizarin (m/z 240) obtained at 70 eV with no collision gas; detected ions: m/z 223 (M-OH), 212 (M-CO), 211 (M-CO-H), 194 (M-CO-H₂O), 184 (M-2 x CO), 183 (M-2 x CO-H), 166 (M-2 x CO-H₂O), 156 (M-3 x CO), 155 (M-3 x CO-H), 138 (M-3 x CO-H₂O), 128 (M-4 x CO), 127 (M-4 x CO-H); b) 70 eV EI spectrum; c) "raw" spectra of the molecular (m/z 240) and the doubly charged molecular ion (m/z 120).

The Quadrupole Mass Spectrometer

Because of the simplicity of its construction and operation, the quadrupole mass spectrometer represents the most popular device utilized as the "on-line" detector for GC and other chromatographic techniques. It operates with a separate ion source of cylindrical symmetry and requires only a small potential to expel ions into a short drift region. Four quadrupole rods, connected in opposite pairs, are powered with a radio-frequency modulated potential. In the oscillating field, ions enter a spiraling path stable for a selected m/z ratio only; consequently, these "stable" ions reach the detector. Scanning can be relatively fast; a second or less for a mass range of 1,000 amu and fast repetition is possible due to minimal interscan time. The resolving power of the quadrupole filter can be adjusted so as to obtain highest sensitivity in a whole mass range. This "unit resolution", low at the low mass and increasing proportionally with the mass range, resolves ions of a mass 1,000 amu or even higher. Originally, such devices were designed to cover a mass range required for the GC operation. Quadrupoles of an extended range, now approaching 4,000 amu, are becoming available and find application as an alternative to sectors in "hybrid-MS/MS" instruments composed of E-, B-, and Q-sectors.

Quadrupole analyzers can be connected in series to give "quadrupole-only" MS/MS operation mode with a versatile control of ion transmission. The third quadrupole, installed between these two analyzers, is not involved in scanning and serves as a gas collision cell. Scanning modes detect parent, daughter, and ions differing by the same neutral fragment. Such systems, connected to a chromatographic instrument considerably increase sensitivity and selectivity of detection.

Improving Sensitivity

Most magnetic sector instruments focus only one ion at the time, and field(s) must be varied in order to obtain the complete spectrum. As a consequence, the increased resolution leads to a situation in which the detector measures mostly the background current. This is a considerable sacrifice for spectra acquired in an extended mass range. Nevertheless, due to a much better economy of the number of ions measured and the time spent for their detection, the high gain of electron multipliers approaching millions permits one to obtain high sensitivity for short scans at lower resolution. Ion detection, especially those with high mass, is further improved by a "post-acceleration" which increases the kinetic energy of ions after the exit slit. In particular, this method

improves the sensitivity of quadrupoles operating at very low acceleration potential but is also beneficial for magnetic instruments.

Focusing of part of a spectrum and the simultaneous registration of all ions in this region has been achieved in the past with instrument geometry suitable for photographic detection. Modern electronic array detectors use the same principle and can cover several per cent of the spectrum with a considerable increase in sensitivity. This is especially important for the high mass MS/MS analysis with four sector instruments. Due to a limited dynamic range, however, array detectors are less useful for spectra acquired directly. When compared to the usual mode of scanning, arrays present a significant challenge for data acquisition, but further perfection of this technology may bring many new applications.⁷

In scanning mass spectrometers (and by the same token other kinds of instrumentation), most of the ions formed in the source are eliminated from detection. When extreme sensitivity is required, the mass spectrometer is either focused on a single ion maximum or scans only a narrow mass range. Fast switching of the accelerating voltage (and electrostatic sector voltage if this is present) measures closely located ions. Magnet switching is much slower for reasons discussed above, but is required for more distant ions. This selected or multiple ion monitoring (SIM), even more easily performed with quadrupole instruments, is of great utility for detection and quantitation by GC-MS and HPLC-MS. Measurements with sector instruments, and certain quadrupoles offering medium resolution, can be performed at either unit resolution or at high resolution, which greatly increases selectivity of ion detection. Obviously, such fast scanning presents its own problems, especially mass stability, but by using a "lock mass" the mass spectrometer can more precisely stay "on top of the peak". Fast scanning of SIM produces more precise information of a peak shape in combinations with chromatographic methods. Full scan conditions are not always able to provide a sufficient number of spectra across the chromatographic peak.

Old Ideas, New Instruments

Mass spectrometers based on different principles of ion separation than those described above can provide considerable improvement in the ratio of ions formed and ions detected. Many such devices utilizing electric and magnetic fields have been proposed. Technological advances have generated renewed interest in some older mass spectrometer designs. Examples include time-of-flight, ion cyclotron, ion trap, and Wien filter mass spectrometers.

Multichannel array detectors represent a modern version of the original photographic and fluorescent screen registration. No doubt, these new developments are made possible by remarkable advancements in electronic and computer technology. Thus, it would not be surprising if a new appearance of the earliest J.J. Thomson parabola spectrograph, employing the parallel electrostatic and magnetic field, were to appear.

Time-of-flight (TOF) mass spectrometry was limited, in its original application, to low molecular weight compounds by its low resolution. However, it offers unlimited mass range and extremely fast detection of all ions formed in the source. Recent application of TOF as almost an ideal detector for laser and plasma desorption induces intensified effort in its perfection.⁸ Additionally, its fast scanning capabilities are well suited for high resolution capillary GC and other chromatographic and electrophoretic methods.⁹

Designed in the late 1940s, the TOF instrument is certainly one of the most simple mass analyzers, composed of an ion source and a detector separated by a flight tube. Ions, accelerated either by a constant potential or a pulse, "fly" to the distant detector. Uniform kinetic energy, gained by the acceleration in the constant potential as formulated in equation [2], can be translated to:

$$z^*V = m^*(L/t)^2/2 \quad [9]$$

where "L" and "t" is the length and time of flight, respectively. This produces the square root dependence of a m/z and time of flight but the additional pulse is required to "gate" the ion beam:

$$t = L * \sqrt{(m/z) * (1/2 * V)} \quad [10]$$

Resolving power of such instrument is proportional to $t/2 * \Delta t$, accounting for the different location of ions in the source and their different thermal energies in the factor " Δt ". These problems were discussed earlier for sector instruments. Notably, as indicated by this dependence, the resolving power ought to increase with mass.

The acceleration by a short pulse "c", on the other hand, produces a pulsed ion beam of the momentum " m^*v " and time of flight "t":

$$m^*v = m^*(L/t) = V * z * c \quad [11]$$

$$t = (m/z) * (L/V * c) \quad [12]$$

Resolving power in this mode is proportional to $t/\Delta t$. The simplicity of the TOF instrument permits easy calibration, i.e time to mass conversion.

With ion velocities in a range of 10^4 - 10^5 m/sec, the time of flight in an analyzer tube of about 1 m permits fast repetition, but requires fast and accurate electronics both for precise ion acceleration and ion current measurement and storage.

The addition by Mamyrin of a "reflectron" electrostatic mirror introducing corrections for unequal velocities of ions of the same m/z has been especially significant for the development of TOF.¹⁰ This additional electrode, or rather a set of equally spaced electrodes provides a homogeneous electric field and is placed between the ion source and detector. Electrostatic reflection increases time of flight of faster ions while shortening it for slower ones. Focusing, or "bunching", of ions with the same m/z value is obtained resulting in resolving power on the order of 10,000. Other electrodes incorporated into the flight tube can also be used for manipulation of ions and neutrals reaching the detector, including detection of metastable ions.^{8,11}

Combination of a TOF spectrometer with a laser ion source is relatively straightforward in providing a start pulse and determining the time interval. The californium-252 plasma desorption (^{252}Cf PD) ion source, however, represents a different problem.¹² Here, ionization results from fission products bombarding the sample, and each such random event must have its own precise start time so as to add and average individual "one pulse spectra". Favorably, of the two fission products travelling in opposite directions one can be used to provide such a start signal, but possible overlapping of spectra limits the amount of radioactivity employed for this ionization. TOF instruments with the ^{252}Cf PD ion source were the first to demonstrate the remarkable formation and measurement of high mass ions under vacuum conditions.¹³ Clearly, before this (originally unplanned) observation, such a possibility was considered to be beyond the realm of mass spectrometry.

Ion cyclotron resonance (ICR) originated from a device invented in the late 1940s, and is now used for the concomitant detection of all ions by Fourier transform analysis (FT-MS).¹⁴ Usually, ions are formed directly in the cubical cell placed in a strong magnetic field, an ion source and analyzer at the same time, and kept between two parallel electrodes (trapping plates) perpendicular to the magnetic field. Ions circle with the orbital frequency of $w=(z/m)*(v/B)$ resulting from equation [3], $R=(m/z)*(v/B)$. The radius of the original orbit is determined by the velocity of thermal motion. Notably, and contrary to the case of the magnetic instrument, the difference in the original positions and thermal velocities are of no significance for this orbital frequency. Two other parallel plates are used to excite orbiting ions, and the remaining two for the detection of their motion. Importantly, this detection is solely based on the image potential generated by circling ions that remain in the cell. In order to fulfill conditions

for such detection, all ions must be excited to larger orbits and synchronized to a coherent motion. This is obtained by a short pulse containing all required frequencies, which is analogous to the 90° pulse used in FT-NMR.

FT-MS is a versatile instrument, resembling NMR in its operation, offering multiple ways of ion manipulation and detection with resolution not achievable by other techniques. Ions may be generated by various ionization methods, including combination with chromatography, especially when either separate cells¹⁵ or external source are employed for ion generation.¹⁶ Most convenient internal sources, electron impact and laser ionization, are fully compatible with the restrictive requirement for very good vacuum. This requirement results from the long time required for preparation and detection of ions and the necessity to avoid ion-molecule interaction of orbiting ions. However, long "incubation" of trapped ions may lead to "self-chemical ionization" and formation of MH^+ ions without the need for introduction of the additional reagent gas.¹⁷ Creation of too many ions produces a considerable space charge and limits not only the dynamic range but also the accuracy of mass determination.¹⁸ FT-MS instruments present an entirely new opportunity for the observation of ion stability.¹⁹ Designed frequency pulses permit expulsion of selected ions while keeping others in the cell.²⁰ These retained ions can be observed, sometimes for an extended time, and their decomposition followed in time. Repetition of this process offers $(\text{MS})^n$ measurement not limited by the number of sectors.

The high resolving power of the FT-MS instrument decreases with mass, and this is considered a drawback in the analysis of high molecular weight compounds. However, ionization methods producing multiple charged ions (see below) shifts m/z to a lower range, where measurement could be performed with optimal resolution and sensitivity. FT-MS is only slowly gaining the acceptance it really deserves. Its demanding vacuum requirements, combined with a substantial time for data processing, especially when compared to certainly more robust and efficient quadrupole or even sector instruments, plays a prohibitive role in this respect. Remarkable potential, presented by ultra-high resolution and multiple MS/MS can be of special significance in structure elucidation of compounds for which mass spectrometry is the only source of information (see example below: 15-hydroxylated bile acids). The resolving power of FT-MS ought to permit, for example, differentiation of ions differing by $^{12}\text{C}^2\text{H}/^{13}\text{C}^1\text{H}$. For such doublets at mass 500 amu the difference of 2.9 mmu requires resolving power of 170,000, which is not offered by other instruments. Isotopic measurements of this type can be of great value in metabolic and biosynthetic studies.

The ion trap, a simple device conceived in the 1950s, uses the same volume for ion formation and analysis enclosed between three electrodes of a

hyperboloid symmetry.²¹ Top and bottom cap electrodes are perforated, the EI filament placed outside produces electrons injected to the trap cavity, while the opposing electron multiplier detects ejected ions. Similar to a quadrupole filter, a direct "U" and a radio-frequency potential "V" [in a form of $U+V\cos(\Omega t)$] is applied to the central ring electrode, and the resulting field traps oscillating ions. Instability of ion trajectory and the consequential ejection of ions with increasing m/z is caused by varying this potential. In the presence of gas, usually helium of a GC instrument at about 10^{-3} Torr, unit resolution in a mass range of over 600 amu can be obtained. The gas restricts the trajectories of oscillating ions and increases the available mass range. A sequence of events, to some extent resembling FT-MS, is required for ion formation and ejection. The simplicity of this ion manipulation also permits one to obtain multiple MS/MS analysis of selectively trapped ions. The ion trap, used as mass detector for GC, is characterized by somewhat limited dynamic range and EI spectra containing ions due to "self-CI". The simplicity of the ion trap and the immense potential for ion storage and manipulation have generated interest in its further developments, including an impressive extension of mass range and combination with other ion sources.²²

The Wien filter originates from an idea conceived at the beginning of this century. It is based on a linear analyzer in which magnetic and electric fields overlap in a perpendicular fashion.²³ Accelerated ions are able to cross this filter only if both these fields are mutually canceled:

$$(m/z) = 2 * (B * V / E)^2 \quad [13]$$

Scanning can be performed by changing E or B (preferably) at any selected accelerating voltage V. High accelerating voltage "V", however, significantly improves resolution and ion transmission while relatively high vacuum is required in order to retain sensitivity of detection.²⁴ Theoretically, an unlimited mass range makes this scanning device very promising.²⁵

COMBINATION OF MASS SPECTROMETRY WITH CHROMATOGRAPHIC METHODS

Certainly, impressive analytical applications of mass spectrometry can not be separated from parallel developments in chromatography. The marriage of both techniques brought to life plentiful off-springs of hybrid techniques. The

combination of GC and MS, unquestionably, is and will remain the principal method for analysis of complex mixtures of low molecular weight compounds. Such compounds certainly constitute the prime focus of many areas of chemical and biological investigations. Study and synthesis of larger polymeric compounds also requires precise identification of their low molecular weight structural sub-units.

The combination of a mass spectrometer with a gas chromatograph posed a series of problems, such as pressure compatibility and the requirement for fast scanning to accurately reproduce in-peak-concentration of eluted substances. The sensitivity of a chromatographic technique is proportional to its resolving power, and the same amount of substance eluted as a broad peak may produce a low level signal comparable to that of the noise. If however, the width of the peak is decreased, the same amount of substance produces a distinct pulse of increased detector response over the same level of background and noise. The difficulty of the introduction of a carrier gas into high vacuum was critical for packed column chromatography, but now the widespread application of capillaries of low flow rate has minimized this problem. Direct introduction of the capillary column to the ion source eliminates concentration changes typical for the earlier jet-separator with only a minimal influence on a quality of chromatographic separation. The requirement of fast scanning, mentioned in the discussion of different mass spectrometers, is fulfilled by several instruments.

Thus, quadrupoles and laminated magnet instruments are able to produce a few full scan spectra in a second, while electromagnets with no core can be scanned ten times in a second. This is sufficient for most capillary GC-MS separations, but high resolution chromatography, producing sub-second peak-width, requires an even faster rate. FT-MS, due to its repetitive mode of operation, does not provide considerable improvement. TOF instruments, however, scanning more than 1,000 spectra a second, and array detectors employed for sector instruments may provide solutions if the problem of very fast data acquisition can be solved. It may be worth remembering, that the very first commercial GC-MS system (LKB-9000), based on a 60°, a 20 cm radius, and a 10 K Gauss magnet, initiated new analytical potentials of great consequences for many areas. It could not compete, however, with faster scanning quadrupoles despite their lower resolution and sensitivity.

Not all organic compounds are suitable for direct GC separation, but for many volatility can be introduced by either derivation or by degradation to smaller molecules. This is illustrated for methyl ester-acetates of glucuronides of bile acids, separated on a medium length non-polar capillary column with no

detectable decomposition (Fig. 2). Multiple ways of derivation are of a great value for the identification of known and unknown compounds, even in complex mixtures. The "on-line" derivation in micro-reactors, either before or after the separation, can also be part of a GC-MS system.²⁶

A simple, two capillary column system, employing either solid or split-splitless injector, with one column introduced to a mass spectrometer and the second to a standard FID detector permits one to compare directly GC and GC-MS profiles obtained from the same injection. This is valuable information because, as already discussed, MS detection may not always provide sufficient time resolution to distinguish closely eluting compounds. Other detectors, including FT-IR,²⁷ could also be employed and give the additional structural and fingerprint information (see example "diterpenoid acids").

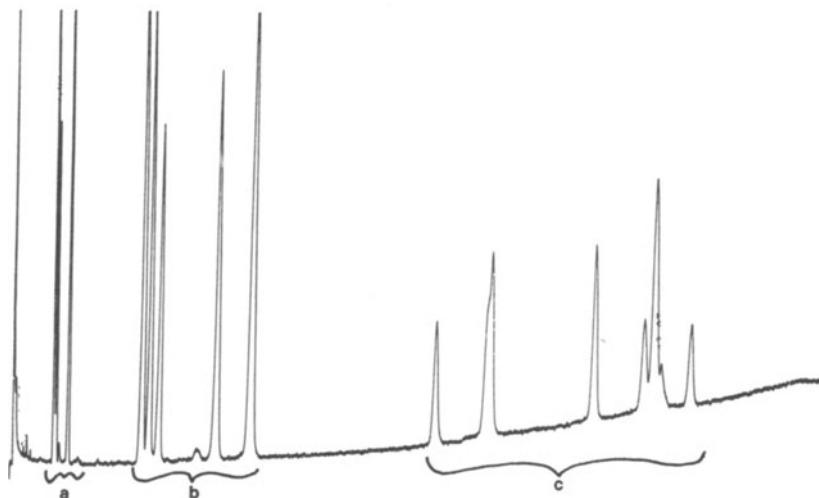


Figure 2. Capillary GC of bile acids and their glucuronides as methyl ester acetate derivatives, 15 m DB-1 column, 0.25 mm ID, 0.1 μ , temperature program from 200°C to 300°C, 2°C per min: Group "a": C₂₀ acids, 3 β ,5 β , 3 α ,5 β , and 3 β ,5-ene; Group "b": C₂₄ acids, 3 α ,5 β (lithocholic), 3 β ,5-ene, cholesterol, 3 α ,7 α ,12 α (cholic) tri- and di-acetates; Group "c": 3-O- β -glucuronides of C₂₀ 3 α ,5 β , C₂₀ 3 β ,5 α , C₂₀ 3 β ,5-ene, C₂₄ 3 α ,5 β , 3 β ,5-ene, C₂₄ 3 α ,7 α (chenodeoxycholic), C₂₄ 3 α ,7 α ,12 α (cholic), and C₂₄ 3 α ,7 α ,12-keto.

Liquid chromatography still presents a considerable challenge for the direct combination with a mass spectrometer, with limitations imposed on flow rate, solvent composition, and ionization modes. Functional "on-line" devices include introduction of residues after solvent removal on a moving-belt or wire, direct liquid introduction, thermo-spray ionization, atmospheric pressure ionization, particle beam, and the continuous supply of column eluate to the fast atom bombardment probe. Combination of mass spectrometry with capillary zone electrophoresis and super critical fluid chromatography poses similar problems.

Direct combination of chromatographic techniques with a fast scanning mass spectrometer as a highly specific detector constitutes a powerful analytical tool for analysis of the complex mixtures typical for natural, biosynthetic, and even synthetic products. This quickly available analytical information considerably enhances the efficacy of product isolation and purification. Thus, in a search for new compounds of a specific class, one may be able to recognize all known compounds by using a library of spectral and chromatographic data of available standards and then to focus only on compounds which may not be identified this way. An alternative is a tedious purification, frequently resulting in isolation of known compounds and their identification only after the complete spectral analysis. Multidirectional combination of several chromatographic techniques permits one to screen for a whole class of compounds. The additional dimension given by a mass spectral "fingerprinting" highly increases the probability of the correct identification. A "fractal" character of mixtures of natural origin is clearly revealed upon the sequential chromatographic fractionation, revealing multiple components frequently present at each level of separation. The high resolution of capillary GC-MS is especially valuable in this respect.

ION FORMATION

Electron Impact Ionization (EI)

This method certainly represents the most popular way of obtaining spectra of organic compounds. The EI ion source is built around a hot filament (cathode) and electron trap (anode) producing an electron beam parallel to slits and a magnetic field. In order to enhance ionization efficiency, small magnets placed on the opposite sides of the ion source induce a spiral path for these electrons. Their energy, measured in eV, is determined by the difference of potential between these two electrodes. Partly isolated from the main vacuum

chamber which is at 10^{-6} - 10^{-7} Torr, the "ion volume" has somewhat higher pressure of $\sim 10^{-5}$ Torr. Gases are ionized by the loss of electrons in a process of low probability (~one in a million). Electron capture, leading to negative ions, is much less probable for most organic compounds. Electrons with sufficient energy, higher than the ionization energy of a compound (on the order of 10 eV) are required for electron removal. Nevertheless, the ion currents of $\sim 10^{-10}$ A usually obtained are sufficient for very sensitive detection. Electron impact is limited to compounds with a sufficient vapor pressure in vacuum. For many compounds it demands increased temperature of the ion source, GC-transfer lines, and heated probe for substances introduced directly. Efficiency of ionization and excess energy delivered to the ion depends upon the energy of ionizing electrons, which is usually sufficient for further, sometimes extensive ion fragmentation. The appearance energy is the minimal energy at which given fragmentation shows in the spectrum. Consequently, spectra measured at a lower eV (<20 eV) usually contain less fragment ions and a proportionally more pronounced molecular ion. However, not only the electron energy, but also the temperature of the ion source, affects fragmentation.

The molecular radical ion $M^{+\cdot}$, formed in the above process by a loss of one electron from a lone-electron pair, π or σ bonds, has an odd number of electrons. Its fragmentation can occur in two ways. In one, $M^{+\cdot} \rightarrow R^{\cdot} + D^+$, a radical R^{\cdot} and even electron daughter ion D^+ are formed, in another, $M^{+\cdot} \rightarrow D^{+\cdot} + N$, a new odd electron radical ion $D^{+\cdot}$ is formed together with a neutral fragment N. The nominal mass of these ions depends upon their elemental composition. For C, H, O, halogen-containing molecules the nominal mass of odd electron ions is always even, whereas even electron ions have an odd nominal mass; this rule is reversed with the presence of an odd number of nitrogen atoms.

Fragmentation

Fragmentation of EI spectra (and to some extent induced by CID), provides a "fingerprint" for identification of known compounds. In this respect, fast searching computer algorithms of large data bases of available spectra are of great help. Spectra, however, may not be unique and the likelihood that isomeric or structurally similar compounds, including even homologous series with undetectable molecular ions, may produce almost identical mass spectra, ought to be always remembered. Moreover, fragmentation, if correctly interpreted, can be used for structural assignments. For many classes of natural products vast experimental information is available as a point of reference.

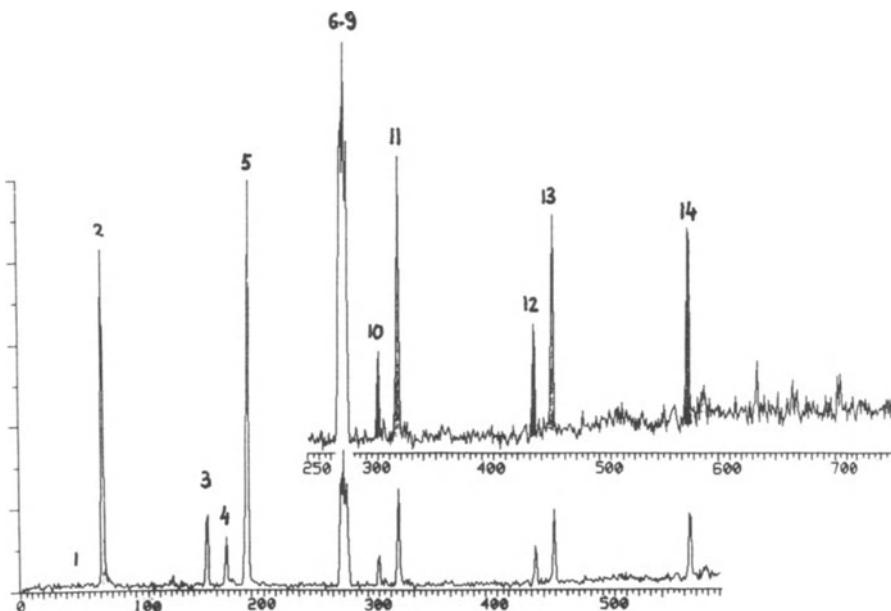


Figure 3. Fragment of capillary GC-MS chromatogram of the silylated neutral aliphatic fraction obtained from feces of the Australian opossum; total ion current 200-700 amu; DB-5 column, 30 m, 0.3 mm ID, 0.1 μ ; temperature program 200°C to 280°C, 5°C per min; EI-MS at 20 eV, 100-600 amu. Components 1 and 3 n-hydrocarbons C₂₇ and C₂₉; 2, 5, 11, and 13 primary alcohols n-C₂₄, n-C₂₆, n-C₂₈, and n-C₃₀; 6 symmetrical n-C₂₉ ketone (15-one); 7-9 n-C₂₉ secondary alcohols (10-ol, 14-ol, and 15-ol); 4, 10, 12, and 14 mono-unsaturated primary alcohols n-C₂₆, n-C₂₈, n-C₃₀, and n-C₃₂.

Aliphatic compounds were isolated from the saponified extract of feces of the Australian opossum and analyzed primarily in order to identify microbial products of a novel bile acid this animal produces.²⁸ The aliphatic fraction was isolated by cocrystallization with urea and analyzed by capillary GC-MS both before and after silylation. At least 14 components were distinguished as shown in Figure 3, and, by assuming their aliphatic character, their identification was based on simple mass spectral fragmentation in addition to the reactivity toward silylation and oxidation. For this herbivorous marsupial, all these compounds were of probable plant origin. Compared to the regular cleavage of a normal

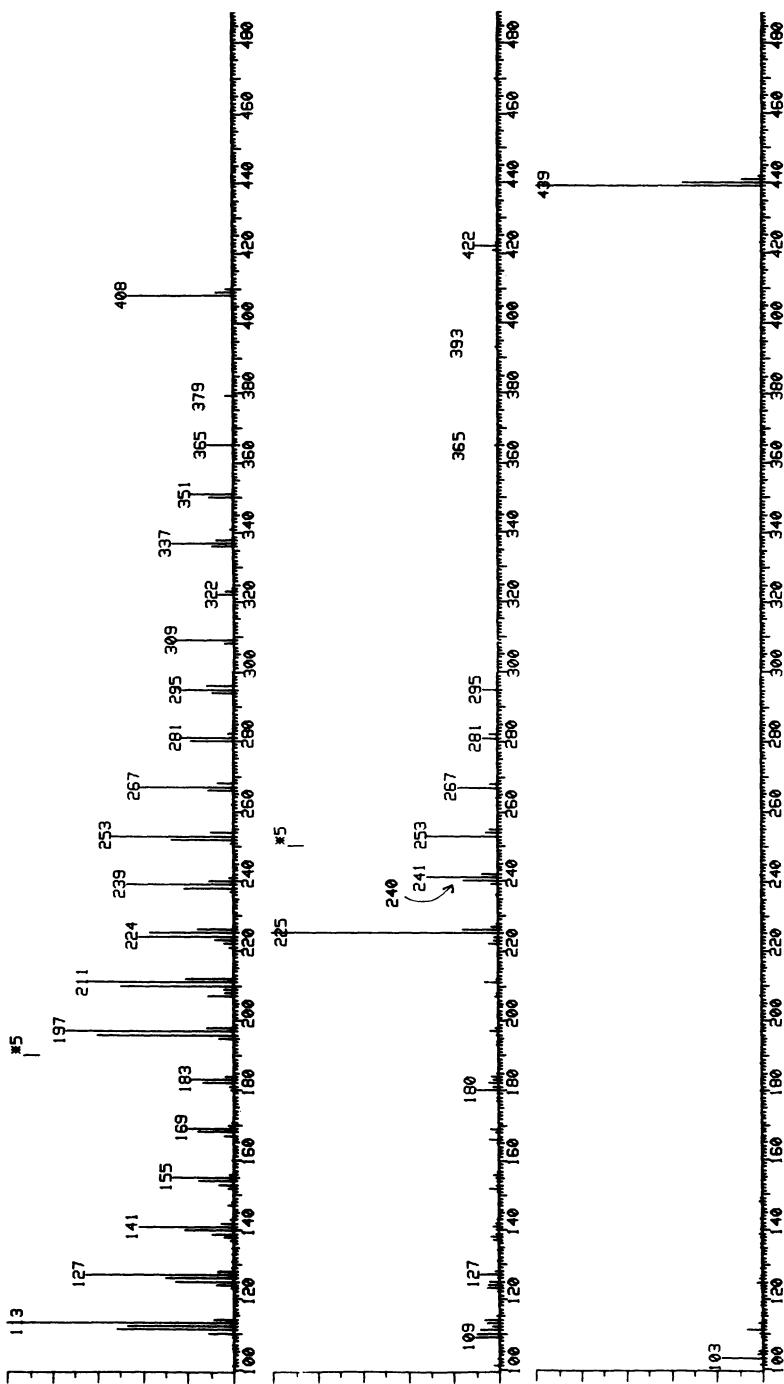
hydrocarbon the introduction of an oxygen function, as exemplified here by ketone and silyl ethers of primary and secondary alcohols, results in a diagnostic fragmentation (Fig. 4).

Monomorin I, the well known ant venom, was identified as one of many related compounds in the methylene chloride wash prepared from several ants of *Monomorium sp.* which abundantly infested my two MS laboratories in Houston, TX. The EI spectrum (Fig. 5) shows simple fragmentation resulting from eliminations of H, Me, and Bu radicals suitably located with respect to the nitrogen atom. Notably, all these fragment ions (with even electron number) are of the even nominal mass due to the presence of one nitrogen atom in the molecule.

Protected glucuronides reveal that if the parent substance is not directly amenable to EI, either due to involatility or instability, derivation frequently may provide a suitable compound. Methylated and acetylated glucuronides of steroidal acid illustrate such a case. Isomeric glucuronides can be formed for bile acids of a different side chain size and differ in the attachment point of the sugar molecule, i.e. either to a hydroxyl or a carboxyl group.²⁹ Their derivatives have different molecular weight and EI fragmentation (Figs. 6, 7) and such an easy distinction with fast atom bombardment is not possible. Moreover, stable methyl ester-acetates of glucuronides are directly accessible from the straightforward chemical synthesis, but only hydroxyl-attached compounds can be deprotected without hydrolysis of the glycoside bond. Thus, for their identification in natural sources, it is sufficient to perform a simple derivation of the extracted material and compare it directly to synthetic standards. This simple protection is also preferable for GC and NMR characterization.^{30,31}

Chemical Ionization (CI)

CI, applied for the first time in 1952 (V.L. Talrose), represents another method of ion formation. For the most part, it is limited to compounds with a sufficient volatility. The ion source, similar to that of EI or even the same, is gas-tight, permitting one to obtain higher pressure, on the order of 0.3-3 Torr. "Reagent gas" introduced to this volume is ionized with an electron beam of several hundred eV. Methane, iso-butane, and ammonia are used frequently. Ions formed from the reagent gas (e.g. for methane, mostly CH_5^+ and C_2H_5^+), prevail and react in the ion-molecule reaction with a vaporized substance introduced to the source. Pseudomolecular (quasimolecular) ions are formed by protonation from compounds which are better proton acceptors than the conjugate base of a reagent gas ions:



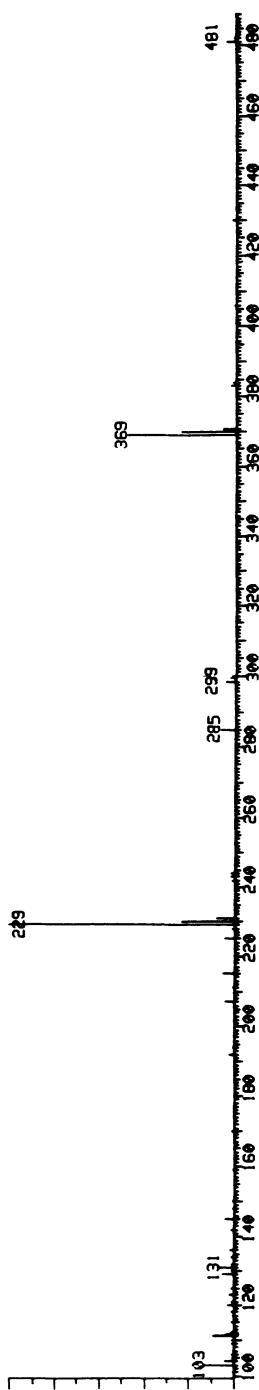


Figure 4: Examples of spectra and major fragment ions used for the structure assignment of aliphatic compounds listed in Figure 3; from the top: n-C₂₉ hydrocarbon (3), m/z 408 (M^+); n-C₂₉ 15-one (6), m/z 422 (M^+), 393 (M-Et), 365 (M-Et), 393 (M-Bu), 240 (product of the McLafferty rearrangement), 225 ($CH_3(CH_2)_13CO^+$); silyl ether of n-C₂₆ primary alcohol (5), 439 (M-Me), 103 (CH_2OTMS); silyl ether of n-C₂₉ 10-ol, 481 (M-Me), 369 (CH₂(CH₂)₁₀CHO⁺TMS), 229 (CH₂(CH₂)₈CHO⁺TMS); ions at 285 and 299 due to impurities of isomeric 14 and 15-TMS ethers.

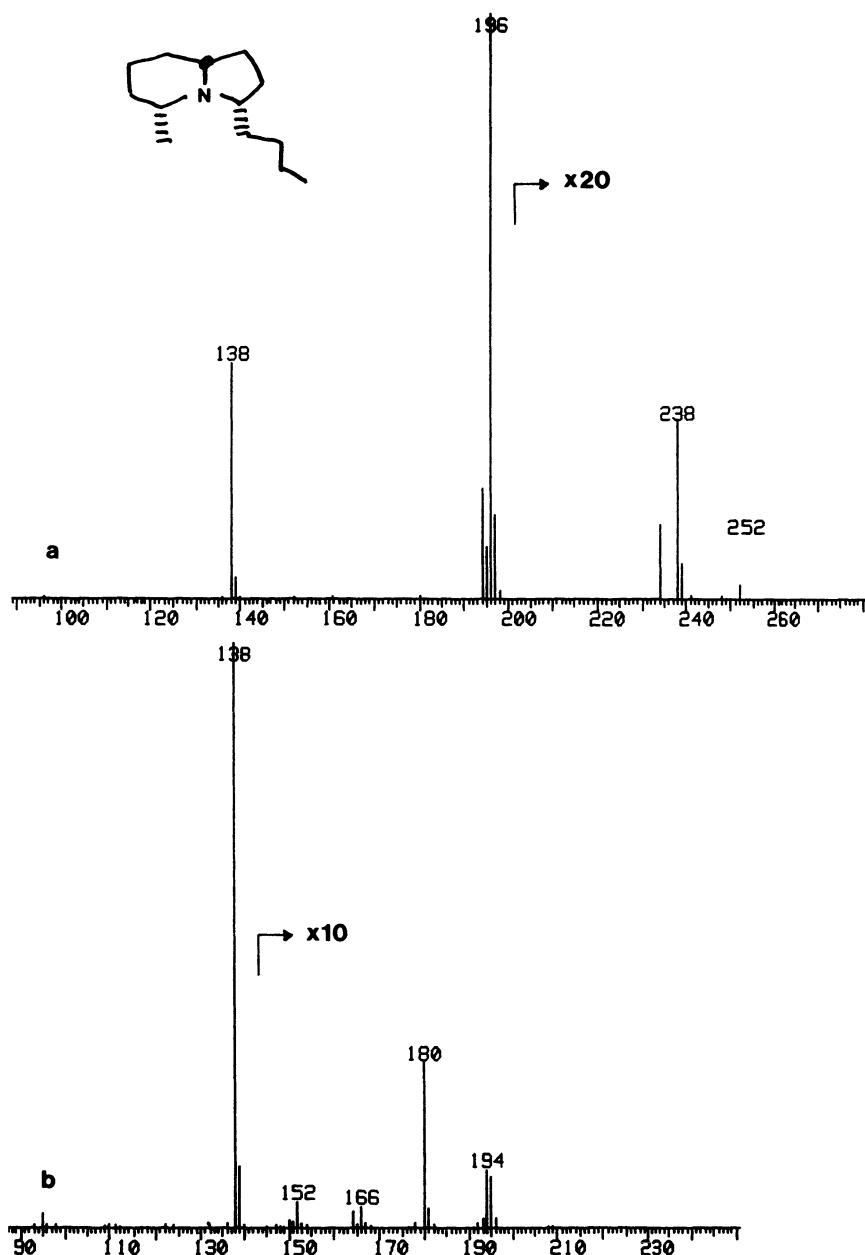
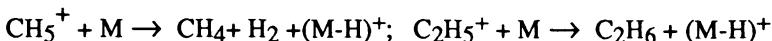


Figure 5. Iso-Butane CI (a) and 20 eV EI (b) spectrum of monomorin I. Both spectra obtained by GC-MS analysis of a complex mixture of related compounds present in the ant venom.



Conversely, compounds which are stronger Brönsted acids transfer hydride to the reagent gas ion:



Because the ionized gas at relatively high pressure is able to "cool" ions due to short mean free path and many collisions lowering the excess of energy, energy transferred to newly formed ions is relatively low and ions are more stable and less prone to fragmentation. The relative stability of pseudomolecular ions also results from even electron character as opposed to the "usual" radical molecular ion. Ions resulting from clustering with reagent gas species can also be formed, as illustrated for the iso-butane CI spectrum of monomerin (Fig. 5). This "reagent-ion-capture" represents another mode of a possible CI, and may not be easily distinguished from products of the "real" chemical reaction occurring with reagent gas molecules. The third mode of ionization is a charge transfer in which a "normal" molecular ion $\text{M}^{+\bullet}$ is formed. If all three ions, $(\text{M}-\text{H})^+$, $\text{M}^{+\bullet}$, and MH^+ , in addition to ion clusters with reagent gas, are formed, a complex pattern results. The "chemical" nature of CI permits one to adjust ion-molecule interaction by selection of a reagent gas or their mixtures and provides a selective and efficient method of ionization. Also a deuterated gas (e.g. ammonia and water) can be used for CI, permitting one to count the number of active hydrogen atoms. However, the formation of strong molecular and pseudomolecular ions may not happen under CI conditions and fragment ions may be observed instead.

Capture of an electron leading to a negative ion, with or without subsequent dissociation, may also take place under CI conditions. For compounds with high electron affinity, such as quinones, nitro, and halogenated compounds, this ionization mode provides an extremely sensitive method of detection. Special derivatives, e.g. esters of halogenated acids, are commonly used in order to introduce such properties to the analyzed compound(s).

Atmospheric Pressure Ionization (API), Thermo-spray (TS) Electrospray (ES) and Particle-beam

The API source is suitable for direct interfacing with many chromatographic and electrophoretic techniques.³² The ionization of volatile compounds

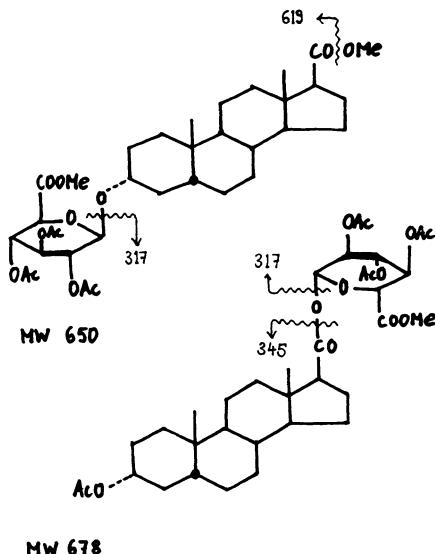


Figure 6. Structures and origin of main fragment ions of methyl-acetyl derivatives of hydroxyl- and carboxyl-linked glucuronides of $C_{20} 3\alpha,5\beta$ steroidal acids; see Figure 9b and 9c for their spectra.

resembles that of CI, but occurs at much higher pressure in a plasma produced either by corona discharge or radiation. "Cool" pseudomolecular ions, in addition to sometimes extensive clusters with air, water and solvent molecules, are formed with high efficiency. Only a small part of the sample, however, can enter the mass spectrometer via a sampling pin-hole. Increased temperature of the source and inlet usually lowers cluster formation. The potential of API is fully realized in the MS/MS system and triple quadrupole spectrometers are currently the most common for this application.

The API source, when combined with a liquid chromatographic interface, may also take advantage of another mode of ion formation, i.e., directly from ions present in a liquid phase. Such a process, although not necessarily understood in depth, can be explained by fast desolvation of micro-droplets containing ionic species which creates strong repulsive forces between ions. Single and multiple charged ions may thus be directly transferred into the gas phase and sampled by a mass spectrometer.

This process explains also the operation of *thermo-spray* (TS) and *electrospray* (ES). The formation of droplets and their desolvation in TS is

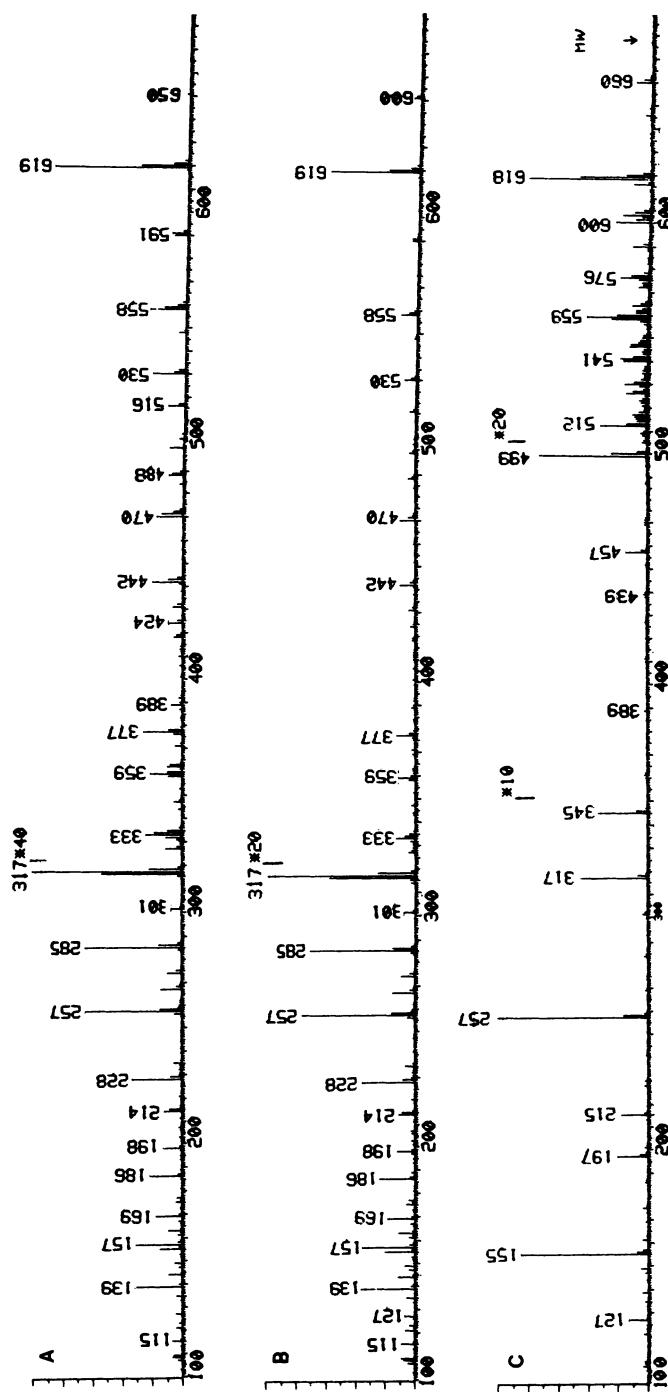


Figure 7: 20 eV EI-mass spectra of methyl-acetyl derivatives of hydroxyl A (3 β ,5 α), B (3 α ,5 β), and carboxy-linked C (3 α ,5 β) C₂₀ steroidal acids. These spectra were obtained from the platinum probe tip directly introduced to the ion source.

facilitated by fast heating and formation of a supersonic jet of vapor and liquid directly in the source.³³ The second method involves formation of micro-droplets by "electrospray" in a potential of a few kV between the outlet of a liquid and the opposite electrode.³⁴ The TS ion source accepts a relatively high flow, ml/min rates of a solvent, which must be a volatile buffer. Ion spray functions with $\mu\text{l}/\text{min}$ flow rates. Positive TS spectra for differently functionalized bile acids (Fig. 8) illustrate types of ions formed and their remarkable dependence on the number and configuration of hydroxyl group (Fig. 9). The formation of multiple charged ions by ES ionization is important.

This revolutionary method (Aleksnadrov³⁵) is directly applicable to large polypeptide (and oligonucleotide) molecules on the order of 100,000 amu, and can accept the number of charges close to that of the number of monomers.³⁶ Consequently, values of m/z , even for large molecules, are in the mass range available even for quadrupole instruments, which despite low resolution can offer relatively precise (0.005 %) mass measurements. This allows calculation of the parent molecular weight from the m/z interval between $(M+nH)^{n+}$ and $(M+nH+H)^{(n+1)+}$ ions. In practice many such pairs are usually available. Even spectra of mixtures can be "deconvoluted" to give parent molecular weights of individual components. With quadrupole instruments, the resolution of isotopic ions for larger molecules, or even ions due to additions of Na^+ and K^+ , may not be possible. Equally, the possibility of CID performed on multiply charged ions, due to the inherent complexity of such fragmentations, will certainly require much better resolving power.

Particle beams are another method of desolvation of a chromatographic eluate, and produces particles which, when transferred into the ion source, may be ionized by different methods, including EI and CI. This is in contrast to the three above methods in which coupling of the chromatographic outlet to the mass spectrometer includes the process of ionization.³⁷

Field Ionization (FI) and Field Desorption (FD)

These are two related methods used for the first time to study compounds difficult to handle by EI and CI.³⁸ Abstraction of an electron to give a molecular ion M^{+*} takes place in a strong electrostatic field (FI). FD, applied to involatile compounds deposited on a heated emitter produces either proton abstraction, to give the M^{+*} ion, or cationization, to give $M\text{H}^+$ or $M\text{Na}^+$. Preexisting ions of organic salts can also be thermally desorbed. When the high

voltage of the emitter is reversed, proton abstraction may lead to negative ions ($M-H^-$). Usually, at a temperature sufficient for desorption, only such molecular ions are present in FD spectra but the increased temperature may lead to additional fragmentation. Despite its relatively low sensitivity FD is suitable for the molecular weight determination, including analysis of mixtures, in a mass range of several thousand amu. FD, in contrast to FAB and SIMS (see below) produces relatively clean spectra with no ions due to matrix substances.

Secondary Ion Mass Spectrometry (SIMS) and Fast Atom Bombardment (FAB)

These are two related techniques utilizing either ions accelerated in a potential of several kV, or atoms obtained by their neutralization. These fast species desorb and produce secondary ions of molecules directly from the condensed phase^{39,40}. Enough energy for this process is supplied by ions (and atoms) of high mass (Cs^+ , Ar, and Xe). The density of the primary ion current, on the order of nA/cm² for "static-SIMS", determines the formation of molecular

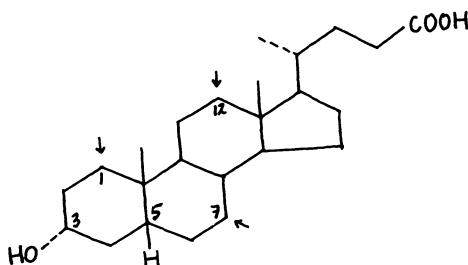
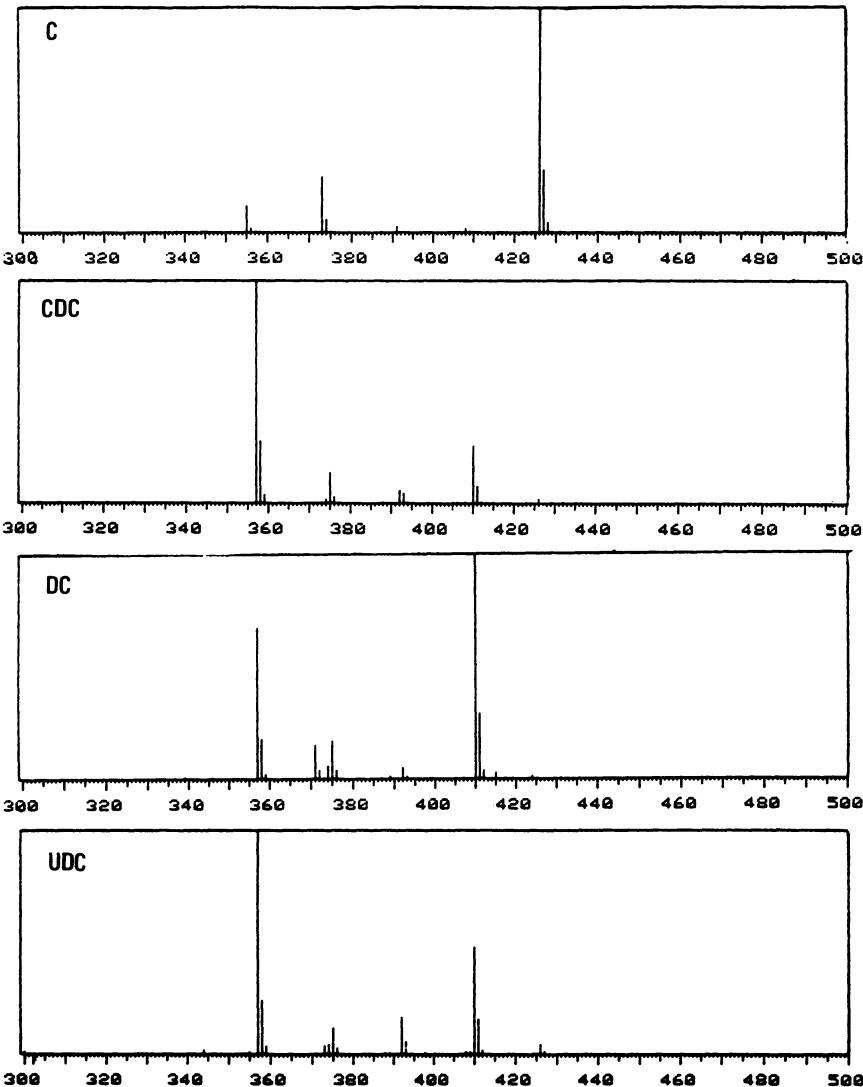


Figure 8. Basic skeletal formula to show structure of major C₂₄ bile acids discussed in the text and Figures 9, 11: LC 3 α -hydroxy-5 β -cholan-24-oic (lithocholic); kLC 3-oxo-5 β -cholan-24-oic; HDC 3 α ,6 α -dihydroxy-5 β -cholan-24-oic (hyodeoxycholic); UDC 3 α ,7 β -dihydroxy-5 β -cholan-24-oic (ursodeoxycholic); CDC 3 α ,7 α -dihydroxy-5 β -cholan-24-oic (chenodeoxycholic); DC 3 α ,12 α -dihydroxy-5 β -cholan-24-oic (deoxycholic); C 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic (cholic); VCA 1 α ,3 α ,7 α -trihydroxy-5 β -cholan-24-oic (vulpecholic).



