1

How to Approach the Isolation of a Natural Product

Richard J. P. Cannell

1. Introduction

It can seem a formidable task, faced with a liter of fermentation broth–a dark, viscous sludge–knowing that in there is one group of molecules that has to be separated from all the rest. Those molecules possibly represent only about 0.0001%, or 1 ppm of the total biomass and are dispersed throughout the organism, possibly intimately bound up with other molecules. Like the proverbial needle in a haystack, you have to remove lot of hay to be left with just the needle, without knowing what the needle looks like or where in the haystack it is.

1.1. What Are Natural Products?

The term "natural product" is perhaps something of a misnomer. Strictly speaking, any biological molecule is a natural product, but the term is usually reserved for secondary metabolites, small molecules (mol wt < 1500 amu approx) produced by an organism but that are not strictly necessary for the survival of the organism, unlike the more prevalent macromolecules such as proteins, nucleic acids, and polysaccharides that make up the basic machinery for the more fundamental processes of life.

Secondary metabolites are a very broad group of metabolites, with no distinct boundaries, and grouped under no single unifying definition. Concepts of secondary metabolism include products of overflow metabolism as a result of nutrient limitation, or shunt metabolites produced during idiophase, defense mechanisms, regulator molecules, and so on. Perhaps the most cogent theory of secondary metabolism has been put forward by Zähner, who described secondary metabolism as evolutionary "elbow room" ***(1)***. If a secondary metabolite has no adverse effect on the producing organism at any of the levels of

From *Methods in Biotechnology, Vol. 4: Natural Products Isolation*
Edited by R J P Cannell © Humana Press Inc , Totowa, NJ

differentiation, morphogenesis, transport, regulation, or intermediary metabolism, it may be conserved for a relatively long period during which time it may come to confer a selective advantage. Secondary metabolism therefore provides a kind of testing ground where new metabolites have the opportunity, as it were, to exist without being eliminated, during which time they may find a role that will give an advantage to the producing organism. This is supported by the fact that secondary metabolites are often unique to a particular species or group of organisms and, while many act as antifeedants, sex attractants, or antibiotic agents, many have no apparent biological role. It is likely that all these concepts can play some part in understanding the production of the broad group of compounds that come under the heading of secondary metabolite.

Isolation of natural products differs from that of the more prevalent biological macromolecules because natural products are smaller and chemically more diverse than the relatively homogeneous proteins, nucleic acids and carbohydrates, and isolation methods must take this into account.

1.2. The Aim of the Extraction

The two most fundamental questions that should be asked at the outset of an extraction are:

1. What am I trying to isolate?
   There are a number of possible targets of an isolation:
   a. An unknown compound responsible for a particular biological activity.
   b. A certain compound known to be produced by a particular organism.
   c. A group of compounds within an organism that are all related in some way, such as by a common structural feature
   d. All of the metabolites produced by one natural product source that are not produced by a different "control" source, e.g., two species of the same genus, or the same organism grown under different conditions.
   e. A chemical "dissection" of an organism, in order to characterize all of its interesting metabolites, usually those secondary metabolites confined to that organism, or group of organisms, and not ubiquitous in all living systems. Such an inventory might be useful for chemical, ecological, or chemotaxonomic reasons, among others.
2. Why am I trying to isolate it?
   The second fundamental question concerns what one is trying ultimately to achieve, for defining the aims can minimize the work required. Reasons for the extraction might be:
   a. To purify sufficient amount of a compound to characterize it partially or fully.
   b. More specifically, to provide sufficient material to allow for confirmation or denial of a proposed structure. As in many cases this does not require mapping out a complete structure from scratch but perhaps simply comparison with a standard of known structure; it may require less material or only par-

tially pure material. There is no point in removing minor impurities if they do not get in the way of ascertaining whether the compound is, or is not, compound X.

c. The generation/production of the maximum amount of a known compound so that it can be used for further work, such as more extensive biological testing. (Alternatively, it may be more efficient to chemically synthesize the compound; any natural product that is of serious interest, i.e., is required in large amounts, will be considered as a target for synthetic chemistry.)

1.3. Purity

With a clear idea of what one is trying to achieve, one can then question the required level of purity. This in turn might give some indication of the approach to be taken and the purification methods to be employed.