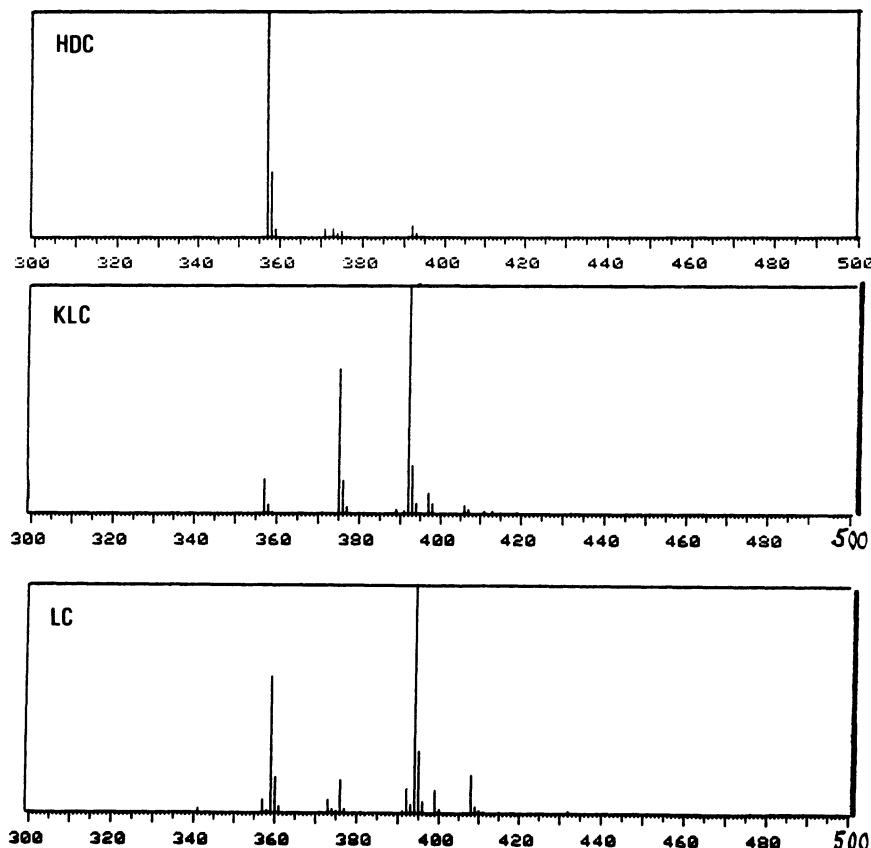


Figure 9. Positive thermospray spectra obtained for bile acid standards;
see Figure 8 for structures of compounds.

ions versus other processes. The formation of secondary ions may not be fully understood yet, but its similarity to CI ionization is quite possible.⁴⁰ The major feature of FAB and "liquid-SIMS" is the presence of a "matrix", usually an involatile liquid, as substrate for dissolution of investigated substances. Most commonly used are glycerol, thioglycerol and other related compounds as well as p-nitrobenzyl alcohol and crown ethers, frequently with additions of acids and salts. Acidic properties of the matrix are important for the extent of protonation leading to MH^+ . Other ions, apart from MH^+ , M^{+*} , and $(\text{M}-\text{H})^-$ for the negative mode, include clusters with alkali metal ions and matrix molecules. This complexity is illustrated for the negative FAB spectra of salicylic and vulpecholic acids with glycerol as matrix (Figs. 10,11). The elimination of this "chemical background" is one of the major advantages of MS/MS. The use of elements with two abundant isotopes permits easy identification of such ions, clusters of MAg^+ , and MTl^+ (compare Table 1) produce easily recognizable doublets in contrast to monoisotopic MH^+ and MNa^+ showing mainly ^{13}C isotopic ions.⁴¹ The appearance of multiple ions corresponding to a single compound in FAB spectra can introduce some difficulties in the interpretation; Figure 12 illustrates the simple addition of a crown ether to a glycerol matrix, which efficiently removes the formation of cluster ions with sodium.

The sensitivity of FAB is influenced by many factors, including the mutual interaction of a compound and matrix molecules.⁴² When mixtures are analyzed, the suppression effect resulting in highly disproportionate response of different components may be especially disturbing. This is illustrated by the negative FAB spectra of taurine conjugated bile acids separated from the bile of the Australian opossum, *Trichosurus vulpecula*. It shows strong suppression of glycerol and tauro-vulpecholic acid by tauro-chendodeoxycholic acid (Fig. 13).

Applied for the first time in 1981,⁴³ FAB due to its apparent simplicity quickly gained popularity as a method applicable to a wide variety of polar and large molecular weight compounds. FAB is one of the principal methods utilized for generation of the molecular ions of polypeptides approaching even several thousands amu.⁴⁴ Fragmentation of such ions under CID conditions, observed in four sector instruments, is used for sequencing.⁴⁵ The first two sectors act as a separatory device selecting one ion only; therefore, a mixture of peptides as obtained from specific enzymatic hydrolysis can be directly investigated without separation. Other large molecules, exemplified by complex lipids and carbohydrates, can also be investigated in this way. Obviously, analysis of pure compounds can be followed by direct spectra in two sector and quadrupole instruments with CID and linked-scanning. The success of such a method of peptide sequencing is primarily due to their linear structure and the

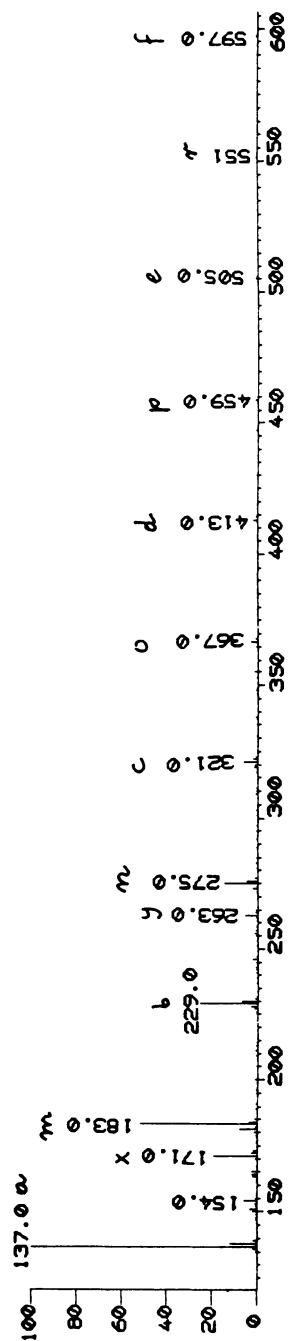


Figure 10: Negative FAB spectrum of salicilic acid with glycerol as matrix; assignment of ion series: a ($(M-H)^-$), b, c, d, e, and f ($(M-H, \text{glycerol}_n)^-$, $n=1$ to 5; m, n, o, p, and r ($(\text{glycerol}-H, \text{glycerol}_n)^-$), $n=1$ to 5.

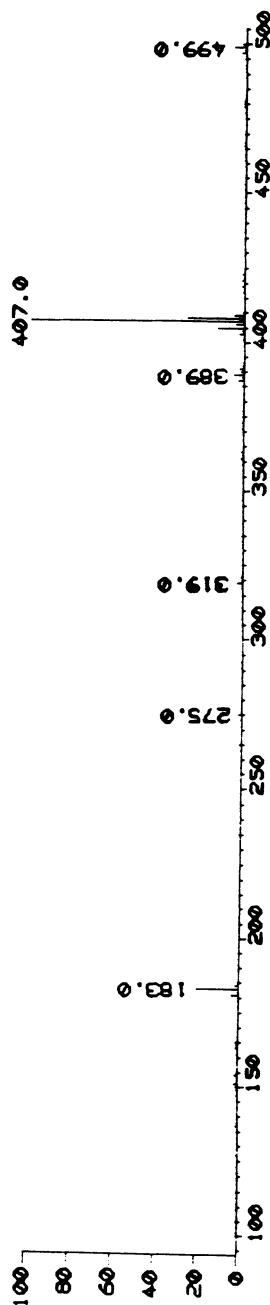


Figure 11: Negative FAB spectrum of vulpecholic acid (see Figure 8) with glycerol as matrix; ion assignment: m/z 183 and 275 as in Figure 10, 389 ($M \cdot H_2O \cdot H^-$), 407 ($M \cdot H^-$), 499 ($M \cdot H^-$, glycerol), ion 815 ($M_2 \cdot H^-$) not shown.

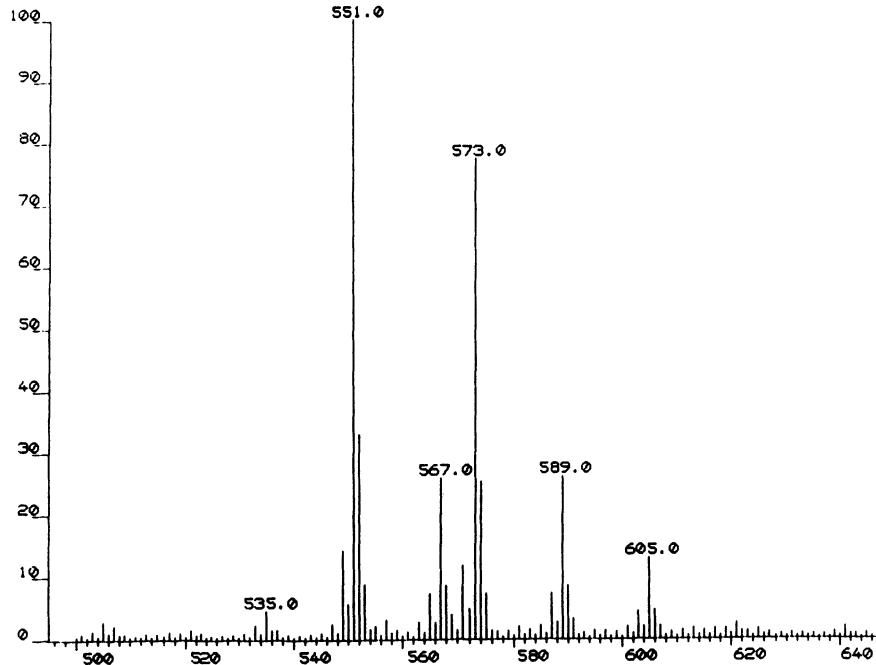
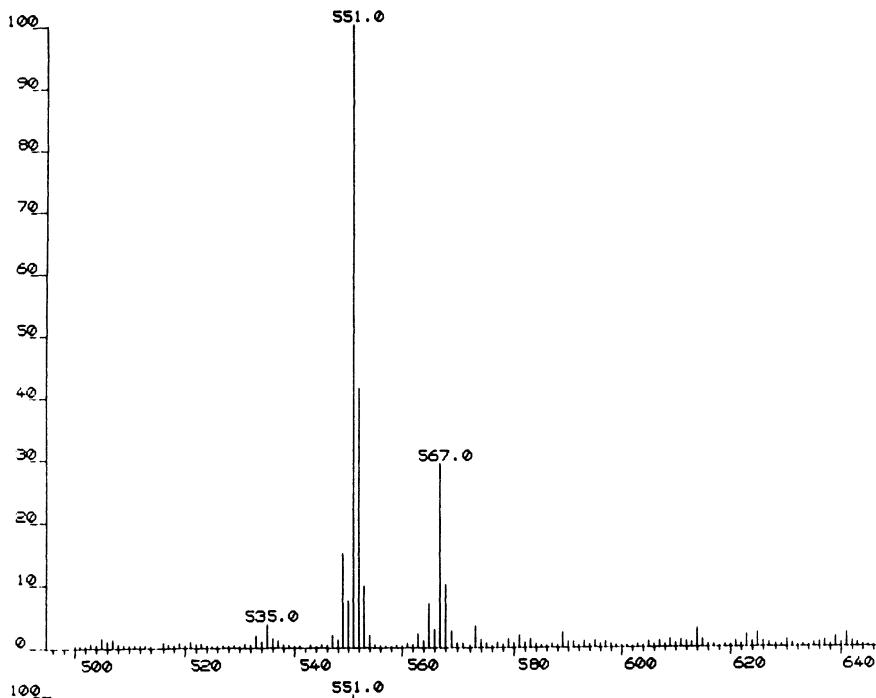
limited number of unique amino acids. Thus, fragmentation of the chain, occurring in regular intervals, is easy to interpret. Simple and specific derivation reactions, as in other cases, make ion assignments easier. In contrast to "wet-sequencing methods," additional modifications of amino acids can be easily disclosed by MS. Complex molecules with branched structures and multiple isomeric units (e.g. sugar fragments), however, present a formidable task for the interpretation of fragmentations. Nevertheless, even a "simple" molecular weight determination for such polar molecules, and the possibility of a confrontation of a suspected structure with observed fragmentation constitutes a significant advancement.

Flow-FAB, the direct introduction of a liquid sample into the FAB target, first as Frit-FAB⁴⁶, is not only a viable method of coupling with known chromatography and electrophoresis but also improves the sensitivity of detection and corrects for suppression effects.⁴⁷

Accurate mass measurement can also be performed with FAB ionization as with other techniques, but is limited by the relatively low sensitivity of this method. Such determination demands addition of a standard to the matrix, e.g. CsI or other similar salts forming extensive high mass cluster ions of the type $(CsI)_nCs^+$, $(CsI)_nI^-$. Also, organic polymeric compounds such as polyethylene glycol can be used as standards. Alternatively, spectra of a standard and the unknown can be acquired in a quick succession from a dual target probe. Obviously, acquisition of the fully resolved spectrum at the mass of several thousands must involve suitable resolving power and very good calibration. The precise mass determination of compounds with high molecular weight creates obvious accuracy problems; the intensity of a molecular ion corresponding to major "low mass" isotopes may be relatively small, obstructing the accuracy of mass determination. The measurement performed on other ions, e.g. M+1, may be effected by the fact that these ions are composed from isotopic ions.⁴⁸

Plasma Desorption Mass Spectrometry (PD-MS)

This has already been mentioned in the discussion of the TOF instrument.¹² Its first application to organic molecules revolutionized the scope of mass spectrometry, demonstrating apparently unlimited possibility in the formation of large ions and their mass analysis in vacuum. The nuclear fission of ^{252}Cf , occurring in addition to α -emission for this radioisotope, produces two smaller nuclides with a huge energy, about 100 MeV. In a PD source, one such fragment supplies the start signal for electronics of the TOF instrument while the second bombards the target, which is a substrate foil with a compound adsor-



bed on its surface. It is assumed that this gigantic energy release in the substrate (e.g. nitrocellulose) ionizes and expels molecules into the gas phase as ions of one or several charges. A broad range of high molecular weight compounds, especially peptides and small proteins, have been analyzed by PD-TOF-MS. Certainly, this technique is now becoming one of the best tools for protein chemists and may supply precise information on purity and molecular weight.⁴⁹ Apart from the relatively precise molecular weight information, many *in situ* reactions can also be performed right on the substrate foil and their products analyzed directly by another determination of the PD spectrum. This is possible because only a small amount of the sample material is used by PD-ionization. Direct enzymatic hydrolysis with proteolytic enzymes may be used for characterization of peptides and terminal sequences. Selective enzymatic and chemical treatment can also be utilized for the detection of sugar and other modifying residues of a polypeptide chain.

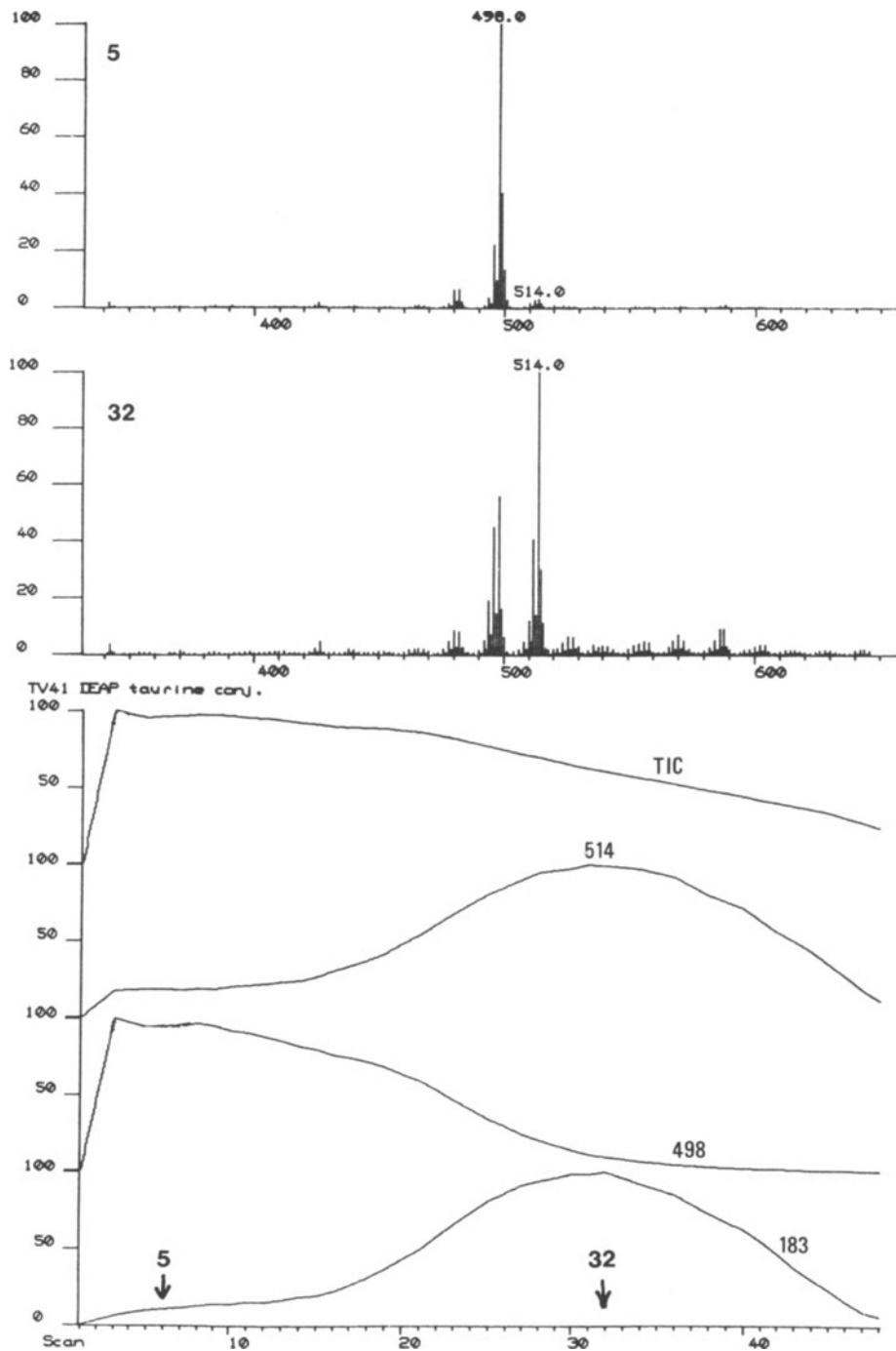
Laser Desorption (LD)

LD can be combined with several different mass spectrometers; FT-MS and TOF-MS are especially important as well adapted to the pulsed ion source. The laser source permits adjustment of wavelength, energy, and time of the pulse. This gives additional possibilities not available for other methods. The introduction of matrix-assisted LD, which involves UV-absorbing matrix molecules (exemplified by nicotinic acid), is especially important for the analysis of peptides and small proteins.⁵⁰

MASS SPECTRA

The quality of a mass spectrum analysis affects its worth for any sort of interpretation. Currently, most of the mass spectra are provided in the form of a computer printout presenting stick diagrams of m/z and their normalized inten-

Figure 12. Negative FAB spectra of glucuronide conjugated fraction obtained from Australian opossum bile; right: in glycerol as matrix; ions m/z 551 lithocholic acids 3-O- β -glucuronide and 567 1 α ,3 α -dihydroxy-5 β -cholan-24-oic acid 3-O- β -glucuronide (M-H)⁻; 573 and 589 corresponding (MNa-H)⁻; 605 (MNa-H)⁻ ion of trihydroxylated bile acid glucuronide; above: same mixture in glycerol-crown ether matrix.



sities. This presentation generally removes additional information contained in the raw data provided by sector instruments. Frequently, such a presentation is misleading and successfully masks major inadequacies of the measurement, since the computer analysis by no means guarantees that the spectrum has been obtained using proper sensitivity, resolution, and calibration. Small deviations in parameters utilized for analysis performed by various MS software may assign nominal masses to either lower or higher value, especially if atomic composition deviates from that of an average organic compound. This is also especially important for FAB spectra obtained for compounds of high molecular weight. Also important is the exclusion from interpretation of any background ions, either from the mass spectrometer itself, of air gasses, residual hydrocarbons and other pump oil residues, stationary phase from gas chromatography columns and ions due to the FAB matrix, as well as ions due to previously run samples (!). A simple background subtraction may not always be fully successful in this respect, especially if sample ion signals are weak and comparable with those of the background, as may frequently occur for GC-MS and HPLC-MS. The proper appearance of a mass spectrum, i.e., the presence of ions of both high and very low intensity, especially isotopic ions +1, +2, and +3 is useful for the quality assessment. Directly disputing the quality of spectral data provided by the MS laboratory, however, may not be the best approach; if knowledgeable questions are asked the quality of data may be drastically improved. The relative simplicity of mass spectral analysis permits everyone to become familiar with the behavior as well as the appropriate derivation and ionization methods of the class of compounds in which one is interested.

Nominal and Accurate Mass

Simple rules enable worthwhile understanding of apparently complex mass spectra. These rules are, for the most part, the play of numbers. In essence, only the knowledge of atomic masses and abundances of isotopes is required for spectral interpretations (Table 1). Integral combinations of atomic

Figure 13. Negative, probe FAB spectrum of taurine conjugated fraction obtained from the bile of the Australian opossum in glycerol as matrix; bottom: total ion current (TIC) and "mass chromatograms" at m/z 183 ($\text{glycerol}_2\text{-H}^-$); 498 tauro-cheno-deoxycholic acid, and 514 tauro-vulpecholic acid ($\text{M}-\text{H}^-$) ions; top: mass spectra of scans 5 and 32.

Table 1: Atomic masses and abundances of isotopes of major elements

Element	unit mass	accurate mass	natural abundance
H	1	1.00783	100
H (D)	2	2.01410	0.015
Li	6	6.01512	8.0
Li	7	7.01600	100
C	12	12.00000	100
C	13	13.00335	0.011
N	14	14.00307	100
N	15	15.00011	0.36
O	16	15.99492	100
O	17	16.99913	0.04
O	18	17.99916	0.2
F	19	18.99841	100
Na	23	22.9898	100
Si	28	27.97693	100
Si	29	28.97649	5.1
Si	30	29.97376	3.4
P	31	30.99376	100
S	32	31.97207	100
S	33	32.97146	0.8
S	34	33.96787	4.4
S	36	35.96709	0.015
Cl	35	34.96886	100
Cl	37	36.96590	31.9
K	39	38.96371	100
K	41	40.96184	7.4
Br	79	78.91835	100
Br	81	80.91634	98
Rb	85	84.9117	100
Rb	87	86.90918	38.6
Ag	107	106.90509	100
Ag	109	108.9047	93
I	127	126.90435	100
Cs	134	133.90509	100
Tl	203	202.9723	41.8
Tl	205	204.9745	100

masses can be done with unit precision (nominal mass, low resolution) or high precision (accurate mass, high resolution). For hydrocarbons, if the number of H atoms exceeds 128 ($C_{63}H_{128}$), the calculated exact mass is 885.0016 but corresponds to the nominal mass of 884, and such proper correction ought to be included in analyzing spectra of larger molecules.

Most ionization methods produce ions with only a single charge but ions carrying two and even more charges can also be formed and the measurement of m/z may not necessarily give the mass of an ion. However, "z" can be deciphered if corresponding isotopic ions are present. The pattern of singly and doubly charged molecular ions observed for EI spectrum of alizarin serves as an illustration (Fig. 1c). Interpretation of multiply charged ions by ES and related methods may also benefit from the direct observation of isotopic patterns if measured at sufficient resolving power.

Molecular Ion

Identification of a molecular and pseudomolecular ion is usually the first, but unfortunately, frequently the only step in the interpretation of mass spectra. A common approach for the characterization of a new compound is either to obtain consistent elemental analysis or to perform a high resolution measurement (HR) on a molecular ion, usually with only 10,000 resolving power. It ought to be remembered that this measurement, under most circumstances, can not be relied on as the sole source of the information for the atomic composition of unknown compounds.

However, if the approximate elemental composition is known, finding the expected value for the accurate mass of a molecular ion dramatically increases the probability of such assignment. Frequently, required information can be obtained from the same mass spectrum. By no means is HR-MS a method of providing purity information. Isomeric compounds are frequent for synthetic reactions, encountered for natural products, and obviously produce the same molecular ion. Even the presence of structurally similar compounds in mixtures may not be directly recognizable from the mass spectrum because differences in molecular weight may correspond to one of the common eliminations.

Prediction of atomic composition solely from the nominal mass of an ion is impossible, because even at low values of m/z many combinations of atomic masses may correspond to the same integral value (Table 1). The classical example is mass 28 of the singly charged ions of $^{12}C^{16}O$, $^{14}N_2$, $^{12}C_2^{1}H_4$, and $^{12}C^{1}H_2^{14}N$.⁵¹ The difference between their accurate mass and 28 is -5.1, 6.1, 31.3, and 18.7 mmu, respectively, and the definitive separation can

be obtained with the resolution of 2500. It is calculated from $M/\Delta M = 28/0.011$, where 0.011 is the smallest difference of 11 mmu between the mass of CO and N₂. Obviously, the differentiation of various atomic combinations corresponding to ions of a larger mass requires much higher resolving power. Combinations of elements producing the same nominal mass and which are especially important for the analysis of organic compounds are listed below, together with the resolving power required for their complete separation at m/z of 500 amu.

		ΔM (mmu)	$M/\Delta M$
¹ H ₁₂	¹² C	-93.9	5,300
³² S	¹² C ₂ ¹ H ₈	-90.5	5,500
¹⁶ O	¹² C ¹ H ₄	-36.4	14,000
¹⁶ O	¹⁴ N ¹ H ₂	-23.8	21,000
³² S	¹⁶ O ₂	-17.8	28,000
¹⁴ N	¹² C ¹ H ₂	-12.6	40,000
¹⁴ N	¹³ C ¹ H	-8.1	62,000
¹³ C	¹² C ¹ H	-4.5	111,000

This demonstrates a formidable difficulty in the unambiguous prediction of the atomic composition utilizing HR-MS as the sole source of information. Again, using alizarin as an example, 55 combinations of C, H, O, and N are listed for the nominal mass 240 in Beynon's original table.⁵¹ This list includes nine C, H, O combinations and two C, H combinations, listed in decreasing order of the accurate mass recalculated to the ¹²C standard. If the presence of other elements is excluded, all these twelve combinations can be distinguished by the mass measurement with the accuracy better than 5 mmu. Their full separation would require the resolving power of 16,000.

C ₁₇ H ₃₆	240.2817	ΔM
C ₁₆ H ₃₂ O	240.2453	36.4
C ₁₅ H ₂₈ O ₂	240.2089	36.4
C ₁₈ H ₂₄	240.1878	21.1
C ₁₄ H ₂₄ O ₃	240.1725	15.3
C ₁₇ H ₂₀ O	240.1514	21.1
C ₁₃ H ₂₀ O ₄	240.1362	15.2
C ₁₅ H ₁₂ O ₃	240.0786	57.6
C ₁₈ H ₈ O	240.0575	21.1
C ₁₄ H ₈ O ₄	240.0423	15.2
C ₁₇ H ₄ O ₂	240.0211	21.2

For the unknown compound, even if the molecular ion represents the base peak in its spectrum, its assignment may not be unambiguous in the absence of an alternative verification. If MS is the sole source of structural information, this verification may be frequently supplied by different ionization methods and analysis of derivatives. Counting (or at least estimating) the number of hydrogen and carbon atoms in the unknown molecule from ^1H and ^{13}C NMR spectra is a good alternative and complement to elemental analysis. Multiplicity of all ^{13}C signals, corrected by the number of hydrogen atoms connected to heteroatoms (N and O), produces the total number of both carbon and hydrogen atoms. The latter may not be easily available from complex and not well resolved ^1H spectra. ^1H NMR can also be used for the determination of a molecular weight of a pure compound, provided signals due to the known number of hydrogen atoms can be identified. This only very rarely used approach is based on the integration of such a signal in comparison to a standard compound for solutions of the known concentration of both. The independent information about approximate molecular weight helps in the interpretation of mass spectra.

Isotopic ions reflect atomic composition. From the natural abundance and number of atoms for each element relative intensities of additional ions due to heavier isotopes can be calculated (Table 1). At low mass the contribution of chlorine and bromine with abundant heavier isotopes is significant. At the high mass, however, even small amount of ^{13}C and ^{18}O create an extended cluster of isotopic ions. Notably, the center of gravity for such a cluster corresponds to the "chemical" molecular weight calculated from the average atomic mass of all elements, and molecular weight information can be obtained even well below unit resolution. Deviation from the calculated isotopic pattern, especially for the molecular ion, indicates contribution of either another compound or an additional loss or gain of hydrogen atom(s). Calculations of ion abundances and HR data make the interpretation more reliable and programs for such calculations are included in a standard MS software.

Such a calculation performed for the molecular ion alizarin $\text{C}_{14}\text{H}_8\text{O}_4$, including up to three atoms of ^{13}C , and one atom of ^2H , ^{17}O and ^{18}O respectively, produces intensities of 100.00, 15.99, 2.01 and 0.15 % for ions at m/z 240, 241, 242, and 243. Accurately measured intensities are 100, 15.7, 2.7, and 0.2 % respectively, but the additional ion at m/z 239 has an intensity of 11.8 %. This ion, evidently due to $(\text{M}-\text{H})^+$, ought to produce its own M+1 and M+2 ions, and, if further correcting calculations are performed, experimental intensities are in agreement with those calculated. The higher intensity of the ion at m/z 242 (+0.7 %) may indicate the presence of hydroquinone impurity in the analyzed sample of alizarin.

The validity of HR-MS measurement for the molecular ion of alizarin (the value of 240.0433 was obtained with a 3 sec. HR scan at 15,000 resolving power) can be discussed in view of the above. The $(M-H)^+$ ion has its own $M+1$ due to one ^{13}C (240.0378) and contributes to the measurement performed at M^+ (240.0423) and the difference of 4.5 mmu (19 ppm) requires resolving power of 50,000. Fortunately, the intensity of this interfering ion is low and does not influence the accuracy of determination at the medium resolving power. HR-MS measurements at medium resolution power, however, in cases of complex molecular and pseudomolecular ion clusters, require careful selection of ions least altered in their position by possible isotopic ions. Such ions, located at the lowest mass side of the cluster may not be sufficiently abundant to enable the precise measurement.

The nominal mass of molecular ions, obtained from low resolution spectra, may be sufficient for a reliable assignment of the molecular formula. Often, specific methods of isolation indicate not only the chemical character but also the structural class of an isolated compound and the prompt determination of its molecular weight, even with unit precision, may lead to advanced structural conclusions. Using underivatized terpenoids as an example, assignment of a class, prediction of the number of oxygen atoms and unsaturation equivalent is easily obtained. The removal of one or more carbon atoms, esterification, substitution with sugar molecules, and other derivations, however, may not be directly recognized from low resolution spectra, unless the number of carbon atoms is unequivocally verified.

Lactucin was the first sesquiterpenoid lactone ever isolated.⁵² It is commonly found in the milky juice of different *Lactuca* species. Analyzing related *Compositae* plants,^{53,54} we used mass spectra for the initial characterization of purified compounds. *L. serriola* produced four components, each with apparently dominant molecular ions at m/z 276, 262, 260, and 410 indicating that the first three represent sesquiterpenoid lactones: $C_{15}H_{16}O_5$, $C_{15}H_{18}O_4$, and $C_{15}H_{16}O_4$. Since the first "276 compound" was identical with lactucin (mp and other spectral data) we assumed that another two are related deoxy-compounds. In fact, compound "262" was identical with jacquilenin, and "260" was identified as a new lactone "deoxylactucin".⁵³ Methanol extract of *Cichorium intybus* L., also produced lactucin, deoxylactucin, compound "410", and another compound with barely detectable M^+ at m/z 442. Compound "410" (Fig. 14), apart from the molecular ion at m/z 410, showed ions at m/z 258, 240, 229, and 212, resembling those of lactucin, in addition to ions 152 (base peak) and 107. This pointed to lactupicrin, a known ester of lactucin with p-hydroxy-phenylacetic acid (MW 152) for which the esterification position was

incorrectly established.⁵³ Mass spectrum of the fifth compound "442", showed also ions M-H₂O, and M-MeOH, otherwise closely resembling that of lactupirin. This indicated the probable artefactual opening of a lactone with methanol during the extraction, which was not the case for other lactones but for lactupicrin only. Subsequent extraction with acetone rather than methanol precluded its formation.

Cyclotrichosantol, the acetate of a compound isolated from the Indian plant *Trichosantes palmata* was purified by argentation TLC.⁵⁵ Polarity of the parent alcohol was intermediate between sterols and monohydroxylated triterpenoids (e.g. amyrins). Thus, it was expected to be a "4-methyl-sterol" containing only one oxygen atom. Moreover, another component of the same polarity, separated as a monoacetate by argentation chromatography, was identified as cycloeucaleanol acetate, the known 4 α -methyl-sterol (C₃₀H₅₀O). Ions at m/z 482, 440, and 438, present in spectra of this acetate, alcohol, and ketone obtained by CrO₃ oxidation (Fig. 15), indicated the presence of 31 carbon atoms and the C₃₁H₅₂O composition of the parent alcohol. For a tetracyclic triterpenoid with 31 carbon atoms, additional methylation of the original C₈ side chain was feasible. Spectra of the acetate, free alcohol, and 3-ketone, identified by side chain elimination as M-139, indicated a C₁₀ side chain with two additional carbon atoms. Oxidation with OsO₄/IO₄⁻ produced a new keto-alcohol in which the above elimination was replaced by M-141 (replacement of =CH₂ by =O). This clearly indicated the presence of only one double bond, being a methylene located in side chain, and a pentacyclic structure. A cycloartenol type structure, with one methyl missing in the nucleus, was in accord with the observation of the known mode of fragmentation of 9,19-cyclocholestanes. This elimination indicated the presence of only one methyl group at C-4. Another fragment ion M-84, replaced by the ion M-86 for the side chain oxidation product, was explained as the result of the cleavage of the terminal part of the side chain (SC). The distant location of the double bond was in accord with the lack of M-SC-2H fragment usually observed for 22- and 24-unsaturated sterols. The structure of cyclotrichosantol, deduced from these mass spectral data, was fully confirmed by NMR involving one of the first applications of europium shift reagents to natural products (Fig. 16).

Pentacyclic triterpenoids in sunflowers. GC and GC-MS analysis of the saponified extract of one of the *Helianthus* species exemplifies the identification of multiple sterol and triterpenoid components (Fig. 17). The combination of 20 eV EI mass spectra, in most cases with the abundant molecular ion, and GC retention times allows easy identification once isolation and characterization from one source has been completed (Fig. 18, unpublished).

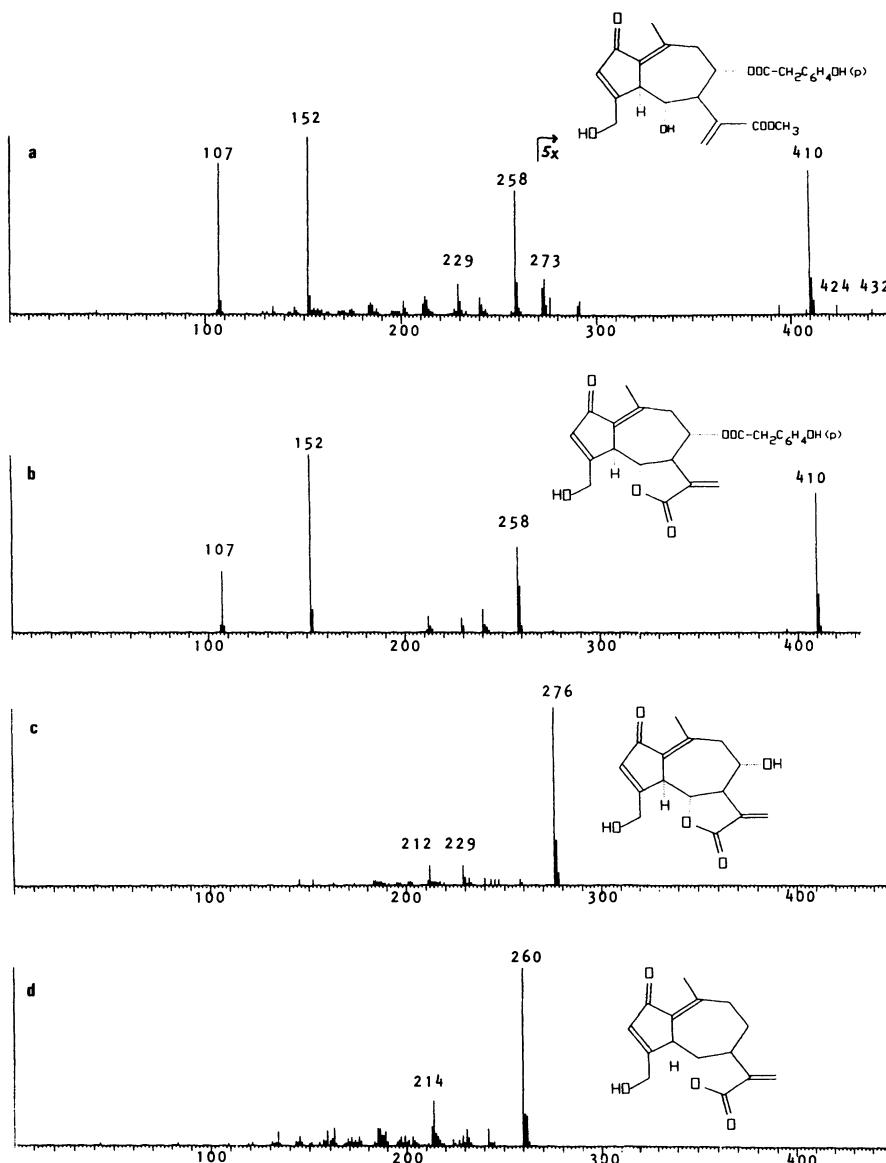


Figure 14. 15 eV EI spectra of deoxylactucin, lactucin, lactupicrin, and "lactupirin-artefact."

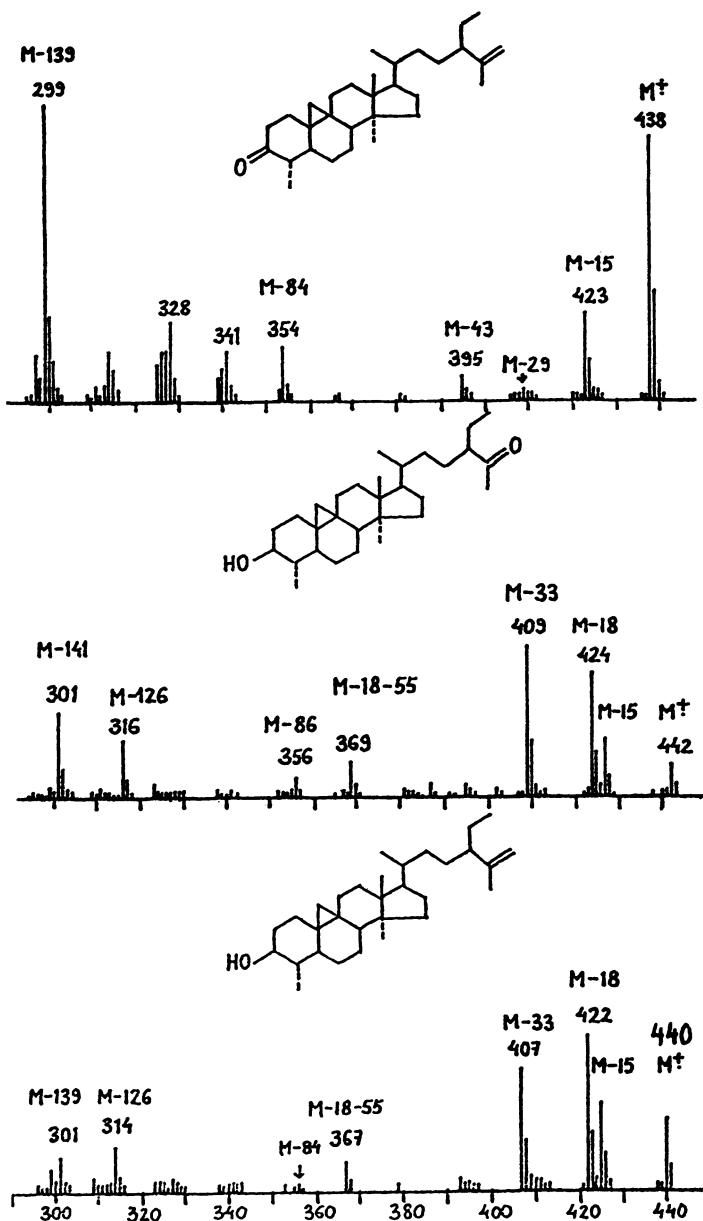


Figure 15. EI spectra of cyclotrichosantol (bottom), and its $\text{RuO}_4/\text{IO}_4^-$, and CrO_3 oxidation products (top).

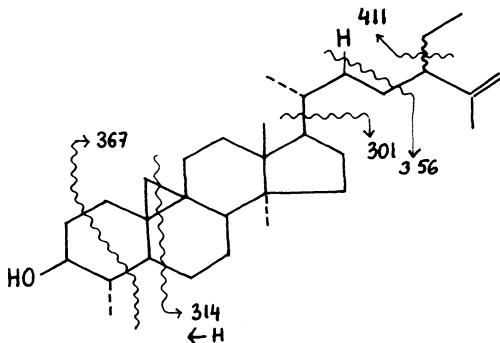


Figure 16. Cyclotrichosantol and its structurally diagnostic fragment ions.

Diterpenoids in sunflowers. Sunflower extracts usually contain various diterpenoids. These compounds are also easily recognized by the GC-MS analysis of crude extracts. Thus the approximate retention time, molecular ion position, and the few immediately following fragment ions are usually sufficient for characterization. The molecular weight 272 indicates hydrocarbons 290 tertiary alcohols as probable hydration products 288 primary alcohols as probable hydroxylation products 286 aldehydes and 302 free acids as their oxidation products. In addition, "274" components are identified as nor-diterpenoids resulting from the auto-oxidation of aldehydes. Once more, since most of these compounds have been isolated and fully characterized from one species,^{56,57} their GC-MS detection in related plants is trustworthy (Fig. 19).

The following set of simple examples illustrates the applications of HR-MS measurements, performed for molecular and fragment ions, in making decisive structural assignments.

Alkaloid from Sida rhombifolia, SR-2-2: a compound isolated from this Indian plant illustrates the frequent confusions encountered in studies of natural products. The first extract sent from India contained a small amount of alkaloids, and their reisolation in quantity was subsequently attempted. As new extracts did not contain a detectable amount of alkaloids, it was concluded that the first isolation resulted from a contamination with another "true" alkaloid containing plant. One extract, however, yielded a small amount of crystalline product, "SR-2-2" mp 211-213°C. Its composition was calculated from the

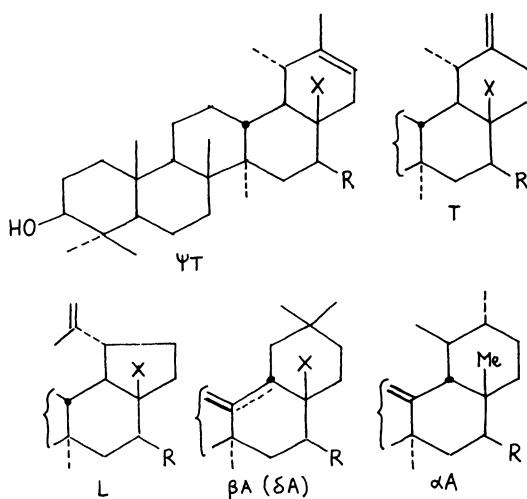
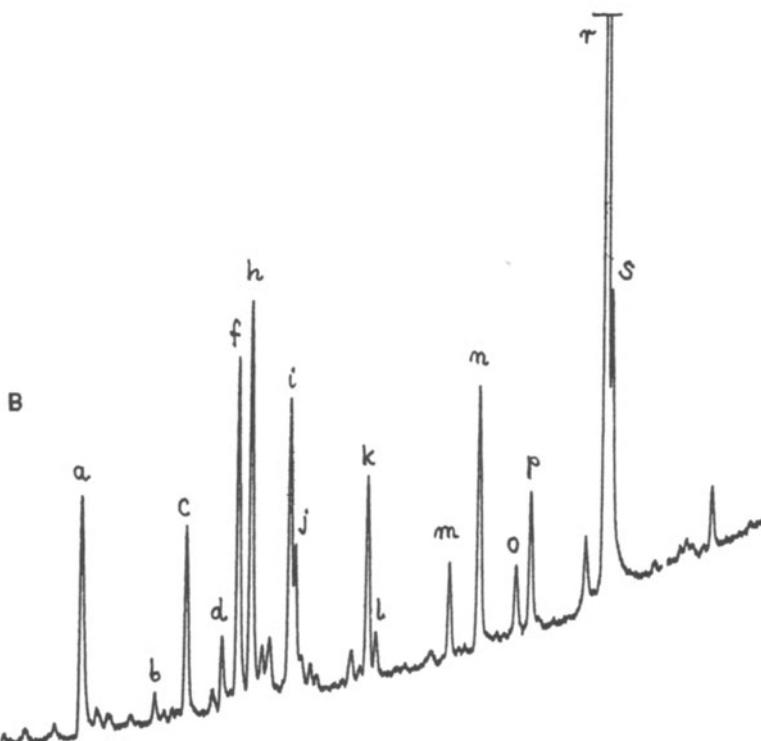
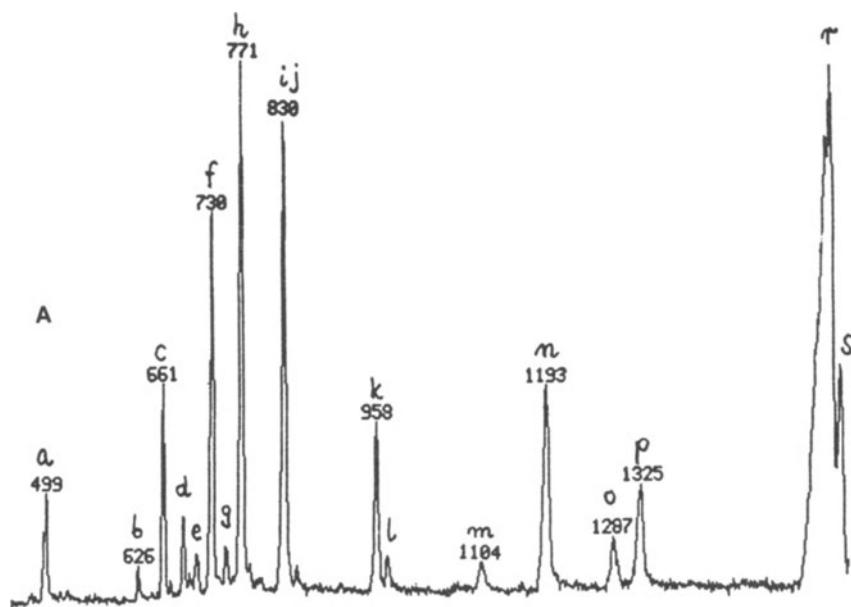


Figure 17. Structure of selected pentacyclic triterpenoids identified in flowers of *Compositae* plants.

Ψ -Taraxasterol	X = Me	R = H
Faradiol	Me	OH
Heterobetulin	CH ₂ OH	H
Heliantriol B ₀	CH ₂ OH	OH
Taraxasterol	Me	H
Arnidiol	Me	OH
iso-Heterobetulin	CH ₂ OH	H
Heliantriol B ₁	CH ₂ OH	OH
Lupeol	Me	H
Calenduladiol	Me	OH
Betulin	CH ₂ OH	H
Heliantriol B ₂	CH ₂ OH	OH
β -Amyrin	Me	H
Maniladiol	Me	OH
Erythodiol	CH ₂ OH	H
Longispinogenin	CH ₂ OH	OH
δ -Amyrin (not-detected)	Me	H
Coflodiol	Me	OH
Coflotriol	CH ₂ OH	OH
α -Amyrin	Me	H
Brein	Me	OH



elemental analysis as $C_{16.23}H_{15.82}O_{2.10}N_{1.00}$ or $C_{16.00}H_{15.60}O_{2.07}N_{0.99}$ (normalized for 1 nitrogen and 16 carbon atoms, respectively). Because an odd number of hydrogen atoms ought to be present per one nitrogen atom, the assumption of one or two nitrogen atoms per molecule suggested that this composition corresponded to one out of five molecular weights of 253, 255, 506, 508, and 510. The EI spectrum indicated that the low intensity ion at m/z 506 ought to be the molecular ion, especially since a probable loss of a benzyl group, producing two fragment ions at m/z 415 and 91, agreed with its position even if mass calibration was not perfect above 500 amu. Subsequent HR-MS measurement corroborated this assignment (measured 506.2195, calc. for $C_{32}H_{30}O_4N_2$ 506.2206). NMR spectra were crucial in this identification and indicated the identity of SR-2-2 with an interesting aromatic metabolite, N-benzoyl-O-[N'benzoyl-L-phenylalanyl]-L-phenylalaninol, mp 210°C, isolated at the same time as a fungal product.⁵⁸ Therefore, mold contamination was blamed for its presence in this plant extract.

Investigation of plant material may frequently reveal contamination. As an example, in the analysis of sterol fractions detection of ergosterol and cholesterol, as minor constituents along with prevalent C₂₉ "plant" sterols, may be due to the very probable fungal or insect contaminations. Large scale extractions, followed by extensive chromatographic separation steps can frequently lead to the isolation of compounds that are not necessarily present in the original material. In this respect, the careful analysis of a small amount of the characterized and homogeneous material with sensitive methods (as GC-MS, HPLC-MS, direct FAB) can be very helpful in conducting time consuming large scale separations.

Figure 18. Section of capillary GC-MS (A) and GC analysis (B) of the neutral, unsaponified fraction obtained from a sunflower flowers (*H. rigidus*), 35 m SE-30 capillary peak identification: **a** and **e** aliphatic compounds **b** campesterol, **c** stigmasterol, **d** unidentified triterpanol, **f** β -sitosterol, **g** avenasterol, **h** β -amyrin, **i** lupeol, **j** α -amyrin, **k** Ψ -taraxasterol, **l** taraxasterol, **m** dammarenediol II, **n** maniladiol, **o** brein, **p** calenduladiol, **r** faradiol, and **s** arnidiol. Figure 19. As in Figure 18 **a** sesquiterpenoid alcohol, **b** nor-diterpenoid alcohol MW=274; **c**, **d**, and **e** diterpenoid alcohols MH=290; **f** aliphatic compound, **g**, **h**, and **i** diterpenoid alcohols MW=288, **j**, **k**, **l**, **m**, and **n** aliphatic compounds.

Mixed esters of terpenoids derived from the saponified extract of sunflower flowers: two isomeric compounds $C_{30}H_{46}O_2$ (measured 438.3486, calculated 438.3498, and confirmed by C, H determination) were isolated.⁵⁷ Although this molecular formula was compatible with that expected for a triterpenoid diketone (compare two examples below) their spectra, except for M-Me, did not show any ions directly following M^+ . Two significant fragment ions, however, one at m/z 302.2240 ($C_{20}H_{32}O_2$, calc. 302.2246), and one at m/z 136.1249 ($C_{10}H_{16}$, calc. 136.1252), could represent two complementing parts of the parent molecule. The ion 136 was followed by a series of ions typical for bicyclic monoterpenoids at m/z 121, 93, and 91, with corresponding metastable ions present. These two C_{30} compounds were suspected, therefore, to be esters of a monoterpenoid alcohol with a diterpenoid acid, especially since abundant diterpenoid acids, kaurenoic and trachylobanoic, were already known to occur in this plant.⁵⁶ The reduction with LiAlH₄ produced primary diterpenoid alcohols (kauren-19-ol and trachyloban-19 ol) and monoterpenoid tertiary alkohol (thujanol) identified by GC, GC-MS and other methods. Significantly, these esters (tertiary alcohol, pivalic type acid) were resistant even to strong alkaline hydrolysis (Fig. 20).

Faradione, originally, two pentacyclic triterpenoids faradiol and arnidiol, occurs in great abundance in flowers of numerous *Compositae* plants (Fig. 17),⁵⁹ were assigned the structure of 3,12-diols.⁶⁰ Faradione was obtained as the common derivative of both these diols by CrO₃ oxidation and double bond isomerization. However, according to our measurement, its HR-MS spectrum did not show any significant fragment ions containing both oxygen atoms and three prominent fragment ions at m/z 150 ($C_{10}H_{14}O$), 149 ($C_{10}H_{13}O$), and 135 ($C_9H_{11}O$), were consistent with the alternative 3,16-substitution (Fig. 21).⁶¹⁻⁶³

Calenduladione has a 3,12-diol structure that was originally proposed for thurberin, a lupane derived diol isolated from the organ pipe cactus.⁶⁴ It was based on the mass spectrum of the corresponding diketone showing a prominent ion at m/z 247 (Fig. 22). However, according to our measurements, this ion showed the accurate mass of 247.2069, indicating the composition $C_{17}H_{27}O$ (calc. 247.2062) rather than $C_{16}H_{23}O_2$ (calc. 247.1700). Subsequently, this diketone and the corresponding diol have been found to be identical with calenduladione and calenduladiol,⁶⁵ a diol accompanying faradiol, arnidiol,^{61,62} maniladiol, brein, and coflodiol⁶⁶ in numerous *Compositae* plants.⁵⁹ All these pentacyclic triterpenoids have been identified as $3\beta,16\beta$ -diols derived from lupene, pseudo-taraxene, taraxene, olean-12-ene, urs-12-ene, and olean-13(18)-ene, respectively (Fig. 17).

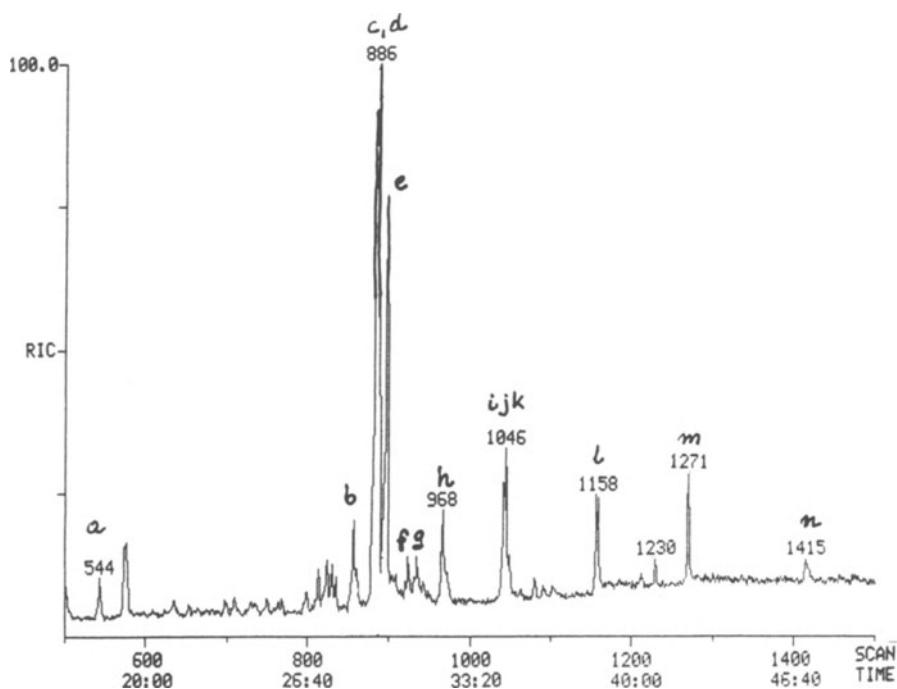


Figure 19. As in Figure 18; **a** sesquiterpenoid alcohol, **b** nor-diterpenoid alcohol MW=274; **c, d**, and **e** diterpenoid alcohols MH=290; **f** aliphatic compound, **g, h**, and **i** diterpenoid alcohols MW=288, **j, k**, **l, m**, and **n** aliphatic compounds.

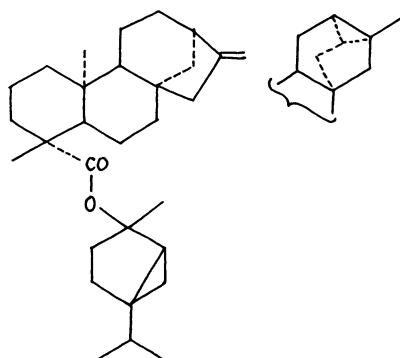


Figure 20. Two diterpenoid acid-monoterpene esters isolated from flowers of the common sunflower *H. annuus*.

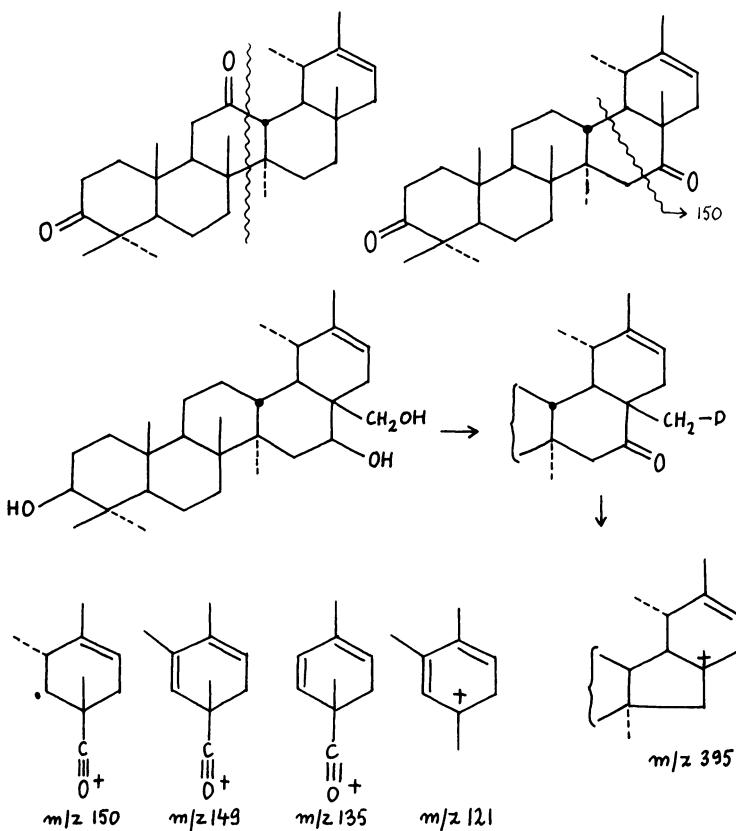


Figure 21. From the top: incorrect and correct structure of faradione labeling of one of methyl groups in faradione by the transformation of heliantriol B₀ in the last step NaI/Zn/D₂O was used for the reduction of p-toluene sulfonate; probable structure of fragment ions corresponding to the loss of Me and CO (*m/z* 395) and the ring E part.

15-Hydroxylated bile acids show us that fast scanning of a double sector instrument, prerequisite for the capillary GC-MS operation, may not permit optimal conditions for HR-mass measurement. FT-MS is a technique with great potential in such applications. Expanding our previous study of bile acids produced by marsupials, several new species were investigated (unpublished data). Two of them, according to capillary GC-MS performed at low resolution with a quadrupole, showed the presence of several new components analyzed as methyl ester-TMS ethers. The fragmentation of one TMS-ether could be explained by

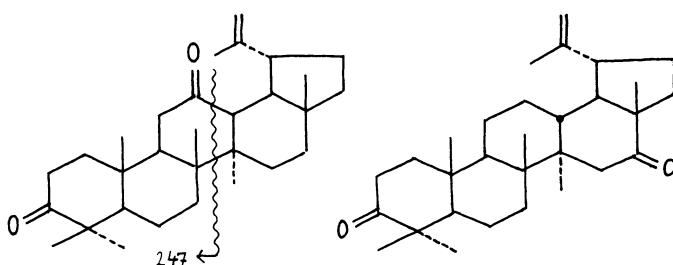
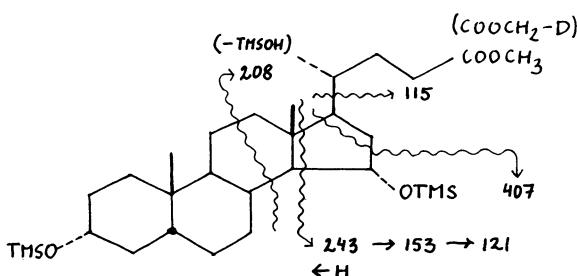


Figure 22. Incorrect and correct structure of calenduladione.

Figure 23. Novel $3\alpha,15\alpha$ -dihydroxy- 5β -cholan-24-oic acid isolated from the bile of two Australian marsupials (wombats); origin of major fragment ions for the methyl-trimethylsilyl derivative.

the unique hydroxylation at C-15 (Fig. 23). The origin of four crucial ions was verified first by the labeling of side chain methoxyl in a simple reaction with diazomethane in methanol-d₁. Ions at m/z 243 and 153, expected to represent the side chain fragment, were shifted by one unit while the ion 121 remained unchanged. Another ion not effected by deuteration, m/z 407, was attributed to the loss of the side chain and C16..17.

The predicted elemental composition of these four important fragment ions was confirmed by capillary GC-MS analysis performed with a FT-MS instrument using 15,000 resolving power at 100 amu (unpublished data obtained in collaboration with R.B. Cody). Ion 243.1423 corresponded to C₁₂H₂₃O₃Si (calculated 243.1416, 3 ppm difference). The ion 153.0921, formed from the above by the loss of TMSOH, deviated by 3.5 ppm from the expected composition C₉H₁₃O₂ (calculated 153.0916), whereas ion 121.0652, expected to be the result of additional loss of MeOH, the major component of a doublet, deviated by only 0.8 ppm from the expected composition C₈H₉O (calculated

121.0653). The minor ion 121.10128 was identified as C₉H₁₃. Ion 407.2818, assigned due to the loss of C_{16..17} and the side chain, contained both TMS groups and deviated by 4 ppm from the composition C₂₃H₄₃O₂Si₂ (407.2801). Other important ions were also measured with good accuracy: m/z 535, M-Me (C₃₀H₅₅O₄Si₂) 255 M-2xTMSOH-SC (C₁₉H₂₇) 208 C-12+ring D+SC fragment (C₁₃H₂₀O₂) and 115 SC fragment (C₆H₁₁O₂). Since this determination, this new bile acid has been isolated and its structure established as 3 α ,15 α -dihydroxy-5 β -cholan-24-oic.

The absence of a molecular ion from the mass spectrum by no means disqualifies its use for structural determination. Frequently, fragment ions are just from simple eliminations, and, with the additional information resulting from derivatives, prediction of the correct molecular weight can be made with certainty. Ions directly following the expected molecular ion, if such is present are first to be assigned. Simple eliminations (e.g., methyl radical, water, carbon monoxide, acetic acid.)⁶⁷ are easily identifiable and frequently characterize the type of functionalization. Moreover, ions which can not be explained by simple eliminations, i.e., producing no rational combination of elements expected in the investigated compound, are of equal importance and may indicate the presence of impurities. The presence of such ions puts in doubt the assumed position of a molecular ion. Ions at the value M \pm 14, as the classical example, indicate a homologue or methyl ester/methyl ether impurity, but ions M \pm 28, due to a homologue different by two methylene groups, can be easily missed as equivalent to the loss of ethylene or carbon monoxide.

Heliantriol E is a compound isolated from flowers of sunflower and purified as the diacetate (NMR) showed the presence of a free hydroxyl group (IR). Its EI spectrum showed, in the high mass region, ions at m/z 529, 526, 484, 466, 451, 424, 409, 406, and 391. Assignment of the 529 ion as a molecular ion led to a series of unreasonable eliminations (-3, -45, -63...). However, assuming that it represents a "M-Me" ion, this permitted the identification of all other ions as M-H₂O, M-AcOH, M-H₂O-AcOH, M-Me-H₂O-AcOH, M-2xAcOH, M-Me-2xAcOH, M-H₂O-2xAcOH, and M-Me-H₂O-2xAcOH respectively, and indicated that the parent triol has composition C₃₀H₅₂O₃. Consequently, this compound was identified as the 3,16-diacetate of 3 β ,16 β ,20 β -trihydroxytaraxane, (unpublished data).

Bis-seco-triterpenoid., through the dehydrobromination of α -bromo-ketone, derived from faradione, produced several rearranged unsaturated ketones.⁶⁸ This rearrangement, unrecognized in the original study of faradiol and arnidiol,⁶⁰ was evidently responsible for the incorrect "3,12"-hydroxylation pattern. The structure of a C₃₀ α , β -unsaturated ketone, formed with an array of basic

reagents, was confirmed by its partial degradation involving a stepwise oxidation with RuO₄/IO₄⁻ reagent (Fig. 24). One of the intermediates, a dicarboxylic acid with 28 carbon atoms in its skeleton, was characterized by 15 eV EI-MS as its mono- and di-methyl ester (Fig. 25). For the first ester, the molecular ion was barely detectable. However, ions at m/z 520, 516, 489, and 488 corroborated the molecular weight of 548, assuming their origin as M-CO, M-MeOH, M-COOMe, and M-HCOOMe/AcOH, respectively. Only 10 significant ions were observed in its spectrum and for eight of them the elemental composition could be easily measured, the other two overlapped with ¹³C isotope ions. In addition, five distinct metastable ions confirmed the sequence of eliminations resulting from simple α -cleavage processes and eliminations of HCOOMe and AcOH with H-migration. Ions observed in the spectrum of the dimethylester, and assigned to the AB part of the molecule, were shifted by 14 amu, as expected for methylation, and the additional ion at m/z 231 appeared to be due to the loss of methanol from ion 263 (Fig. 26). The loss of the two carbon atoms as result of the degradation sequence applied, and the straightforward fragmentation pattern of these and other products, confirmed that only one methyl group was involved in the original rearrangement. Therefore, more extensive skeletal transformation was excluded (unpublished data, in collaboration with E. Czyzewska).

Diagnostic Ions

Diagnostic ions, present frequently in spectra of parent compounds as well as purposely created by planned derivatization, can be used for identification. Examples of such ions can be seen in simple spectra of silyl esters discussed before (Fig. 4). A good example is supplied by the retro-Diels-Alder fragmentation of amyrin derivatives. In the spectrum of daturadiol derivative (see also example below),⁶⁹ two such fragmentations, one due to a 12:13, another due to a 5:6 double bond, are present (Fig. 27).

Silylation, frequently used as the derivation method for GC and GC-MS, is especially useful for the induction of specific fragmentation processes.⁷⁰ This can be exemplified by ions of well-understood structure and origin resulting from localization of the initial charge: m/z 103 [CH₂-O(⁺)Si(Me)₃] diagnostic of a primary hydroxyl group, 117 [CH₃-CH=O(⁺)-Si(Me)₃, or CO(⁺)OSi(Me)₃], 129 [CH₂=CH-CH=O(⁺)-Si(Me)₃] and M-129 present in spectra of 5-unsaturated sterols and steroids, 191 [Si(Me)₃-CH=O(⁺)Si(Me)₃] resulting from migration of closely located silyl groups, 243 [Si(Me)₃-O(⁺)=CH-CH=CH-CH=CH-O-Si(Me)₃] present in spectra of 3,7-dihydroxylated bile acids. Several ions characterize hydroxylation of the sterol side chain, m/z 131 [Si(Me)₃(⁺)O=C(Me)₂],

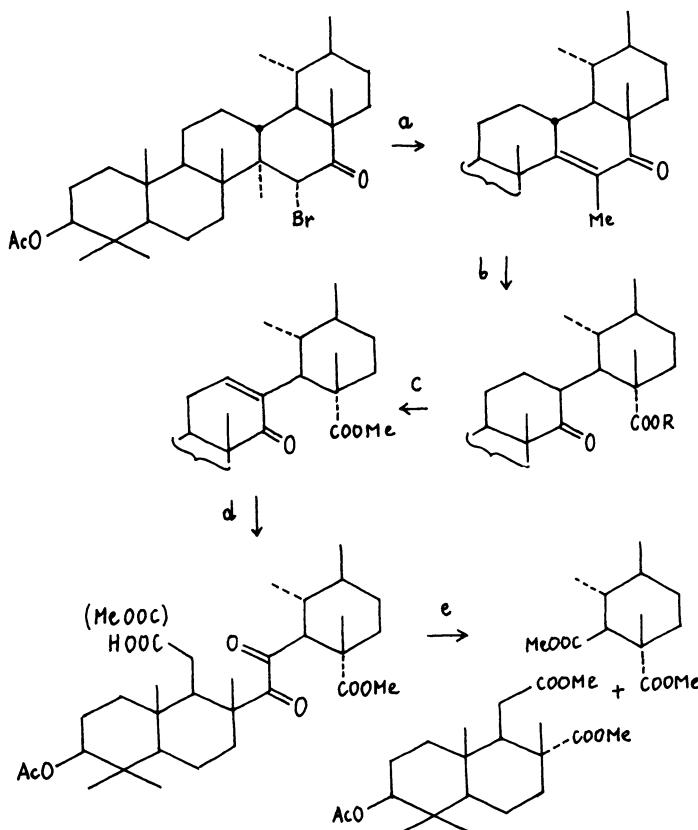


Figure 24. Dehydrobromination of α -bromoketone derived from dihydrofaradiol ((a) AcOH/AcONa and other reagents) produces a rearranged α,β -unsaturated ketone. Its degradation proved that the rearrangement was limited to the migration of 14α -Me group; (b) $\text{RuO}_4/\text{IO}_4^-$, (c) CH_2N_2 ; Br_2 ; $\text{Li}_2\text{CO}_3/\text{DMF}$, (d) $\text{RuO}_4/\text{IO}_4^-$, (e) H_2O_2 , OH^- .

and 145 [$\text{Si}(\text{Me})_3-(^+) \text{O=CH-C(Me)}_2$]. Ions common for many silylated compounds are present at m/z 73 [$\text{Si}(\text{Me})_3^+$, $[\text{Si}(\text{Me})_2^+-\text{OH}]$, and 147 [$\text{Si}(\text{Me})_3-\text{O-Si}^+(\text{Me})_2$], whereas the elimination of silanol(s) produces $M-n^*(90)$ ions. Recognition of such fragments can be greatly facilitated by the application of deuterated reagents (e.g. these with $d_9 \text{ Si}(\text{Me})_3$].

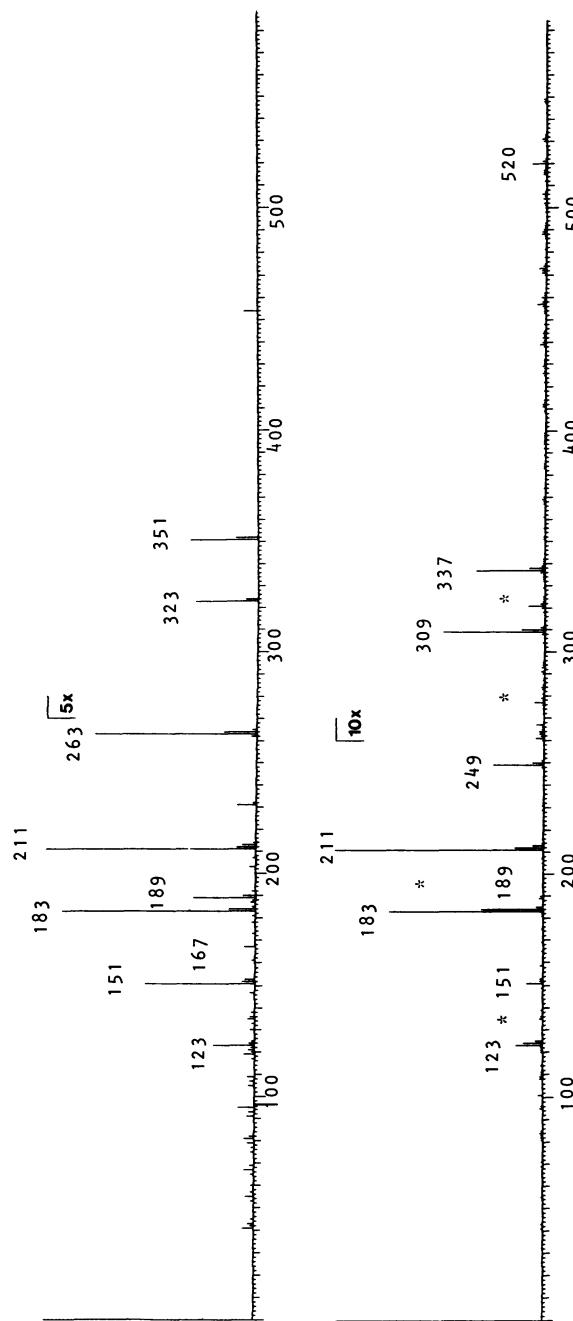


Figure 25. 15 eV, EI mass spectra of the α -diketone obtained from degradation outlined in Figure 26 as dimethyl (top) and monomethyl esters (bottom). Metastable ions identified for major eliminations (*).

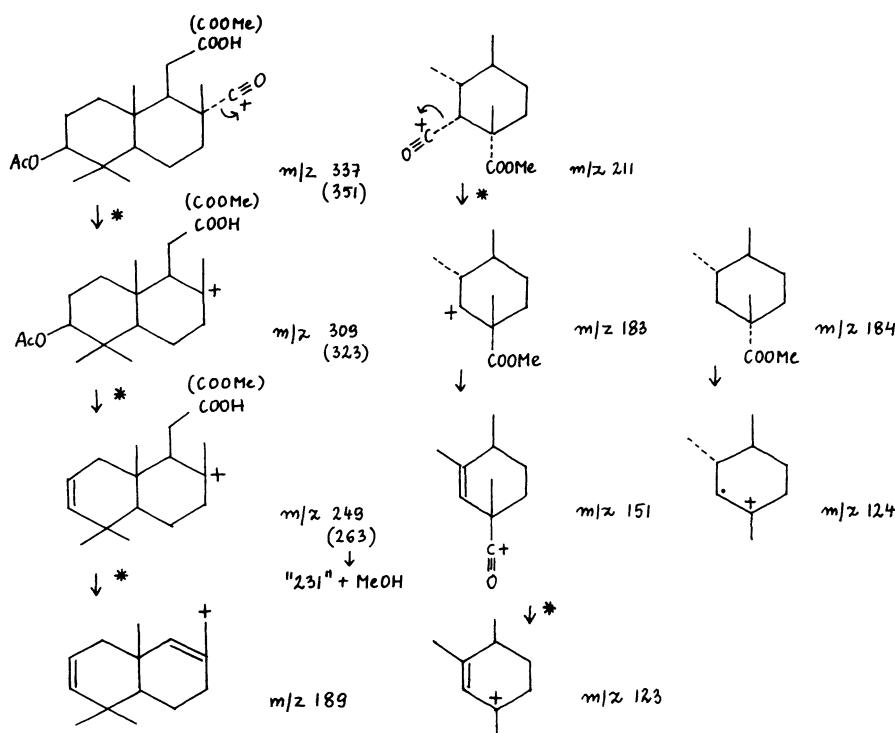


Figure 26. Origin of fragment ions identified in spectra of two α -diketones (Figures 24 and 25).

Vulpecholic acid, a new bile acid isolated from the Australian opossum, *Trichosurus vulpecula* (Lesson), produced a tris-TMS derivative, with MW of 638 and the dominant fragment ion at m/z 217.²⁸ This diagnostic ion indicated the presence of a 1,3-diol structure (Fig. 28) and it was subsequently identified as $1\alpha,3\alpha,7\alpha$ -trihydroxy- 5β -cholan-24-oic acid. Two other derivatives involved in this identification also displayed straightforward fragmentation. They were first obtained in reactions performed without isolation with only a μg quantity of bile material.

Common Eliminations

Common eliminations such as the loss of water from alcohols, methyl radical, and acetic acids from acetates, are of great help in spectral analysis,

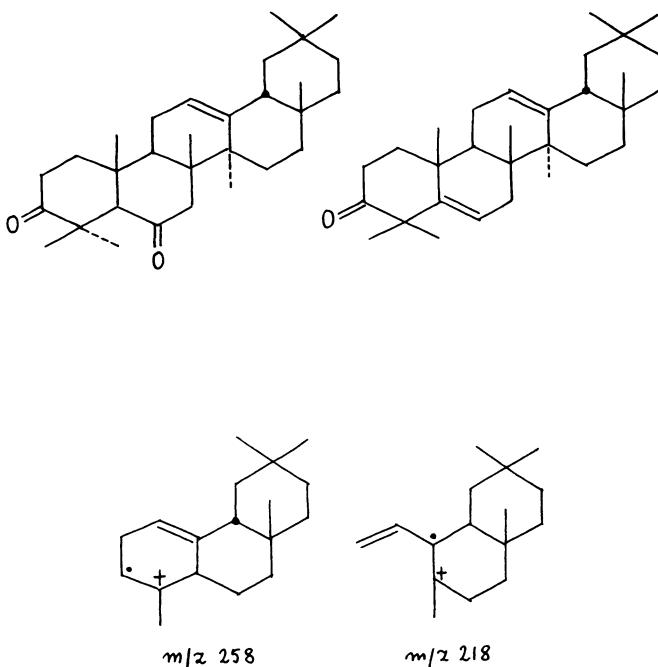


Figure 27. Daturadione and its derivative produce fragment ions due to the retro-Diels-Alder fragmentation in ring B ($m/z\ 25/z\ 218$).

including verification of the molecular ion (example of heliantriol E above). Frequently, in the absence of any other facile fragmentation processes such eliminations are the only ones observed. This was the case for the 15 eV spectra of lactucin and related sesquiterpenoids (Fig. 14).

Fasciculoloss are enta- and hexa-hydroxylated triterpenoid alcohols isolated from a toxic mushroom, *Naematoloma fasciculare* (Hudson ex. Fr.) Karst. They present an example of spectra with fragmentation limited to the loss of water and Me radical.⁷¹ The only fragmentation observed was the elimination of the side chain (Fig. 29). Long lists of ions due to $M-n^*(H_2O)$, $M-n^*(H_2O)-Me$, $M-n^*(H_2O)-m^*(AcOH)$, and $M-n^*(H_2O)-m^*(AcOH)-Me$ were present for these compounds and their partly acetylated derivatives. Importantly, all these compounds showed relatively abundant molecular ions at 15 eV. This was not the case, however, for the two esterified derivatives containing an interesting residue of hydroxymethylglutaric acid amide of glycine methyl ester. Confirmation of the molecular weight was obtained from the FD spectrum.

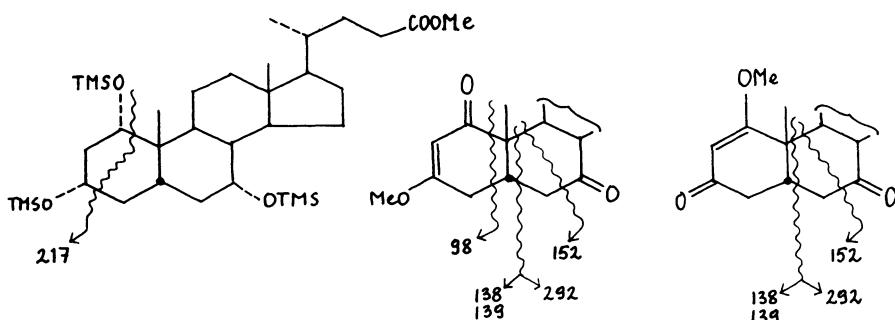


Figure 28. Origin of major fragment ions in spectra of vulpecholic acid methyl ester-trimethyl silyl ether and the two isomeric enol ether obtained by the methylation (CH_2N_2) of the triketone derivative.

ISOTOPES

Detection and analysis of elements and their stable and radioactive isotopes, the very first front and great success of MS, is still a very important branch. A notable alternative, but limited to selected isotopes only, is the NMR detection and even precise localization of labeled atoms. Since MS measurement detects the presence of isotopes in a single molecule, therefore, multiple labeling can be followed unambiguously. For radioactive isotopes this exact information is not provided by radioactivity measurement, whereas the NMR detection of multiple labeling is limited only to the exceptional case of the direct coupling.

Distinct mass differences of isotopes (Table 1) permits detection, counting and quantitation of introduced isotopic atoms directly from the mass spectrum of the investigated compound. The spectrum of an unlabeled compound provides good reference for calculations, especially if the ionization method used produces a molecular ion cluster with intensities difficult to predict. There are cases, however, when this is not possible. Apart from the obvious case when the useful mass spectrum can not be obtained, the isotope level may be too small for its unambiguous detection and measurement above the background of ions due to naturally present heavier isotopes. Degradation to a simple low molecular weight, preferably gaseous compound, permits precise measurement when compared to standards of the known abundance, a method with long tradition for deuterium, carbon, nitrogen, and oxygen isotopes.

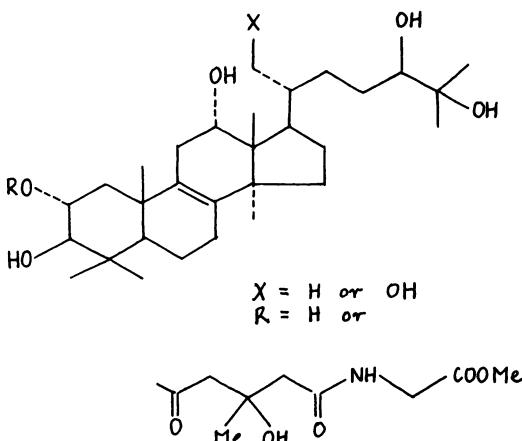


Figure 29. Structure of lanostane derivatives isolated from a toxic mushroom, *Naematoloma fasciculare* (Hudson ex. Fr.) Karst.

In metabolic and biosynthetic studies with stable isotopes, direct detection by mass spectrometry is relatively straightforward only if sufficient incorporation of labeled precursor takes place. Introduction of several heavier atoms shifts the position of the labeled ion away from the clusters of M+1, M+2, and M+3 and permits detection of lower level of labeled molecules with less precise methods. Such labeled products can be directly detected in mixtures, e.g. by GC-MS, especially if the so called "twin-ion" technique is utilized.⁷² This simple approach is based on the application of a mixture of labeled and unlabeled precursors of known ratio, and permits much easier detection of products showing doubled ions with the expected separation. Losses of the specific label can also be detected this way. Such detection provides relatively high sensitivity, particularly if metabolites are not known prior to the experiment and are obscured by other components. The sensitivity of high resolution capillary GC-MS measuring full scan spectra for the detection of compounds labeled with ¹⁴C well compares to counting methods. Thus, as an example, if good quality full scan EI spectrum of a keto-sterol TMS ether with molecular weight 472 can be obtained for 0.2 ng per GC-peak, 10-20 % enrichment with ¹⁴C, corresponding to only 6-12 DPM, is easily detected in the M+2 ion. Even better sensitivity can be obtained with SIM acquisition. It is not the case, however, for ³H of a much higher specific radioactivity.

The possibility of isotope fractionation in metabolic reactions, especially prominent for hydrogen isotopes due to the high isotope effects, ought

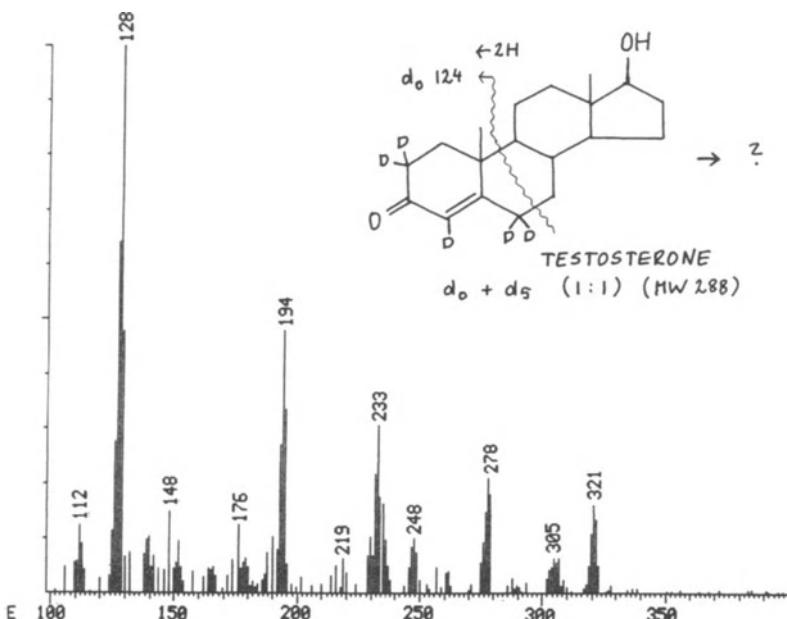


Figure 30. 20 eV, EI mass spectrum of an unidentified metabolite of testosterone only deuterated (d_5) material was transformed to this compound from the 1:1 mixture of d_0 and d_5 testosterone.

to be seriously considered. This is exemplified by the detection of a partly identified d_4,d_5 product (Fig. 30, unpublished data) completely devoid of fast metabolizing d_0 species from the Sertoli cell culture incubation of 1:1 mixture of $d_0:d_5$ testosterone labeled at C-2,2,4,6,6.⁷³

A significant improvement of the sensitivity of isotope detection in labeled compounds present in complex mixtures can be obtained by the combination of capillary GC separation with the "on-line" conversion to low molecular weight compounds directly monitored by a mass spectrometer.^{74,75} Such a specialized instrument permitting precise measurement of ^{13}C with an isotope ratio mass spectrometer is commercially available. More versatile instruments, with an "on-line" thermal or microwave chemical converter have been recently constructed. With the latter, the incorporation of only about 1 % of the ^{13}C , ^{15}N , and ^2H label was detected in even very complex mixtures.⁷⁴ For labeled, especially deuterated compounds, due to isotope effects, a partial

separation of labeled and non-labeled molecules frequently occurs on capillary columns, and this effect further enhances the chromatographic detection of labeled molecules.

Another important area of the application of labeled compounds is their use as internal standards for quantitation, especially with GC-MS in the SIM mode of operation, and represents the extension of radioactive-isotope-dilution method. Such labeled of internal standards may also serve as carriers for the detection of trace components, which otherwise are not easily detectable in complex mixtures due to unfavorable chromatographic conditions. We utilized this method, using two different deuterated internal standards, to demonstrate the natural occurrence of 3β -hydroxy- 15α -cholest-8(14)-ene, a strong inhibitor of cholesterol biosynthesis, in rat skin lipids. Its TMS-ether, d_0 MW 472, and d_5 and d_9 species were used as separate internal standards and carrier.⁷⁶

Specific isotope labeling permits one to gain better understanding of fragmentation. Deuterium, introduced into organic molecules in an array of reactions including even a simple exchange, is especially useful. Such labeling not only pinpoints the specific origin of fragment ions, if different possibilities exist (see: 15-hydroxylated bile acids), but also permits one to follow migrations of hydrogen atoms involved in fragmentation reactions. Excellent examples of such applications are available especially from detailed studies of MS of steroids.⁷⁷ The convenient use of deuterated derivation reagents, offering the direct comparison of spectra for d_0 and d_n species, is also of a great value in structural analysis.

2-Hydroxylated diterpenoid. is a new diterpenoid diol isolated from the saponified extract of the common sunflower which could be oxidized to a hydroxy-ketone with molecular ion at m/z 304, along with M-15, M-18, M-15-18, M-58, and M-58-15 ions. Its dehydration and the Wolff-Kishner removal of the ketone produced kaurene and isokaurene. This identified the skeleton and indicated the presence of a 16-hydroxyl group leaving the secondary alcohol to be

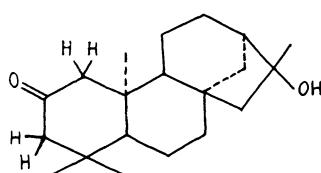


Figure 31. Hydroxy-ketone derivative of a new diterpene diol isolated from the common sunflower formation of d_4 species upon exchange unambiguously confirms the location of the ketone function.

localized at one of seven available positions. The exhaustive deuteration of the above hydroxy-ketone under alkaline conditions followed by removal of hydroxyl deuterium with d₀ alcohol shifted all these ions by 4 amu. Position 2 was the only one compatible with incorporation of four D atoms by the enolization exchange (Fig. 31, unpublished results).

Daturadione, a β -amyrin derived pentacyclic triterpenoid diol isolated from *Datura innoxia*, was transformed into the diketone, daturadione (M^{+} at m/z 438), by Jones's oxidation.⁶⁹ Its deuteration under forced alkaline conditions produced deuterated daturadione in addition to its isomer, each with the incorporation of five deuterium atoms (M^{+} at m/z 443). Ions resulting from the retro-Diels-Alder fragmentation and corresponding to ring DE part of molecule (m/z 218 and 203) remained unchanged upon this treatment. 3,6-Dione was the only pattern of oxygenation compatible with incorporation of five deuterium and epimerization (Fig. 27).

Faradione and *calenduladione* provide another example as already mentioned, the original structures proposed for the parent diols, faradiol and calenduladiol, involved 3,12-hydroxylation (Figs. 21, 22). This, however, was subsequently corrected to a 3 β ,16 β -hydroxylation pattern. For corresponding diketones, the strong evidence against the "3,12" alternative was obtained from the deuteration under alkaline conditions introducing only four, and not five, deuterium atoms.⁶²

In the spectrum of faradione the elimination of 43 amu from the molecular ion produced a dominant fragment ion. Its origin was assigned as a one stage loss of a methyl group and carbon monoxide (HR-MS), the elimination of ketene and H· (C15..16) was ruled out from the spectrum of the above mentioned d₄-faradione, which showed also the unchanged position of fragment ion at m/z 150 and 135. Out of eight methyl groups present, two located closest to the C-16 keto-group were first to be suspect. One of them, at C-17, i.e. Me-28, could easily be labeled as -CH₂D by the selective removal of C-28 hydroxyl group of heliantriol B.⁷⁸ This group was found to be the sole one taking part in the above one-stage loss (Fig. 21).⁷⁹ Reaction of labeled compounds and incorporation of label from the reaction medium can be frequently followed by mass spectrometry in a quanti-tative fashion. This information, especially if combined with the more direct determination of the label's position by NMR, permits insight into the reaction mechanism.

19-Epifaradione, obtained by the acid rearrangement (AcOH-H₂SO₄ 5:1) of faradione, differs only in the configuration of one methyl group (Fig. 32) and its spectrum is almost identical to that of faradione. If this rearrangement is performed in AcOD-D₂SO₄ extensive exchange takes place and the product has

up to 12 deuterium atoms incorporated. The same result is also obtained by the equilibration of d_0 -19-*epi*-faradione in AcOD-D₂SO₄. Since four hydrogens can be exchanged by enolization at C-2 and C-15, eight are evidently incorporated in other positions. In the spectra of deuterated 19-*epi*-faradione, the already mentioned fragment ions at m/z 150 and 135 (Fig. 21) are shifted to 158, 157, and 140, 139 indicating the localization of these eight deuterium atoms in ring E. A similar conclusion is obtained from the spectrum of deuterated 19-*epi*faradione after deuterium from positions 2 and 15 is removed by enolization in alkaline medium. Importantly, these spectra indicated that the elimination M-Me-CO, producing the major fragment ion, involves non-deuterated Me, whereas fragmentation "150 → 135" involves, probably exclusively, the deuterated methyl group. This could be rationalized by the extensive participation in this rearrangement of hydrogen atoms at C-19, C-21, and methyl groups 29 and 30. This deuteration pattern is in agreement with the ¹H NMR spectrum which excluded also deuteration of C-22.

Coflodione (Fig. 32b) was another product of the above isomerization performed in the medium containing more sulfuric acid (2:1). It was identical with 3,16-dioxoolean-13(18)-ene, the oxidation product of cofloiol, the diol mentioned above (Fig. 17). The compound isolated from the deuterated medium, after back-enolization, contains up to ten deuterium atoms. Major ions resulting from the fragmentation of ring C, and present in the d_0 diketone at m/z 205, 219, and 232, are present in the deuterated diketone at 205, 219, 228, 229, and 241, 242. This confirms the double origin of ion 219 and even more extensive deuteration of ring E when compared to 19-*epi*faradione (unpublished results, in collaboration of E. Czyzewska).

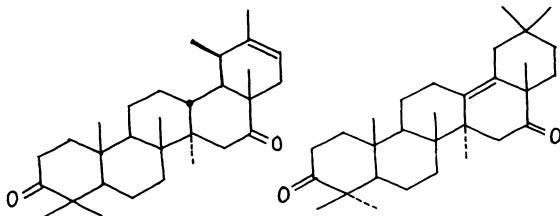


Figure 32. 19-*Epi*-faradione and coflodione, obtained as products of the acid rearrangement, incorporate eight and ten deuterium atoms from the medium into ring E.

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Chapter Seven

STRUCTURE ELUCIDATION OF NATURAL PRODUCTS BY MASS SPECTROMETRY

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INTRODUCTION

For over three decades, mass spectrometry has played a central role for the structure elucidation of natural products because it can be used to determine the molecular weights and elemental compositions of substances with a wide range of chemical and physical properties. In addition, if the molecular ion is formed with sufficient excitation energy, it will undergo a series of unimolecular reactions to give fragment ions, the relative abundances of which are normally unique to the structure of the molecular ion. By understanding the general rules governing the fragmentation reactions, the types of functional groups present, as well as the manner in which they are joined to form a unique molecular structure may be deduced from the mass spectrum.

Molecular weight is only the first step of a general approach to structure elucidation by mass spectrometry. This initial step is followed by

determining the elemental compositions of the molecular ion and important fragments from their exact masses (actually mass-to-charge ratios). Peaks just below the molecular ion are frequently due to the loss of simple molecules such as water, ketene or methanol. Elemental composition information may be combined with knowledge of common neutral losses to indicate which functional groups comprise the molecular ion. At some point in the analysis, tentative structures consistent with the elemental composition, as well as features deduced from other spectroscopic methods, are proposed. The general features of mass spectra of the tentative structures are predicted from our knowledge of charge and radical induced cleavage reactions, especially as applied to substances with structures homologous to the tentative structure. This cyclic process of proposing tentative structures and comparing their predicted fragmentation patterns with the mass spectrum of the unknown is continued until only a few structures consistent with the mass spectral data remain. Choosing the correct structure normally requires using other spectroscopic information, and may require synthesizing and characterizing all of the tentative structures. Thinking of all possible tentative structures, and correctly predicting their fragmentation patterns may be the most difficult part of the structure elucidation process.

Despite the difficulty in accurately predicting the fragmentation patterns of tentative structures, mass spectrometry continues to play a major role in structure elucidation problems because it is more sensitive than other spectroscopic methods, and because it is a unique source of certain types of information. For example, a complete mass spectrometric analysis, including several ionization methods, low and high resolution, and chemical derivatization, can normally be performed with less than 50 µg of sample. This rather large amount of sample should not be confused with the quantity required for detection after the mass spectrometric features of a compound have been determined. Sub-nanogram, and in many cases sub-picogram quantities can be detected. However, high sensitivity is not sufficient for most trace analyses because of problems relating to interferences from impurities in a sample. Mass spectrometry has been especially successful for trace analyses because a particularly high degree of selectivity is achieved by combining gas and liquid chromatography with mass spectrometry. Selectivity may also be increased by special mass analysis techniques, such as high resolution and MS/MS.

In this contribution, we describe general mass spectrometric procedures used in our laboratory to assist in the determination of structures of natural products. Applications of these procedures are illustrated with a series of biologically active compounds, acetogenins, the mass spectra of which have been studied extensively in our laboratory.¹⁻⁶ Chemically, the acetogenins have

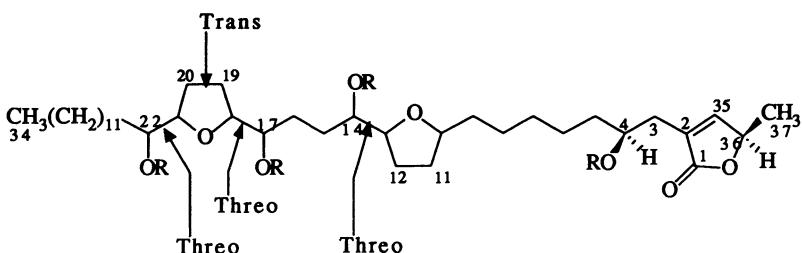


Fig. 1. Structure of gigantecin, a representative acetogenin.

a single chain backbone of 35 to 38 carbons with one or two tetrahydrofuran rings, a γ -lactone, and several hydroxyl groups, as illustrated for gigantecin in Figure 1.⁷ Some acetogenins have a carbonyl in place of a hydroxyl.⁸ Although the structures of the functional groups present in specific acetogenins were determined principally by other spectroscopic methods, their locations along the carbon backbone were established by mass spectrometry.

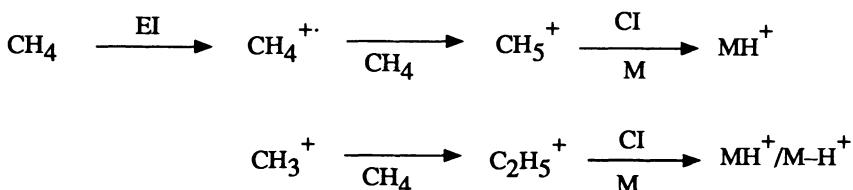
Mass spectrometry refers to the production, separation according to mass-to-charge, and detection of ions. Although there are often several acceptable approaches to each step, some may be equivalent, one usually must work within the constraints imposed by the types of instrumentation that are readily available. Most laboratories have access to electron, chemical, and fast atom bombardment ionization, as well as high resolution mass spectrometry. The discussion that follows will focus on these methods because they are readily available, and because it would be imprudent to adopt a highly specialized approach before trying conventional methods.

ELECTRON AND CHEMICAL IONIZATION

Conversion of a portion of the sample into an ensemble of gaseous ions is the first step in any mass spectrometric analysis. For materials which can be volatilized by heating without thermal degradation (including intramolecular rearrangements), electron and chemical ionization are usually the preferred ionization methods. For many substances, electron ionization (EI) gives a mass spectrum rich in fragment ions which are characteristic of the chemical structure of the substance. Fragment ions are formed through a combination of series and parallel intramolecular reactions which occur on the time scale from 10^{-6} to 10^{-13} seconds, and are highly dependent on the excitation

energy in the molecular ion. In favorable cases, fragmentation gives a relatively small number of prominent peaks that can be related to the structure of the starting material. In less favorable cases, fragmentation appears to occur randomly, giving a large number of fragments that can be reconstructed into the structure of the parent ion only with considerable difficulty. The family of acetogenins may be classified as an unfavored case. Their EI mass spectra contain many peaks that are not easily related to the structure of the starting material. We have some evidence suggesting that intramolecular rearrangement reactions occur either during thermal vaporization or after ionization to give ions consistent with incorrect structures. As a result, the EI mass spectra of underivatized acetogenins have not proved very useful for structure elucidation.

Chemical ionization (CI) can be used in place of EI to reduce fragmentation and increase the relative abundance of the molecular ion. As a result, CI is preferred for determining molecular weights and the analysis of mixtures. Instrumentally, EI and CI have similar requirements. For CI, the ionization chamber is filled with a reagent gas to a pressure of approximately 0.5 Torr. The reagent gas is ionized by an electron beam to form ions that undergo ion-molecule reactions to give reagent ions that react with the gaseous neutral molecules to give ions characteristic of the sample. Methane, isobutane, and ammonia are frequently used as reagent gases. This sequence of reactions is illustrated below for methane. Electron ionization of methane gives primarily CH_4^+ and CH_3^+ , which react with methane to give the reagent ions CH_5^+ and C_2H_5^+ , respectively. Upon collision with sample molecules with higher proton affinities (i.e., sample molecules that are more basic than CH_4 or C_2H_4), a proton is transferred to give an MH^+ ion. It is important to note that C_2H_5^+ may also react by accepting a hydride ion (H^-) from the sample to give an $\text{M}-\text{H}^+$ ion.



Methane or isobutane CI mass spectra of acetogenins are dominated by peaks corresponding to MH^+ and a series of peaks due to multiple losses of

water. These spectra are used to determine the molecular weight, elemental composition, and purity of acetogenins. Although methane and isobutane CI mass spectra afford little structural information, ammonia CI mass spectra give important information regarding the relative locations of hydroxyl groups. Acetogenins do not contain nitrogen, and hence are generally not sufficiently basic to take a proton from the ammonium ion. As a result, ammonium adduct ions, MNH_4^+ , may dominate the ammonia CI mass spectrum. However, if a pair of hydroxyl groups is oriented such that they can share a proton, the basicity increases and proton transfer occurs in the gaseous phase to form an MH^+ ion. Hence, whether a pair of hydroxyl groups is sufficiently close to share a proton can be determined from the relative abundances of the MH^+ and MNH_4^+ peaks.⁹ These reactions are illustrated in Figure 2, which also gives the dependence of the proton transfer vs. adduct ions on the structure of acetogenins and partial structures of representative acetogenins. For asimicin and bullatacinone, none of the hydroxyls are sufficiently close that they can share a proton; hence their ammonia CI mass spectra are dominated by a single peak corresponding to MNH_4^+ . For structures in which hydroxyl groups are separated by 2 to 4 methylenes, proton transfer becomes a major, if not dominant process. Since the reactions responsible for formation of MH^+ and MNH_4^+ have different dependencies on the pressure and temperature of the reagent gas, the MNH_4^+ / MH^+ ratio obtained for the same compound but for different source conditions may vary somewhat. Ammonia CI is attractive because it gives the molecular weight as well as structural information.

DESORPTION IONIZATION

Various desorption techniques, including particle (with either ions or atoms), laser, and field, have been used to analyze substances that are not sufficiently volatile for analysis by EI or CI. These desorption techniques have the apparent commonality that vaporization and ionization occur as a single step. The most frequently used desorption method, fast atom bombardment mass spectrometry (FABMS), also referred to as liquid secondary ion mass spectrometry (LSIMS), has greatly expanded the role played by mass spectrometry in the analysis of polar substances.¹⁰⁻¹² For example, useful mass spectra of peptides with 50 amino acids, oligosaccharides with 15 residues, and oligonucleotides with 8 units are routine. In addition, highly polar low molecular weight substances with ionic functional groups such as sulfate or phosphate are amenable to analysis by FABMS. Although FABMS has generally replaced

Acetogenin	MNH_4^+/MH^+	Partial Structure
ASIMICIN	100	
BULLATACINONE	100	
ANNONACIN	4	
GIGANTECIN	1.4	

$\text{NH}_3 \xrightarrow{\text{EI}} \text{NH}_3^+ \xrightarrow{\text{NH}_3} \text{NH}_4^+ \xrightarrow[\text{M}]{\text{Cl}} \text{MH}^+ \text{ Proton Transfer}$
 $\xrightarrow[\text{M}]{\text{Cl}} \text{MNH}_4^+ \text{ Adduct Formation}$

Fig. 2. Relative intensities of peaks corresponding to proton transfer or adduct formation for ammonia CIMS of selected acetogenins. Reactions involved (proton transfer and adduct formation) are included.

field desorption mass spectrometry (FDMS) for the analysis of biopolymers, FDMS remains attractive for certain classes of natural products which give weak FAB mass spectra.

FABMS analyses, which can normally be made on mass spectrometers that are used for EI and CI, are easy to perform. The sample (one microgram) is dissolved in a liquid matrix, such as glycerol, and bombarded with a beam of high energy (6-10 KeV) atoms. The continuous impact of atoms on the matrix removes molecules, matrix as well as sample, from the surface of the matrix. Ions, which were either preformed in the matrix or in the gas phase after

desorption, are analyzed according to their mass-to-charge ratios. FABMS is most successful when the sample is preferentially concentrated on the vacuum/matrix surface where it is likely to be desorbed by the impacting beam of high energy atoms. For example, oligosaccharides, which are very soluble in glycerol and are therefore uniformly distributed throughout the matrix, give weaker spectra than the corresponding permethyl derivatives which are less soluble and are concentrated on the vacuum/glycerol interface. Matrices frequently used for analysis of natural products include glycerol, thioglycerol, a mixture of dithiothreitol and dithioerythritol, and m-nitrobenzyl alcohol. For most modern mass spectrometers, one also has the option of looking for negative or positive ions. The general features of positive ion FAB mass spectra of acetogenins are illustrated in Figure 3, which was obtained with 300 ng of gigantecin. The large peak at m/z 639 is the MH^+ ion, indicating that the molecular weight of gigantecin is 638. Most of the other peaks in the mass spectrum are due either to ions from the matrix or fragment ions of gigantecin. The small peak at m/z 645 is probably due to an impurity. Because acetogenins do not form ions in solution, their FAB mass spectra are generally weak. Similar results have been obtained using m-nitrobenzyl alcohol as the matrix. Since FAB mass spectra are obtained without heating the sample, they are ideal for determining molecular weights of thermally labile substances.

CHEMICAL DERIVATIVES

Electron ionization is usually the preferred ionization method for structure elucidation of volatile substances because, in favorable cases, it gives a mass spectrum with many fragments which may be assembled as the pieces of a puzzle to give the appropriate chemical structure. An EI mass spectrum may not lead to a correct structure if the material is involatile, or if it is volatile but fragments into small, nondescript pieces. In either case, chemical derivatization may be used to make the material more volatile and to direct fragmentation to give ions that are diagnostic of the structure. Although many different chemical derivatives have been used,^{13,14} trimethylsilylation (TMS) and acetylation (AC) have been used most extensively, and will be discussed here.

TMS derivatives are easy to form and generally give very high quality mass spectra, as long as they are not exposed to moisture. These derivatives can usually be formed by drying 25 μg of acetogenin in the bottom of a 100 μl conical vial for 24 hours in a vacuum desiccator which contains P_2O_5 as a drying agent. Twenty μl of BSA (N,O -bis(Trimethylsilyl)acetamide) and 2 μl of dry

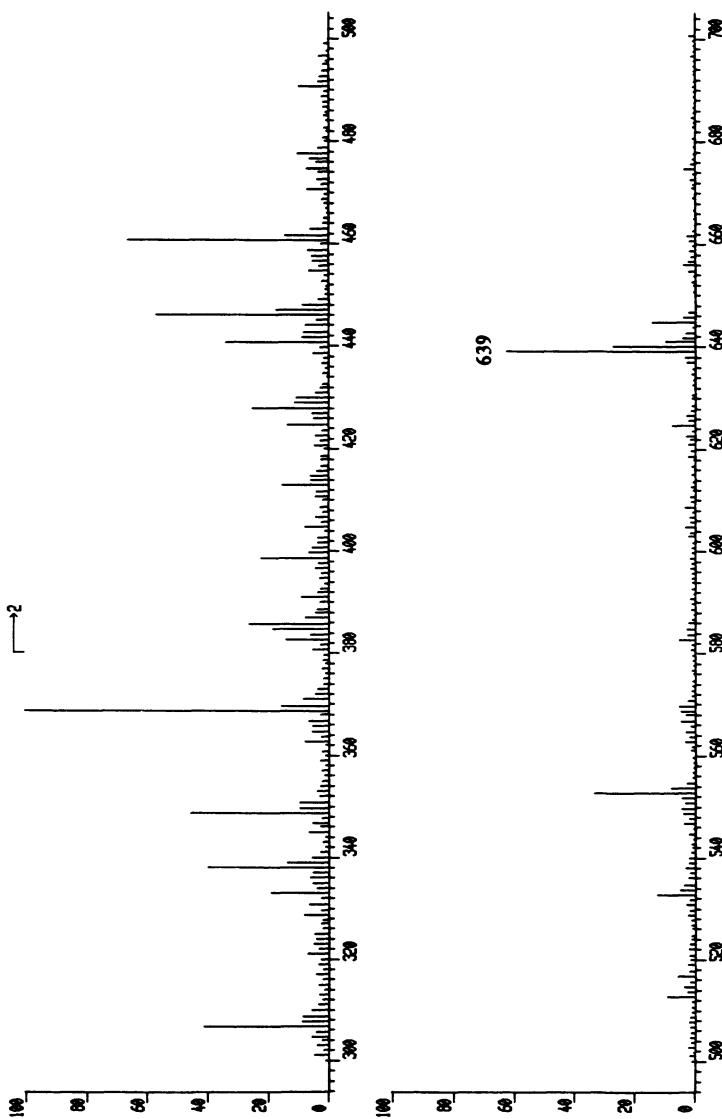


Figure 3. FAB mass spectrum of 300 ng of gigantecin obtained using a 1:1 mixture of glycerol and 1-thioglycerol as the matrix.

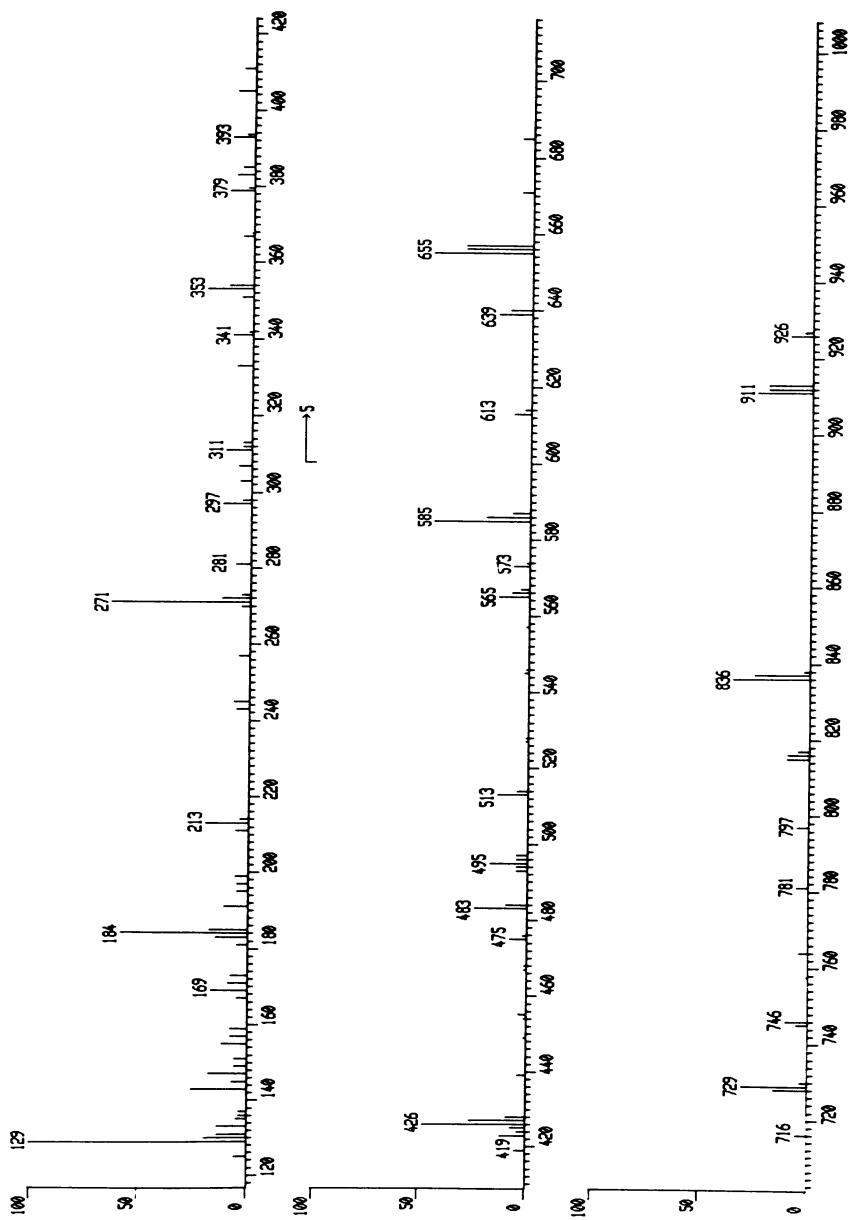
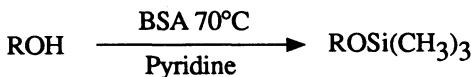


Figure 4. EI mass spectrum of the TMS derivative of gigantecin.

pyridine are added to the vial, which is sealed with a teflon lined cap and heated to 70° for 30 min. When the reaction is complete, all hydroxyl groups are converted into trimethylsilyl ethers, as illustrated below. The highest quality spectra are obtained when the derivatized sample is analyzed without purification as soon as it cools to room temperature. Although we have not used them for analyses of acetogenins, t-butyldimethylsilane derivatives are sometimes preferred because they give an intense M-57 peak, and because they are less sensitive to moisture.



The general features of the EI mass spectra of TMS derivatives are illustrated in Figure 4, which is the mass spectrum of the TMS derivative of gigantecin. The molecular ion of TMS derivatives of acetogenins is normally a prominent peak in the EI mass spectrum, and may be identified as the highest member of a high-mass series of ions formed by consecutive losses of TMSO or TMSOH (89 or 90 mu), respectively. For gigantecin, this series consists of the peaks at m/z 565, 656, 746, 836, and 926, suggesting that the peak at m/z 926 is the molecular ion. This assignment is confirmed by a peak 15 mu lower (m/z 911), corresponding to a loss of ·CH₃. Since each of the four TMS groups adds 72 mu, the molecular weight of underderivatized gigantecin is 638. This result is consistent with the MH⁺ ion at m/z 639 found in both the isobutane CI and the positive ion FAB mass spectra (Fig. 3). Should the number of TMS groups be uncertain, another sample may be derivatized with a deuterated silylating reagent. Since the mass of the deuterated TMS groups (d9) is 9 mu greater than the nondeuterated form, the number of TMS groups in any peak may be determined from the mass shift. For example, the molecular ion of the deuterated TMS derivative of gigantecin appears at m/z 962. This 36 mu increase is consistent with four TMS groups. All of the major peaks in the EI mass spectrum of the TMS derivative of gigantecin (Fig. 4) can be assigned to specific portions of the proposed structure (Fig. 5). The peaks at m/z 426 and 729 are due to ions formed by an intramolecular transfer of a TMS (or TMS+H) group to the ions at m/z 353 or 655, respectively.

Although the EI mass spectra of acetyl derivatives (AC) of acetogenins have fewer fragments ions diagnostic of molecular structure, they do give some useful information that complements information derived from the mass spectra of TMS derivatives. Peracetylated derivatives of acetogenins are formed by drying 50 µg of sample in a conical vial, adding 20 µl of acetic anhydride and 6

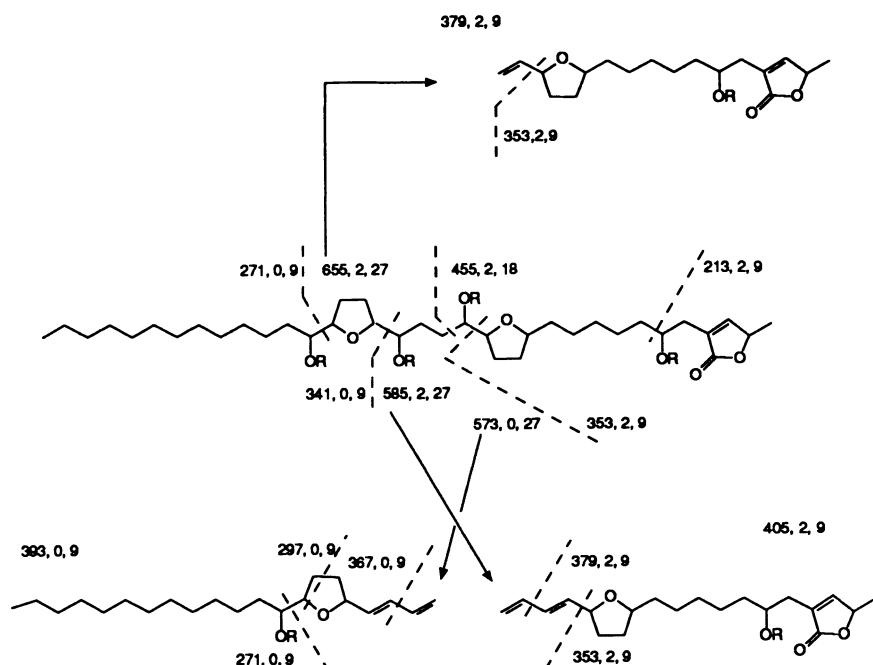
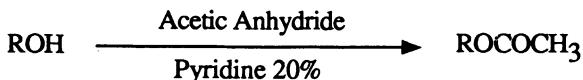


Fig. 5. Structure and fragmentation scheme of the TMS derivative of gigantecin. The nominal mass and mass shift found for the TMS d9 derivative are indicated for fragments diagnostic of the molecular structure. Sequential fragmentation reactions were deduced from the MS/MS daughter ion mass spectra of the parent ions.

μl of dry pyridine, and allowing the reaction to proceed at 20° for 12 hr. As for TMS derivatives, the acetyl derivative is analyzed without any purification.



The EI mass spectrum of the acetyl derivative of gigantecin (Fig. 6) may be used to illustrate the general features of EI mass spectra of acetyl derivatives. Since each acetyl group increases the molecular weight by 42 mu, the molecular weight of the acetyl derivative of gigantecin is 806. Although the EI mass spectrum of this derivative does not have a peak for the molecular ion

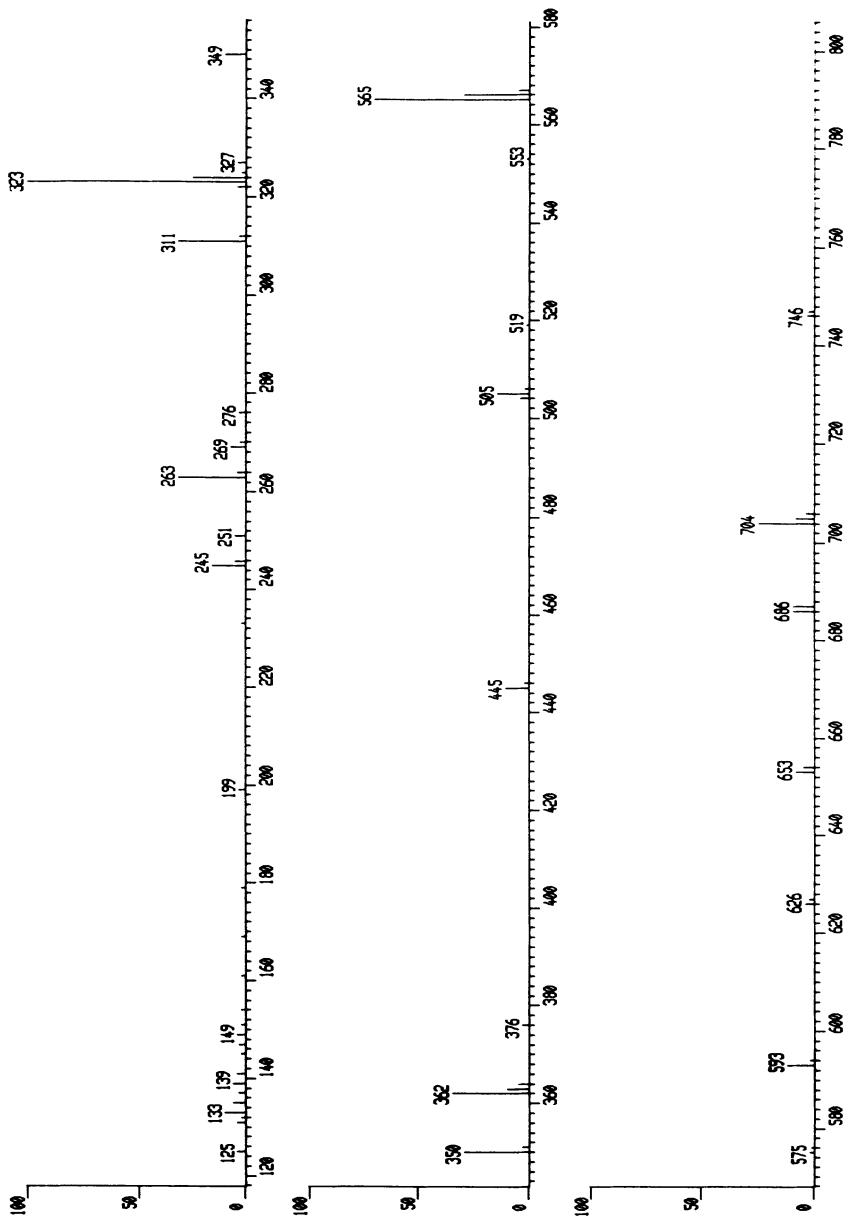


Figure 6. EI mass of the acetyl derivative of gigantecin.

(m/z 806), there is a series of fragment peaks (m/z 746, 686, 626) corresponding to multiple losses of 60 mu (acetic acid). Peaks separated by 42 mu may be due to loss of ketene or incomplete derivatization. The ions at m/z 311, 323 and 565 in Figure 6 correspond to ions at m/z 341, 353 and 655 in Figure 4. If the molecular weight of the acetyl derivative cannot be deduced from the EI mass spectrum, it can be easily determined from the isobutane CI mass spectrum, where the molecular ion (MH^+) is the base peak. As for TMS derivatives, acetyl derivatives can be made with deuterated acetic anhydride to form the deuterated derivative. The mass shifts for ions in the mass spectra of the h_3 and d_3 derivatives can be used to determine the number of acetyl groups added in the derivatization step.

STRATEGIES FOR STRUCTURE ELUCIDATION

The collection of simple rules used to rationalize fragmentation of small molecules are useful but not sufficient for determining the structures of large molecules with several functional groups. Although the primary fragmentation paths for each functional group can be predicted with considerable accuracy, the dominant fragmentation paths for molecules with several functional groups usually cannot be predicted reliably. This pessimistic view becomes less true for compounds with the same functional groups, differing only in their number and position. For example, acetogenins have the common features of 1 or 2 tetrahydrofuran rings, a γ -lactone, and several hydroxyl groups. With three different functional groups, the TMS derivatives of acetogenins give EI mass spectra that are unlikely to be predicted accurately *a priori*. However, having studied the mass spectra of more than a dozen acetogenins, we are now able to deduce major features of new forms just from the EI mass spectra of the TMS derivatives.

The molecular weight of acetogenins can usually be most easily deduced from the CI mass spectrum of either the underderivatized or derivatized material. Although both methods have been reliable for acetogenins, they could give misleading results for some types of substances. For example, water may be lost during the vaporization process to give an apparent molecular ion 18 mu too low, or the derivatization reaction may not proceed to completion. Alternatively, the molecular weight may be determined by FABMS. After establishing the molecular weight, it is desirable to determine the elemental compositions of the major peaks in the EI mass spectra of the TMS and AC derivatives. Since all but carbon of the common elements (H, N, O, etc.) have

fractional atomic weights (1.007825, 14.0034, 15.9959 etc.), the exact mass-to-charge ratio (m/z) of an ion is directly related to the elemental composition. For example, both molecular oxygen and methanol have nominal molecular weights of 32 but different exact molecular weights (31.9918 and 32.0272, respectively). As a result, the elemental compositions of ions can be determined from their m/z if it is determined with high accuracy. High resolution, ($m/\Delta m = 10,000$) is used because the centroid of the peak can be found more accurately when the peaks are narrow, and because ions of the same nominal mass but different elemental composition can be resolved.

The process and pitfalls for relating the accurate value for the m/z of an ion to its elemental composition can be illustrated with the ion at m/z 585 in Figure 4. An accurate measurement of this ion, using a mass resolving power of 10,000, indicated that the m/z is 585.3454. The error for such measurements is normally less than 10 ppm. Hence, all compositions with a m/z within 10 ppm of the determined value must be considered as viable candidates. A list of all compositions consistent with this m/z , calculated with the aid of a computer, are given in Table 1. From these results, it is apparent that, within the normal uncertainty of mass determination, several different compositions must be considered. This problem is especially severe for ions that contain mass deficient elements (silicon, sulfur, and phosphorous) and for high-mass ions. From the TMS derivative, the number of moles of silicon can be determined from mass-shifts observed in the TMS h_9 and d_9 mass spectra. The ion at m/z 585 was 27 mu higher in the mass spectrum of the TMS d_9 derivative, indicating that this ion has three TMS groups. Hence, four of the tentative compositions in Table 1 can be removed from consideration. Furthermore, for TMS derivatives, the number of silicons must be equal to or less than the number of oxygens (or nitrogen). It follows that only one of the tentative compositions listed in Table 1 ($C_{29}H_{57}O_6Si_3$) is acceptable.

It is important to note that the list of compositions given in Table 1 is incomplete. Specifically, only those compositions that have no more than 50 carbons, 100 hydrogens, 8 oxygens, and 6 silicons were considered. The number of elements, as well as the number of moles of each element is normally restricted to eliminate compositions that are not compatible with information derived from other sources. For example, the presence of nitrogen was excluded. Had the possibility of as many as 5 moles of nitrogen been allowed, the number of tentative compositions would have been 26. One of the greatest pitfalls encountered when using exact mass data occurs when a substance has an unexpected element. For example, TMS derivatives of phosphates are easily formed and give excellent EI mass spectra. Since phosphorous is not normally

Table 1. Possible elemental compositions consistent with m/z 585.3454 if it is assumed that the error is less than 10 ppm, and that only carbon, hydrogen, oxygen, and silicon are present

Calculated m/z	Error (mmu)	C	H	O	Si
585.3426	2.6	34	49	8	0
585.3430	2.3	33	53	5	2
585.3403	5.0	36	53	1	3
585.3461	-0.7	29	57	6	3
585.3434	1.9	32	57	2	4
585.3465	1.3	28	61	3	5

included in the element list, a computer generated list of potential compositions for the exact mass of the molecular ion of such a compound would likely have many entries, none of which are correct!

A useful approach for structure elucidation of unknown substances has been to determine the mass of the molecular ion by the highly accurate, but time and sample consuming peak-matching mode, and the masses of the fragments by the less accurate scanning mode. Since the mass of the molecular ion is determined with very high accuracy (1 ppm), a relatively unrestricted list of elements is used to generate a list of tentative assignments, of which only a small number (usually 1 or 2) of compositions are consistent with the experimentally determined mass. Having established the elemental composition of the molecular ion, a highly restricted list of elements can be used to generate a list of tentative compositions for the fragments. The results presented in Table 1 are typical of this approach.

Having determined the elemental composition from exact mass and TMS d9 data, likely structures for the fragments can be proposed. This is a difficult step requiring considerable imagination, and is facilitated by additional spectroscopic information. For the acetogenins, NMR analysis indicated the number of hydroxyl groups, tetrahydrofuran rings, and γ -lactones. Assigning structures to the fragments usually proceeds in concert with determining how smaller fragments join to form larger fragments. This process of joining small pieces to give larger pieces, always within constraints of the elemental compositions of the pieces, is continued until one or more tentative structures of the molecular ion evolve.

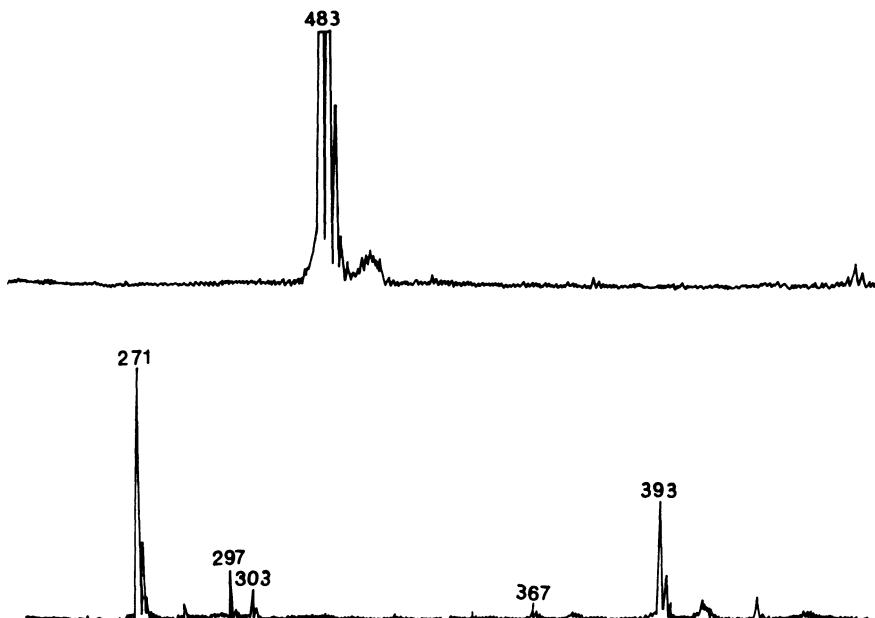


Fig. 7. E/B linked scan spectrum of the peak at m/z 573 in Figure 4.

Fragment ions are formed by a combination of series and/or parallel fragmentation reactions. The genesis of each fragment ion may be viewed as a family tree, with the molecular ion as the precursor of all fragments. Some features of a family tree can usually be determined from the elemental compositions of the fragments. For example, an ion with three oxygens can only be formed from a parent ion with three or more oxygens. Alternatively, the branches of a family tree can be determined by mass spectrometry / mass spectrometry (MS/MS).¹⁵ This form of analysis may be viewed as two-dimensional chromatography. A specific fragment ion is isolated from all other ions in the mass spectrum, and fragmented to give an ensemble of daughter ions which are recorded and interpreted as a conventional mass spectrum.

There are several types of mass spectrometers, such as triple quadrupole and four-sector instruments, which were designed specifically for MS/MS measurements. Similar results, though of generally less quality, may be obtained with two-sector instruments in which the electric sector voltage (E) and

the magnetic sector field (B) are scanned such that the ratio E/B is held constant. The E/B linked scan of the fragment ion at m/z 573 of the TMS derivative of gigantecin (from Fig. 4) is given in Figure 7. Consecutive losses of 1, 2, and 3 TMSOH groups is indicated by peaks at m/z 483, 393, and 303. The relationship of other peaks in the spectrum to the structure of gigantecin is indicated in the fragmentation scheme (Fig. 5). The power of using MS/MS daughter ion mass spectra for structure elucidation can be illustrated with the peak at m/z 271, which is due to the hydrocarbon chain indicated in Figure 5. It follows that ions which undergo fragmentation to give a peak at m/z 271 must include the left-hand side of the structure as it is drawn in Figure 5. This procedure was used in the original structure elucidation of gigantecin to determine how smaller ions were joined to form larger ions, that is, the family tree of the TMS derivative of gigantecin.

CONCLUSION

Mass spectrometry has played an important role in the structure elucidation of natural products because of its high sensitivity, and because it can be used to obtain information, such as molecular weight and elemental composition, which is not readily available by other methods. A complete and reliable analysis usually requires using different ionization methods. Electron ionization gives fragment ions indicative of molecular structure, whereas chemical ionization is most useful for determining molecular weight and purity. For materials that may undergo thermal degradation when heated, fast atom bombardment mass spectrometry may be the most reliable method for determining molecular weight. Alternatively, the electron and chemical ionization mass spectra of trimethylsilyl and acetyl derivatives of thermally labile substances can often be analyzed for molecular structure determination.

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Chapter Eight

MODERN NMR METHODS IN PHYTOCHEMICAL STUDIES

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INTRODUCTION

The last two decades have seen major new developments in the application of nuclear magnetic resonance (NMR) techniques applied toward structural organic chemistry and biochemistry problems in general, and structural as well as biosynthetic studies of natural products, in particular. The

development of superconducting magnets together with the application of pulse techniques and Fourier transformations by faster and more powerful computers have dramatically increased the versatility of NMR analysis.¹⁻⁷

In contrast to the continuous wave (CW) NMR measurements, which are carried out in the frequency domain, modern NMR spectroscopy uses the pulsed Fourier transform (FT) technique to record data after a pulse measuring the amplitude as it evolves with time (time domain).³ In the pulsed mode, a nucleus placed in a magnetic field is irradiated for several microseconds with an intense radiofrequency which causes reorientation of the nuclei into a nonequilibrium state. Between pulses the nuclei precess at frequencies which are influenced by their spin-spin interactions and their chemical environment (chemical shift). Chemically equivalent nuclei precessions decay exponentially, which can be detected in a receiver coil surrounding the sample. The complex sinusoidal, time-dependent wave forms detected by the receiver coil are referred to as "free induction decay" (FID). Fourier transformation of the sum of decreasing sine waves of different frequencies can be recorded on the frequency scale, which represents the NMR spectrum. Figure 1 shows the FID signal of the terpene parthenolide (a) as well as the Fourier transform of (a) in the frequency mode, which represents the ¹H NMR spectrum (b) of parthenolide.

One significant advantage of pulsed FT-NMR techniques over CW NMR measurements lies in the fact that pulsed FT-NMR experiments simultaneously record all frequencies instead of one after another as done in the CW NMR mode. This dramatically reduces experiment time, allowing multiple spectral scannings which significantly reduce the signal-to-noise ratio, thus enhancing the sensitivity of the technique.

Two important nuclei in organic chemistry, ¹²C and ¹⁶O, have no nuclear spin and cannot be used in NMR spectroscopy. However, ⁸H and ¹³C have nuclear spins of 1/2 and are therefore the most common nuclei used in NMR studies of natural products. The main disadvantage in the use of ¹³C NMR spectroscopy is the low natural abundance of the ¹³C nucleus, which is only 1.1% of ¹²C. Consequently, the sensitivity of ¹³C NMR, which is also influenced by its low gyromagnetic ratio, a characteristic constant for each nucleus, is nearly two orders of magnitude less (1.59%) than that of ¹H NMR. This gives an overall sensitivity ratio of ¹³C/¹H of about 1/5700. It is the availability of Fourier transformation and the application of pulse techniques that permits the use of ¹³C NMR⁴⁻⁶ as a powerful tool in organic structure determination.

A wide range of highly specialized NMR techniques has been introduced in recent years, which can provide a wealth of structural information. After the

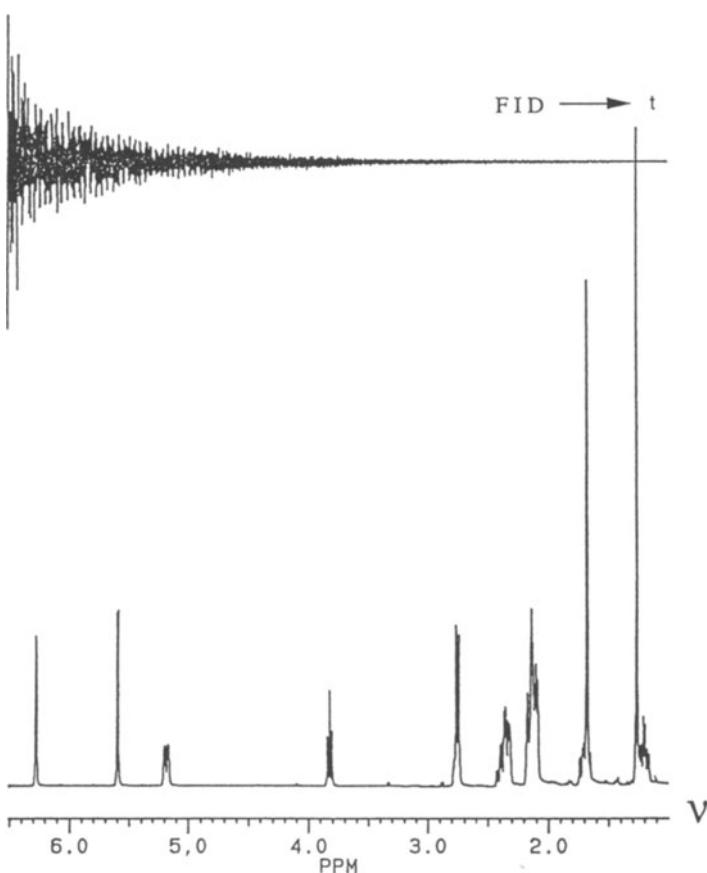


Fig. 1. (a) FID signal of parthenolide 200; MHz CDCl_3 . (b) Fourier transform of (a), the ^1H NMR spectrum of parthenolide.

introduction of two-dimensional (2D) NMR methods involving ^1H and ^{13}C , over 500 pulse sequences have been reported in the literature.^{8,9} Although it is understood that all these new powerful NMR techniques are based on a solid foundation of quantum mechanics and spin physics, this chapter will mainly emphasize the application aspects of selected NMR methods, which are commonly used in structure determination. Since natural products chemists are generally interested in obtaining structural and/or biosynthetic information from an NMR experiment, techniques will be discussed that have proven to be particularly useful in solving structural and biosynthetic problems of relatively

complex molecules with little emphasis on the physical foundation of these methods. The reader is referred to recent reviews and books for more detailed coverage of the basic principles of FT-NMR in general¹⁻⁷ and pulse sequence techniques in particular.^{8,9} Below, several useful one-dimensional (1D) and two-dimensional (2D) pulse sequences will be presented and examples for the use of these methods in solving structural problems of natural products will be outlined.

In the following section, examples for the most commonly used classical and modern homo- and heteronuclear correlations will be discussed. Figure 2 summarizes the major correlations of ¹H and ¹³C nuclei that are useful in the study of carbon and hydrogen connectivities within the molecular framework of a natural product. Substructures of a carbon-hydrogen framework can be conveniently established by 2D proton-proton (Fig. 2a), carbon-carbon (Fig. 2b) and carbon-proton shift correlations (Fig. 2c).

HOMO- AND HETERONUCLEAR SHIFT CORRELATIONS AS AN AID FOR STRUCTURE ASSIGNMENTS

Major types of ¹H - ¹H interactions (Fig. 2a) include geminal, vicinal and allylic couplings. In the past, CW NMR techniques involved specific ¹H-¹H decoupling experiments, which were tedious and time consuming.^{1,2} The homonuclear proton-proton shift correlation also known as COSY (for Correlation Spectroscopy)⁹⁻¹¹ is a 2D correlation in which the COSY matrix is quadratic. Since proton-proton couplings lie in a small range, the proton shift correlation spectrum exhibits all couplings within a molecular framework. This includes geminal, vicinal and long-range ¹H,¹H-couplings in a molecule.

Within the contour plot of a COSY experiment the one-dimensional ¹H NMR spectrum is projected on the diagonal resulting in a set of diagonal signals. In addition, due to the homonuclear magnetization exchange of coupled protons, cross signals in the square are generated. Protons without couplings will give diagonal signals only, but coupled protons also provide cross signals. This method establishes all spin couplings within a molecule. Examples for COSY spectra of natural products and other organic compounds are found in reference 9. Figure 3 shows the expanded ¹H-¹H COSY spectrum of parthenolide. The non-coupled proton signals (e.g. H-15) exhibit diagonal signals only, whereas the coupled absorptions also give cross signals indicating that they are coupled to each other.

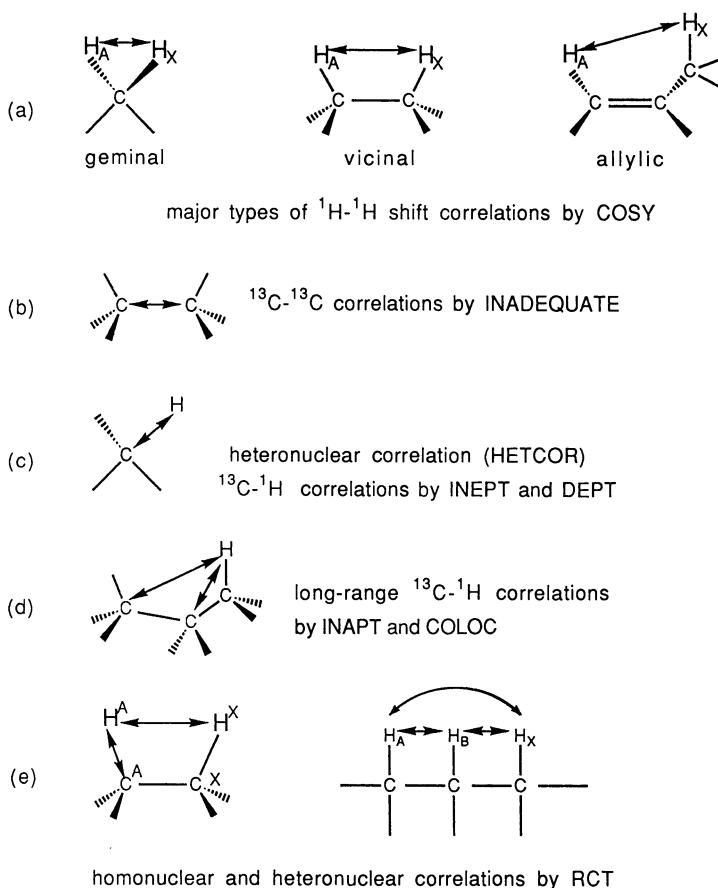


Fig. 2. Homo- and heteronuclear shift correlations commonly used in the structure determination of organic natural products.

Proton broadband decouplings are the most commonly used experiments in ^{13}C NMR spectroscopy. This simple and sensitive method records ^{13}C NMR spectra without the C-H couplings. Broadband decoupling is achieved by a decoupling field covering the range of all proton Larmor frequencies while the ^{13}C spectrum is recorded. These large noise-modulated frequency band irradiations collapse all multiplets due to ^{13}C - ^1H coupling, which results in the appearance of single lines for each ^{13}C absorption. This methodology, which is referred to as noise or broadband decoupling, is applied for two major reasons: simplicity as well as sensitivity. Broadband decoupling experiments increase the sensitivity of ^{13}C signals by accumulation of the multiplet signals into singlets.

However, an additional enhancement of sensitivity is observed which is due to dipolar coupling known as the nuclear Overhauser enhancement (NOE).¹² This effect is caused by through-space dipolar coupling of magnetic moments of two spins, which influence each other and therefore do not relax independently. Deviation from equilibrium of the spin state of one nucleus is transferred to the spin state of the other nucleus, thus causing deviation of its equilibrium, which generally results in signal enhancements. In broadband decoupling experiments, irradiation of the proton absorptions of C-H bonds contribute to proton relaxation (spin lattice relaxation). As a consequence, ¹³C nuclei attached to these protons

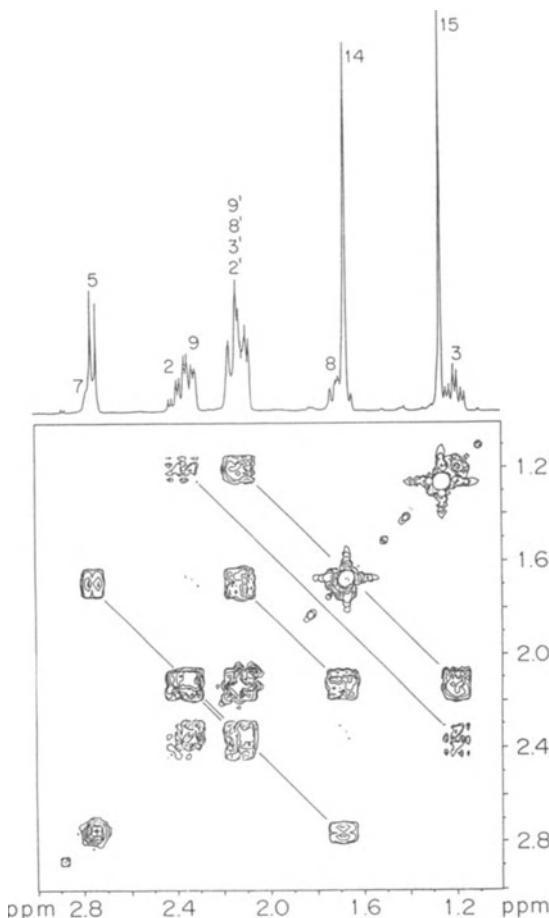


Fig. 3. Expanded 2D proton-proton shift correlation (COSY) of parthenolide (region 1-3 ppm).

also relax due to dipolar ^{13}C - ^1H coupling, causing an intensity increase of the carbon signals by a maximal factor of nearly two.¹³

NOE effects involving through space dipolar interactions between protons can provide valuable information on the configuration and conformation of organic compounds.^{12,13} In recent years, the applications of NOE effects in structural studies of natural products have become a standard technique in configurational as well as conformational assignments. 2D-COSY type spectra are frequently obtained to simultaneously determine all ^1H - ^1H NOE effects within a molecule. This method, which is commonly referred to as NOESY,³ will be applied later in configurational and conformational assignments of parthenolide (see Fig. 11).

Carbon-Carbon Connectivities by 2D INADEQUATE

Measurements of carbon-carbon connectivities (Fig. 1c) in an organic molecule without ^{13}C enrichments involves determination of the ^{13}C - ^{13}C couplings ($^{2}\text{J}_{\text{C,C}}$). Identical $^{2}\text{J}_{\text{C,C}}$ values indicate bonding between these carbons. Since the natural abundance of ^{13}C isotopes is only 1.1% of ^{12}C (10^{-2}), the probability of a ^{13}C - ^{13}C linkage is approximately 10^{-4} . Therefore, ^{13}C - ^{13}C couplings in natural abundance ^{13}C NMR spectra disappear as very weak satellite signals in the noise of the spectrum. However, suppression of the central ^{13}C signals, under proton-broad-band decoupling to remove the ^{13}C - ^1H couplings, could allow detection of the natural abundance ^{13}C - ^{13}C couplings. A pulse sequence known as 2D-INADEQUATE (Incredible Natural Abundance Double Quantum Transfer Experiment) permits detection of C-C-connectivities.¹⁴⁻¹⁶ In spite of improvements to reduce measuring time, and the data matrix and give COSY-like square correlation,¹⁷ the 2D INADEQUATE experiments still remains a highly insensitive technique which requires many hours of instrument time and at least 100 mg/ml of sample. Complete ^{13}C -assignments of the sesquiterpene lactone $11\beta\text{H},13$ -dihydroparthenolide from *Ambrosia artemisiifolia* were made and C-C-connectivities were determined by this technique as will be discussed later (see Fig. 14).

Polarization Transfer Experiments (DEPT, INEPT, INAPT, and COLOC)

Comparing the population difference between the ^1H and the ^{13}C precession states, ^{13}C nuclei are less polarized by a magnetic field due to the

small gyromagnetic ratio γ ($\gamma_{\text{H}} \approx 4\gamma_{\text{C}}$), resulting in weaker ^{13}C signals. Transfer of strong proton magnetization to the weakly polarized ^{13}C nucleus can be used to enhance ^{13}C signals, a method generally known as polarization or population transfer. In a ^1H - ^{13}C spin system, C-H coupling occurs between the nucleus with small population difference (^{13}C) and the one (^1H) with the larger population difference. Pulsed irradiation of the nucleus with large population difference (^1H) causes selective polarization transfer (SPT) to the weakly polarized ^{13}C nucleus. These types of experiments are useful for the observation of insensitive nuclei such as ^{13}C .

Pulse techniques for non-selective polarization transfer are known by the acronym INEPT¹⁸ (Insensitive Nuclei Enhanced by Polarization Transfer). They are useful for multiplicity selection as well as signal enhancements. A modified version of the INEPT method was introduced by Bax¹⁹ this technique with the acronym INAPT (Insensitive Nuclei Assigned by Polarization Transfer) is a simple and sensitive one-dimensional NMR method for determining two- and three-bond ^1H , ^{13}C -connectivities (Fig. 2d). INAPT is particularly useful in establishing correlations between protons and quaternary carbons via two- and three-bond couplings ($^2J_{\text{C},\text{H}}$, $^3J_{\text{C},\text{H}}$). Several examples of this powerful method in establishing structural connectivities will be presented later.

DEPT (Distortionless Enhancement by Polarization Transfer)²⁰ represents an improved polarization transfer technique that shows low sensitivity to the C-H couplings in a molecule with full signal enhancement and excellent multiplicity selection. It is particularly useful for generating subspectra of CH_3 , CH_2 and CH groups, allowing an unambiguous analysis of CH_x multiplets.

The magnitude of the polarization transfer is dependent on the pulse angle θ as shown in Figure 4. For instance, CH carbon signals give a maximum polarization transfer at $\theta = 90^\circ$. At this pulse angle the resulting magnetization is zero for CH_2 as well as CH_3 carbon signals. Consequently, experiments with $\theta = 90^\circ$ generate a subspectrum only for CH carbon signals in a molecule. Selection of an experiment with $\theta = 135^\circ$ give positive CH and CH_3 carbon signals but negative CH_2 ^{13}C -absorptions. In summary, DEPT spectra provide CH_x multiplicities with subspectra for CH, CH_2 and CH_3 groups. An example for this powerful technique, which provides C-H connectivities within a molecule, is given in Figure 7 for parthenolide.

A two-dimensional carbon-proton shift correlation denoted as COLOC (Correlation via long-range coupling) allows for identification of carbon-proton bonds via $^1J_{\text{C},\text{H}}$ and long-range carbon-proton interactions via $^2J_{\text{C},\text{H}}$ and $^3J_{\text{C},\text{H}}$ parameters.^{8,21} This method is particularly useful for localizing quaternary carbon substructures by correlation via long-range coupling, as in Figure 2d.

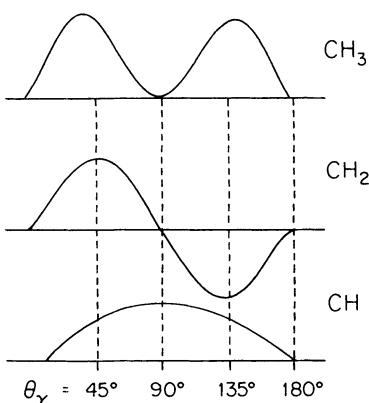


Fig. 4. The dependence of ^{13}C signal intensities in DEPT spectra of CH, CH_2 , and CH_3 groups as a function of the polarization transfer angle θ .

Before discussing specific examples of the above mentioned 1D and 2D NMR methods, the field strength dependence of signal separations and resolutions of ^1H NMR spectra needs to be re-emphasized. In Figure 5 the ^1H NMR spectrum at 100, 200 and 400 MHz of subcordatolide C, a known 7,8-lactonized eudesmanolide-type sesquiterpene lactone from *Calea subcordata*²², is shown. The 100 MHz ^1H NMR spectrum gives good separations of the proton signals between 3.0 and 6.5 ppm but strong overlaps of multiplets are seen between 1.5 and 3.0 ppm. At 200 MHz, all signals are well separated between 2 ppm and 6.5 ppm, but considerable overlap still exists between 1.3 and 1.9 ppm, a problem that is overcome by recording the spectrum of 400 MHz. The 400 MHz spectrum provides well separated signals which is highly desirable for further analysis by the above mentioned 1D and 2D FT-NMR techniques.

APPLICATIONS OF 1D AND 2D NMR EXPERIMENTS IN THE STRUCTURE DETERMINATION OF PARTHENOLIDE

We have chosen a well known natural product, parthenolide, to demonstrate the advantages and limitations of the most commonly used modern 1D and 2D NMR techniques all of which were performed on standard equipment. Parthenolide represents a terpenoid that was originally isolated from *Chrysanthemum parthenium*²³ and later found in other members of the families Asteraceae

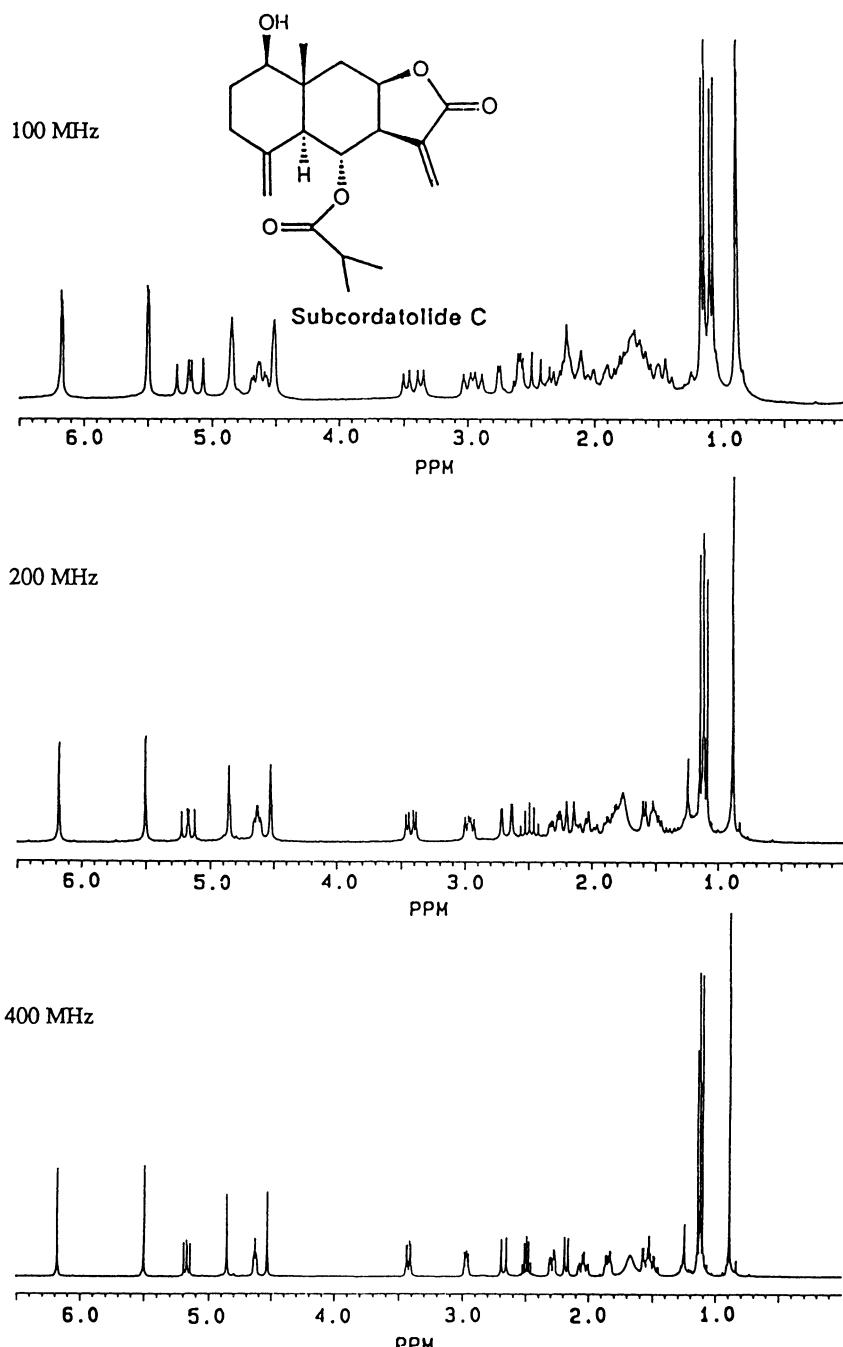


Fig. 5. ¹H NMR spectra of subcordatolide C at 100, 200, and 400 MHz.

and Magnoliaceae.²⁴ Recently, this compound attracted considerable attention since it is presumed to be the major active ingredient in European feverfew (*Tanacetum parthenium*), preparations of which are now marketed in a number of countries for prophylactic treatment of migraine headaches.²⁵ We recently found that parthenolide and closely related compounds are potent germination stimulants, at the ppb level, of witchweed (*Striga asiatica*), a parasitic weed of several major graminoid food crops including sorghum, corn, millet and sugar cane.²⁶

In order to develop our structural and stereochemical arguments, let us pretend at this point that the structure of parthenolide is unknown. This crystalline compound exhibits a strong UV end absorption near 210 nm and shows intense IR peaks at 1767 and 986 cm⁻¹, indicating the presence of an α,β -unsaturated γ -lactone and epoxide(s), respectively. The low resolution mass spectrum

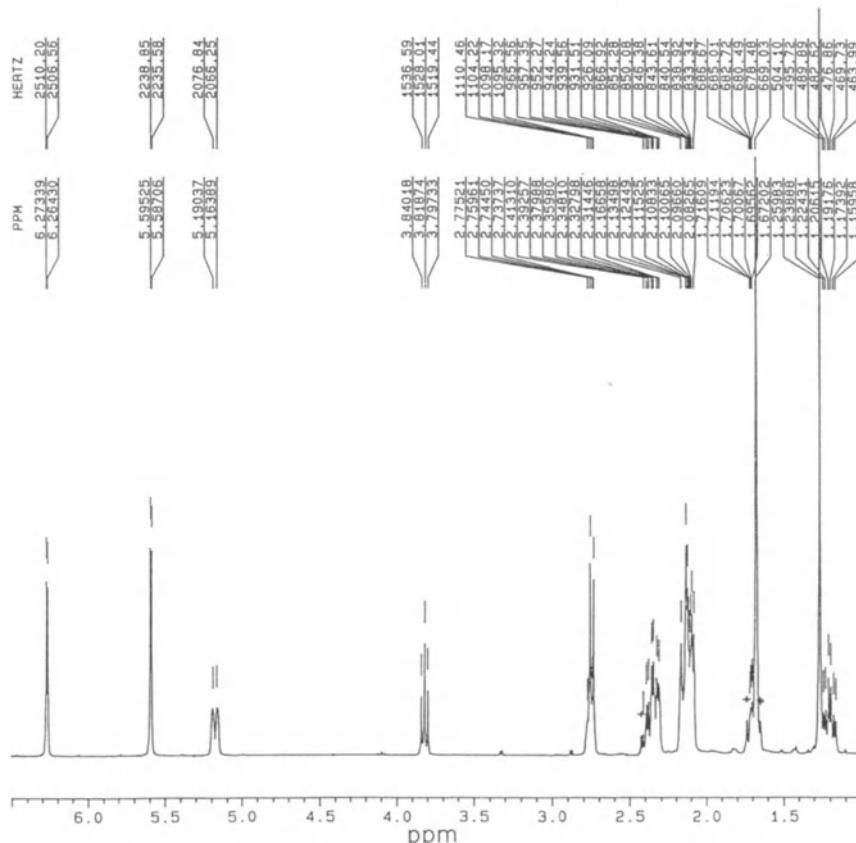


Fig. 6. ^1H NMR spectrum of parthenolide (CDCl_3 ; 400 MHz).

gives a parent peak at *m/z* 248. The 400 MHz ^1H NMR spectrum of parthenolide is shown in Figure 6. Below 3 ppm all peaks are well resolved but considerable signal overlap occurs above 3 ppm. Electronic integration clearly indicates that parthenolide contains 20 hydrogens.

The broad band proton-decoupled ^{13}C NMR spectrum at 100.6 MHz (Fig. 7a) shows 15 well separated carbon signals. Tentative chemical shift considerations suggest the presence of one conjugated carbonyl ester group near 170 ppm (C-4), four olefinic carbons between 120 and 140 ppm (C-13, C-1, C-10, and C-11) and three carbons (C-4, C-5, C-6) with a carbon-oxygen single bond (60-90 ppm). The subspectrum obtained by DEPT 45 CH_x (Fig. 7b) indicates that four carbons bear no hydrogens [C-4 (C-O), C-10, C-11 (C=C), and C-12 (C=O)]. Subspectrum c in Figure 7, which represents the $\theta = 90^\circ$ for C-H selection, shows that four carbons in parthenolide bear a C-H group [C-1 (-CH=), C-5 and C-6 (-CHO-) and C-7 (possibly-CH-C=)].

DEPT experiments with $\theta = 135^\circ$ (Fig. 7d) provide positive signals for CH and CH_3 groups and negative CH_2 absorptions. There are four aliphatic CH_2 groups (C-2, C-3, C-8, C-9) and one olefinic (C-13). In addition, two closely spaced CH_3 -absorptions appear near 18 ppm. In summary, editing the DEPT experiments of parthenolide provided highly important information about the types of carbons based on chemical shift considerations as well as the proton substitution pattern derived from the various subspectra. From this experiment we learned that the molecule must contain:

1 carbonyl carbon	
4 olefinic carbons:	two quaternary, one C-H bearing and one CH_2 -bearing carbon
3 oxygen-bearing carbons:	one quaternary and two CH-O-type carbons
5 methylene carbons:	one olefinic and four aliphatic CH_2 -groups
2 methyl carbons:	one at a carbon bearing an oxygen and one attached to an olefinic carbon

Taking into account that the molecular weight of parthenolide is 248 and that the molecule contains 15 carbons (180 m.u.) and 20 hydrogens (20 m.u.), it must contain 3 oxygens (48 m.u.), thus resulting in an empirical formula of $\text{C}_{15}\text{H}_{20}\text{O}_3$. Application of the unsaturation equivalent rule shows that this compound contains six unsaturation equivalents ($\text{C}_{15}\text{H}_{32}$ vs $\text{C}_{15}\text{H}_{20}$ in parthenolide): two olefinic bonds and one carbonyl were clearly established by the DEPT spectra. In addition, the IR spectral band at 1767 cm^{-1} strongly suggested an α,β -unsaturated γ -lactone. If the other two oxygen-bearing carbons,

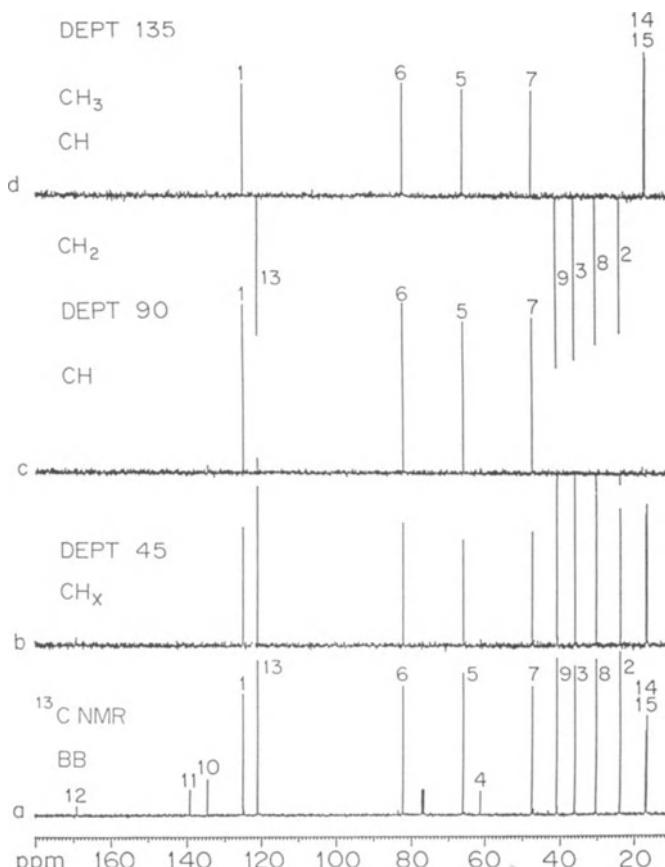


Fig. 7. Proton broadband decoupled ^{13}C NMR spectrum of parthenolide (CDCl_3 ; 100.6 MHz). (a) proton broadband decoupled spectrum; (b) DEPT subspectrum of ^1H -bearing ^{13}C nuclei; (c) DEPT subspectrum of CH nuclei; (d) DEPT subspectrum of CH, CH_3 (positive) and CH_2 nuclei (negative).

as determined by the ^{13}C NMR spectrum, represented an ether (possibly an epoxide), the remaining 6th unsaturation equivalent must be due to a cyclic ring structure. This would suggest a germacranolide-type sesquiterpene- γ -lactone, which typically contains a ten-membered ring.²⁴

Having considered the ^{13}C NMR spectrum and the DEPT subspectra of

parthenolide, it is now important to correlate ^1H - ^1H couplings by COSY and ^{13}C - ^1H connectivities of INAPT to learn about the C,H-framework of the molecule. As outlined above, the COSY spectrum is a quadratic auto-correlated experiment which provides information via couplings between the proton resonances. Responses on the diagonal correspond to all proton signals in the molecule. Coupled protons also give off-diagonal responses, which have a symmetrical relationship with reference to the diagonal. To analyze the data contained in the contour plot, it is important to select a starting point. In Figure 8, convenient starting points are the two downfield proton signal (H-13 and H-13'). They exhibit no couplings to each other, but both correlate with an overlapping signal near 2.8 ppm. Since one of the signals appears as a sharp doublet (H-5), the small couplings of H-13 and H-13' must be with the underlying broad multiplet (H-7).

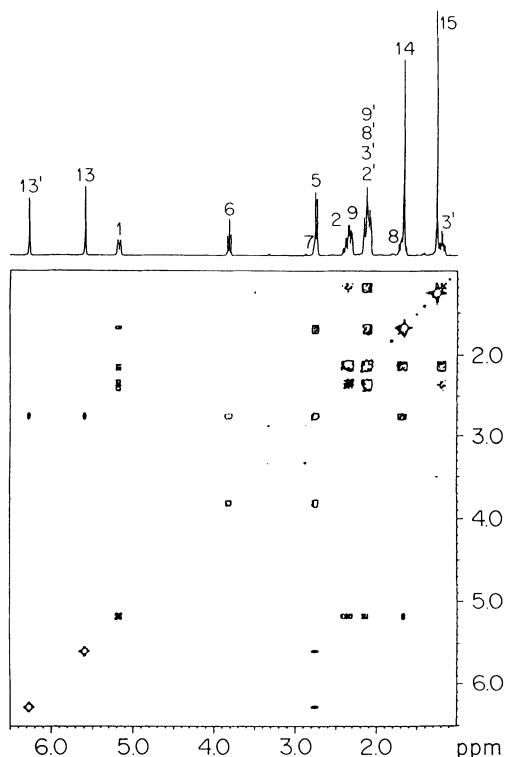


Fig. 8. 2D Proton-proton shift correlation (COSY) with ^1H NMR spectrum for reference (400 MHz; CDCl_3).

We can also observe that this overlapping signal exhibits a cross peak with the triplet at 3.82 ppm, presumably the lactonic proton (H-6). Since H-6 shows no coupling to other proton absorptions and the magnitude of the coupling constant ($J_{5,6} = J_{6,7} = 8.8$ Hz) corresponds to the coupling of the sharp doublet in the complex at 2.8 ppm, we can safely assign this doublet to H-5. In return, H-7 cross-correlates with a multiplet near 1.7 ppm (H-8) which partially overlaps with a broadened methyl singlet (H-14). In addition, H-8 correlates with a cluster of four overlapping signals near 2.2 ppm which on the basis of the J-value (> 12 Hz) must include a second geminal proton, H-8'. H-8 and H-8' are further correlated with multiplet absorptions near 2.3 and 2.1 ppm (H-9 and H-9'), which are geminally coupled judging by the splitting of the multiplet at 2.3 ppm ($J_{9,9'} \approx 13$ Hz).

Assuming that parthenolide indeed contains an α -methylene- γ -lactone moiety as indicated by the IR spectrum and diagnostic doublets near 5.6 and 6.3 ppm,²⁷ structural fragment A in Figure 9 can be drawn. Another starting point for cross correlations of proton signals is the signal at 5.18 ppm, (H-1), which correlates with three upfield signals including the methyl absorption near 1.7 ppm. This suggests that the broadened H-1 doublet is allylically coupled to the methyl group (H-14) and shows vicinal couplings to two further protons (H-2, H-2') which in turn are further coupled to two additional protons, H-3 (near 2.2 ppm) and H-3' at 1.20 ppm. The structural fragment B in Figure 9 can be assembled from the above data. This accounts for all carbons and hydrogens except the carbon bearing the second methyl group and the ether oxygen. The chemical shift at 1.22 ppm for this methyl (H-15) is in agreement with its attachment to an oxygen-bearing carbon as shown in fragment C. Fragments A-C can be assembled to provide the biogenetically reasonable structural framework D in Figure 9 for parthenolide. This arrangement of atoms is in full agreement with the ^1H NMR, ^{13}C NMR and their COSY and DEPT analysis.

An extension of the COSY experiment is the 2D Homonuclear Relayed Coherence Transfer (RCT) experiment.^{28,29} This technique allows to follow the spin system beyond the single spin-spin coupling by providing cross peaks between protons that are not coupled to each other (eg. H-13a and H-6/H-8) but are coupled to a common third proton (eg. H-7).

Further verification of the internal consistency of the previous assignments was obtained from the 2D ^{13}C - ^1H correlation experiment (Fig. 10). The two most downfield doublets assigned to the lactonic methylene protons (H-13 and H-13') are attached to the same olefinic carbon and H-1 is also attached to an olefinic carbon. The H-6 triplet near 3.8 ppm is correlated with the carbon bearing the lactonic oxygen and most importantly, the proposed epoxide carbon

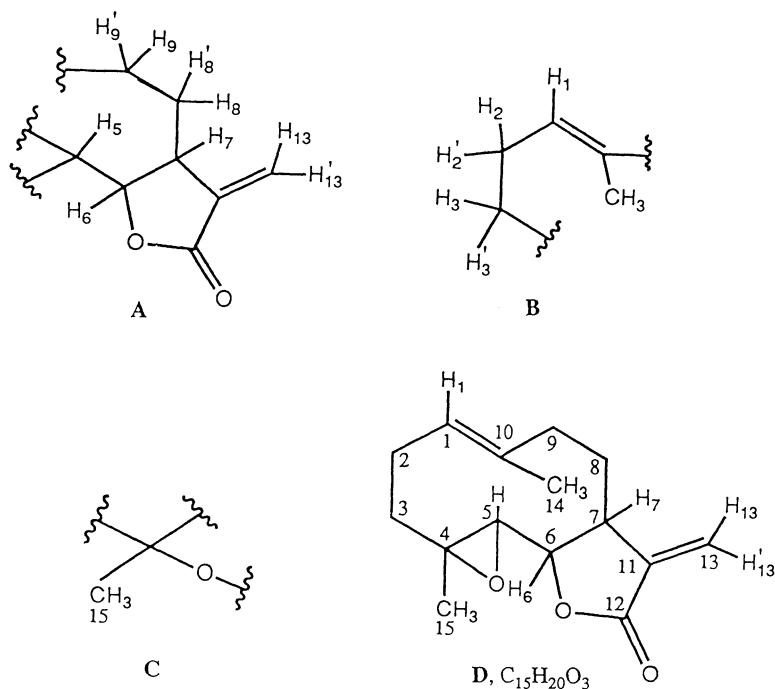


Fig. 9. Structural fragments A-C and the proposed molecular framework (D) of parthenolide.

signals 4 and 5 clearly exhibit chemical shifts near 60 ppm in agreement with ^{13}C NMR absorptions typical for epoxides.^{5,6} Also, H-5 is indeed attached to one of the oxygen-bearing carbons (C-5), part of the epoxide moiety.

It would be desirable, however, to unambiguously demonstrate that fragments A, B and C are connected in the arrangement shown in assembly D. Unfortunately, these connectivities could not be derived from the above NMR spectral data. Unambiguous demonstration of connectivities between C-9 and C-10 as well as C-3/C-4 and C-4/C-5 was accomplished by the use of INAPT¹⁹ and COLOC.^{8,9,21} Both methods allow for correlations between protons and quaternary carbons via two- and three-bond couplings (²J_{C,H}, ³J_{C,H}). Figure 11 shows the one-dimensional INAPT spectra of parthenolide involving the distinct proton absorption H-6 and H-1 appearing near 3.8 and 5.2 ppm, respectively. Using a coupling of J_{C,H} = 7 Hz, selective polarization of H-6 (Fig. 11b) generated resonances for C-8 at 30.5, C-4 at 61.44, C-5 at 66.23 and C-6 at 82.39 ppm. Polarization of H-1 (Fig. 11c) with a coupling J_{C,H} = 10 Hz gave

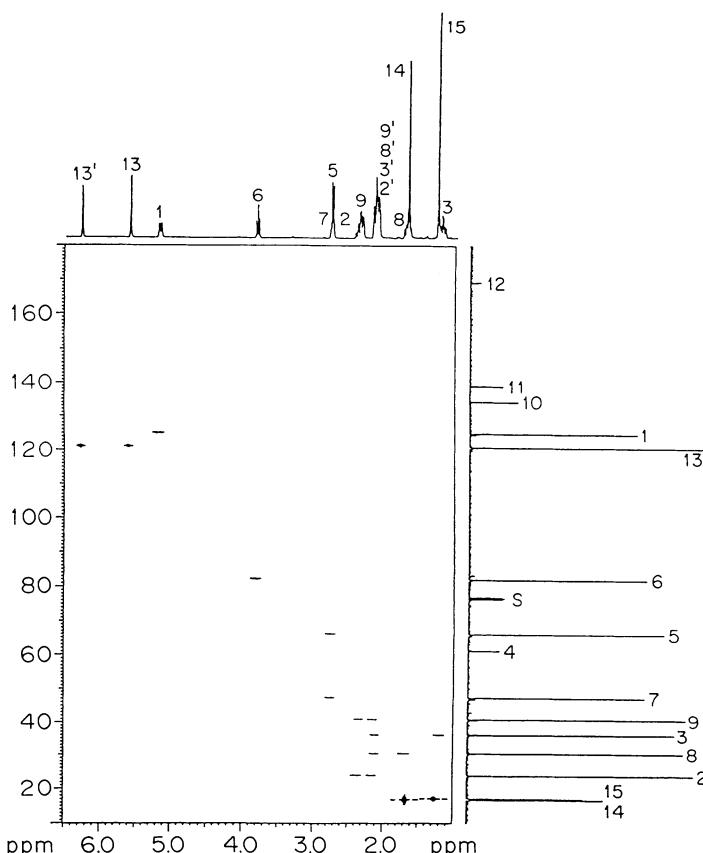


Fig. 10. 2D ^{13}C - ^1H shift correlation of parthenolide in CDCl_3 .

resonances for the methyl carbon C-14 at 16.80 and C-9 at 41.04 ppm. The above data clearly established the connectivities between C-4, C-5 and C-6 as well as between C-9 and C-10. This also requires that C-3 of fragment **B** must be connected to C-4 of fragment **C** in Figure 9, thus providing unambiguous evidence for the structural framework **D** for parthenolide (Fig. 9), exclusive of stereochemistry and conformation.

Figure 12 represents the COLOC spectrum of parthenolide. The 2D ^{13}C - ^1H shift correlations via long-range ^{13}C - ^1H couplings ($^2\text{J}_{\text{C},\text{H}}$, $^3\text{J}_{\text{C},\text{H}}$) support the INAPT results for parthenolide and shows that the previous assignments are self consistent. At this point it is not known whether the configuration of the 1(10)-double bond in parthenolide is *E* or *Z* and the 4,5-epoxide is

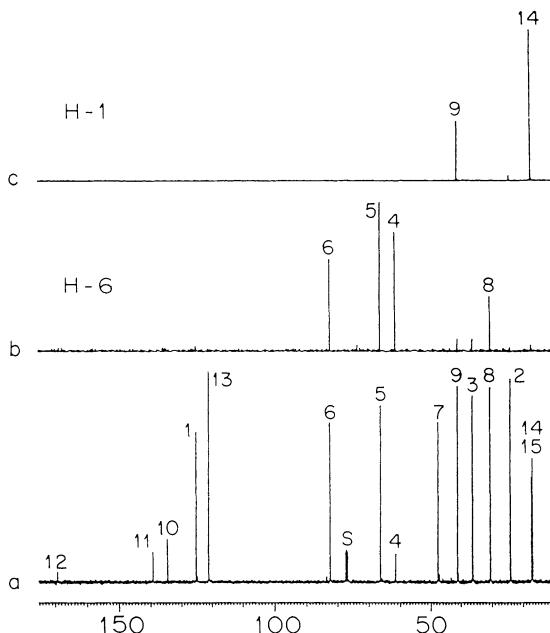


Fig. 11. Selective 1D ^{13}C - ^1H shift correlation of parthenolide via long-range ^{13}C - ^1H coupling (INAPT).

derived from an *E* or *Z* double bond. Furthermore, the configurations at C-6 and C-7 need to be established. The large couplings ($^2J_{5,6} = ^2J_{6,7} \approx 9$ Hz) suggest an antiperiplanar (trans) arrangement of these three protons (H-5/H-6 and H-6/H-7). Since in all sesquiterpene lactones from higher plants the C-7 proton is without exception α -oriented,^{24,27} H-6 had to be β and H-5 α . This fact was used as a starting point for NOE studies of parthenolide to establish its conformation. As shown by the cross-correlations of the NOESY in Figure 13, the two lactonic methylene protons show the expected dipolar interaction. More importantly, H-1 and the C-10 methyl group (H-14) as well as H-5 and the C-4 methyl (H-15) signals give no correlation suggesting an (*E*)-1(10)-double bond and a 4,5-epoxide with the C-4 Me and H-5 being trans. Furthermore, the correlation of H-1 with the H-5/H-7 cluster indicates an orientation of H-1 below the plane of the medium ring. This orientation requires that the methyl groups at C-10 (H-14) and C-4 (H-15) reside above the plane of the medium ring in proximity of H-6 β . Cross correlations between the H-6 signal and the respective C-4 and C-10 methyl signals establish their relative relationship in

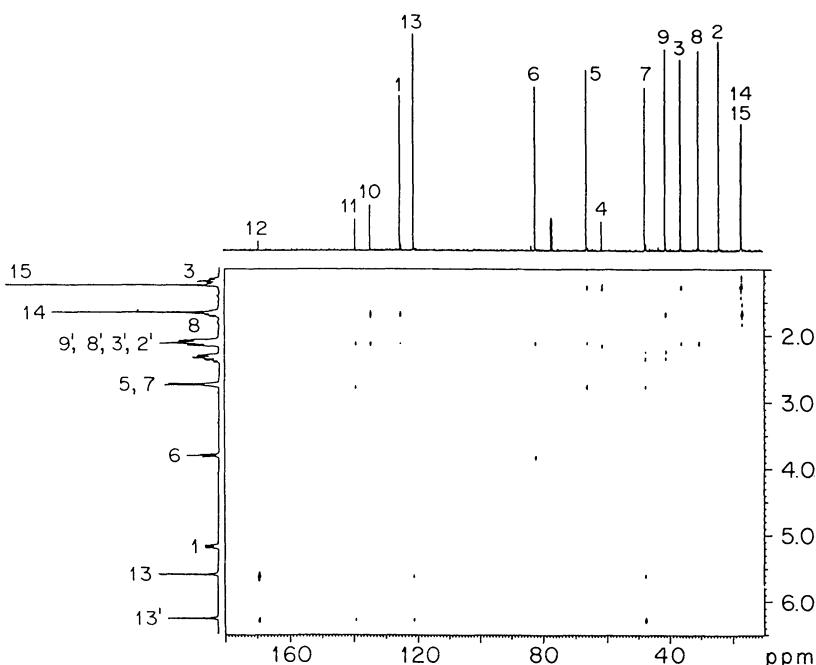


Fig. 12. 2D ^{13}C - ^1H shift correlation of parthenolide via long-range ^{13}C - ^1H coupling (COLOC).

space with the conformational structure shown in Figure 13. The structure and solid state conformation of parthenolide was previously established by single crystal X-ray analysis³⁰ and the presented NMR data are in complete agreement with the reported molecular structure.

CARBON-CARBON CONNECTIVITY OF $11\beta\text{H},13\text{-DIHYDRO-PARTHENOLIDE BY 2D-INADEQUATE}$

The pulse sequence known as 2D-INADEQUATE permits detection of carbon-carbon connectivities.¹⁷ Although this technique is insensitive and requires large sample sizes (~100 mg/ml or more) and considerable instrument time, it was successfully applied toward the unambiguous assignment of all ^{13}C absorptions leading to the molecular framework of $11\beta\text{H},13\text{-dihydroparthenolide}$, a constituent of the aerial parts of common ragweed (*Ambrosia artemisiifolia*).³¹ As parthenolide, this compound is also highly stimulatory toward the germina-

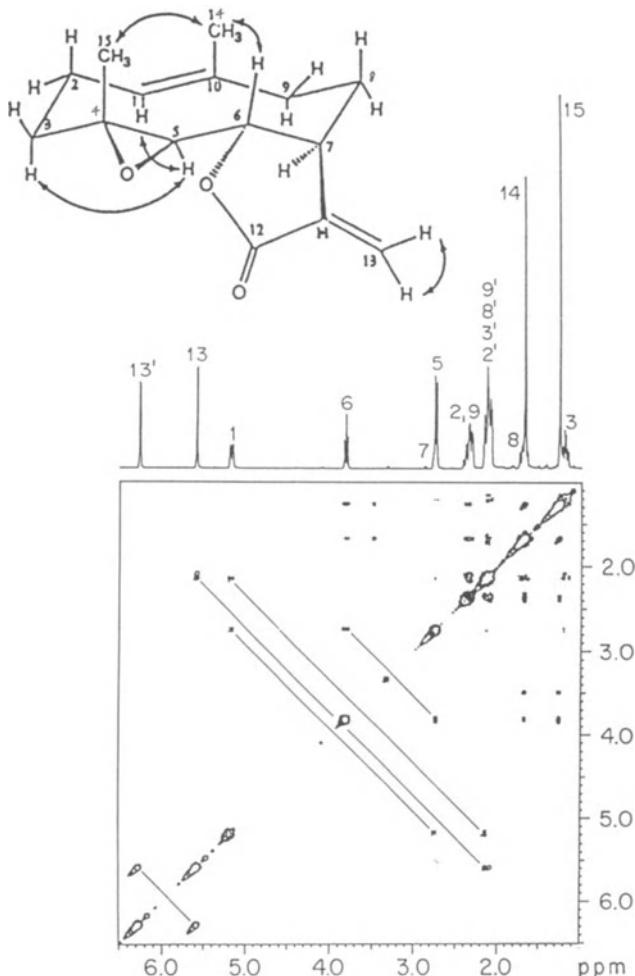


Fig. 13. 2D ¹H-¹H NOE correlation (NOESY) of parthenolide (CDCl₃; 400 MHz).

ation of witchweed at the 10⁻⁷ - 10⁻⁹ molar level.²⁶ Figure 14 shows the carbon-carbon connectivity plot of 11 β H,13-dihydroparthenolide. The vertical axis represents the "f₁" dimension (double quantum frequency) and the horizontal axis gives the chemical shift (f₂ dimension). If two carbons are directly bonded they will show the same double quantum frequency f₁ and appear equally spaced on both sides of the f₁ line. Inspection of the accentuated carbon-carbon connect-

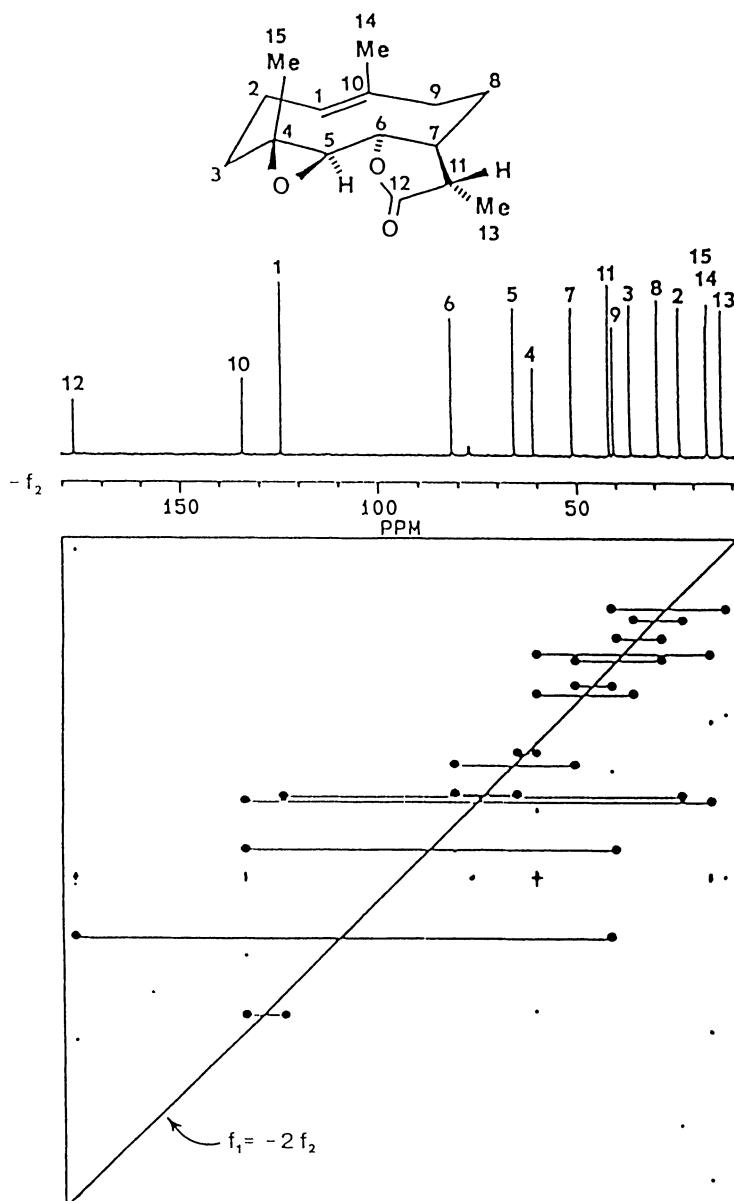


Fig. 14. Carbon-carbon connectivity plot of 11,13-dihydroparthenolide. Carbons sharing the same quantum frequency f_1 appear equally spaced on both sides of the line f_1 . (From Spectroscopy Letters 1987, 20: 445-450; with permission).

ivities in Figure 14 result in complete carbon assignments and the molecular framework of 11β H,13-dihydroparthenolide.

STRUCTURAL ASSIGNMENTS OF FLAVONOIDS AND POLYACETYLENES BY THE INAPT METHOD

We have recently applied INAPT for the unambiguous assignment of ^1H and ^{13}C NMR signals as well as the positions of hydroxyls and methoxy groups of a flavonoid isolated from *Calamintha ashei* (Lamiaceae).³² A major difficulty in the structure determination of highly substituted flavonoids resides in the fact that there is no reliable general method to distinguish between 5,6,7- and 5,7,8-substituted flavonoids. It is generally also difficult to distinguish between the above two structural types and 5,6,7,8-substituted flavonoids. In addition, unpredictable effects of hydroxyls and methoxy substituents, in particular upon the ^{13}C NMR chemical shifts of carbons in the A-ring of flavonoids, do not allow unambiguous ^{13}C signal assignments.

This study describes the application of INAPT to be used as a complementary method in solving structural problems of flavonoids. It is known that the hydrogen-bonded C-5-hydroxyl hydrogen of flavonoids exhibits long-range couplings to C-6, C-10, and C-5.^{33,34} This common C-5-hydroxyl substituent present in most flavonoids can be very useful in the application of INAPT as an aid in structural assignments.

Figure 15 illustrates how INAPT experiments can be designed to distinguish between different types of C-5-hydroxylated flavones and flavonols with one unsubstituted carbon in ring A. In principle, it should be applicable regardless of the kind of substitution at the other carbons. For flavones and flavonols with H-6 or H-8, polarization of the C-5-OH alone should be sufficient (Fig. 15a). Compounds bearing a hydrogen at C-6 would transfer from the C-5-OH proton to quaternary carbons C-5 and C-10 and to one hydrogen-bearing carbon appearing as a doublet (C-6). Alternatively, flavonoids with an H-8 and any non-hydrogen substituent at C-6 would transfer to three quaternary carbons (C-5, C-10, and C-6).

To determine whether a proton singlet is due to an H-8 or an H-3, two INAPT experiments should be performed. The expected INAPT results are illustrated in Figure 15b,c. In a 5,6,7-substituted flavonol, polarization of H-8 would transfer to quaternary carbons C-6, C-10, C-7, and C-9 while polarization of C-5-OH would affect quaternary carbons C-5, C-6, and C-10 (Fig. 15b). In 5,6,7-substituted flavonols, both INAPT spectra would show polarization

transfers to C-6 and C-10. For a 5,6,7,8-substituted flavone (Fig. 15c), polarization of H-3 would transfer to C-2, C-10, and C-1' while polarization of C-5-OH would affect C-5, C-6, and C-10. In this flavone substitution pattern both INAPT spectra would show polarization transfers to C-10. Since the chemical shifts of C-10 and C-2 are very distinct, assignments could be performed unambiguously.

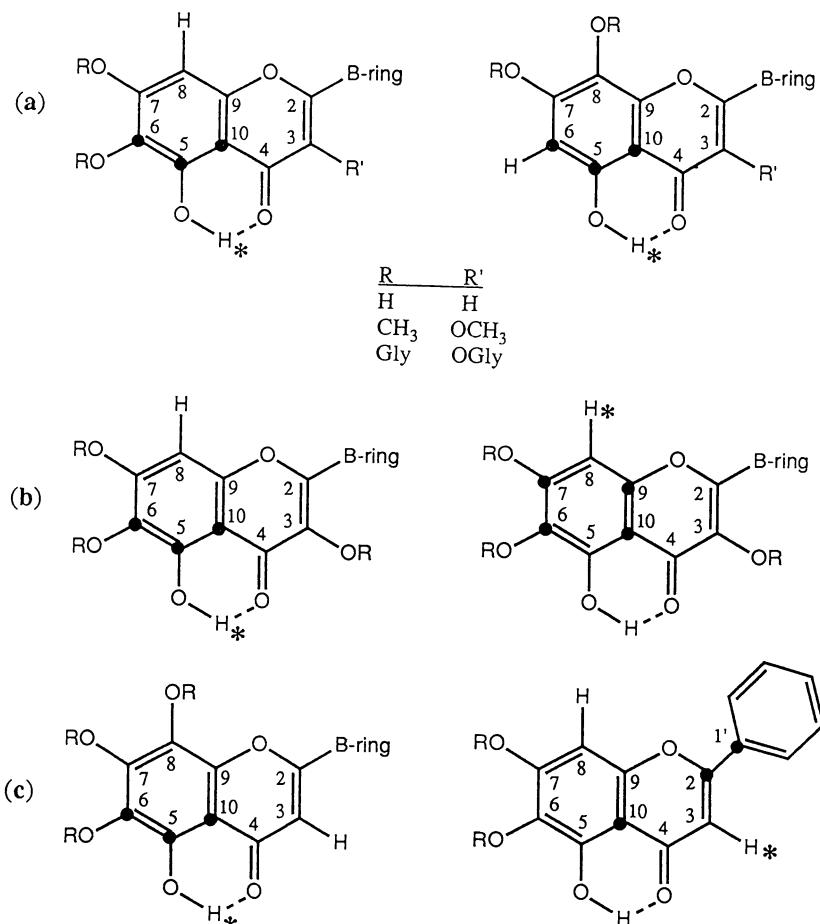


Fig. 15. (a) Expected INAPT results to distinguish between H-6 or H-8 of flavones as well as flavonols; (b,c) Expected INAPT results of flavonols with H-8 and flavones with fully substituted A-rings. Stars denote the polarized proton; dots denote the carbons that would be affected in the INAPT spectrum.

Figure 16 shows the ^1H NMR (a), the broad band ^{13}C NMR (b) and the INAPT spectra (c-f) of the diacetate of 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (thymonin).³⁵ The distinctly low-field hydrogen-bonded C-5-hydroxyl proton of flavonoids appearing near 12.5 ppm exhibit long-range couplings to C-6 and C-10³³ as well as C-5³⁴, a fact very useful for the application of INAPT experiments in the structural assignments.

The INAPT experiments of the diacetate of thymonin provided conclusive results on the attachments of the various substituents due to the well-resolved proton chemical shifts (Fig. 16a). As shown in Figure 16c, polarization of the C-5 hydroxyl proton (~12.5 ppm) transferred to C-5, C-6 and C-10. Selective polarization of the singlet at 6.68 ppm generated resonances for C-2 and C-10 which confirmed the assignment of this proton as H-3 (Fig. 16d). To verify the type of substitution at C-6, the INAPT subspectra of the methoxy groups were recorded. As shown in Figure 16e and f, the methoxy groups caused no polarization transfer to C-6, thus indicating that C-6 in thymonin bears no methoxy group. Instead, signals appeared for C-7 and C-8 which confirmed that this flavone bears methoxy moieties at these two carbons and therefore has to be 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone.

The above example demonstrates that INAPT can be used as a powerful tool in solving structural problems in highly substituted flavonoids. Polarization of the well-separated C-5 hydroxyl proton would provide information on the presence or absence of a proton at C-6. Furthermore, this technique permits to distinguish between protons H-6 and H-8 of the A-ring as well as H-3 of a flavone. In case of low polarization selectivity due to overlap of proton signals, derivatization such as acetylation and/or change of solvent may lead to well-resolved ^1H NMR spectra.³²

The assignments of carbon signals in polyacetylenes represent another example for the usefulness but also the limitations of the INAPT technique. Figure 17 gives the INAPT spectra of (*Z*)-dehydromatricaria lactone. Polarization of the methyl absorption at 2.02 ppm (H-10), using a coupling parameter of 10 Hz, transferred to three quaternary carbons: a strong signal at 85.8 (C-9) and two weaker ones at 86.5 and 64.5 ppm which most probably belong to C-8 and C-7. Irradiation of the same signal (H-10) using a 4 Hz coupling ($^3\text{J}_{\text{C},\text{H}}$)³⁶ also enhanced the two signals at 86.5 and 64.5 ppm. This result could not distinguish between C-8 and C-7 since the couplings $^3\text{J}_{\text{C},\text{H}} \approx 4$ Hz and $^4\text{J}_{\text{C},\text{H}} \approx 3$ Hz are too similar.³⁶ Polarization of the methyl signal (H-10) using a C,H coupling value of 3 Hz ($^5\text{J}_{\text{C},\text{H}}$) transferred polarization to two quaternary carbon absorptions at 86.5 and 67.9 ppm. This allowed unambiguous assignment of C-7 at 86.5 ppm, since it gave a strong signal when the $^5\text{J}_{\text{C},\text{H}}$ value

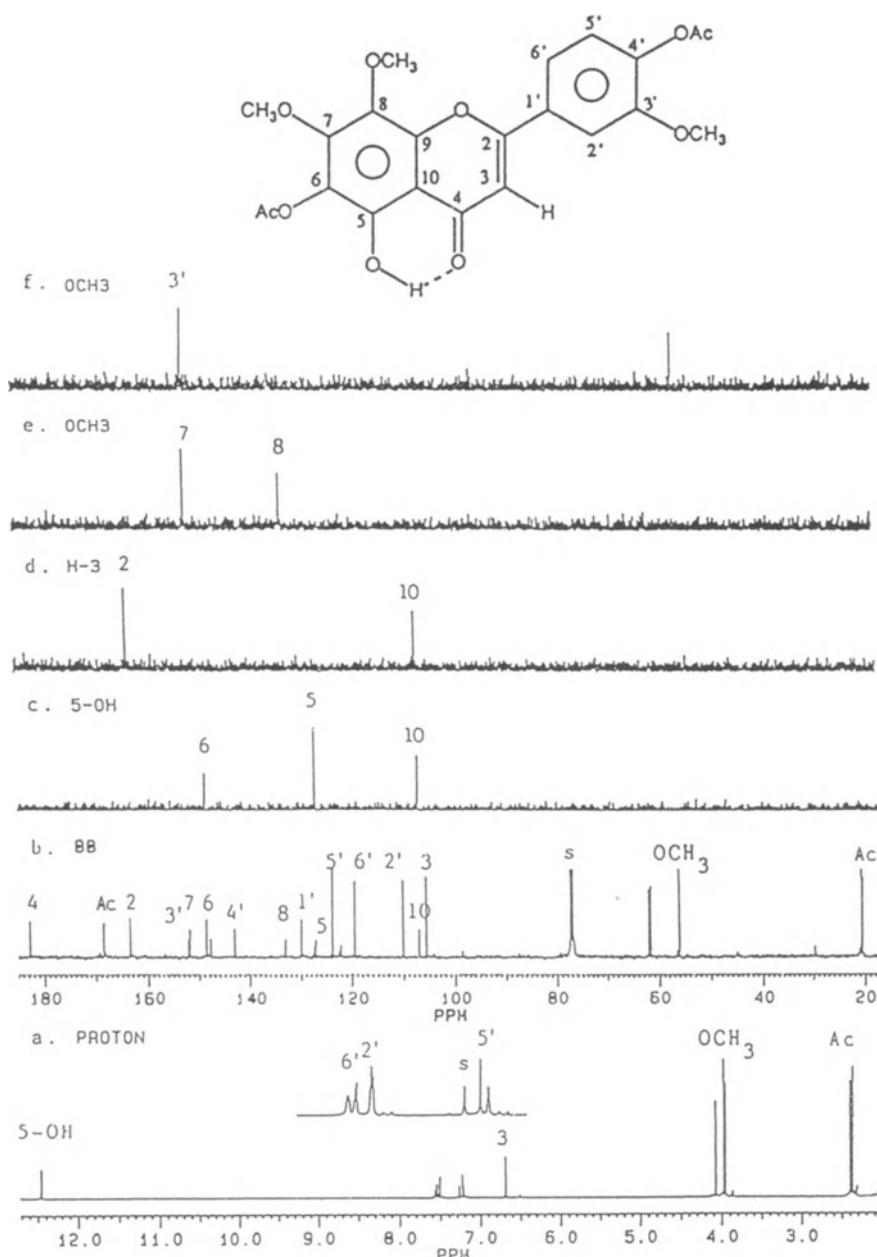


Fig. 16. ¹H NMR (a) and broadband ¹³C NMR (b) and INAPFT spectra (c-f) of the diacetate of 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (thymo-nin). (From Spectroscopy Letters 1988, 21: 927-934; with permission).

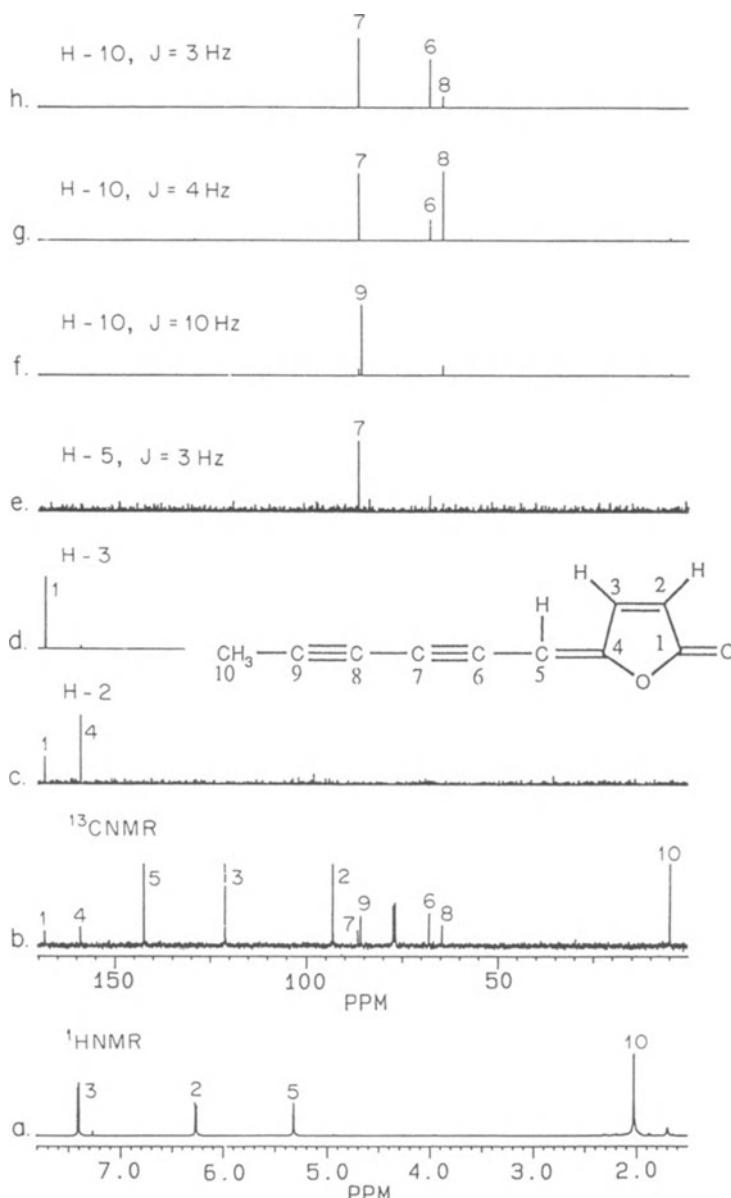


Fig. 17. ^1H NMR (a) and broadband ^{13}C NMR (b) and INAPT spectra (c-f) of 4,5(*Z*)-dehydromatricaria lactone.

was applied. The other carbon signal at 64.5 ppm can now be assigned to C-8 based on the INAPT experiment with a $^4J_{C,H} = 4$ Hz. The COLOC experiment clearly showed that the methyl protons (H-10) couple to all four acetylenic carbons (C-9 to C-6).

The appearance of the signal at 86.5 ppm, after irradiation of H-5 using a coupling of 6 Hz, confirmed the assignment of C-7. Polarization of H-3 (Fig. 17d), using a coupling of 5 Hz, transferred to C-1 at 168.2 ppm. Finally, the INAPT spectrum resulting from polarization of H-2 (Fig. 17c) showed a strong signal at 158.9 ppm (C-4) and a weak one at 168.2 ppm (C-1).

Although the INAPT experiment is a very powerful technique in the assignment of quaternary carbons through ^{13}C - ^1H couplings, its limitations in the structure elucidations of polyacetylenes with two or more conjugated triple bonds arise from the fact that all acetylenic carbons couple to the protons of interest. Also, the different ^{13}C - ^1H coupling constants are very similar, giving INAPT spectra with residual signals which make the interpretation of the spectra more difficult.

The stereochemistry of the 4,5-double bond of dehydromatricaria ester was assigned by NOE difference experiments of protons 5 and 3. Irradiation of H-5 gave an NOE of H-3 showing that H-5 and H-3 are on the same side. Clear NOE effects on H-5 and H-2 were also observed upon irradiation of H-3, thus confirming the previously assigned (Z)-configuration of the 4,5-double bond.³⁷

BIOSYNTHETIC STUDIES OF BITHIOPHENE I IN HAIRY ROOT CULTURES OF *TAGETES PATULA* USING ^{13}C -LABELED ACETATES

A wide range of structural types of acetogenins with multiple triple bonds (polyacetylenes) are found as natural products they are very common constituents in many members of the large sunflower family (Asteraceae).³⁸ Recently, these relatively unstable, often highly conjugated compounds have attracted considerable attention due to their broad spectrum of bioactivity.³⁹ Previous biosynthetic studies of polyacetylenes and the derived thiophenes were performed with radioactive ^{14}C - and tritium-labeled precursors.^{40,41} Based on these results, it is now generally accepted that polyacetylenes are formed by linear combination of acetate units.⁴⁰ The thiophenes are presumably derived from pentaynenes by addition of sulfide to the triple bonds, a process that can be mimicked *in vitro* by the addition of H_2S to diynes.³⁸

The introduction of pulsed Fourier transform NMR techniques in the

early 1970s permitted the use of ^{13}C NMR labeling experiments in biosynthetic studies.⁴² The advantage of the use of ^{13}C -labeled precursors is the gain of a wealth of biosynthetic information without specific degradation experiments, which are required in ^{14}C - and tritium-labelling studies. A disadvantage is its low sensitivity, necessitating relatively high incorporations of the ^{13}C -labels.

The use of hairy root cultures in biosynthetic studies is unexploited and has considerable potential. We therefore attempted the incorporation of [1- ^{13}C]-, [2- ^{13}C]- and [1,2- $^{13}\text{C}_2$]-labeled acetates into bithiophenes using hairy root cultures of *Tagetes patula*. It was of particular interest to learn whether biosynthetic incorporations of ^{13}C -labels into constituents of hairy root cultures are sufficiently effective to make use of the ^{13}C NMR methodology. Biosynthetic ^{13}C NMR experiments with live plants are generally limited due to the low yield of incorporation of precursors, thus excluding this powerful but insensitive ^{13}C NMR method.⁴²

Our biosynthetic studies of bithiophenes using hairy root cultures of *T. patula* demonstrated that hairy root cultures can be highly suitable media for biosynthetic studies of root constituents, using the ^{13}C NMR methodology. Complete assignments of the carbon signals of bithiophene I (Fig. 18) were achieved by the use of DEPT 135° and DEPT 90° experiments as well as carbon-hydrogen (C-H) correlations, INAPT experiments and by spectral comparison with published results.⁴³ The ^{13}C NMR spectra of the unlabeled and the singly labeled experiments were recorded on the same scale to allow for easy comparison of the relative intensities of the peaks thus permitting better evaluation of the magnitude of ^{13}C -incorporations. The ^{13}C NMR spectra of (a) unlabeled, (b) acetate-[1- ^{13}C]-, (c) acetate-[2- ^{13}C]- and (d) acetate [1,2- $^{13}\text{C}_2$]-enriched bithiophenes I are shown in Figure 18.⁴⁴ [1- ^{13}C]-Acetate enriched bithiophene I (Fig. 18b) exhibited enhanced signals for carbons 2,4,6,8,10 and 12 while the acetate-[2- ^{13}C] enriched sample (Fig. 18c) showed enhancements of carbon signals 1,3,5,7,9 and 11. The ^{13}C NMR spectrum of acetate-[1,2- $^{13}\text{C}_2$]-enriched bithiophene I is shown in Figure 18d. The carbons peaks derived from intact incorporated acetate units appear as triplets. The center peaks are due to the natural abundance signal and the two satellite signals are due to ^{13}C - ^{13}C couplings from incorporation of intact acetate units.⁴² Couplings of 88.5 (C-2/C-3), 106.8 (C-4/C-5), 58.0 (C-6/C-7), 71.7 (C-8/C-9) and 58.0 Hz (C-10/C-11) were observed, indicating that these positions are derived from intact acetate units. When compared to the signals of the natural abundance ^{13}C NMR spectrum, C-1 and C-12 appeared as significantly enhanced singlets with very small satellite peaks, suggesting that C-1 and C-12 are derived from acetate units

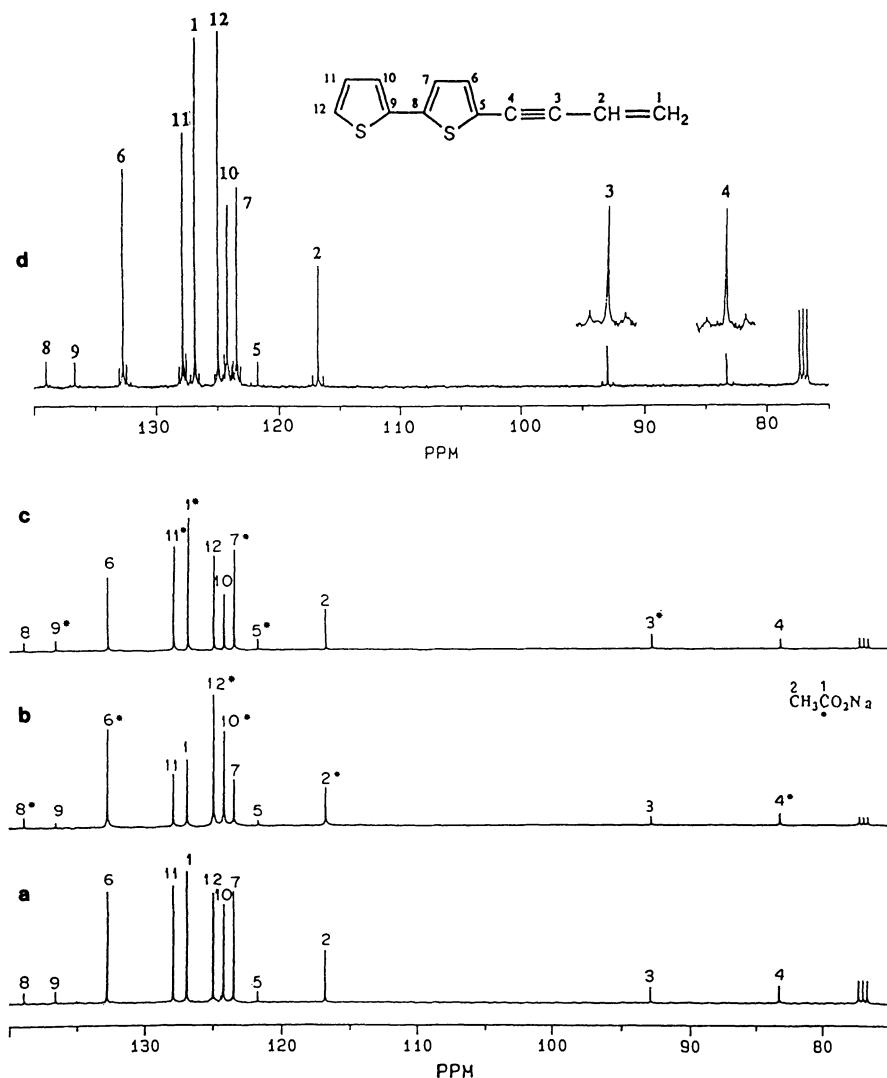


Fig. 18. Broadband ^{13}C NMR spectra of ^{13}C -labeled acetate enriched bithiophene I. (a) natural abundance spectrum; (b) acetate-[1- ^{13}C]-enriched ^{13}C NMR spectrum; (c) acetate-[2- ^{13}C]-enriched ^{13}C NMR spectrum; (d) acetate-[1,2- $^{13}\text{C}_2$]-enriched ^{13}C NMR spectrum. (From Spectroscopy Letters; with permission).

Table 1. Applications of 1D and 2D NMR techniques toward mono-, sesqui- and diterpenes

Struc. No.	Name	NMR Methods	Ref.
Monoterpene			
1	geraniol	Gd(fod)3 shift reagent; DEPT; INEPT	45
2	limonene	INADEQUATE	46
3	carvone	^1H - ^{13}C long-range couplings	47
4	camphor	2D-Heteronuclear NOE	48
5	asperuloside	COSY; ^{13}C DEPT; NOE	49
6	globularin	DEPT; ^{13}C - ^1H -HETCOR	50
Sesquiterpenes			
7	mortonin C	COSY; HETCOR	51
8	caryophyllenes	NOESY	52
9	(-)-caryophyl- lene oxide	2D-HETCOR	53
10	spatulenol	2D-HETCOR	53
11	cantabric acids	DEPT; ^{13}C - ^1H COSY	54
12	leucanthanolide	COSY	55
13	guaianolide	2D Heteronuclear Relayed Correlation	56
Diterpenes			
14	isolongirabdiol	COSY; H-NOESY	57
15	stevioside	INAPT	58
16	14-dehydro- agrostistachin	INAPT	59
17	pygmaecin A	NOESY	60
18	grayanotoxin I	INADEQUATE; NOE; COLOC	61
19		COLOC	62
20	pseudolaric acid		63
21	maleopimamic acid	NOE	64

Table 2. Application of 1D and 2D NMR techniques toward structural assignments of triterpenes and steroids

Struc. No.	Name	NMR Methods	Ref.
2 2	dammaran-20(S)-ol	INEPT; 2D-INADEQUATE; COLOC	65
2 3	ganodermanondiol	DEPT; COSY; ^1H - ^{13}C -HETCOR	66
2 4	hopane hydrocarbons	INEPT	67
2 5	lanostan-3 β -ol	DEPT; ^1H - ^{13}C HETCOR	68
2 6	cycloartenol	SFORD	69
2 7	soyasapogenol B	DEPT; ^1H - ^1H and ^{13}C - ^1H COSY	70
2 8	steroid	^1H - ^1H and ^{13}C - ^1H COSY NOESY; COLOC	71
2 9	sterol	COSY; ^{13}C - ^1H HETCOR	72
3 0	bryophyillin B	^1H - ^1H and ^{13}C - ^1H COSY; NOE; COLOC	73

Table 3. Application of 1D and 2D NMR methods toward structural assignments of phenolic natural products

Struc. No.	Name	NMR Methods	Ref.
3 1	lignane	INADEQUATE	74
3 2	phyllanthostatin A (dilignane glycoside)	^{13}C - ^1H COSY; COLOC	75
3 3	coumarin glycoside	COSY; NOESY	76
3 4	dihydrochalcone	INAPT	77
3 5	dichamanetin	COLOC	78
3 6	2,3-dihydro- auriculatin	INAPT	79
3 7	ambonin, hepta acetate	HETCOR	80
3 8	intricatinol	COLOC	81

Table 4. Application of 1D and 2D NMR methods towards the structural elucidation of alkaloids

Struc. No.	Name	NMR Methods	Ref.
3 9	australine	HETCOR	82
4 0	echinatine	HETCOR	83
4 1	anonamine	COSY; HETCOR	84
4 2	norharmane	DEPT; COLOC; HMBC	85
4 3	glaucine	COSY; HETCOR	86
4 4	1,11-dimethoxy-canthin-6-one	NOESY	87
4 5	camptothecine	INAPT	88
4 6	13-epi-yenhusomine	INAPT	89
4 7	isoquinoline alkaloids	NOE; HECTOR Lanthanide-ind. shifts	90
4 8	spirasine	COSY; HETCOR	91
4 9	tatsidine	2D techniques	92
5 0	strychnine	INADEQUATE	93
5 1	stemoninine	COSY; COLOC; NOESY	94

Table 5. Application of 1D and 2D NMR methods in structural assignments of mono-, di- and oligosaccharides

Struc. No.	Name	NMR Methods	Ref.
5 2	D-fructose	HETCOR; 2D-Relay	95
5 3	D-sucrose	2D Heteronuclear NOE	96
5 4	disaccharide stachyose (tetrasaccharide)	Long-range ^{13}C - ^1H coupling const. COSY	97 98
5 5	acarbose, antibiotic	INADEQUATE	99

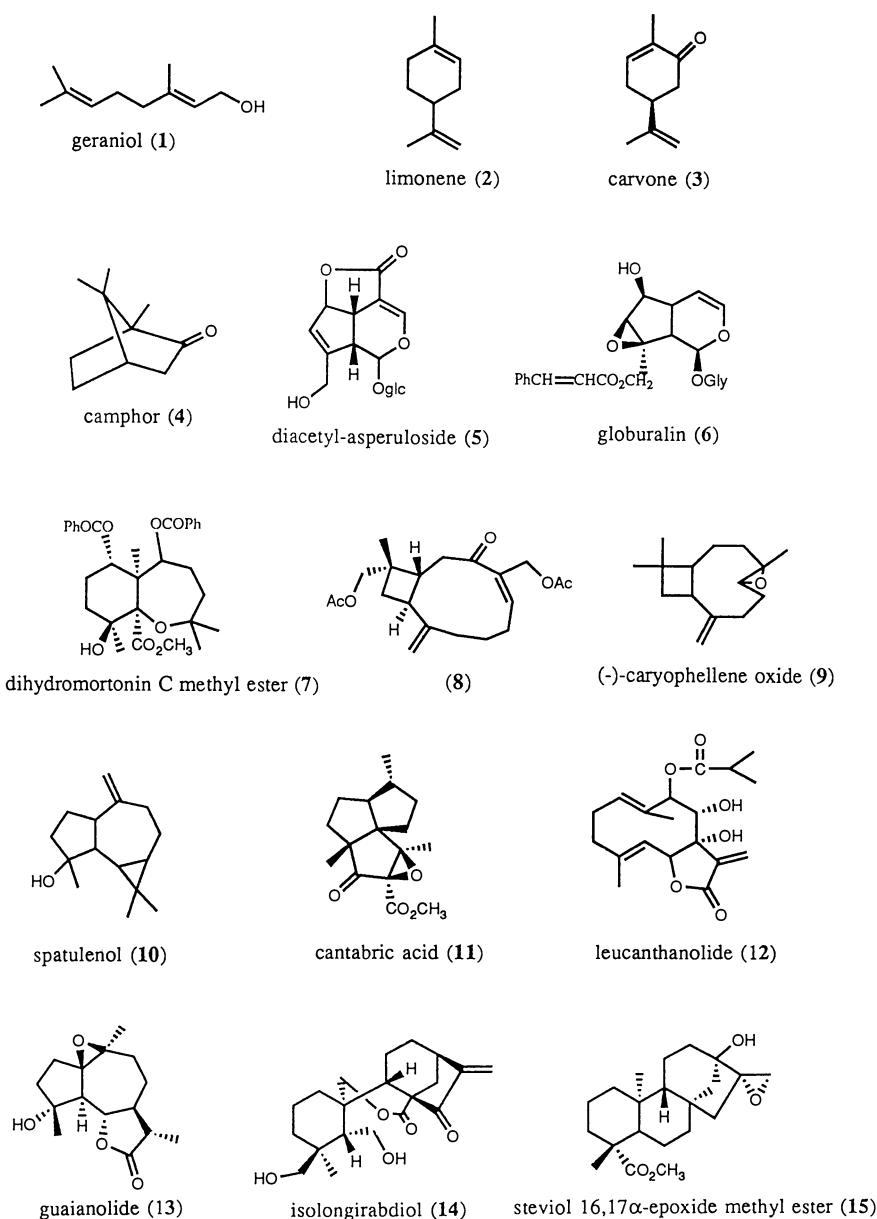


Fig. 19a. Miscellaneous natural products studied by modern NMR methods.

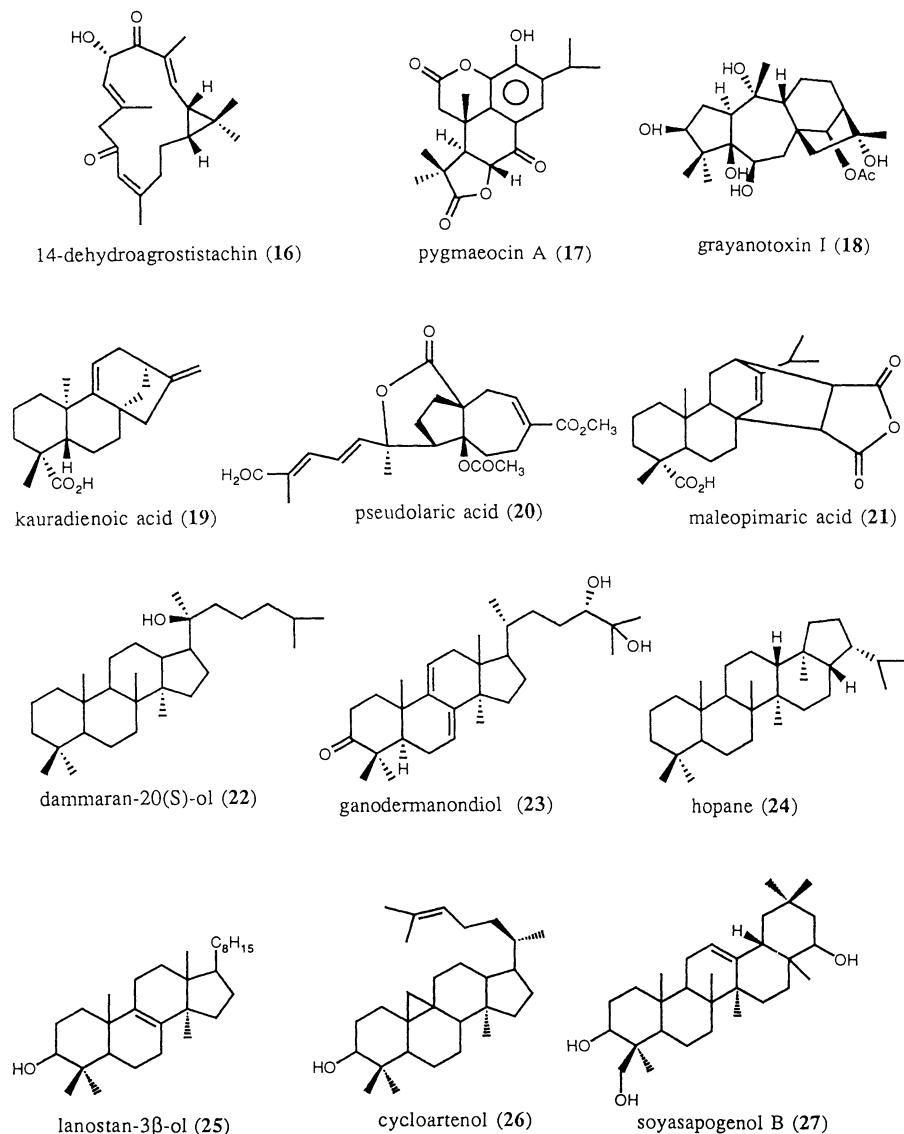


Fig. 19b. Miscellaneous natural products studied by modern NMR methods.

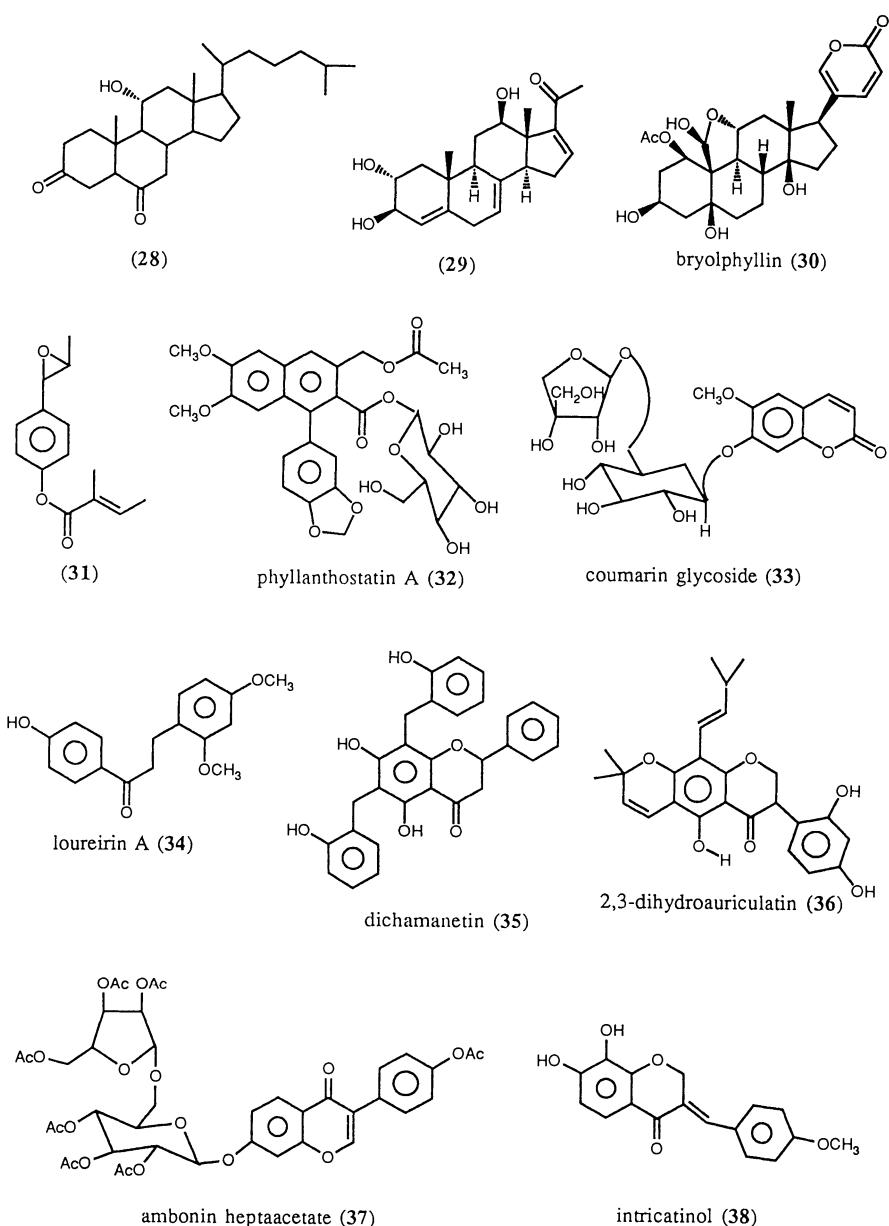


Fig. 19c. Miscellaneous natural products studied by modern NMR methods.

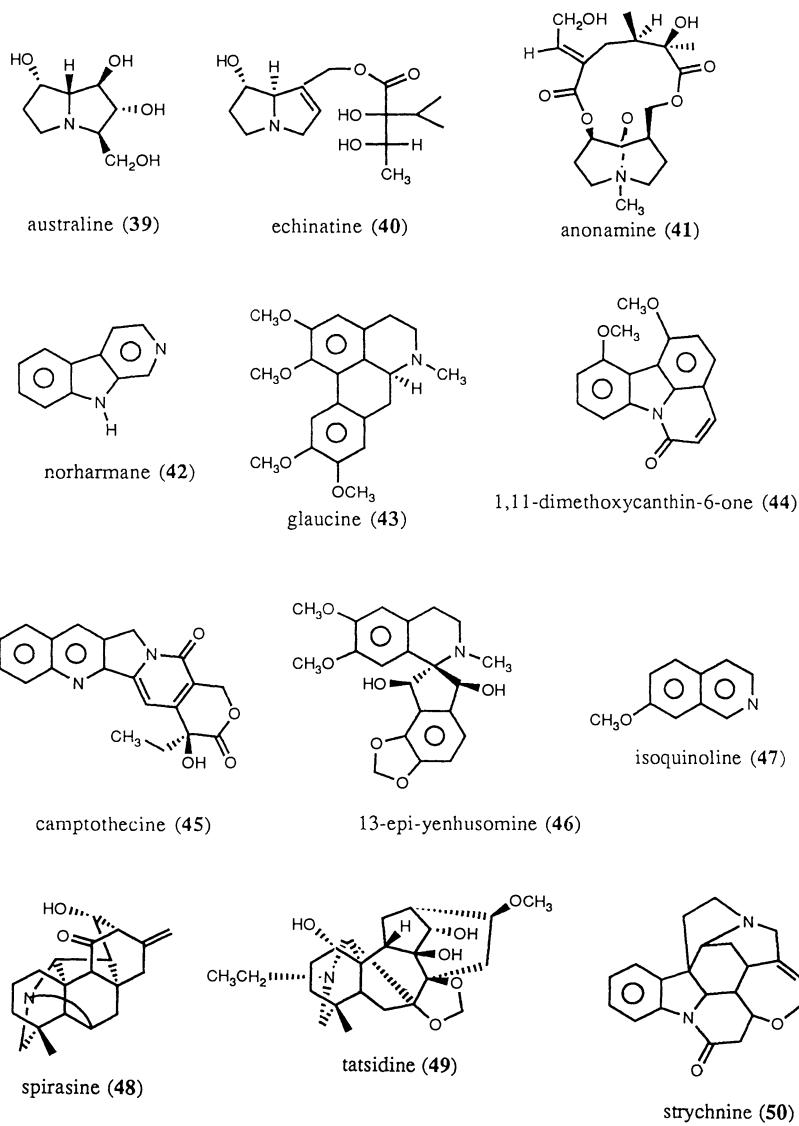


Fig. 19d. Miscellaneous natural products studied by modern NMR methods.

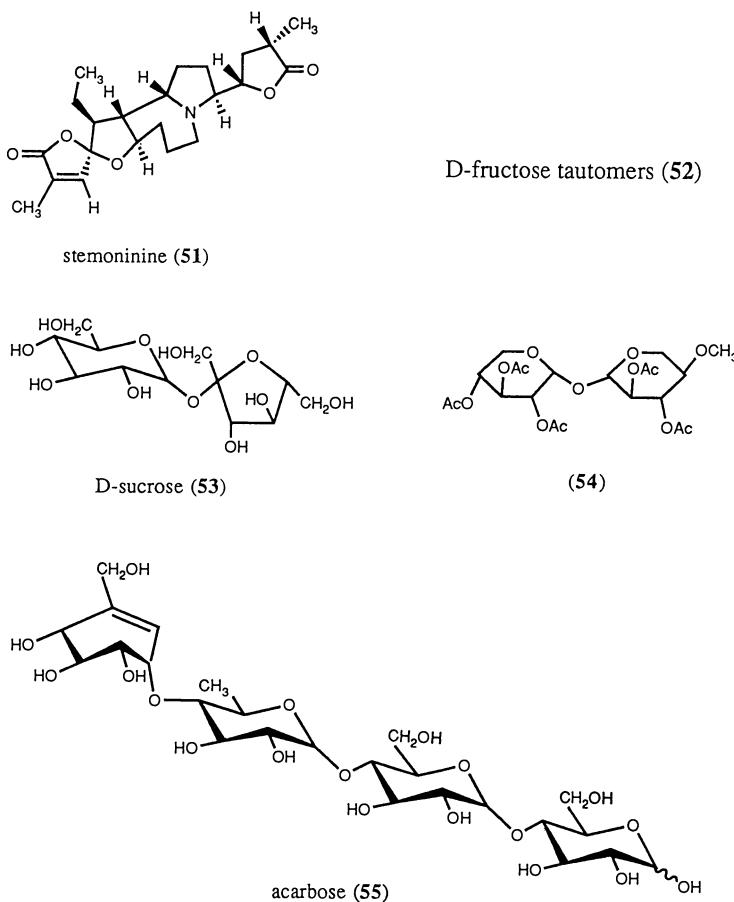


Fig. 19e. Miscellaneous natural products studied by modern NMR methods.

which lost their adjacent carbon. These results are in agreement with the biogenetic proposal that suggests that the twelve-carbon polyacetylenes and their thiophene derivatives are derived from fourteen-carbon precursor followed by loss of one carbon moiety at each end of the carbon chain.³⁸

APPLICATIONS OF 1D AND 2D NMR TECHNIQUES IN STRUCTURAL STUDIES OF MISCELLANEOUS NATURAL PRODUCTS

An increasing number of papers related to the use of 1D and 2D NMR methods in structure elucidations of natural products appear in the literature. We have tabulated a variety of different types of natural products from the recent literature which were studied by the use of modern NMR methods. In Tables 1-5 specific compounds with structural numbers are listed together with the applied NMR techniques, here followed by the literature references. Figure 19 (a-e) shows the structures of the representative natural products listed in Tables 1-5.

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Chapter Nine

TRICHOME MICROSAMPLING OF SESQUITERPENE LACTONES FOR THE USE OF SYSTEMATIC STUDIES

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INTRODUCTION

Over the last two decades few classes of secondary plant metabolites have attracted as much attention from phytochemists as sesquiterpene lactones (STL), which are characteristic metabolites of the Asteraceae. While Fischer et al.¹ reviewed some 900 hundred different structures of naturally occurring STL in 1979, Seaman² counted over 1300 only three years later and their number has increased at a rate of 200 to 250 a year since then (Fig 1).¹⁻⁶ Interest in the

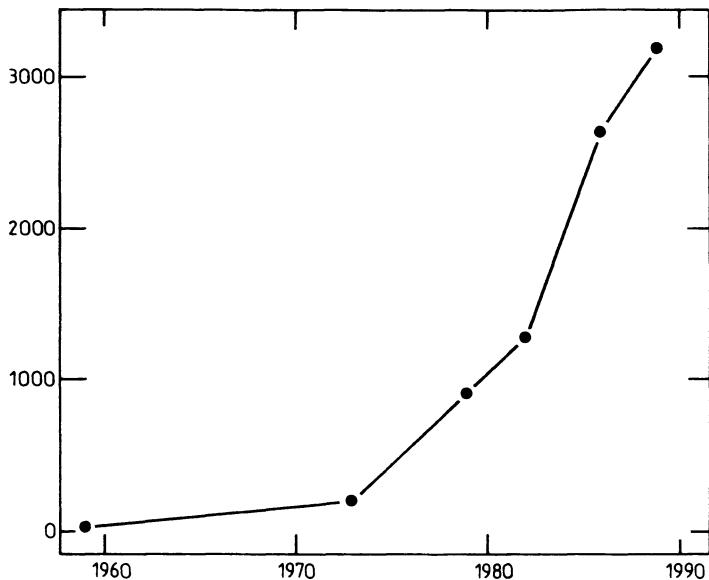


Fig. 1. Number of different STL structures as reported during the past 30 years according to the cited reviews¹⁻⁶.

broad spectrum of biological activities of these compounds (for reviews see ^{7,8}) is probably the major driving force for this development.

Although often mentioned as a possible application of STL studies, the use of chemical data for plant systematic investigations has not developed with the same engagement. Reasons for this are plentiful and have been discussed recently.⁹ The employment of time-consuming and expensive techniques for the extraction and purification of STL is mostly responsible for the lack of sufficient data in almost all genera of the *Asteraceae*. Based on the data reviewed by Seaman² (1300 different STL from ca. 900 different taxa), approximately 2200 taxa have been investigated to date. This is only about 10 % of the species of this huge plant family. So far, few reports exist in which the majority of the species within a genus (or an even smaller taxonomic unit) were investigated and in which the amount of data allows reliable conclusions on chemotaxonomic relationships.¹⁰⁻¹⁵

A presupposition for the use of STL as a taxonomic character is a low intraspecific variability in terms of genetic stability and environmental modification. Standard chemical procedures to obtain the STL pattern of a plant

by extraction, purification and identification, are inappropriate to deal with the amount of different samples that are necessary for taxonomic comparison. Broad sampling, therefore, requires new methodical approaches in phytochemistry that should have the following properties:

- a) reduced time between extraction and identification of compounds;
- b) reduced consumption of plant material;
- c) reduced cost and technical facilities.

Attempts have been made in this direction by Seaman and Mabry.¹⁶⁻¹⁷ They used NMR spectroscopy to identify compounds by characteristic signals in crude plant extracts. Some reasons for the low acceptance of this method may be that gram amounts of plant material still need to be extracted, that only major compounds of a sample can be detected in crude extracts, and that access to state-of-the-art NMR equipment is certainly limited to a few laboratories. A solution to this problem appeared when Polites¹⁸⁻²⁰ observed the formation of "bitter tasting" metabolites in glandular trichomes of *Cnicus* and *Artemisia*; similar species were consequently reinvestigated, using the possibilities of the technological progress of almost 40 years.

SESQUITERPENE LACTONE COMPARTMENTATION AND DISTRIBUTION

Several authors, in the late 1940s and early 1950s, observed a special type of capitate glandular trichome in various *Asteraceae* (e.g. *Artemisia*, *Achillea*) that produced bitter tasting substances.^{18,21,22} This was consistent with the observation that STL could be extracted from *Parthenium hysterophorus*, using short leaf rinses with organic solvents.²³ The best way to demonstrate the accumulation of STL in such trichomes, however, was to harvest the hairs mechanically, to extract them and to identify the content by means of analytical methods. This was achieved with glands from *Helianthus annuus*²⁴ and *H. maximiliani*.¹¹

The common sunflower, under microscopical investigation, reveals the existence of three different types of epidermal trichomes on leaves (Fig. 2).²⁴ Selective harvesting and extraction of hairs demonstrated that only glands forming a distinct cuticular globe on top of the apical cells (capitate glandular trichomes) contain detectable amounts of STL.²⁴ A subsequent investigation on *H. annuus* documented the development of the glands.²⁵ While trichomes are

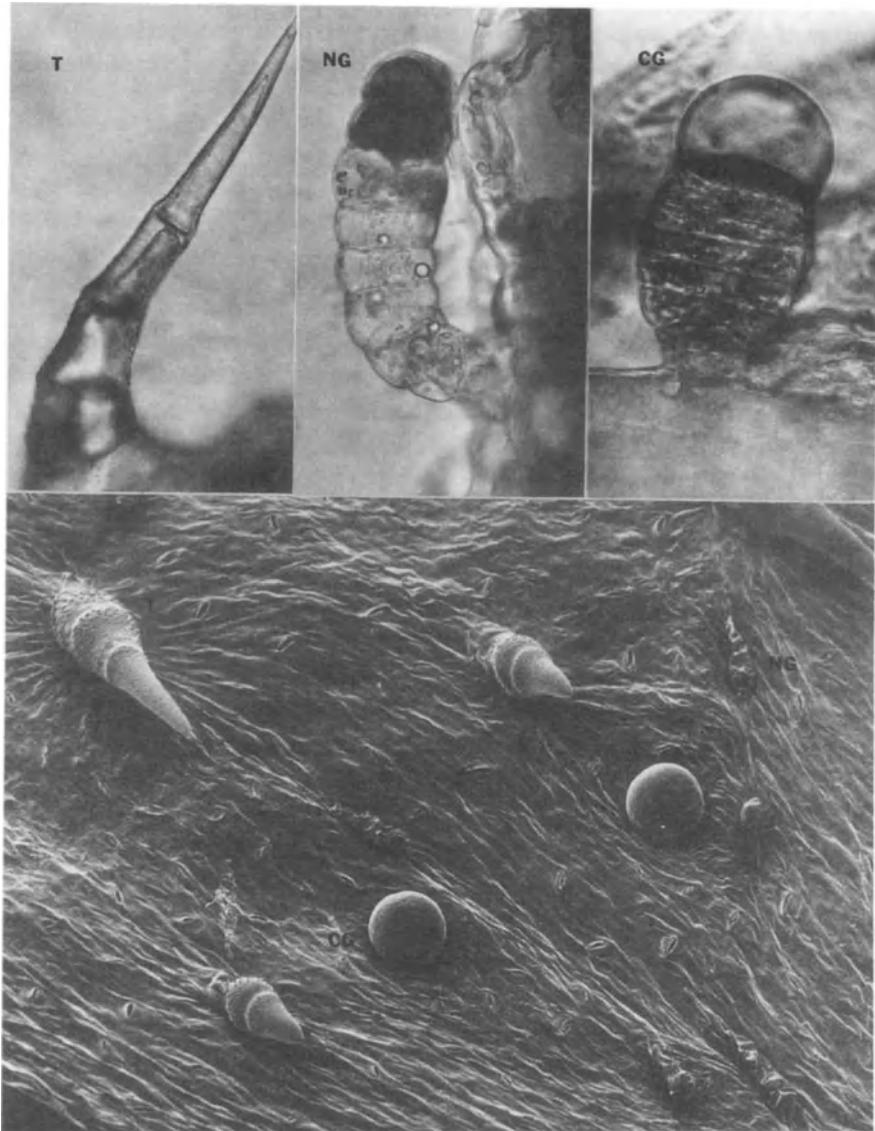


Fig. 2. Photomicrographs of trichomes from sunflower leaves: T, nonglandular trichome; NG, noncapitate multicellular glandular hair; CG, multicellular capitate glandular hair. (From Spring *et al.*²⁴).

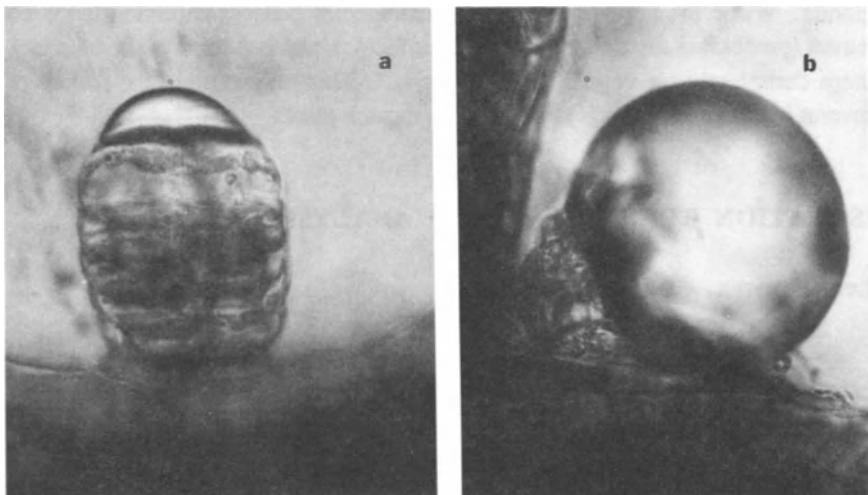


Fig. 3. Influence of light on trichome development. Photomicrographs of capitate glandular hairs from primary leaves of a sunflower seedling, cultivated three weeks under low intensity light of (a) 14 W per m^2 , and high intensity light of (b) 100 W per m^2 (From Spring and Bienert²⁵).

formed at a very early stage of leaf development without photomorphological influence, biosynthesis of the STL starts only after light irradiation. The phytochrome system seems to be involved in this process, but light intensity also influences the growth of the cuticular globe (Fig. 3).

Once formed in the young leaf, the density of glands per area decreases with the onset of leaf expansion. Investigation of the number of glands in different vegetative parts of the plant showed significant augmentation towards the apical bud. Both the high density in the early stages of leaf development and an increasing number towards the plumule may be indicators of the chemical defense strategy of the plant against insect predation.²⁶

Gershenson¹¹ also showed the presence of capitate glands in parts of the flowerhead of *H. maximiliani*, especially on anther appendages. Meanwhile, such glands were found in all but one species of *Helianthus* and in plants of many other genera of the Asteraceae.⁹ On green plant parts, however, individual as well as interspecific differences can be observed in the distribution of capitate

glands. While the majority of *Helianthus* species possess capitate glands on leaves (predominantly on the lower leaf surface), about one third of the taxa lack them completely on vegetative plant parts.⁹ This may explain the failure of several biochemical reports to detect STL in such plants.

ISOLATION TECHNIQUES AND ANALYSIS

Leaf Rinses

Short leaf rinses with organic solvents were often used in the past to extract STL from plant material. Although this is a good technique for examining the range of STL and for subsequent purification of compounds for spectroscopic measurements, such extracts contain a multitude of unspecific metabolites, not derived from the STL-accumulating compartment. For chemotaxonomic use of STL, a more selective technique is desirable, for several reasons:

- a) The purification of substances for spectroscopic identification is too time consuming. Substance identification could be performed better by indirect comparison with known reference compounds in the same analytical system.
- b) The quality of such analysis improves with the purity of the sample. In other words, the lower the number of different substances in the extract, the higher the resolution of the analysis.
- c) Leaf rinses can destroy the plant material and therefore rare plants from herbaria may not be accessible for sampling.
- d) The lack of glands on the leaf surface of a species may prohibit information on the STL chemistry, whereas glands could be gained from parts of the inflorescence.

Trichome Microsampling and Separation Methods

The direct and selective extraction of capitate glandular trichomes can overcome these shortcomings. Fully developed capitate glandular trichomes have a diameter of 20 to 80 μm , depending on the plant. They are large enough to be collected with an insect pin or a fine pair of forceps, under a dissecting microscope (Fig. 4). With fresh plant material, the cuticle is very fragile and

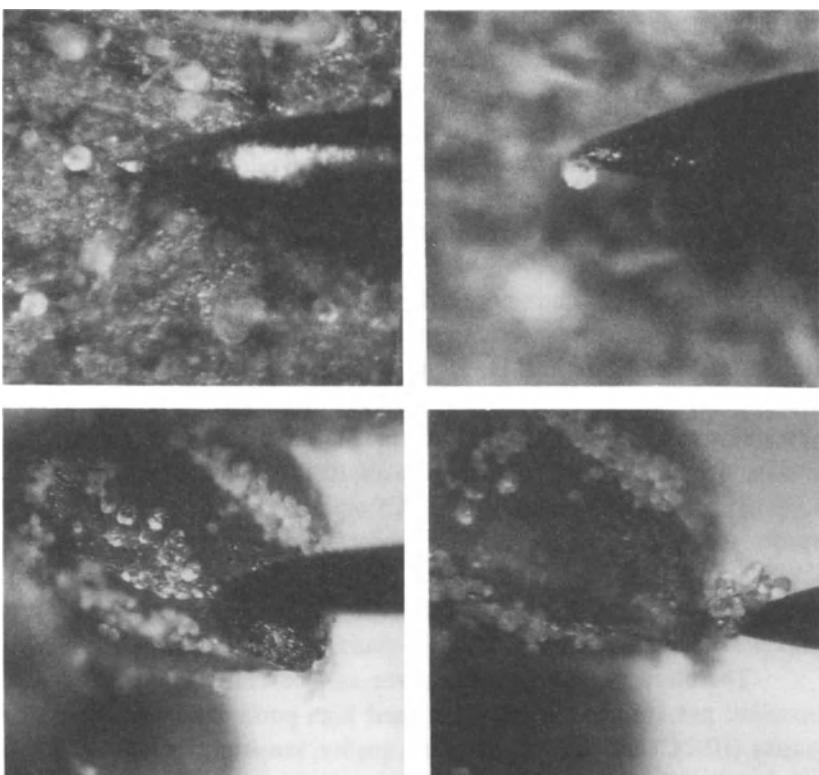


Fig. 4. Trichome harvesting as performed under a dissection microscope; above, from leaf surface; below, from anther appendages.

generally opens when the gland is touched with the needle. Part of the resinous content of the globe sticks to the needle tip and can be rinsed off by dipping the pin into an organic solvent. A more convenient method is to use dried plant parts for sampling. The basal cells of the glandular trichomes are shrunken and easily break off when slightly disturbed by the needle. With this technique, the entire cuticular globe can be harvested and transferred into the vial for extraction.

Previous investigations have shown no significant differences between fresh collected glands and those from air dried material.²⁴ Moreover, recent studies demonstrated an extremely high stability of the chemical constituents within the glands, compared to the fast turnover of some STL, when they were

purified and stored in solvent.^{13,27} In many cases, STL profiles of plants that were collected 30 or 40 years ago did not show signs of degradation. A possible reason for the low turnover of STL in trichomes could be the protection against photooxidation by co-occurring flavonoids, recently detected in the same glands.^{11,28} Finally, it was shown that glands from different parts of the plant (including the flower head) gave very similar compound patterns, though some degree of quantitative differences between leaf trichomes and those from anther appendages could be observed.⁹

This raises the question whether such glands contain enough STL for compound detection in a reasonably small number of trichomes that have to be collected, and further, what is the appropriate analytical method for very small amounts of STL. Since all spectroscopic techniques are incapable of detecting minor components in crude extracts, the need for chromatographical separation seems inevitable. Simple methods, however, like the frequently used thin layer (TLC) or column chromatography (CC) are not sensitive enough for this purpose. Fluorescence detection on precoated TLC plates still requires μg quantities of STL. Staining reactions,²⁹⁻³⁰ though somewhat more sensitive, cannot be used for all kinds of structures. To detect at least the major STL of a plant via TLC, a minimum of 200 to 300 glands need to be collected.

Therefore, only two alternatives can be considered for analytical separation: gas chromatography (GC) and high performance liquid chromatography (HPLC). Although separation quality, sensitivity and the possibility for an easy coupling of the system with mass spectrometry would clearly favour the GC technique, no reports for the successful use of this method in STL analysis of plant extracts exist. One exception is the paper of Jamieson et al.,³¹ but only a single major STL (bakkenolide-A) in leaf lipid extracts of *Petasites* was investigated here. The majority of STL are not sufficiently volatile to be analyzed by GC without prior derivatization. Recently, it was shown that a single hydroxyl group in the molecule can be responsible for the failure to separate a STL via GC (Spring, O., Jordan, E.D., Fischer, N.H., unpublished results). While the germacranolide atripliciolide³² was separable on the system used (DB 1 column), its 15-hydroxy derivative, budlein A,³³ was not volatile enough to be eluted (Fig. 5). Lee,³⁴ attempted to solve this problem by methylation of the compounds in the crude extract. However, this increased the number of structures and derivatives in the sample to such an extent that identification of even major components became difficult.

HPLC therefore seems to be the more promising technique for STL analysis. Encouraged by the report of Strack et al.³⁵ we developed an isocratic separation method for sunflower STL.³⁶ Owing to the relatively wide range in

polarity of the different compounds, only reverse phase chromatography (e.g. Hypersil ODS) gave satisfactory separation. The peak UV-absorption of many STL at wavelengths close to 200 nm precludes the use of most solvents with the exception of alcohol/water or acetonitrile/water mixtures. Good results can be obtained with methanol/water (1:1, v/v). The limit of detection for STL with this system is as low as 10^{-8} and 10^{-9} g per injection, which is far less than the amount of major compounds in a single glandular trichome.²⁵ The total STL content of a gland in *H. annus* ranges between 100 and 150 ng. Thus, an extract of 10 to 20 glands allows detection of even the minor constituents and it takes less than an hour to establish the STL profile of a plant.

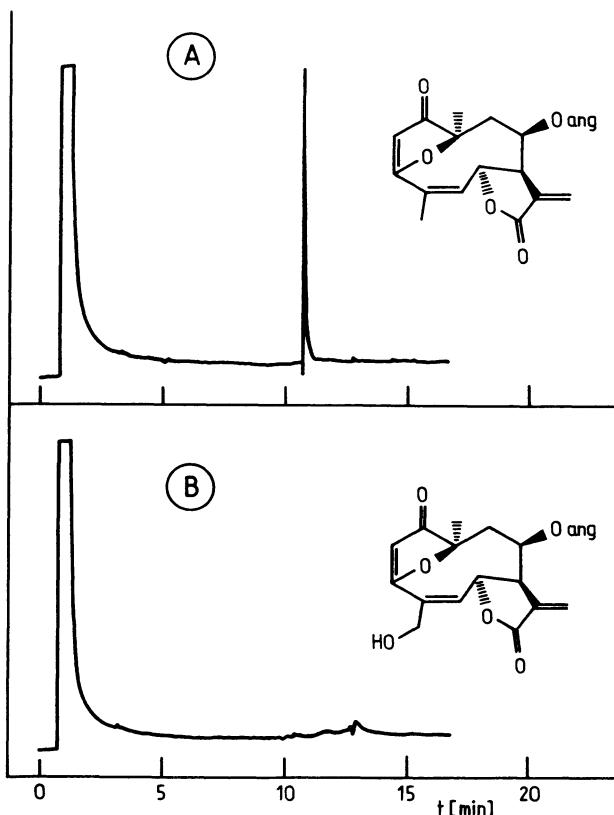


Fig. 5. Gas chromatography of atripliciolide angelate (A) and budlein A (B). Identical amounts (140 ng) of both compounds were injected (DB-1 column; 125°-285°C, 15°C per min).

Summarizing the advantages of the microsampling technique, the following point can be mentioned: the localization of STL in glandular trichomes allows a much more selective extraction of the compounds in comparison to standard procedures. Even if present in only few parts of a plant (e.g., anther appendages) it is possible to establish the STL profile. The glands are easy to collect and the content of only a few of them is sufficient for an HPLC analysis. The stability of the compounds offers the possibility of using air-dried plant material collected many years ago. Since removal of a few glands causes almost no destruction, the method is applicable for the investigation of valuable herbarium specimens. The analysis requires no further preparative steps apart from dissolving the glands' content in an appropriate solvent (e.g. MeOH), diluting the sample with water for HPLC analyses and injecting the solution. This reduces the time and costs involved and allows broad sampling, a prerequisite for chemosystematic investigation.

COMPOUND IDENTIFICATION

The use of an analytical method for STL investigation necessitates alternative approaches to substance identification as well. Structure elucidation in STL chemistry is usually performed by intensive spectroscopic measurements, particularly via NMR spectrometry. This requires milligram quantities of the pure compound. The very low quantities of STL that can be obtained with the microsampling technique is insufficient for NMR spectroscopy. At best, UV and MS data can be obtained, but both are unable to provide sufficient information for the unambiguous identification of a substance. UV spectra of most STL consist of a single peak close to 210 nm and therefore are highly uncharacteristic for differentiation. On the other hand, too many STL have identical molecular weight (for instance, approximataly 70 % of the almost 100 STL detected in *Helianthus*¹³⁻¹⁵ show a molecular peak within the range of 350 to 380). Therefore, substance identification in the analytical range must depend on an indirect method, such as comparisons based on physico-chemical behaviour (solubility, chromatography, spectroscopy, reactivity, etc.) of reference compounds, which must be obtained by standard preparative procedures.

The isocratic HPLC separation is a simple but reliable way to characterize substances by their retention time. Since slight alterations in solvent mixtures or intrinsic variation in the pumping system can easily shift the elution time of a compound from one analysis to another, the use of internal standards is recommended. Dimethylphenol (DMP), for instance, has been a

useful tool for that purpose. Peak assignment in an unknown sample, can thus be made by comparison of the retention time (relative to DMP) with the same parameter of pure STL of known chemical structure. Starting with plant material that has already been investigated with standard procedures, the major compounds are mostly known and can often be obtained from the laboratories which have performed structure elucidation. In such cases, where the whole set of purified STL is available as a reference, substance identification in the unknown extract becomes a matter of finger printing (Fig. 6). As shown for *Helianthus maximiliani*, the natural STL profile of a plant can even be reconstructed with compounds that were purified many years ago from completely different plants of the same species.

Unfortunately, this is not the standard situation and the availability of references can be a major limitation for such chemosystematic studies. Many STL are so unstable that they degrade shortly after their purification. Their stability within the natural compartment, however, could help in these cases. Plant material with very characteristic and prominent compounds could easily be used to get access to certain STL, if such vouchers were available.

Another problem prohibiting unequivocal peak assignment can result from the lack of resolution of certain compounds. In some cases, the large number of STL that may occur in different species of just one genus, is beyond the resolution capabilities of the isocratic HPLC system. From *Helianthus* species, at least six STL are known, which show a HPLC retention time of 0.60-0.61 (relative to the internal standard DMP) in the separation system used (Hypersil ODS 5 μ m, 4 x 250 mm; MeOH/H₂O 1:1, 1 ml/min). As a consequence, it may be impossible in a single elution sequence to assign a peak of an unknown sample with a similar chromatographical behavior of a known compound. This can be overcome, however, by combination of different data sets (Table 1).

Some STL, like the 1-oxo-2(3)-en-3,10-furanoheliangolides (e.g. budlein isovalerate, Table 1), have additional chromophore regions that are responsible for a second maximum in UV absorption at 260 to 270 nm. This allows detection of such compounds via HPLC at a wavelength where STL with the usual UV endabsorption are undetectable. Modern equipment like multi-wavelength or diode-array detectors could help to obtain such information without further HPLC runs. For five of the six substances in Table 1, however, additional experiments are inevitable. A simple solution is the alteration of the mobile phase in chromatography. By using acetonitrile instead of methanol in the solvent mixture, the retention time of all but one compound shifts to a new characteristic value.

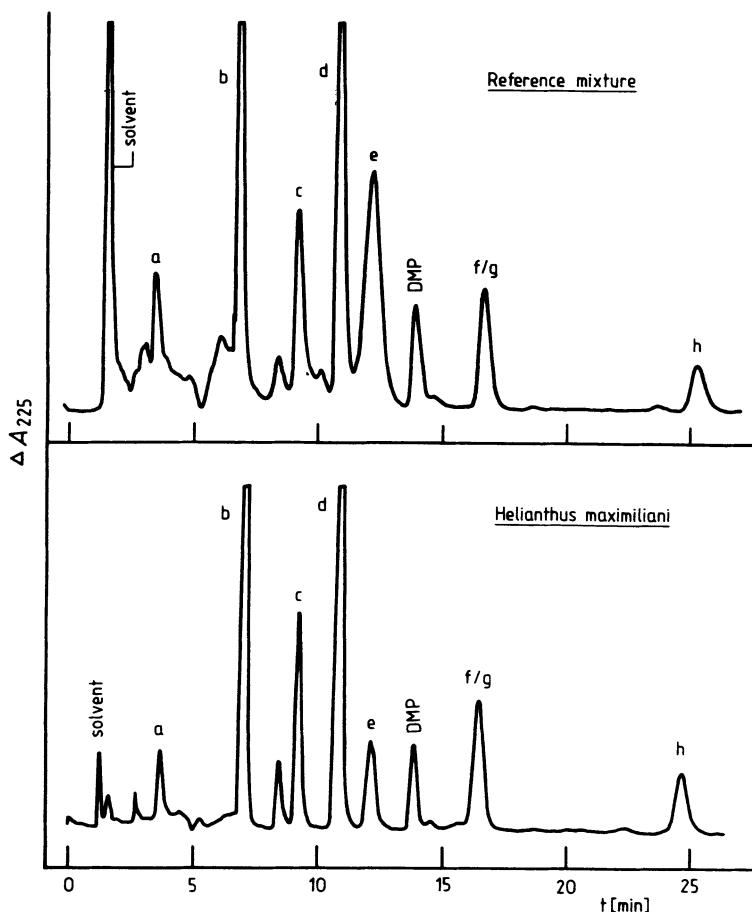


Fig. 6. HPLC elution diagram (Hypersil ODS, 5 μ m, 4 x 250 mm; 50 % aqueous MeOH, 1 ml/min; UV-A₂₂₅) of a mixture of STL reference samples in comparison to an extract of 10 capitulate glandular trichomes from *Helianthus maximiliani* (collected Sep. 1988 at Bloomington, Indiana; O. Spring # 43). Compound purification from a bulk collection of *H. maximiliani* (collected Sept. 1975, Waverley, Coffey Co., Kansas; N.C. Henderson and G.D. Anderson # 75-106) was performed by Herz and Kumar³⁷. Compound assignment: a) eupatolide, 2 α -hydroxy (eupaserrin, des-O-sarracenate); b) tifruticin, 1-hydroxy; c) tifruticin, desoxy-1,2-dihydro; d) niveusin C, des-3-hydroxy; e) tifruticin, desoxy; f) tifruticin; g) niveusin C; h) tifruticin, acetyl.

Table 1. HPLC retention time (relative to DMP, RT=1) and UV absorption of some STL from *Helianthus*^{27,38,40,41} and *Viguiera*³⁹ species. Separation was performed on Hypersil ODS (5μm, 4x250 mm column) with solvents: RT1: 50 % aqueous MeOH , 1ml / min; RT2: 30 % aqueous CH₃CN, 1.3 ml / min

Compound	UVmax	RT1	RT2	Ref. No.
ternifolin angelate	210 nm	0.608	0.613	38
niveusin A 2-methylbutyrate	206 nm	0.604	0.478	39
niveusin A, 1,2-anhydrido	208 nm	0.609	0.355	27
heliangine	207 nm	0.598	0.600	40
budlein A isovalerate	207+265	0.600	0.676	41
costunolide, 8,14-dihydroxy (= desacetyllovatifolin)	207 nm	0.608	0.418	41

The possibilities of obtaining additional data sets in this way are numerous, and, besides the solvent system, the stationary phase can also be changed. A highly sophisticated technique of combining separation and spectroscopic measurements has recently been developed with LC-MS coupling. Although being capable of providing very specific information for compounds, the cost of this device will limit its application.

APPLICATIONS OF TRICHOME MICROSAMPLING

Whilst the above mentioned sections were primarily dedicated to more or less technical aspects, the following will focus on the applicability of STL for systematic studies and on application of the microsampling technique.

Sesquiterpene Lactones, a Reliable Taxonomic Character?

Seaman² has described in detail the requirements for the use of biochemical parameters in plant systematics. A basic presupposition is genetic stability and independence from environmental influences.⁴² Although STL have been used for systematic purposes almost since the beginning of their investigation,^{43,44} few reports exist that specifically evaluate the principle usefulness of these compounds in taxonomy.

Seaman and Mabry,^{10,16,17} in their studies on *Ambrosia* were among the first to test the quality of STL in plant systematics by investigation of numerous populations of a species with different geographical distributions. Recently, the reliability of STL as a taxonomic character was also established in an investigation of twenty-five independent specimens of *Helianthus annuus*,⁹ using the microsampling technique. In comparison to some morphological characters, the qualitative intraspecific variation of STL in *H. annuus* was shown to be very low. Six of the structurally known compounds of this species²⁷ could be detected in at least 90 % of the samples. The similarity of the STL profiles of different specimens can be demonstrated with the HPLC elution diagrams of two plants, collected at different times at different locations (Fig. 7).

This, however, does not exclude differences in the ratio of compounds from one plant to the other, or even within different organs of the same plant (e.g. anther appendages/leaves). Environmental influences may be responsible for such quantitative effects. The stimulation of STL biosynthesis through high intensity light irradiation has already been discussed (see Fig. 3) and might also be an explanation for the results of Kelsey et al.,⁴⁵ who reported quantitative differences from winter to summer in *Artemisia* species. So far, no modification in STL patterns through different types of soil (e.g., *H. bolanderi* race bolanderi and *exilis*, the latter one growing on serpentine) or nutrition (various concentrations of nitrogen, phosphate and potassium were tested on sunflowers)(Spring, O. unpublished data) were observed. These data from *Ambrosia*, *Artemisia* and *Helianthus* in general left no doubts about on the genetic stability and low intrinsic variability of STL.

Chemotaxonomy of *Helianthus* Species

For the first time, trichome microsampling offered the possibility of investigating the species of a whole genus in one experimental series. Within a few months all known 63 taxa of *Helianthus*¹³⁻¹⁵ were screened for STL. A total of over 300 specimens were investigated to obtain data of at least three, and usually more, independent collections of a species. Such broad sampling was only possible because the applied technique allowed sampling from herbarium materials. Although leaf glands were lacking in almost one third of the taxa, STL patterns could be established due to the presence of trichomes on anther appendages in all but one species, *H. paradoxus*. Approximately 70 % of the peaks detected in HPLC analysis could be tentatively assigned by means of reference compounds, including almost all major constituents.

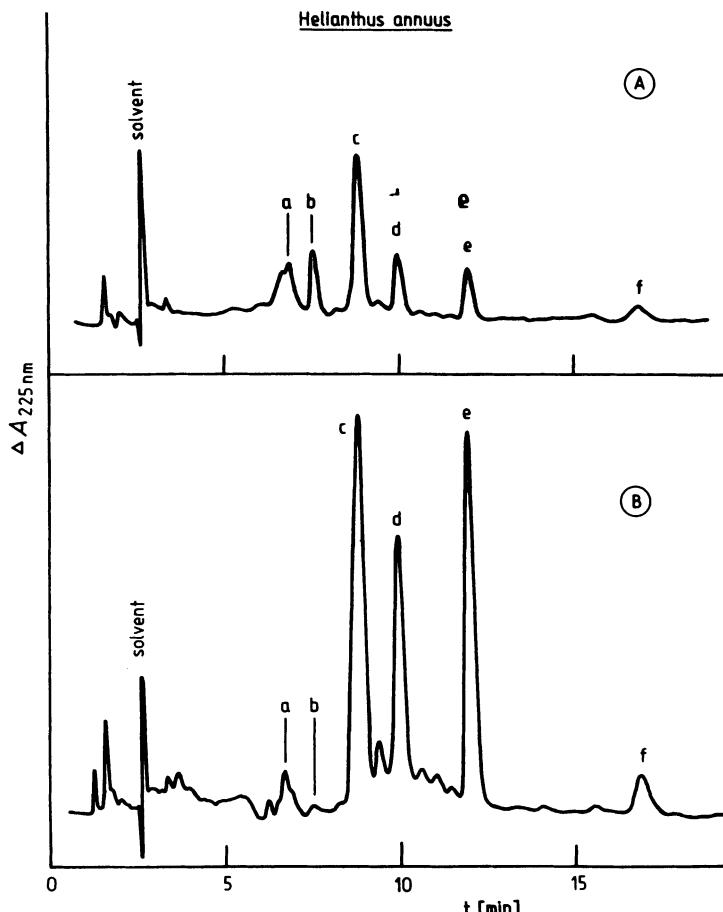


Fig. 7. HPLC analysis (conditions as given in Fig. 6) of two independent specimens of *Helianthus annuus*; (A) collected 1986, Missouri, St. Louis, Heiser s.n.; (B) collected 1966, Massachusetts, Hampshire Co., Ahles #64710. Compound assignment: a) niveusin A, 1,2-anhydrido-4,5-dihydro; b) argo-phyllin B; c) niveusin A, 1,2-anhydrido; d) tifruticin, desoxy-3-dehydro-15-hydroxy; e) niveusin A, 1-methoxy-4,5-dihydro; f) niveusin B; g) niveusin C.

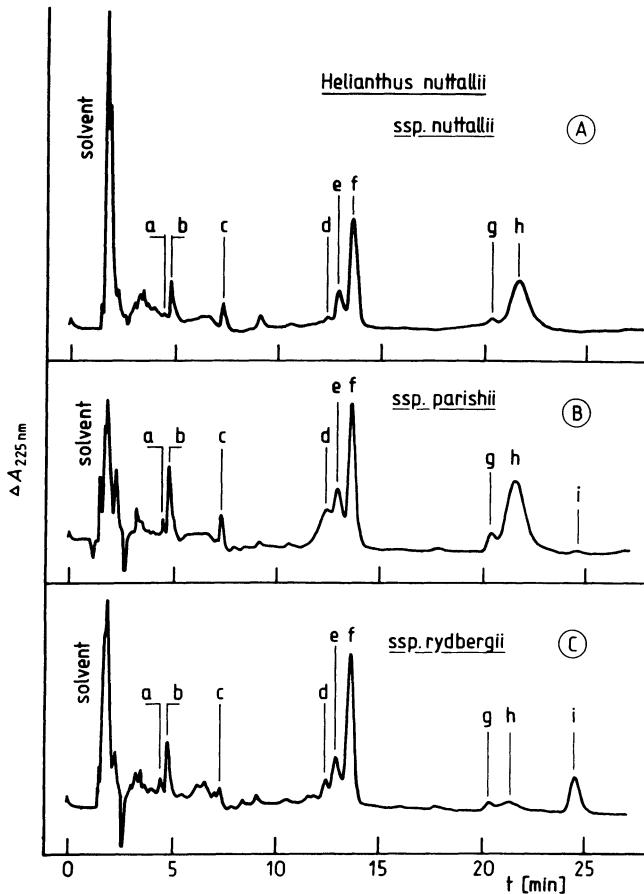


Fig. 8. HPLC analysis (conditions as given in Fig. 6) of the three subspecies of *Helianthus nuttallii*: (A) ssp. *nuttallii*, 1964, Utah, Sevier Co., Burton #453b; (B) ssp. *parishii*, 1951, California, San Bernardino Co., Roos #5196; (C) ssp. *rydbergii*, 1962, N. Dakota, Griggs Co., Stevens #2634. Compound assignment: a) pinnatifidin, 1,2-dihydroxy; b) pinnatifidin, 1-hydroxy; c) budlein A; d) tifruticin, desoxy; g) atripliciolide angelate; e, f, h and i unknown.

Diploid species, in general, gave characteristic STL profiles that permitted taxonomical assignment of unassigned specimens based on their chemistry (Spring, O., Schilling, E.E., unpublished results). While qualitative differences in compounds were evident throughout the species level, some morphologically well characterized subspecies showed nearly complete homology in STL (e.g., *H. debilis* ssp. *debilis*, *cucumerifolius*, *tardiflorus*, and *vestitus*).

On the other hand, specific compounds characterized the three subspecies of *H. niveus* and a relatively low proportion of shared STL was found in three morphologically similar groups of *H. maximiliani*, for which the existence of chemical races was recently proposed.^{37,46,47} It should be mentioned at this point that trichome microsampling from herbarium material not only provided access to chemical data of some rare species⁴⁸ of the genus like *H. anomalus*, *H. deserticola* or *H. eggertii*, but also permitted investigation of the STL pattern of *H. nuttallii* ssp. *parishii* (Fig. 8), a taxon considered to be extinct since the late 1950s.⁴⁹ Although collected nearly 40 years ago, the specimen showed almost all compounds of the other two subspecies, *nuttallii* and *rydbergii*.

Seaman² has pointed out different possibilities of the use of chemical data in taxonomy. A common method of analyzing the similarity between a pair of taxa is the calculation of the proportion of shared to total number of compounds in both plants (Table 2). This affinity index can be used for cluster analysis as shown in a phenogram (Fig. 9) for the relationships among species of *Helianthus sect. Ciliares*.¹⁴ According to STL profiles, the species of the section segregate into two groups, one combining *H. arizonicensis*, *H. laciniatus* and both subspecies of *H. ciliaris*, the other consisting of *H. cusickii*, *H. gracilis* and *H. pumilus*. This agrees with the previously proposed taxonomy which divided the section into series *Ciliares* and series *Pumili* on the basis of morphological criteria.⁵⁰

A more biogenetic approach can be undertaken using skeletal types and substituents instead of the structures themselves. This reduces the number of different parameters and allows comparison of the results on the level of taxonomic units. To give an example, Table 3 summarizes the results for *Helianthus* sections *Helianthus* versus *Ciliares*. Eupaserrin-type compounds (2α -hydroxy-*trans, trans*-germacra-1(10),4(5)-dien-12,6-olides) clearly separate section *Ciliares* from section *Helianthus*. On the other hand, niveusin-type STL (3,10-furanoheliangolides) are found in both sections. Budlein derivatives, in particular, indicate a similarity between species of the series *Ciliares* and the annual species *H. debilis*, *H. petiolaris* and *H. praecox*.

The entire genus is characterized by 6,7-lactonized germacranolides of the germacrolide and heliangolide type and partly by 7,8-lactonized eudesmano-

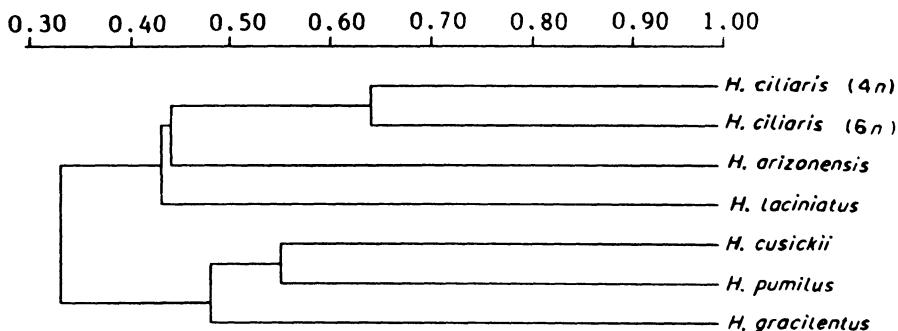


Fig. 9. Phenogram illustrating relationships among species of *Helianthus* sect. *Ciliares* based on sesquiterpene lactone data. Scale represents affinity index in %. (From Spring and Schilling¹⁴).

Table 2. Comparative sesquiterpene lactone profiles of *Helianthus* sect. *Ciliares*. Below diagonal, shared/distinctive compounds; above diagonal, proportion of shared to total compounds (affinity index in %). Taxon names abbreviated by first three letters of species epithet. (From Spring and Schilling¹⁴).

	ARI	LAC	4n-CIL	6n-CIL	CUS	GRA	PUM
ARI	X	30	44	33	23	19	15
LAC	7/16	X	42	43	25	30	16
4n-CIL	11/14	10/14	X	64	24	33	17
6n-CIL	8/16	9/12	14/8	X	19	23	11
CUS	6/20	6/18	7/22	5/22	X	45	55
GRA	5/22	7/16	9/18	6/20	10/12	X	48
PUM	4/23	4/21	5/25	3/25	11/9	10/11	X

Table 3. Distribution of major compound types in *Helianthus* sections *Helianthus* and *Ciliareas*

Taxon	Compound types (a)							Side Chains (b)								
	E	Et	B	N	T	A	Ed	1	2	3	4	5	6	7	8	9
<i>Sect. Helianthus</i>																
<i>H. annuus</i>			*	+	+			*								
<i>H. argophyllus</i>			*	+	+			*								
<i>H. deserticola</i>	x		+	+				+								
<i>H. bolanderi</i>			*	+				*								+
<i>H. anomalous</i>			*	+		*		*								
<i>H. neglectus</i>			*	*			+	*								
<i>H. niveus</i>	+		*					*								+
<i>H. debilis</i>			*	+			+	*	+						+	+
<i>H. petiolaris</i>			*	+				*								+
<i>H. praecox</i>			*	+	+			*	+							+
<i>Sect. Ciliareas</i>																
<i>H. arizonicensis</i>	+		*	+	+			+	+	+	+	+	+			*
<i>H. ciliaris</i>	+		*	+	+			+	+	+	+	+	+			*
<i>H. laciniatus</i>	+		*	+				+	+			+				+
<i>H. cusickii</i>	*			+	+			+	+		+	+	*			
<i>H. gracilentus</i>	*		*		+			*	+		+	+	*			x
<i>H. pumilus</i>	*	x			+			+	+		+	+	*			

a) Germacrolides: E, eupaserrin; Et, eupatolide; Heliangolides: B, budlein; N, niveusin; T, tifruticin; A, argophyllin; Ed, eudesmanolide; *, major; +, detectable; x, based on previous literature report only.

b) Side chain: 1) angelate; 2) epoxyangelate; 3) tiglate; 4) 2-hydroxyethylacrylate; 5) sarracinate; 6) acetylsarracinate; 7) 2-methylbutyrate; 8) isovalerate; 9) isobutyrate.

lides.¹³⁻¹⁵ More advanced skeletal types (comp. Fischer et al.¹) don't play a role, although guaianolides and melampolides were reported from two and one species, respectively.^{46,51,52} This combination of basic structures in *Helianthus* is generally shared by two other closely related genera, *Vigueira* and *Tithonia*, but is not a unique feature of the subtribe *Helianthinae*. Similar skeletal types have been reported from other subtribes as well.^{1,2}

Helianthus species typically possess compounds with a five carbon ester side chain cleaved to C-8, of which angilic acid is by far the most common. However, as long as the biosynthetic pathway of the compounds remains largely unclear, it is questionable whether side chains or other substituents can be regarded as a significant taxonomic character.

These few examples should be sufficient to provide insight into the possibilities and problems of the use of STL data for the investigation of plant relationships. Biochemical data of the *Helianthus* species in most cases supported previous systematic relationships, based on morphological characters and hybridization experiments.^{49,50} Among the few exceptions, *Helianthus microcephalus* is remarkable, because it was the only species of the genus in which only guaianolides could be detected.

The Origin of Hybrids and Polyploid Species

In contrast to the diploid species, the polyploid taxa of *Helianthus* showed considerable intraspecific variability. This is similar to the inconstancy of morphological characters in these plants. If autopolyploidy is considered to be the reason for the increased chromosome number of a species, a relatively high degree of homology in STL patterns of the different genetic levels could be expected. This was not observed in *Helianthus*. None of the diploids had a high affinity index with any of the six tetraploid species, so that simple chromosome doubling could not explain the situation. Interspecific hybridization, a frequently occurring process in *Helianthus*,⁴⁹ is more likely to be responsible for the origin of the polyploids.

A solution to this question might come from the observation that F1-hybrids show mostly the additive STL patterns of their parents.^{9,53} In a recent study this effect was used to investigate the origin of the triploid taxon *H. x multiflorus* and the hexaploid *H. x laetiflorus*.⁵³ HPLC analysis of the extracts of the polyploids were compared to those of the postulated parents and of artificial hybrids between the possible progenitors (Table 4). *H. x multiflorus*, for instance, showed the germacrolide desacetylepaserrin, a major compound of the diploid form of *H. decapetalus*. Although the tetraploid race of *H.*

Table 4. Affinity index (in %) of *H. x multiflorus* and *H. x laetiflorus* in comparison to additive patterns of putative progenitors and artificial hybrids (Data from Spring and Schilling⁵³)

(a)		ANN/	ANN/	STR/	STR/	TUB/	TUB/	
Taxon	AD1	DA7	DEC-2n	DEC-4n	SUB	PAU	SUB	PAU
MUL	22	18	77	35				
(b)	4/14	4/18	10/3	6/11				
LAE					74	54	88	61
(b)					14/5	13/11	15/2	14/9

a) MUL) *H. x multiflorus*; AD1) DA7, hybrids between *H. annuus* and 4n *H. decapetalus* alternatively used as egg and pollen donor; DEC) *H. decapetalus*, diploid (2n) and tetraploid (4n); LAE) *H. x laetiflorus*; SUB) *H. pauciflorus* ssp. *subrhomboideus*; PAU) *H. pauciflorus* ssp. *pauciflorus*; STR) *H. strumosus*; TUB) *H. tuberosus*.

b) Number of shared/different compounds; facultatively occurring compounds of the putative progenitors not counted.

decapetalus was previously favored as one of the likely parents⁴⁹, the absence of desacetylepaserrin from its STL complement makes it appear that this race is not a progenitor of *H. x multiflorus*.

For the origin of *H. x laevigatus*, the affinity index suggests involvement of *H. tuberosus* and *H. pauciflorus* ssp. *subrhomboideus* rather than the proposed *H. strumosus* and *H. pauciflorus* ssp. *pauciflorus*.⁴⁹ This technique of determining ancestral species was recently also applied to tetra- and hexaploid species of *Helianthus*⁴⁷, but so far with less clear results. Fertile polyploids, in contrast to the triploids, are not genetically trapped and may undergo further hybridization, backcrossing or other processes through which the initially acquired chemical pattern can be modified with time.

Sesquiterpene Lactone Analysis in Uninvestigated Taxonomic Units

Even today, less than 10 % of the species of many *Asteraceae* genera have been investigated for their STL chemistry. This results in a lack of refer-

ence compounds that could be used for a comprehensive study of such a taxonomic unit in the above described way. Trichome microsampling, nevertheless, can be performed. It functions in this case as a fast screening method to select plant material for subsequent extraction and STL purification by means of standard procedures.

In a recent study (Spring, O., Panero, J., Schilling, E.E., unpublished results) this method was chosen to investigate systematic relationships of the South American "sunflower" genus *Pappobolus*⁵⁴ (formerly *Helianthopsi*⁵⁵). A single STL (budlein A 2-methylbutyrate) from one of the 35 species had been reported before.⁵⁶ HPLC analysis of glandular extracts of the species showed that the entire genus contains a total of approximately 35 different compounds. As a consequence of this screening, bulk samples of the three taxa that promised to contain the most unknown substances were chosen for extraction. In this way, about 30 compounds were purified and structurally elucidated.⁵⁸ These STL now serve for peak assignment in the previously performed trichome analysis.

CONCLUSION

Modern plant systematics has reached a point where morphological characters are insufficient to provide information for the resolution of controversial issues among taxa. Biochemical data can contribute additional information for the comparison of the species relationships. Previous attempts to use STL for the taxonomic characterization of *Asteraceae* were limited by the incompleteness of data. If only 10 % of the species have been investigated, the conclusions that can be drawn from the results are consequently limited. The enormous number of species in the *Asteraceae* overwhelms the commonly applied preparative techniques for STL investigation and new methodological approaches are required.

Trichome microsampling seems to be a possible way of closing this gap, at least for a large number of species. Owing to the concentrated location on easily accessible plant parts, STL can be extracted and analysed with a minimum of time, a minute amount of plant material and affordable instrumentation. The potential and speed of this method has been demonstrated in an investigation of a complete genus, for which it took more than 25 years of intensive research with traditional techniques to describe little more than 50 % of the taxa. Of course, standard procedures are still necessary to extract and analyze compounds from plants. Without reference samples all analytical methods will

fail to identify substances. However, the use of large amounts of plant material should be limited to purposes other than chemotaxonomy. Much more effectively than in the past, the use of STL for systematics could be efficiently achieved in a multidisciplinary collaboration. In such a project, the contribution of the plant material, the biochemical screening and the structure elucidation of reference compounds are independent parts that could be performed in different laboratories.

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Chapter Ten

BIOSYNTHETIC METHODS FOR PLANT NATURAL PRODUCTS: NEW PROCEDURES FOR THE STUDY OF GLANDULAR TRICHOME CONSTITUENTS

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INTRODUCTION

Many types of plant natural products, including terpenoids, phenolics and sucrose esters, accumulate in modified epidermal hairs known as glandular trichomes.¹⁻⁴ These substances have long been of interest to phytochemists

because of their structural complexity and their importance as flavoring, perfumery and pharmaceutical agents. In addition, a number of glandular trichome constituents are thought to have a role in plant defense because of their toxicity and deterrence to herbivorous insects, and their anti-fungal and anti-bacterial activity.²⁻⁵

The natural products that accumulate in glandular trichomes have been generally assumed to be biosynthesized *in situ* since gland cells frequently display many ultrastructural features that suggest the active metabolism of gland products.^{1,6} Direct evidence for the biosynthetic capabilities of gland cells has recently become available. Studies with tobacco (*Nicotiana tabacum*), the glandular trichomes of which secrete principally duvane-type diterpenes and sucrose esters, have demonstrated that gland cells incorporate basic metabolic precursors into both of these types of glandular substances.^{7,8} In spearmint (*Mentha spicata*), which accumulates in its glandular trichomes an essential oil containing principally monoterpenes, the enzymes of monoterpene biosynthesis were shown to be located only in the cells of the glandular trichomes and not in the remainder of the leaf.⁹ Thus, to study the biosynthesis of glandular trichome constituents, it seems essential to use preparations containing glandular material.

In the last few years, we have developed several procedures for preparing plant extracts enriched in the contents of glandular trichome cells.^{10,11} Here, we describe further advances in this methodology, the development of procedures for obtaining highly-purified preparations of the cells of glandular trichomes, and discuss how these preparations can be used in several different types of biosynthetic investigations.

Biosynthetic investigations, at least in their initial phases, are usually directed at elucidating the actual sequence of intermediates in a given pathway. Such information was once obtained primarily by administering simple, isotopically-labeled precursors to intact tissue, isolating the products, and determining the position of the label by chemical degradation. However, in recent years, *in vivo* studies of this sort have been largely superseded by *in vitro* studies involving the use of cell-free preparations to analyze the conversion of immediate precursors to products. Cell-free preparations allow a more detailed examination of the individual steps in the pathway and frequently result in better incorporation of biosynthetic precursors because compartmentation at the tissue and cellular levels is eliminated. Once the basic sequence of intermediates has been determined, biosynthetic investigations often proceed to examine the mechanisms of the individual enzymatic reactions, the cellular and subcellular sites of synthesis, and how metabolic flux through the pathway is regulated. In this chapter, we present examples of how intact and cell-free preparations of

purified glandular trichome cells can be used to obtain information about the sequence, mechanism, regulation and site of biosynthesis.

EXPERIMENTAL PROCEDURES

In the course of biosynthetic studies of monoterpenes and sesquiterpenes in members of the Lamiaceae (the mint family), we previously devised several methods for preparing extracts enriched in the contents of glandular trichomes. The glandular trichomes of the Lamiaceae are composed of several different kinds of cells (Fig. 1). Each trichome has from one to 18 secretory cells, one to six stalk cells and a single basal cell embedded in the epidermis.^{1,12-20} The secretory cells appear to produce the terpenoid-rich secretion of the gland and discharge it into a sub-cuticular cavity which forms between the cuticle and the underlying secretory cells. Two basic types of glandular trichomes are found in this plant family: capitate (clavate) glandular trichomes, in which the secretory products are eventually extruded outside of the gland; and peltate (sessile) glandular trichomes, where the secretion accumulates in the sub-cuticular cavity and is not released unless the cuticle is damaged (Fig. 1).^{1,12,13,18,19,21-25}

In our initial efforts to obtain extracts enriched in the contents of glandular trichomes, peppermint (*Mentha piperita*) leaves were submerged in chilled buffer and their surfaces gently brushed with a soft-bristle toothbrush.^{10,26} This method afforded extracts with high levels of monoterpene biosynthetic enzymes and low levels of competing activities, but was of limited preparative value. In addition, it was difficult to carefully brush the small, young leaves in which terpenoid synthesis typically occurs at the greatest rate.

Next, we developed mechanized procedures for abrading the leaf surfaces of peppermint, sage and other species with glass beads in a cell homogenizer.¹¹ Gentle abrasion was found to extract the contents of the glandular trichomes while causing only slight damage to the underlying leaf or stem surface. This technique permitted the recovery of high yields of glandular enzymes from large amounts of plant material with only minor amounts of contamination from other tissue. Our method employed a commercial cell homogenizer (Bead-Beater, Biospec Products, Bartlesville, OK). The finned 300 ml polycarbonate grinding chamber of this apparatus was filled with leaves, glass beads and extraction buffer, and fitted with a Teflon rotor. Typically, 10-20 grams of plant tissue and 100-300 grams of 0.5 mm diameter glass beads were placed in the chamber and extraction buffer was added to full volume. Polyvinylpyrrolidone (PVP, $M_r \sim 10,000$ or $40,000$) (0.5-2.0 grams) and beaded polystyrene resin (XAD-4) (10-40

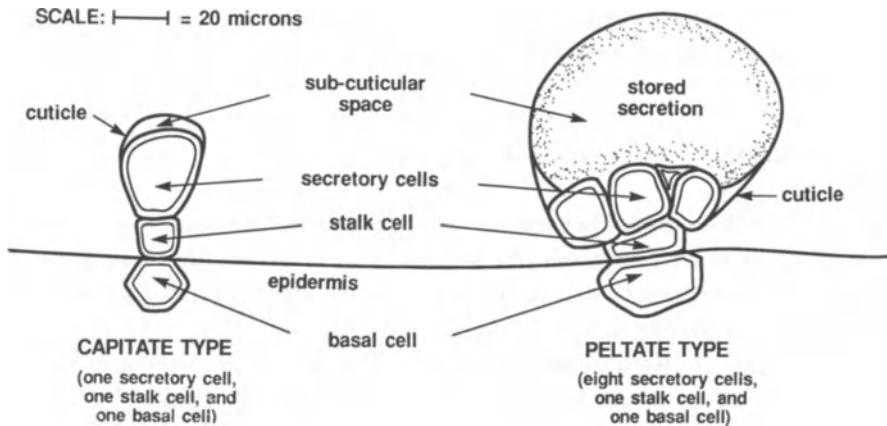


Fig. 1. Sketches of cross-sections of peltate and capitate glandular trichomes from the leaf of peppermint, illustrating the three kinds of cells present in these secretory structures. Glandular trichomes of these two basic types are found in many other species of the Lamiaceae. In peltate glandular trichomes the secretion is stored in the sub-cuticular cavity and is not released unless the cuticle is damaged, whereas in capitate glandular trichomes the secretion apparently volatilizes to the atmosphere through pores in the cuticle.

grams) were included to adsorb the terpenoids and phenolic materials released during surface abrasion.^{27,28} Since the shearing forces generated by the single-speed motor were too high for the selective removal of epidermal glands, the rotor speed was reduced with a rheostat set at 80-110 volts. Gland extraction was usually complete after 0.5-2.0 minutes of operation. This technique could be adapted to leaves of varying size, toughness and surface topography by altering the size of the glass beads used, the proportions of leaves to beads in the grinding chamber, the viscosity of the extraction buffer, the speed of the rotor, and the time of extraction.

Mechanized abrasion of the leaf surfaces of species of the Lamiaceae thus afforded extracts with high activities of monoterpene biosynthetic enzymes, but these preparations also contained significant quantities of material from non-glandular tissue. When peppermint leaves were extracted in this manner, clusters of intact glandular trichome cells were sometimes noted in our extracts. These were secretory cells from peltate glandular trichomes. They occurred as disc-like clusters approximately 60 microns in diameter that contained eight cells each (Fig. 2). Since there are eight secretory cells in each peltate glandular trichome

of peppermint, these disc-like cell clusters were apparently each derived from the fragmentation of a single glandular trichome. We exploited the presence of intact gland cells in our extracts to develop a method for obtaining much purer preparations of glandular material.

Isolation of Cell Clusters from Peltate Glandular Trichomes

The mechanized surface abrasion techniques described in the previous section were modified to enhance the recovery of secretory cell clusters from

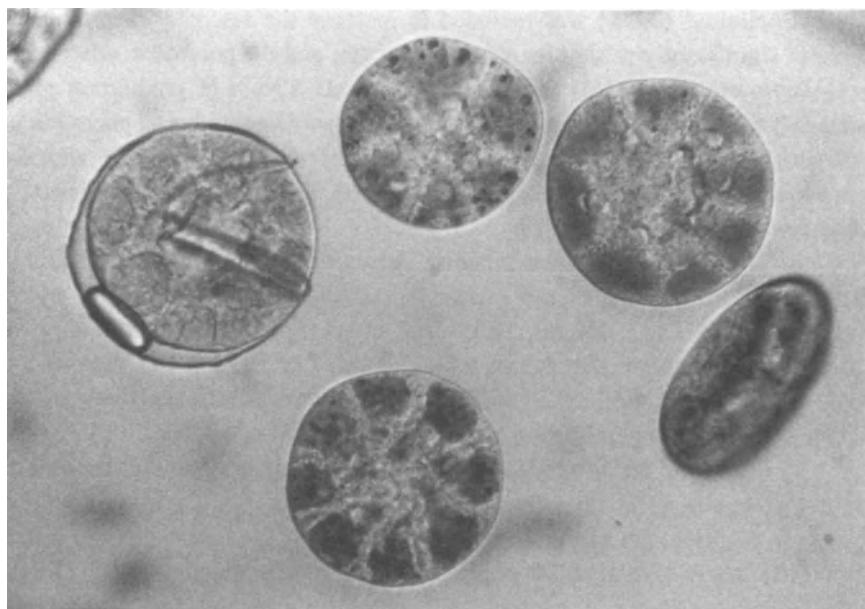


Figure 2. Secretory cells isolated from the peltate glandular trichomes of young peppermint leaves as seen under the light microscope. Cells have been stained with Neutral red, a vital stain taken up by acidic intracellular compartments such as vacuoles. Secretory cells were isolated as disc-like clusters of eight cells each, approximately 60 microns in diameter. Each cluster was derived from fragmentation of a single glandular trichome, and is apparently held together by a sheath of cuticle. In the cluster at the far left, a small oily droplet of glandular secretion has been retained under the cuticle. When viewed edge-on (cluster at lower right), one face often shows a depression formerly occupied by the stalk cell.

young leaves of peppermint. The leaves were first soaked in ice-cold distilled water for one hour immediately prior to extraction to maximize their turgidity. Approximately 15-18 grams of leaves and 100 grams of 0.5 mm diameter beads were loaded into the grinding chamber of the homogenizer for each extraction. Since the isolated secretory cells were very permeable to low molecular weight, water-soluble compounds (as discussed below), the extraction buffer (25 mM Hepes, pH 7.3, 200 mM sorbitol, 10 mM sucrose, 5 mM dithiothreitol, 0.5 mM potassium phosphate, 5 mM magnesium chloride and 10 mM potassium chloride) was formulated to mimic the pH, osmotic strength and ionic composition of native cytoplasm in order to help maintain cell viability. Methyl cellulose (0.6%) was included to increase the viscosity of the buffer without significantly raising its osmotic strength, and the polymeric adsorbents polyvinylpyrrolidone (1%) and polystyrene XAD-4 resin (1 gram/gram plant material) were also added to minimize the binding of terpenoids and phenolics to cell surfaces. Abrasion was carried out at 4°C for four 30 second pulses with the rotor speed controlled by a rheostat set at 85 volts. Between pulses, the grinding chamber was allowed to cool.

The isolated glandular trichome secretory cell clusters were separated from other constituents of the extract by sieving through a series of nylon meshes (Small Parts, Miami, FL). The clusters, being approximately 60 microns in diameter, readily passed through meshes of 350 and 105 microns and were collected on a mesh of 20 microns. They were washed extensively on the 20 micron mesh using extraction buffer without methyl cellulose.

Following this protocol, we were able to obtain purified preparations of peltate glandular trichome secretory cells from peppermint leaves on a routine basis. In addition, the methodology was successfully applied, with only minor modifications, to several other species, including spearmint, garden sage (*Salvia officinalis*), and American wormseed (*Chenopodium ambrosioides*). Other types of cell clusters and tissue debris, such as fragments of capitate glandular trichomes and non-glandular trichomes, were found in these preparations at about 10% of the abundance of the peltate gland cell clusters. The overall recovery of secretory cells from starting plant material was also excellent. Over 90% of the peltate gland secretory cells present on young peppermint leaves were recovered as intact cell clusters. The remainder were either not removed from the leaf (3-5%) or were disrupted during the abrasion procedure. In a typical preparation from young peppermint leaves, 300,000-500,000 secretory cell clusters were obtained per gram of leaves abraded.

The facile detachment of clusters of intact secretory cells from leaf peltate glands, and the tight adhesion of the secretory cells to each other, are

critical to the success of this procedure. However, the reasons for these fortuitous phenomena are still unclear. The thickened walls of the adjacent stalk cell (Fig. 1) may help direct breakage to the area between the stalk cell and the secretory cells. In addition, clusters of secretory cells may be partially held together by a layer of thickened cuticle.

Preparations of Intact Gland Cells

The peltate glandular trichome secretory cells removed from young peppermint leaves by the methods described above seemed eminently suitable for *in vivo* investigations of monoterpene biosynthesis, since this cell type is specialized for monoterpene production. Under the light microscope, nearly all the isolated cells appeared cytologically normal (Fig. 2), having retained their cell walls, cytoplasm, central vacuoles and other organelles. Staining showed that over 95% of the cells were still viable since they were able to accumulate Neutral red and exclude Evan's blue.²⁹ Buffers with an osmolarity of 250-300 mM and with a pH of 7.3 (isotonic to typical plant cytoplasm and of similar pH) seemed to best maintain cell viability.

Several experiments were undertaken to determine the utility of these cell clusters for biosynthetic studies. Clusters were suspended in extraction buffer (see above) without methyl cellulose or polyvinylpyrrolidone and incubated with various radiolabeled precursors of monoterpenoid biosynthesis. Precursors included [$U\text{-}^{14}\text{C}$]sucrose, [$2\text{-}^{14}\text{C}$]mevalonic acid, [$1\text{-}^3\text{H}$]isopentenyl pyrophosphate and [$8\text{-}^3\text{H}$]geranyl pyrophosphate. Preliminary trials showed evidence for very efficient incorporations of prenyl pyrophosphate precursors and rapid uptake of cofactors, such as NADPH, suggesting that isolated secretory cells were permeable to low molecular weight, water-soluble substances. Thus, the incubation buffer was supplemented with 0.5 mM manganese chloride, 1 mM NADPH, 1 mM NAD, 2 mM ATP, 3 mM ADP and 1 mM coenzyme A. A typical incubation contained 1-2 million clusters suspended in a volume of 3 ml buffer and was allowed to proceed for three hours at room temperature. Air was bubbled through the suspension of clusters during incubation and a 2 ml pentane overlay was used to trap volatile monoterpene products.

All administered precursors were incorporated into monoterpenes at an easily measurable rate (Table 1). As might be expected, precursors that were farther along the biosynthetic sequence (see Fig. 3), gave higher levels of percent incorporation. The radiolabeled monoterpene products formed in these incubations consisted primarily of limonene and menthone, with smaller amounts of pulegone, menthol and neomenthol, all typical monoterpenes of young peppermint leaves.²⁶

Table 1. The incorporation of various biosynthetic precursors into monoterpenes in intact secretory cells isolated from the peltate glandular trichomes of peppermint. Experimental details are given in the text. Precursors were administered at a concentration of 2 μM (sucrose), 120 μM (mevalonic acid), 40 μM (isopentenyl pyrophosphate) and 6 μM (geranyl pyrophosphate), and incubated with secretory cells for three hours.

Monoterpene precursor	Incorporation (% of total administered)
[U- ^{14}C]sucrose	0.6 %
[2- ^{14}C]mevalonic acid	1.1 %
[1- ^3H]isopentenyl pyrophosphate	1.9 %
[8- ^3H]geranyl pyrophosphate	34.0 %

The most significant aspect of these results is the relatively high levels of incorporation achieved. The *in vivo* systems used in previous monoterpene biosynthetic studies (cut stems, leaves and leaf discs)^{30,31} have nearly always afforded very low levels of precursor incorporation. For example, the efficiency of incorporation of mevalonic acid in previous studies was usually no greater than 0.01%, whereas, in our experiments with secretory cell clusters, we obtained an incorporation of over 1% (Table 1). Our success can be attributed at least in part to the fact that since secretory cells are specialized for monoterpene biosynthesis there should be less diversion of precursor to other pathways. In addition, studies in progress in our laboratory suggest that, after isolation, the plasmodesmata of the secretory cells remain open, a feature also observed in the isolated bundle sheath cells of several species of C₄ plants.^{32,33} This behavior undoubtedly enhances the uptake of the small, water-soluble precursors administered.

Researchers in several other laboratories have described methods for obtaining preparations of viable, intact glandular trichome cells. Sloane and Kelsey³⁴ isolated clusters of secretory cells from sagebrush (*Artemisia tridentata* ssp. *vaseyana*) by homogenizing leaf and floral tissue in a Waring blender, filter-

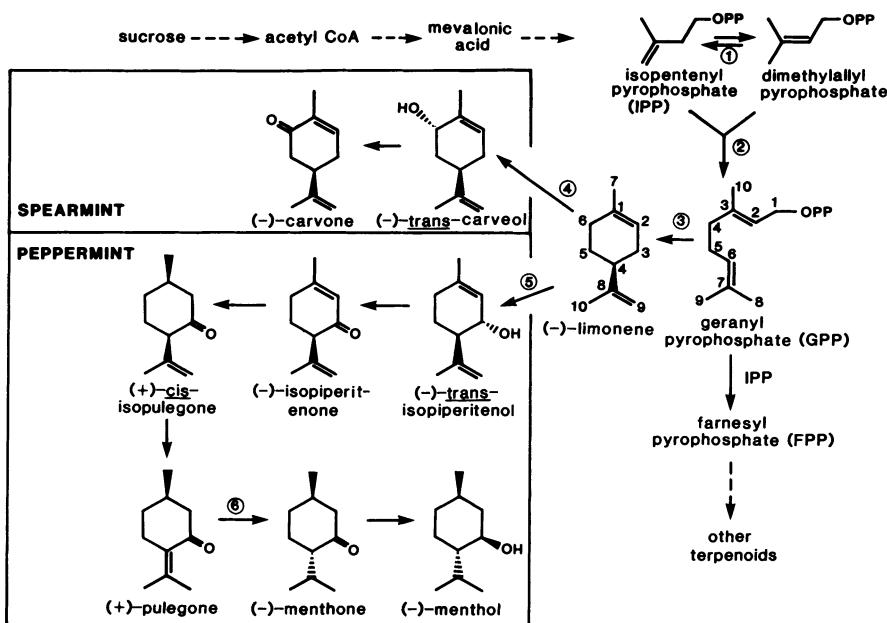


Fig. 3. Outline of the principal pathways of monoterpene biosynthesis in peppermint and spearmint. Enzymes mentioned in the text include: 1) isopentenyl pyrophosphate isomerase, 2) GPP synthase, 3) (-)-limonene cyclase, 4) (-)-limonene-6-hydroxylase, 5) (-)-limonene-3-hydroxylase, 6) (+)-pulegone reductase.

ing and centrifuging the extract to recover intact cells, and separating the gland heads (which contain the secretory cells) on a Percoll density gradient. Since species of sagebrush accumulate large amounts of monoterpenes and sesquiterpene lactones in glandular trichomes,³⁵ these preparations could have considerable utility for studies of terpenoid biosynthesis, although they have not yet been exploited for such purposes. Keene and Wagner⁷ reported a procedure for removing gland heads from the leaves of tobacco simply by touching a microscope cover glass to the leaf surface. Gland heads adhere to the cover glass, apparently because of the sticky exudate coating their surfaces. Detached gland heads were capable of biosynthesizing diterpenes and sucrose esters from basic metabolic precursors, such as carbon dioxide and sucrose, and were used to investigate the role of light and photosynthesis in the formation of these gland products.^{7,8}

Cell-free Enzyme Preparations from Purified Gland Cells

Over the last fifteen years, considerable progress has been made in understanding monoterpene biosynthesis using cell-free systems.³⁶ However, since the preparation of cell-free systems from whole-leaf homogenates has some serious shortcomings (e.g., high levels of competing activities and other interfering substances), we evaluated various techniques for obtaining such systems from the isolated secretory cells of peppermint peltate glandular trichomes. Sonication of a concentrated suspension of secretory cell clusters from peltate trichomes (1 million clusters/ml) successfully disrupted most of the cells, based on microscopic examination, and gave excellent yields of several enzymes of monoterpene biosynthesis. The best recovery was obtained using a microprobe (Braun-Sonic 2000) at maximum power for three one-minute bursts. Sonication afforded much higher levels of enzyme activity than other methods of cell disruption, such as the use of a hand-held glass tissue grinder (Ten-Broeck homogenizer), treatment with a vibrating glass bead mill, mechanized homogenization with a high-speed rotor (Tekmar Tissumizer), blending at high speed with glass beads (Bead-Beater), high-speed centrifugation or repeated freeze-thaw cycles. When secretory cell clusters were frozen in liquid nitrogen and ground with a mortar and pestle, high yields of enzyme activity were also obtained, but this technique was less suitable than sonication since it gave higher yields of competing phosphohydrolase activities (see Table 3).

Sonication was carried out in a buffer consisting of 25 mM potassium phosphate, 1 mM dithiothreitol, 1 mM EDTA and 10% glycerol designed to maximize the yield of enzyme activity. For preparations in which monoterpene cyclase activities were to be measured, polyvinylpyrrolidone (1%) and sodium metabisulfite (10 mM) were also added to the buffer and the pH was adjusted to 6.0 in order to adsorb the protonated phenolic compounds released and minimize their interaction with extracted enzymes.²⁷ For investigations of cytochrome P-450-dependent monoterpene hydroxylase activity, the sonication buffer lacked polyvinylpyrrolidone and sodium metabisulfite but also included 5 µM FAD, 5 µM FMN and XAD-4 resin, and was adjusted to a pH of 7.4. After sonication, the extract was filtered through 20 micron mesh and the filtrate centrifuged, first at 3000-27,000g, then at 195,000g for 90 minutes. Next, depending on the enzyme activities to be measured, the supernatant or pellet was adjusted to the appropriate assay conditions.

For the investigation of monoterpene biosynthesis, these sonicated secretory cell extracts are the most active cell-free preparations that we have yet obtained, and should prove extremely useful for future research with peppermint

and other species. Table 2 shows the comparative activities of some key enzymes of monoterpene biosynthesis in several different types of cell-free preparations derived from peppermint and spearmint leaves. (-)-Limonene cyclase catalyzes the conversion of the ubiquitous isoprenoid intermediate, geranyl pyrophosphate (GPP), to the olefin, (-)-limonene, while (-)-limonene-3-hydroxylase and (-)-limonene-6-hydroxylase are cytochrome P-450-dependent monooxygenases which convert (-)-limonene to (-)-*trans*-isopiperitenol and (-)-*trans*-carveol, respectively (Fig. 3). These enzymes were assayed as previously described.^{26,37} Limonene cyclase activity in sonicates of peppermint secretory cells was over 30 times that in extracts prepared by mechanized abrasion of leaf surfaces and over 300 times that found in whole-leaf homogenates. Limonene hydroxylase activity in the microsomal fraction of secretory cell sonicates was 2-5 times more than that found in the corresponding fractions of leaf surface extracts and much higher than that detectable in whole-leaf homogenates. As might be expected, the specific activities of these enzymes in secretory cell sonicates were also much higher than those measured in the other types of preparations (Table 2).

The high activity of monoterpene-synthesizing enzymes in extracts derived from intact secretory cells is most likely due to the relatively low levels of interfering substances present. In preparing these extracts, the secretory cells are disrupted only after they have been separated from most of the other cells of the leaf, so much lower levels of phenolic substances and bulk protein are present than in the other types of extracts. Another potential source of interference is that due to phosphohydrolases. These enzymes, which are ubiquitous in higher plants, compete effectively for the monoterpene cyclase substrate geranyl pyrophosphate (GPP) in cell-free preparations, converting GPP to geraniol and leading to substantial underestimates of cyclase activity.^{38,39} However, sonicates of peppermint secretory cells have less than 10% of the phosphohydrolase activity of corresponding leaf surface extracts and less than 1% that of whole-leaf homogenates (Table 3). Therefore, secretory cell extracts provide much more suitable experimental systems for investigation of monoterpene cyclases than do other types of cell-free preparations.

Secretory cell extracts are also much more useful experimental systems for the investigation of monoterpene hydroxylases than other types of cell-free preparations. Monoterpene hydroxylases are cytochrome P-450-dependent monooxygenases that are localized in the microsomal fraction of the cell. Microsomes are typically isolated in the light membrane fraction, pelleting between 18,000 g and 195,000 g by differential centrifugation. In our previous studies of monoterpene hydroxylases using whole-leaf homogenates and leaf surface extracts,^{37,40}

Table 2. Comparative activities of some enzymes of monoterpene biosynthesis in different types of cell-free extracts prepared from peppermint and spearmint leaves. Activities are highest in the extract of isolated secretory cells. Preparation of the various kinds of extracts is described in the text.

Enzyme (and species)	Activity ($\text{nmol} \cdot \text{h}^{-1} \cdot \text{g fresh weight}^{-1}$)		
	Whole leaf homogenate	Leaf surface extract	Extract of isolated secretory cells
(-)-limonene cyclase (peppermint)	0.82	8.6	301
(-)-limonene-3-hydroxylase (peppermint)	trace	8.9	20
(-)-limonene-6-hydroxylase (spearmint)	trace	7.1	42

Enzyme (and species)	Specific activity ($\text{nmol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$)		
	Whole leaf homogenate	Leaf surface extract	Extract of isolated secretory cells
(-)-limonene cyclase (peppermint)	0.11	4.3	727
(-)-limonene-3-hydroxylase (peppermint)	ND*	41.1 [#]	1470
(-)-limonene-6-hydroxylase (spearmint)	ND*	40.8 [#]	2040

* Not determined

[#]Data from reference 37.

Table 3. Comparison of geranyl pyrophosphate (GPP) phosphohydrolase activity in several different types of cell-free extracts derived from peppermint leaves. This activity, which interferes with measurements of monoterpene cyclase activity, is lowest in extracts of secretory cells. Preparation of the various kinds of extracts is described in the text.

Extract type	GPP phosphohydrolase activity (nmol · h ⁻¹ · g fresh weight ⁻¹)
Whole leaf homogenate	20.4
Leaf surface extract	1.3
Extract of isolated secretory cells	0.12

characterization was hindered by the fact that a large proportion of the extracted activity pelleted below 18,000g in a dense membrane fraction that contained protein-bound pigments and that tended to aggregate after suspension. However, in secretory cell extracts, over 95% of the total monoterpene hydroxylase activity was present in the light membrane fraction, and thus fully amenable to study.

EXAMPLES OF THE USE OF GLAND CELL PREPARATIONS IN BIOSYNTHETIC INVESTIGATIONS OF MONOTERPENE BIOSYNTHESIS

We have begun to use preparations of secretory cells from the peltate glandular trichomes of peppermint for various types of biosynthetic studies. In this section, we describe some recent results obtained with both intact cells and cell-free extracts.

Elucidation of Biosynthetic Sequences

One of the most important aims of biosynthetic research is to establish the sequence of intermediates in the pathway under investigation. In the formation of oxygenated, cyclohexanoid monoterpenoids in peppermint (*p*-menthane-type), (-)-limonene is thought to be the first cyclic intermediate (Fig.

3). Previous work in our laboratory with peppermint shoot tips fed radiolabeled sucrose suggested that (-)-limonene was an early monoterpenoid product that was then converted to oxygenated products, such as menthone.²⁶ Cell-free systems prepared by brushing leaf surfaces with a toothbrush converted geranyl pyrophosphate to (-)-limonene as the major cyclic product.²⁶

We were able to confirm the sequence "geranyl pyrophosphate (GPP) → (-)-limonene → oxygenated monoterpenoids" using both intact and cell-free preparations of peppermint secretory cells. When clusters of intact cells were incubated with [8-³H]GPP as described earlier, limonene was one of the major monoterpenes formed (45% of total monoterpenoids) and the only olefin produced. In cell-free preparations from purified secretory cell clusters, (-)-limonene was essentially the only product formed from GPP in the absence of reduced pyridine nucleotides. Figure 4 shows a radio-gas-liquid chromatogram of the metabolites of [1-³H]GPP produced by a sonicated extract of peppermint secretory cells. The dominant product is clearly (-)-limonene, which was shown by chemical degradation to be labeled at C-3 as expected (see Fig. 3 for numbering scheme).

Mechanistic Studies of a Key Enzymatic Step in the Pathway

The cyclization of GPP to limonene is a complex process catalyzed by a single enzyme that appears to consist of several discrete reactions. GPP cannot cyclize directly to cyclohexanoid monoterpenes, such as (-)-limonene, because of its *trans* double bond, but has been hypothesized to isomerize first to the enzyme-bound intermediate linalyl pyrophosphate (LPP), which is capable of cyclizing.³⁶ To study the mechanism of this reaction in more detail, it was necessary to have an ample supply of purified (-)-limonene cyclase. A sonicated extract of peppermint secretory cell clusters made an excellent starting point for such a purification because of the high specific activity of limonene cyclase in this preparation (Table 3). We subjected peppermint secretory cell sonicates to anion-exchange chromatography on DEAE-cellulose. In the fractions that contained the bulk of (-)-limonene cyclase activity, this enzyme was the major protein present, as determined by SDS-polyacrylamide gel electrophoresis, and competing activities were essentially non-existent.

The characterization of (-)-limonene cyclase from peppermint secretory cells showed that this enzyme had a molecular weight of 55,000 (by gel permeation chromatography), a pH optimum of 6.7, and an isoelectric point of 4.35 (by isoelectric focusing). The K_m value for the substrate GPP was found

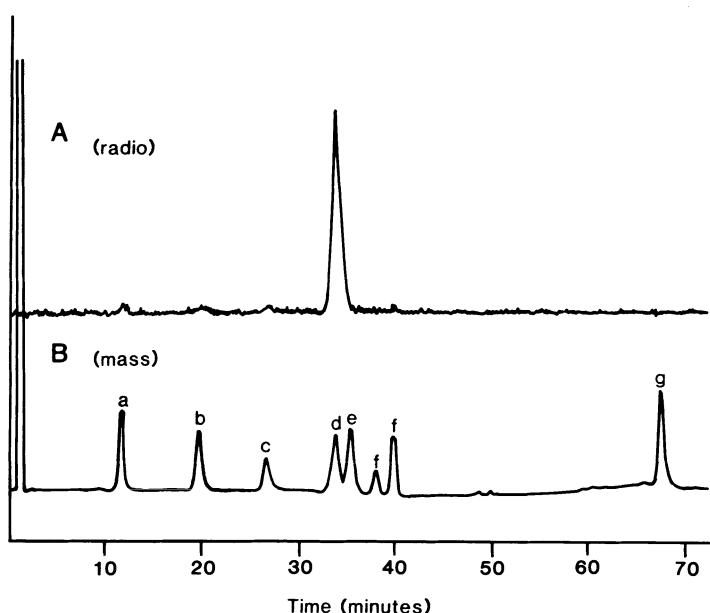


Fig. 4. Radio-gas-liquid chromatogram of the metabolites of GPP produced by a cell-free extract prepared from the isolated secretory cells of peppermint. As seen in the radio trace (A), limonene is essentially the only product formed. The smooth, lower trace (B) represents the response of the mass detector to coinjected standards of a) alpha-pinene, b) beta-pinene, c) myrcene, d) limonene, e) cineole, f) *cis*- and *trans*-ocimene and g) geraniol. Isolated secretory cells from peltate glandular trichomes were sonicated, filtered and centrifuged as described in the text, and assayed according to previously published procedures.²⁶ Chromatography of the pentane extract of the assay was performed on a Gow-Mac 550P gas chromatograph (He carrier gas at 35 ml/min, injector- 150°C, thermal conductivity detector- 200°C and 150 mA) attached to a Nuclear Chicago 7357 gas proportional counter. The column was 15 % AT-1000 on Gas-Chrom Q (12 feet x 0.125 inch stainless steel) programmed from 80°C (30 minute hold) at 5°C/min to 220°C (15 minute hold). Thermal conductivity and radioactivity output channels were monitored with a SICA 7000A Chromatogram Processor, and radioactivity measurements were externally calibrated with [³H]toluene.

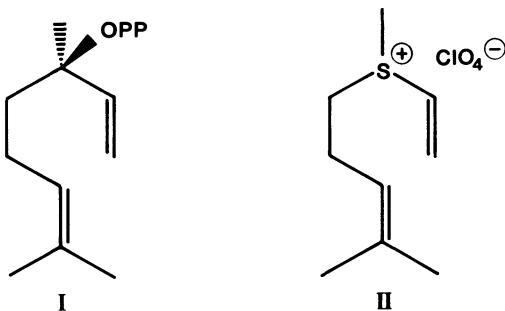


Fig. 5. Structures of (+)-3S-linalyl pyrophosphate (I), a postulated enzyme-bound intermediate in the conversion of geranyl pyrophosphate to (-)-limonene, and an analog of the linalyl carbocation, methyl-(4-methylpent-3-en-1-yl)vinyl-sulfonium perchlorate (II).

to be $1.9 \mu\text{M}$, and catalytic activity required a divalent metal ion, either Mn^{2+} ($K_m = 250 \mu\text{M}$) or Mg^{2+} ($K_m = 2 \text{ mM}$), with Mn^{2+} giving slightly higher levels of activity. Catalysis was inhibited by cysteine- and histidine-directed reagents, and protection studies showed that both of these types of residues are present at or near the active site. In general, the properties of (-)-limonene cyclase resemble those of other monoterpene cyclases.³⁶

The reactions catalyzed by monoterpene cyclases have been hypothesized to proceed from GPP through a series of enzyme-bound intermediates, one of which is linalyl pyrophosphate (LPP) (Fig. 5).³⁶ We have obtained two lines of evidence with (-)-limonene cyclase that are consistent with this suggestion. First, (+)-(3S)-LPP, the enantiomer of LPP that would be the stereochemically-predicted intermediate between GPP and (-)-limonene,⁴¹ was kinetically even more efficient than GPP; K_m was similar to that for GPP ($2.2 \mu\text{M}$ vs. $1.9 \mu\text{M}$), but V was over twice as high ($204 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$ vs. $78 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$). Second, a sulfonium ion analog of the linalyl carbocation, thought to be formed by ionization of LPP in the course of the reaction,⁴² was a very strong inhibitor of (-)-limonene cyclase activity, and effectively protected the enzyme against inhibition by histidine- and cysteine-directed reagents. Not only is LPP likely to be an intermediate in the cyclization of GPP to (-)-limonene, but comparing the kinetic data for GPP and LPP indicates that the isomerization of GPP to LPP proceeds at a slower rate than the subsequent cyclization of LPP to (-)-limonene.

Regulation of Biosynthesis by Changes in Enzyme Activities

Monoterpene biosynthesis in plants seems to be a carefully regulated process, since monoterpene accumulation is usually restricted to specific secretory structures that appear only in certain organs at particular stages of development. For example, in members of the Lamiaceae, monoterpene biosynthesis is restricted to glandular trichomes found on leaves and some floral parts,⁹ and occurs at a much higher rate in young, expanding leaves than in mature leaves.^{43,44} Regulation of the location and timing of monoterpene formation could be accomplished by a wide range of mechanisms operating at the organ, cellular, subcellular and enzyme levels.^{45,46} We are currently interested in how changes in the activities of certain key enzymes could control the rate of monoterpene biosynthesis. The monoterpene cyclases discussed above seem especially good candidates for catalyzing the rate-limiting steps, because they carry out the first committed reaction in monoterpene biosynthesis (Fig. 3).

Using cell-free preparations of secretory cells from peltate glandular trichomes, we have begun to investigate changes in the activity of several enzymes of monoterpene biosynthesis during leaf development in peppermint: isopentenyl pyrophosphate isomerase, GPP synthase, (-)-limonene cyclase and (+)-

Table 4. Changes in the activities of some enzymes of monoterpene biosynthesis during leaf development in peppermint. Activities were determined in secretory cell extracts from young, expanding leaves less than 2 weeks old (young) and fully-expanded leaves 7 weeks old (mature). Details of the enzyme assays can be found elsewhere.^{26,48,49} Activities are expressed on a per cell basis to facilitate comparison of secretory cells at different stages of development.

Enzyme	Activity ($\text{nmol} \cdot \text{h}^{-1} \cdot 10^6 \text{ cells}^{-1}$)	
	Young	Mature
Isopentenyl pyrophosphate isomerase	7.8	3.8
GPP synthase	21	1.9
(-)-Limonene cyclase	39	2.4
(+)-Pulegone reductase	128	128

pulegone reductase (Fig. 3). Young, expanding peppermint leaves accumulate monoterpenes at a rate 50-100 times that of fully-expanded leaves.⁴⁷ Preliminary comparisons of secretory cell extracts made from young, expanding leaves (less than two weeks old) with extracts prepared from fully-expanded leaves (seven weeks old) show dramatic decreases in both GPP synthase and (-)-limonene cyclase activity with age (Table 4). Isopentenyl pyrophosphate isomerase activity also declined somewhat, but the level of pulegone reductase activity showed no change. Thus, either (-)-limonene cyclase or GPP synthase might be important in regulating monoterpene production in peppermint, since the activities of these enzymes are well-correlated with changes in the overall rate of monoterpene biosynthesis.

Monoterpene cyclases have been implicated as regulatory enzymes in other studies.^{44,46,50} GPP synthase is a prenyltransferase, an enzyme that catalyzes the condensation of isopentenyl pyrophosphate with an allylic pyrophosphate generating the next-higher C₅-homologue of the allylic substrate. Prenyltransferases could also represent important regulatory catalysts in monoterpeneoid biosynthesis, since these enzymes catalyze reactions at pathway branch points.⁵¹ Regulation of metabolic pathways at branch points allows flux to be selectively directed among several possible routes. The rate of terpenoid formation *in vivo* shows a close relationship with prenyltransferase activity in a variety of other experimental systems.⁴⁶

Localization of Biosynthesis

Knowledge of the site of monoterpene formation in plants can be an important prerequisite to understanding how monoterpene biosynthesis is regulated. For instance, the location of monoterpene synthesis could affect the availability of biosynthetic precursors, and thus permit the rate of monoterpene formation to be regulated by the supply of substrate.⁴⁶

In a previous study with spearmint, we used extracts prepared by mechanical abrasion of leaf surfaces to establish the fact that monoterpene biosynthesis in this species takes place exclusively in the glandular trichomes.⁹ The availability of isolated cells from the glandular trichomes of peppermint allowed us to check the location of monoterpene formation in this species as well, using the presence of (-)-limonene cyclase, the first step unique to monoterpene biosynthesis in peppermint, as a marker. We isolated secretory cell clusters from the glandular trichomes of peppermint leaves and compared the

level of (-)-limonene cyclase in cell-free preparations of these clusters to the level of (-)-limonene cyclase in the remainder of the leaf. As expected, essentially all (> 99%) of the limonene cyclase activity which could be recovered from peppermint leaves was found in extracts of the secretory cells, indicating that monoterpene biosynthesis in this species, as in spearmint, is restricted to the glandular trichomes. In almost all plants investigated, monoterpene formation appears to occur exclusively in the cells of glandular trichomes^{9,43,52} or other specialized secretory structures, such as resin ducts or resin cavities.⁴⁶

CONCLUSION

The natural products that accumulate in glandular trichomes appear to be produced in the cells of the glandular trichome itself. As a consequence, for investigations of the biosynthesis of glandular trichrome constituents, it is necessary to use preparations containing glandular material. We have developed a new procedure for isolating secretory cells from the peltate glandular trichomes of peppermint in high purity and excellent yield. In this method, the leaf surface is gently abraded with glass beads in a way that removes nearly all of the glandular trichomes and provides clusters of intact secretory cells. From these isolated glandular trichome cells, it was possible to obtain both intact and cell-free preparations that were very active in monoterpene biosynthesis. These preparations are currently being employed in our laboratory to investigate various aspects of the sequence, mechanism, regulation and site of monoterpene biosynthesis. The procedures described in this chapter seem adaptable to a variety of plant species. Since many different types of plant natural products are found in glandular trichomes, these methods should find fairly wide application in preparing *in vivo* and *in vitro* systems for biosynthetic studies.

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Chapter Eleven

QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP ANALYSIS OF NATURAL PRODUCTS: PHOTOTOXIC THIOPHENES

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INTRODUCTION

The wide diversity of secondary metabolites known to exist throughout the plant kingdom has been partially attributed to plant-pest coevolution,^{1,2} although aspects of the process remain controversial.³ It is also known that fungal infections can elicit the production and accumulation of toxic plant secondary metabolites.⁴ The elaborate design of chemical defences in plants can involve mixtures of related structures in one biosynthetic class or multiple lines of chemical defence in a single plant species.⁵ For example, *Chrysanthemum* spp. contain several phototoxic thiophenes and acetylenes, cytotoxic sesquiterpene lactones, and insecticidal pyrethrins and isobutylamides.⁶

In many cases the mechanism of action of a toxic phytochemical is known, at least in part. However, the reasons for differences in biological activity between closely related compounds are usually less well understood. To answer the fundamental question "Why is one allelochemical more potent than its congeners?", the relationships between their chemical structures and their biological activity can be studied, employing a series of naturally occurring or synthetic derivatives of the model compound. Such studies of course also have an applied aspect, since they may lead to the discovery of potent compounds with economic potential as pharmaceuticals or agrochemicals.

Structure-activity relationships have been examined for a variety of natural products. The most common approach is qualitative, in which the biological activity is reported to be enhanced or diminished by the addition or subtraction of a chemical functionality from the parent molecule. Such work is very important for establishing which functional groups are essential for activity, and it provides insight into the possible mechanism of action. For example, in sesquiterpene lactones, the α -methylene- γ -lactone moiety, the β -unsubstituted cyclopentenone ring, and the α -epoxy cyclopentenone system all contribute significantly to anti-inflammatory,⁷ antihyperlipidemic,⁸ and anti-tumor⁹ activities. The reaction of the α,β -unsaturated lactone by a Michael addition to thiol groups of key enzymes is a part of the proposed mechanism of action common to all these activities, but the possession of this functional group is not sufficient to fully explain the activity of the compounds.

Quantitative structure-activity relationship (QSAR) analysis is a complementary approach based on statistical methods of correlation analysis.

QSAR lends itself to the development of models predictive of the level of activity and even degree of specificity of the compounds studied (see Fig. 1).

QSAR has been widely applied in the fields of synthetic medicinal chemistry,¹⁰ toxicology,^{11,12} and pesticide development.^{13,14} However, QSAR studies on natural products are not as common, probably because of the difficulty in isolating a series of related natural products large enough to allow exploration of the various structural factors contributing to the activity. At least five compounds are needed for each factor to be analyzed.¹⁵ The alternative approach of synthesizing enough derivatives of a prototype bioactive natural product is sometimes hampered by the molecular complexity of many natural products. Nevertheless, for some classes of natural products, such as sesquiterpene lactones, flavonoids, and thiophenes, a wide range of homologues has been isolated or synthesized, and QSAR analysis may contribute significantly to our understanding of their biological activities.

BASIC PRINCIPLES OF QSAR

Hansch¹⁶ has recently reviewed his pioneering research in QSAR and important directions for future applications. QSAR analysis can be expressed as an equation:

$$\text{BIOLOGICAL ACTIVITY} = f(\text{HYDROPHOBIC} + \text{STERIC} + \text{ELECTRONIC EFFECTS})$$

where biological activity is considered to be a function (*f*) of physicochemical properties of the compound. While this approach may appear to be simplistic, its utility has been demonstrated repeatedly when applied appropriately. Each of the terms in this equation will be briefly discussed below.

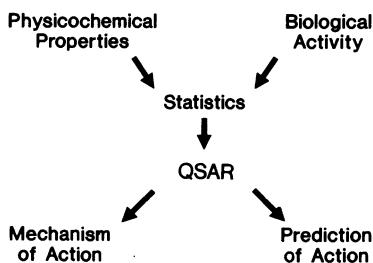


Fig. 1. The quantitative structure-activity relationship paradigm.

Biological Activity

Every organism at every level of organization (i.e. body, organ, tissue, cell, etc.) consists of an incredibly complex array of interconnected and interactive systems. No one compound can possibly have just one effect, so defining the biological activity of a compound is highly artificial. Practically, we can only deal with certain interactions considered in isolation, using multiple replicates to account for the natural variability in response exhibited by every biological system. The simpler the biological system, the easier it should be to derive a relationship between chemical structure and activity, although successful models have been developed for gross effects such as lethality in mice.¹⁷

For QSAR analysis, biological activity is generally expressed in terms of the molar concentration of an organic compound producing a standard biological response in a well-defined system within a constant time interval. Examples would be LD₅₀, ED₅₀, or IC₅₀: the lethal dose, effective dose, or inhibitory concentration required to have the specified effect on 50 % of the population of the test organism or reduce activity to 50 % of the maximal activity. These values should be determined by appropriate statistical methods, such as probit analysis.¹⁸

Hydrophobicity

In considering the physicochemical properties of a biologically active compound, it should first be stated that, like biological systems, these properties are interrelated and affected by their environment. For example, the hydrophobic, electronic, and steric effects of substituents on the parent molecule may be significantly collinear in a particular data set, making it impossible to consider more than one factor in one regression equation.¹⁹ Hydrophobicity will be affected by the degree of ionization of the compound, so the pH of the medium and the pKa of the molecule's ionizable groups must also be taken into account.

Hydrophobicity is a primary factor influencing passive transport processes for a biologically active compound from its source to its site of action. In addition, hydrophobicity has been shown to be very important in regulating the interaction of bioactive compounds with their bioreceptors.²⁰ The role of hydrophobicity in drug activity and QSAR analysis has been extensively reviewed.²¹⁻²³

Hydrophobicity is generally described in terms of the compound's partition coefficient between octanol and water. Octanol/water partition coefficients can be measured using the traditional equilibration methods, followed

by spectrophotometric or gas chromatographic determinations.^{15,22} Partition coefficients may also be measured by high-performance liquid chromatography.^{23,24}

Partition coefficients can be predicted from published tables of hydrophobic substituent constants (π),^{22,23} or calculated by the fragment addition method using the program CLOGP3, included in MedChem Software.²⁵ However, it is important to realize that all the methods used to measure or estimate partition coefficients have problems when applied to extremely hydrophobic or hydrophilic compounds.

The π constant for the contribution of a substituent to the hydrophobicity of the molecule is derived from the equation:

$$\pi_X = \log P_X - \log P_H$$

where P_X is the partition coefficient of a derivative and P_H that of the parent compound. A positive value for π means that, relative to H, the substituent confers greater hydrophobicity, while a negative value indicates that the substituent confers less hydrophobicity. All π values are not truly constant, e.g. OH, OCH₃, and NH₂ groups' π values are raised by electron-attracting NO₂ or CN substituents and lowered by electron-releasing groups, and halogen π values are also strongly affected. However, since the effects on hydrophobicity of substituents are generally considered to be additive, the log P of the derivatives can be calculated from a knowledge of the log P of the parent molecule.²²

Steric Parameters

Every molecule has a three-dimensional size and shape which influences its ability to interact with other molecules. Most molecules are not rigid, and may have many conformations, although there is a minimum-energy conformation that may be "preferred." Adding a substituent changes the molecule's size and conformation, which may enhance, hinder, or have no effect on its interaction with a particular molecule such as a receptor protein. These steric effects of substituents have long been studied for their influence on the rates of chemical reactions, but the complexity of steric effects on the interaction of organic molecules with macromolecules and drug receptors is far greater. Nevertheless, good qualitative agreement has been achieved between statistical correlation models based on enzymic kinetic parameters from reactions occurring in solution and molecular graphics models based on information obtained from x-ray crystallographic analysis of bioreceptors.^{19,20}

Molar refractivity (*MR*) is the steric parameter most commonly used in QSAR studies.^{15,19,22,26} The molar refractivity of a substance is defined by the Lorenz-Lorenz equation:

$$MR = ([n^2 - 1]/[n^2 + 2]) \times MW/d$$

where *n* = refractive index, MW = molecular weight, and *d* = density of the compound. Since the refractive index does not vary much for most organic compounds, and MW/d gives the molar volume, *MR* is a measure of gross volume.¹⁹ This conclusion is supported by detailed studies using QSAR and molecular graphics of enzyme-substrate interactions.²⁰ Extensive tables with *MR* values for a variety of substituents have been published,^{15,22} and *MR* values for additional substituents can be calculated using the CMR program included with MedChem software.²⁷ Steric effects can also be modelled using Taft's steric parameter (*E*_s) and Verloop's parameters (L,B₁₋₄),^{15,22} or by direct computation of molecular volumes and sizes.²⁰

Electronic Parameters

The classical parameters used to describe electronic effects in QSAR analysis are Hammett's σ constants.^{27,28} The σ electronic constants were initially developed to model chemical reactivity, and are defined by the equation:

$$\sigma = \log K_X - \log K_H$$

where *K*_H is the ionization constant for benzoic acid in water at 25° and *K*_X is the ionization constant for a *meta* or *para* derivative under the same experimental conditions. Positive values of σ represent electron withdrawal by the substituent from the aromatic ring, while negative σ values indicate electron release to the ring. These values cannot be applied reliably to aliphatic compounds or aromatic compounds with *ortho* substituents, due to steric interference, or to compounds with substituents directly conjugated with the reaction centre, due to through-resonance. One of the first applications of Hammett constants to QSAR analysis of natural products involved a study of the biological activity of phenoxyacetic acid plant growth regulators and chloromycetin-analogue antibiotics.²⁹ A list of Hammett constants for a wide variety of substituents has been published,²² and modifications to the constants to account for *ortho* substitutions have also been described.^{30,31}

Recent advances in the area of electronic parameters used in QSAR

include the use of quantum mechanics-derived indices, such as HOMO (Highest Occupied Molecular Orbit) or LUMO (Lowest Unoccupied Molecular Orbit) energies.³² The use of such calculations of quantum mechanics is potentially a more powerful approach than the use of Hammett constants, since it allows greater flexibility in the construction of the data set for study, e.g. it could be possible to include more than one kind of aromatic ring in the same data set.

Statistical Models

The strength of the QSAR technique lies in the use of statistics to describe structure-activity relationships. The simplest model is described by a linear relationship between biological activity and a physicochemical parameter, x . Such a relationship, described by the equation:

$$\log 1/C = a x + b$$

where C is the active molar concentration (e.g. EC₅₀), can often explain the activity of a given series of compounds, but cannot logically be extended to infinity, since there are no compounds with infinite activity.

For hydrophobicity the relationship is not linear, since biological activity will increase with increasing hydrophobicity until a maximum is reached, after which the activity will decrease with any further increase in hydrophobicity. A parabolic model relating biological activity to $\log P$, described by the equation:

$$\log 1/C = a \log P + b (\log P)^2 + c$$

has often been used successfully, and may serve as a good first approximation, but the curvature of the left side of the parabola fails to accurately describe the linearity of the activity-hydrophobicity relationship.

A further refinement made by Kubinyi¹⁷ was the development of the "bilinear" model, described by the equation:

$$\log 1/C = a \log P - b \log (\beta P + 1) + c$$

where β is a nonlinear term, usually in the range of 1 to 10⁻¹⁰, and which must be determined by iteration. The model is derived from a hypothetical four-phase biological system, such as may occur in a bacterial cell or isolated tissue cell, consisting of an outer aqueous phase, a lipid (membrane) phase, an inner aqueous

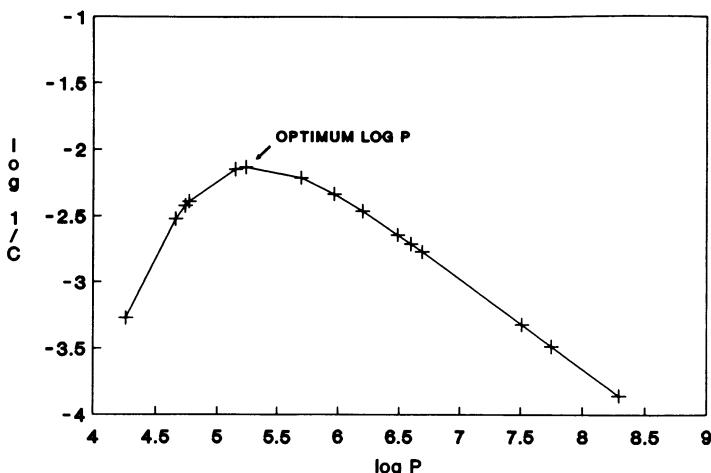


Fig. 2. The bilinear model for hydrophobicity-activity relationships.

phase, and a receptor phase. The bilinear model provides asymmetric curves with linear ascending and descending sides, connected by a parabolic portion within the range of optimum hydrophobicity (see Fig. 2).

The ascending slope is given by the linear coefficient "a," the descending slope by $(a - b)$, and when $b > a$, the maximum of the curve, representing the optimum $\log P$ for biological activity in that system, is given by the equation:²¹

$$\log P_o = \log (a / \beta [b - a]).$$

A comparison of models in more than 300 examples of non-linear structure-activity relationships suggested that in most cases the bilinear model gives a better fit to experimental data.²¹

The relationship between biological activity and factors other than hydrophobicity may be linear or nonlinear, and these factors may interact with hydrophobicity in a simple additive or more complex fashion. Thus the final QSAR model often involves use of multiple regression analysis. As additional factors are considered, the degrees of freedom in the regression equation will be reduced, necessitating a larger number of compounds in the series to ensure statistical significance.

THIOPHENE STRUCTURE-PHOTOTOXICITY RELATIONSHIPS

Background

To illustrate the application of QSAR analysis to natural products, the following is a detailed description of research that lead to the formulation of an equation describing the relationship between physicochemical properties of a homologous series of tricyclic thiophenes, including natural products and synthetic derivatives, and their phototoxicity to nauplii of a marine crustacean, the brine shrimp (*Artemia salina* Leach).

Certain genera of the plant family Asteraceae, such as *Adenophyllum*, *Chrysactinia*, *Dyssodia*, *Eclipta*, *Flaveria*, *Nicolletia*, *Porophyllum*, and *Tagetes*, produce characteristic acetylenic and thiophenic secondary metabolites that are light-activated toxins.³³ These substances are undoubtedly important in the chemical defence of plants against herbivorous insects^{34,35} and other pests, and the plants themselves have been used traditionally as insecticides or antimicrobials for treatment of skin infections.³⁶ In addition, there is now growing interest in these photosensitizing substances as chemotherapeutic and pesticidal agents. In particular, different phototoxic thiophenes of the Asteraceae exhibit highly effective antiviral³⁷ or mosquito larvicidal activities.³⁵

The most promising lead compound is alpha-terthienyl (α -T) which, in the presence of sensitizing ultraviolet light from the sun, is toxic to mosquito larvae at concentrations as low as 4 parts per billion.³⁸ These laboratory results have been verified in field trials in Canada and Africa, which suggest that the compound has comparable efficacy to synthetic pyrethroids.³⁵ In addition, it is rapidly photodegraded in the environment (a half-life of approximately 4 h in sunlight)³⁹ and shows virtually no cross resistance to other pesticides because of its novel mode of action.⁴⁰

The phototoxicity of thiophenes, both *in vitro* and *in vivo*, is known to involve an oxygen-dependent⁴⁰ type II mechanism, as illustrated in Figure 3. A ground-state sensitizer (0S), such as α -T, is promoted to an excited singlet state (1S) following absorption of a photon ($h\nu$) in the UV-A wavelength (320-400 nm). The singlet state sensitizer may return to its ground state through the emission of light (fluorescence, $h\nu_f$) or heat (by internal conversion), but also readily undergoes intersystem crossing (ISC) to produce a more stable triplet-excited state (3S). The triplet-excited sensitizer may also return to the ground state through the emission of light (phosphorescence, $h\nu_p$) or heat (by ISC). However, in the type II phototoxic mechanism, the sensitizer transfers energy to

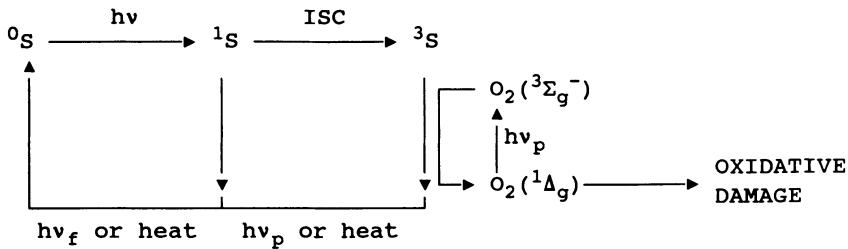


Fig. 3. The type II mechanism of phototoxicity.

ground-state oxygen ($O_2[{}^3\Sigma_g^-]$) to produce highly reactive singlet oxygen, $O_2({}^1\Delta_g)$, which can oxidize unsaturated fatty acids (e.g. in membranes), sterols (e.g. cholesterol), free amino acids, and amino acid residues in proteins (e.g. in enzymes). The sensitizer returns to the ground state, and may be excited by another photon, thus acting as a catalyst for the production of $O_2({}^1\Delta_g)$.^{41,42} By contrast, in the type I phototoxic mechanism, the triplet-excited sensitizer binds covalently to another molecule (e.g. DNA, tRNA, or cellular proteins).⁴³

Preliminary studies of structure-function relationships in the phototoxicity of acetylenes and thiophenes established the importance of their absorption spectra, extinction coefficients,⁴⁴ and of the hydrophobicity of these compounds.⁴⁵ A more detailed qualitative study of their structure-activity relationships was done for polyacetylenes and thiophenes with a variety of biological activities by Marchant and Cooper.⁴⁶ They found that among aliphatic acetylenes, at least three conjugated carbon-carbon triple bonds are required for phototoxic activity, with further unsaturation significantly improving activity. Aromatic acetylenes are in general more toxic than aliphatic acetylenes, and require at least two conjugated triple bonds for phototoxicity. Thiophenes are in general more phototoxic than aromatic acetylenes.

Among the thiophenes, Arnason *et al.*³⁵ found that there was no clear relationship between the Hammett constants for electron donating or withdrawing substituents and phototoxicity to larvae of the mosquito *Anopheles gambiae*. A positive correlation was observed between hydrophobicity and phototoxicity, and a small positive correlation was also found between the quantum yield of singlet oxygen and phototoxicity, although the small variation in quantum yield of the thiophene derivatives was not sufficient to explain the large variation in phototoxicity.

In order to gain a better understanding of quantitative aspects of the structure-phototoxicity relationships of the thiophenes, 51 tricyclic thiophene

analogues have been synthesized. This includes 44 terthienyl derivatives and 7 derivatives in which one or two of the thiophene rings were replaced by a benzene, pyridine, or naphthalene moiety, and also 11 bithienyl derivatives. Biological testing with a variety of organisms, including insects, crustaceans, fungi, yeasts, bacteria, viruses, and mammalian cancer cell lines, is underway. A subset of these results, dealing with tricyclic thiophene phototoxicity to brine shrimp, a well established biological system for pesticidal^{47,48} and structure-activity relationship analysis,⁴⁹ is provided below.

Methodology

The large-scale synthesis of α -T and analogues was accomplished by a Grignard-Wurz coupling reaction, and purification was simplified by use of a soxhlet extraction method, with verification of purity by HPLC, as previously described.^{41,50}

The $O_2(^1\Delta_g)$ generating efficiency of α -T and its analogues were previously measured by means of laser flash photolysis.^{43,51} Recent technological advances in the detection of long-wavelength emission by use of a germanium diode now allow more accurate direct determination of $O_2(^1\Delta_g)$ quantum yields (Φ_Δ) by means of time-resolved measurements of its infrared phosphorescence at λ_{max} 1270 nm.^{52,53} This technique was employed as previously described,⁴⁴ to determine the quantum yield of $O_2(^1\Delta_g)$ for each of the compounds tested.

The Φ_Δ of $O_2(^1\Delta_g)$ is a constant for each phototoxin, and does not reflect significant wavelength or concentration dependence, nor does it vary greatly with organic solvents.⁴⁴ The more biologically-relevant actual rate of $O_2(^1\Delta_g)$ production under the experimental conditions used was also calculated.

Integration of the emission spectrum of a black-light-blue (BLB) lamp⁵⁴ indicated that 97.1 % of the energy of the lamp is emitted in the photo-sensitizing UV-A region of 320-400 nm. A bank of four BLB tubes (Westinghouse, 20 W, F20T12/BLB) was used, and the UV-A intensity of 4 W/m² at 15 cm sample distance was measured with a Y.S.I. radiometer. The sum of the absolute intensities (ΣI_0) for 320-400 nm was therefore:

$$4 \text{ W/m}^2 \times 0.971 = 3.884 \text{ W/m}^2 = 3.884 \text{ J/s/m}^2.$$

Relative intensities (I_r) were determined from the emission spectrum at 5 nm intervals and interpolated to 1 nm intervals. The absolute intensity (I_0) value for each wavelength was calculated by the formula:

$$I_0 = \Sigma I_0 \times I_r / \Sigma I_r.$$

The UV spectrum of each thiophene was determined at a concentration that would provide an optimal spectrum with an absorbance (A) at λ_{\max} of between 1.0 and 2.0. The absorbance of the compound at each wavelength was then corrected to a typical standard spectroscopic concentration by a constant factor (50/concentration in μM) so that every measurement related to the same number of molecules ($50 \mu\text{M}$) of the compound. The light intensity absorbed (I_a), in J/s/m^2 , at each wavelength, relative to the output of the BLB lamp was determined by the formula:

$$I_a = I_0 \times (1 - [1/10^A]).$$

The energy (E) in units of J/mole of photons at each wavelength (λ) was calculated by the formula:

$$E = (N_A \times h \times c) / \lambda$$

where N_A is Avogadro's number, h is Planck's constant, and c is the velocity of light. The photon dose (D) in units of moles of photons/ s/m^2 , for each wavelength, was determined by the formula:

$$D = I_a / E.$$

Multiplying the sum of the photon doses at 320-400 nm by the Φ_Δ , which is a measure of the efficiency of $\text{O}_2(^1\Delta_g)$ production, gave the rate of $\text{O}_2(^1\Delta_g)$ production, expressed in units of $\mu\text{M/s/m}^2$ (referred to as RATE hereafter), under actual experimental conditions.

These calculations thus provided a comparative value for each phototoxin, in terms of production of singlet oxygen by a given number of molecules under experimental conditions, that could be directly related to the LC₅₀ observed under UV-A irradiation for the compound.

The thiophenes (dissolved in a small volume of methanol before addition to the brine) were tested for phototoxicity to 24 hour old brine shrimp nauplii according to the methods of McLaughlin,^{50,55} with minor modifications as described previously.⁵⁶ Two replicates of each concentration (and solvent control) were used, and each experiment was repeated twice. Previous experience with mosquito larvae suggested a preincubation period longer than the experimental preparation time would not significantly alter the results.⁴⁰ UV-A

irradiation, as described above, was for 4 hours, followed by a further 20 hour incubation under ambient room light and temperature. For minus-UV controls, UV light was excluded with a UV filter (Kodak CP2B). To prevent excessive evaporation under the lamps, all vials were covered with a plastic film (Saran Wrap) determined spectoscopically to be transparent to UV-A light. Determination of the LC₅₀ values was by probit analysis.

Octanol/water partition coefficients and molar refractivity values were calculated using the programs CLOGP3 and CMR included in version 3.54 of MedChem software.²⁷ The calculated log *P* (CLOGP) values correlated very well (*r* = 0.95) with log *P* values obtained experimentally by the high-performance liquid chromatography method cited above.^{25,26} The regression analyses were performed using the Pomona College QSAR program. A VAX-8530 computer was used for the calculations.

The QSAR Model

The phototoxicity (LC₅₀ to brine shrimp nauplii) and physicochemical properties of a homologous series of 21 tricyclic thiophenes, for which all the pertinent biological and structural parameters were available, are presented in Table 1, with reference to their chemical structures, shown in Figure 4. Efforts are underway to expand the data set to include all the compounds synthesized. Certain general trends are evident from a qualitative examination of the results. For example, identically disubstituted thiophenes are less active than the monosubstituted derivatives. Increasing the length of a side chain decreases activity. Replacing a thiophene ring with another aromatic group also decreases activity. Several of the synthetic derivatives are more phototoxic to the brine shrimp larvae than the naturally occurring α -T and methyl- α -T, compounds 1 and 2.

A first attempt at a QSAR model for these compounds involved plotting the logarithm of 1/LC₅₀ (the inverse is used so that a larger number indicates greater toxicity, making the graph easier to interpret) versus log *P*. The observed values produce a widely scattered plot. No correlation was observed between log 1/LC₅₀ and Hammett constants such as σ_p (appropriate for *para* substitution), σ_m (appropriate for *meta* substitution), or σ_p^+ (appropriate for *para* substitution on heterocycles). For this data set, there was a significant degree of collinearity between CLOGP and CMR (*r* = 0.59, *P* < 0.005).

A step-wise forward regression analysis of the data demonstrated that parameters related to the production of O₂(¹ Δ_g) were the most significant in explaining thiophene toxicity. Of these parameters, RATE of O₂(¹ Δ_g) correlated

Table 1. Structure, phototoxicity to brine shrimp nauplii, and physicochemical properties of tricyclic thiophenes

NO.	R ₁ , R ₂ GROUPS ^a	LC ₅₀ ^b	CLOGP ^c	CMR ^d	Φ _A ^e	RATE ^f
1.	H, H	0.65	5.70	7.14	0.71	7.40
2.	CH ₃ , H	0.57	6.20	7.60	0.79	8.66
3.	CH ₃ , CH ₃	0.69	6.69	8.07	0.66	6.91
4.	SCH ₃ , H	0.51	6.26	8.49	0.81	8.59
5.	SCH ₃ , SCH ₃	0.88	6.81	9.68	0.52	5.28
6.	Si(CH ₃) ₃ , H	0.31	8.29	9.42	0.84	9.29
7.	CO ₂ H, Si(CH ₃) ₃	0.49	8.08	10.07	0.86	9.45
8.	CH ₂ CH ₂ CO ₂ CH ₃ , H	2.18	5.64	9.18	0.42	4.36
9.	CH ₂ OH, H	2.44	4.66	7.76	0.65	6.93
10.	CH ₂ CH ₂ OH, H	3.28	4.74	8.22	0.65	6.79
11.	CN, H	1.21	5.24	7.67	0.61	6.59
12.	CH=CB ₂ , H	32.07	7.68	9.66	0.15	1.42
13.	CH=CHCO ₂ CH ₃ , H	25.63	6.02	9.31	0.31	1.72
14.	CONH ₂ , H	10.16	4.26	8.01	0.73	8.03
15.	CHO, CHO	27.40	4.59	8.14	0.76	5.66
16.	CH ₂ CH(NH ₂)CO ₂ CH ₂ CH ₃ , H	28.66	5.04	10.02	0.58	6.18
17.	see structure 17	68.90	5.63	7.33	0.56	0.06
18.	see structure 18	11.80	5.63	7.33	0.31	1.87
19.	see structure 19	41.54	4.62	7.12	0.27	1.09
20.	see structure 20	1745.70	5.06	6.70	0.58	0.24
21.	see structure 21	88.54	5.06	6.70	0.58	0.55

^a R₁ and R₂ substituents for structures 1-16, shown in Figure 4.

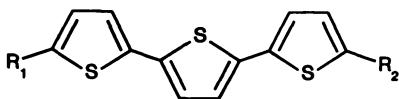
^b LC₅₀ values are expressed in nM concentration.

^c Computer-calculated log P values.

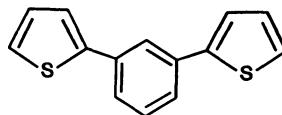
^d Computer-calculated MR values.

^e Quantum yield of singlet oxygen.

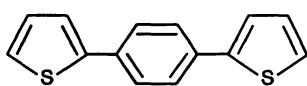
^f Rate of O₂(¹Δg) production, expressed in μM/s/m².



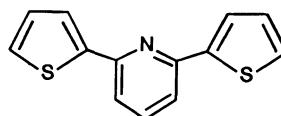
Structures 1-16



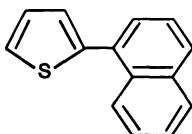
Structure 17



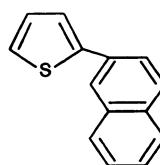
Structure 18



Structure 19



Structure 20



Structure 21

Fig. 4. Chemical structures of naturally occurring and synthetic tricyclic thiophenes studied by QSAR analysis. R₁ and R₂ groups are defined in Table 1.

better with $\log 1/\text{LC50}$ ($r = 0.83$, $P < 0.001$) than quantum yield data (Φ_Δ) ($r = 0.68$, $P = < 0.05$).

A multiple regression equation with RATE and a bilinear function with CLOGP produced the best correlation:

$$\log 1/\text{LC}_{50} = 0.25 (\pm 0.07) \text{ RATE} + 0.76 (\pm 0.47) \text{ CLOGP} - 1.10 (\pm 0.91) \log (\beta_{10}^{\text{CLOGP}} + 1) - 6.03 (\pm 2.49)$$

$r = 0.91$, $n = 21$, $s = 0.454$, $P < 0.0001$, $\log \beta = -6.28$

This equation succeeds in explaining 83 % of the variance of the observed data ($r^2 = 0.83$). Figure 5 shows the highly significant correlation between the observed LC₅₀ values and those predicted by the model.

As described above in the discussion of statistical models for QSAR, the maximum of the bilinear curve can be determined from the line equation:

$$\begin{aligned}\log P_0 &= \log (a / \beta [b - a]) \\ &= \log (0.76 / 10^{-6.28} [1.10 - 0.76]) = 6.63\end{aligned}$$

This value of $\log P_0$ represents the optimum hydrophobicity for tricyclic thiophene phototoxicity to brine shrimp nauplii. To illustrate how RATE interacts with the bilinear model of partition coefficients and phototoxicity, the regression line for the above equation is plotted in Figure 6, when CLOGP = 4, 10, or 6.63 ($\log P_0$). From Figure 6 it can be seen that at any given RATE, phototoxicity is enhanced by a $\log P$ which is optimal for that biological system.

The QSAR model described above is actually a three-dimensional relationship, and the cross-sectional aspect is illustrated in Figure 7, with RATE modifying the regression line for the bilinear function of CLOGP. These QSAR models allow a reasonable prediction of the phototoxicity of untested tricyclic thiophenes, provided that their RATE and $\log P$ values are known. Since the $\log P$ can be calculated, RATE is the only physicochemical measurement necessary to get an estimate of the phototoxicity of a given compound.

Due to anatomical, physiological, and behavioural differences, various organisms differ in their susceptibility to thiophene phototoxicity.^{35,36,40} In

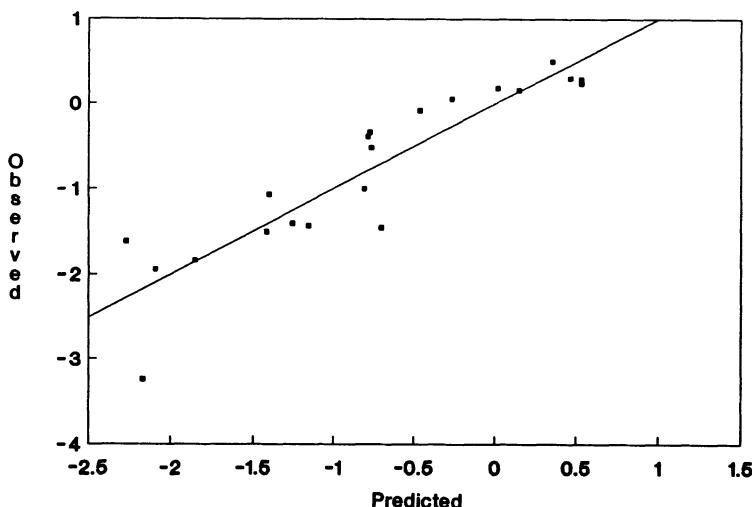


Fig. 5. Plot of observed $\log 1/\text{LC}50$ versus $\log 1/\text{LC}50$ values predicted by multiple regression with the rate of singlet oxygen production and the bilinear function of $\log P$.

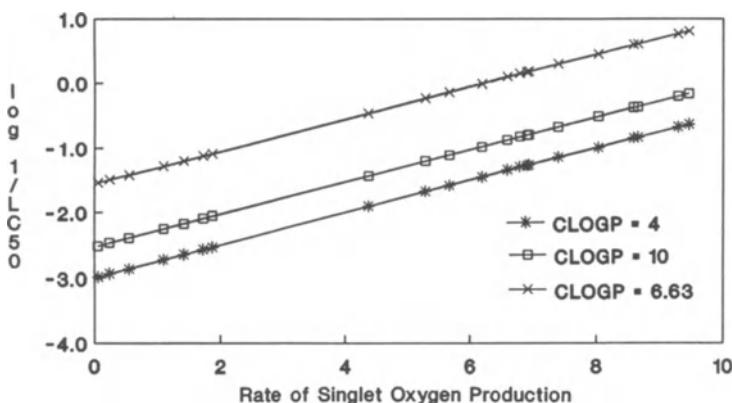


Fig. 6. Plot of $\log 1/LC_{50}$ versus RATE, with the effect of including the bilinear function of CLOGP, where the values of 4, 10, and the $\log P_o$ of 6.63 are substituted into the regression equation.

addition, we have noted that the $\log P_o$ for the same group of thiophenes against different organisms differs significantly. For example, the $\log P_o$ of tricyclic thiophenes against larvae of the mosquito *Aedes atropalpus* is 5.31, and from preliminary results against a mammalian cell line, the P815 murine mastocytoma, and a membrane-bound virus, the murine cytomegalovirus (unpublished data), the $\log P_o$'s for these systems appear to be much lower. Thus, from the $\log P_o$ value an indication of the specificity of the phototoxin can be obtained, allowing synthetic schemes to be directed toward achieving optimal activity against one class of organisms and minimal toxicity to nontarget organisms.

The above models explain why cyano- α -T (compound 11), which has a lower RATE than α -T, is more toxic to mosquito larvae and less toxic to crustaceans; in the laboratory, the ratio of LC₅₀'s against mosquito larvae (α -T / cyano- α -T) is 163.0 nM / 78.64 nM (= 2.07), and the ratio against brine shrimp nauplii is 0.65 nM / 1.21 nM (= 0.54). This has also been demonstrated to hold true in field trials against larvae of the mosquito *Aedes intrudens*, against which cyano- α -T was 1.4 times more phototoxic than α -T and 2.4 times more phototoxic than methyl- α -T, at an application rate of 50 g/ha.⁵⁷

Concern has been expressed over the broad spectrum of activity of phototoxic thiophenes.^{58,59,60} While the toxicity of α -T to non-target organisms, at concentrations necessary for adequate mosquito control, is within

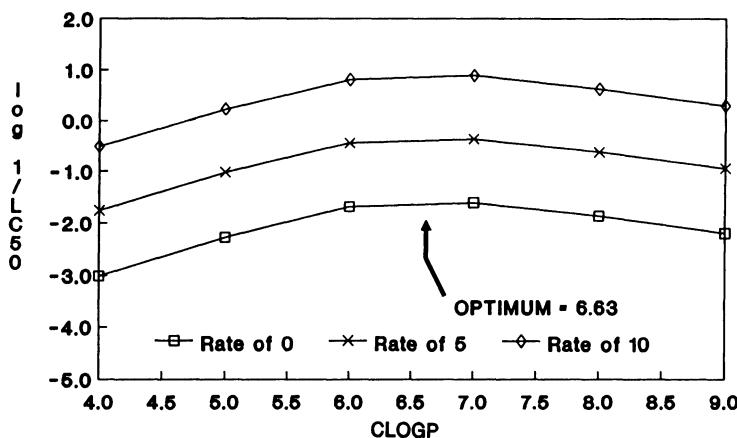


Fig. 7. Plot of $\log 1/LC_{50}$ versus the bilinear function of CLOGP, with the effect of including the RATE of singlet oxygen production, where RATE is 0, 5, or $10 \mu\text{M}/\text{s}/\text{m}^2$. The "optimum" refers to $\log P_o = 6.63$. The model is extrapolated from the observed CLOGP values of 4 - 8 to extremes of 1 - 10 for predictive purposes.

acceptable limits,^{61,62} it is a problem that will require attention when testing novel thiophene analogues for pest control or pharmaceutical applications.

Due to the apparent species dependence of $\log P_o$ values, their determination is a logical step toward determination of the specificity of novel phototoxic thiophenes for particular applications. Efforts are underway to further refine our model of thiophene phototoxicity by consideration of molecular orbital indices.

Finally, thiophenes also possess toxicity to brine shrimp nauplii which is not light-dependent (referred to hereafter as dark toxicity). The ratio of phototoxicity to dark toxicity for 45 thiophenes we have tested, under conditions where UV-A light was excluded with a filter during sample preparation and the full incubation period, ranged from <1 to >1000, with an average of $417.9(\pm 194.8)$ (unpublished data). Work is continuing toward the optimization of a QSAR model for thiophene dark toxicity.

CONCLUSION

Phototoxicity is undoubtedly a complex molecular process, so it is not surprising that our correlations account for only part of the variation observed in

the biological response. However, these correlations are still highly significant despite the relatively large degree of variation in the chemical structures of the thiophenes studied. That a relatively simple model involving only factors of rate of singlet oxygen production and hydrophobicity can account for more than 80 % of the observed variation in phototoxicity relates in part to the non-receptor-mediated mechanism of action, i.e. oxidation of membrane lipids and other biomolecules. Nevertheless, application of QSAR methodology has clarified the relative significance of singlet oxygen generating capacity and hydrophobicity in the varying phototoxicity of tricyclic thiophene derivatives. A further benefit of QSAR modelling is provided by the predictive value of the models for phototoxicity, and the determination of $\log P_o$ values, which provide a quantitative expression of specificity of phototoxicity for a particular biological system.

The application of QSAR analysis to the study of biologically active natural products will not answer all our questions, nor will it always be an appropriate method, given the complex nature of many biochemical interactions. As a tool employed in a broad-based analysis of structure and function, however, QSAR analysis can often provide unique and valuable insights into this relationship. The technique should therefore prove useful in a wide variety of applications, including pharmacognosy, plant and animal physiology and pathology, and chemical ecology.

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