For example, if you are attempting to characterize fully a complex natural product that is present at a low concentration in an extract, you will probably want to produce a compound that is suitable for NMR. The purity needed is dependent on the nature of the compound and of the impurities, but to assign fully a complex structure, material of 95–100% purity is generally required. If the compound is present at high concentration in the starting material and there already exists a standard against which to compare it, structure confirmation can be carried out with less pure material and the purification will probably require fewer steps.

The importance of purity in natural products isolation has been highlighted by Ghisalberti *(2)*, who described two papers that appeared at about the same time, both reporting the isolation from plants of *ent*-kauran-3-oxo-16,17-diol. In one paper, the compound has a melting point of 173–174°C and $[\alpha]_D$–39.2°($CHCl_3$); in the other, no melting point is reported, but the compound has an $[\alpha]_D$–73.1°($CHCl_3$). Either the compounds are different or one is significantly less pure than the other.

If a natural product is required for biological testing, it is important to know at least the degree of purity and, preferably, the nature of the impurities. It is always possible that the impurities are giving rise to all or part of the biological activities in question. If a compound is to be used to generate pharmacological or pharmacokinetic data, it is usually important that the material be very pure (generally >99% pure), particularly if the impurities are analogs of the main compound and may themselves be biologically active.

In some cases, a sample need only be partially purified prior to obtaining sufficient structural information. For example, it may be possible to detect the absence of a certain structural feature in a crude mixture–perhaps by absence of a particular ultraviolet (UV) maximum–and conclude that the mixture does not contain compound A. In other cases, such as X-ray crystallography studies, material will almost certainly be required in an extremely pure state, generally >99.9% pure.

It is worth bearing in mind that the relationship between the degree of purity achieved in a natural product extraction, and the amount of work required to achieve this, is very approximately exponential. It is often relatively easy to start with a crude, complex mixture and eliminate more than half of what is not wanted, but it can be a painstaking chore to remove the minor impurities that will turn a 99.5% pure sample into one that is 99.9% pure. It is also probably true to say that this exponential relationship also often holds for the degree of purity achieved versus the yield of natural product. In the same way that no chemical reaction results in 100% yield, no extraction step results in 100% recovery of the natural product. Compound will be lost at every stage; in many cases it may be that, to achieve very high levels of purity, it is necessary to sacrifice much of the desired material. In order to remove all the impurities it may be necessary to take only the cleanest "cuts" from a separation, thus losing much of the target material in the process (though these side fractions can often be reprocessed).

These factors may, of course, have some bearing on the level of purity deemed satisfactory, and it is useful to ask at each stage of the extraction, whether the natural product is sufficiently pure to answer the questions that are to be asked of it.

At present, there are two main reasons why scientists extract natural products: to find out what they are and/or to carry out further experimental work using the purified compound. In the future, it may be easy to determine structures of compounds in complex mixtures; indeed, it is already possible to do this under some circumstances, but at present, most cases of structural determination of an unknown compound require that it be essentially pure. Similarly, to obtain valid biological or chemical data on a natural product usually requires that it be free from the other experimental variables present in the surrounding biological matrix.

1.4. Fractionation

All separation processes involve the division of a mixture into a number of discrete fractions. These fractions may be obvious, physically discrete divisions, such as the two phases of a liquid–liquid extraction, or they may be the contiguous eluate from a chromatography column that is artificially divided by the extractor into fractions.

The type of fractionation depends on the individual sample and the aims of the separation. Typically, a column is run and the eluate divided into a manageable number of even-sized fractions, followed by analysis of the fractions to determine which contain the desired compounds. (So, the eluate from a silica column with a bed volume of 10 mL becomes, perhaps, 20 × 5-mL fractions.) Obviously, collecting the eluate as a large number of very small fractions means that each fraction is more likely to contain a pure compound, but it requires

more work in analyzing every fraction. This also runs the risk of spreading the target compound over so many fractions that, if originally present in only low concentrations, it may evade detection in any one of the fractions. If the separation process is relatively crude, it is probably more sensible to collect only a few large, relatively crude fractions and quickly home in on those containing the target.

Alternatively, one may monitor "on-line" and fractionate the eluate accordingly. This is generally used at the later stages of separation for separations of less complex mixtures, typically on high-performance liquid chromatography (HPLC) separations monitored by UV, where one can identify and isolate material corresponding to individual peaks.

1.5. Assays

A point that may seem fairly obvious, but worth reiterating, is that, with a complex mixture from which one or a few specific compounds are to be isolated, a means of keeping track of the compound through the extraction process is needed. There are two main ways to follow a compound: (1) physical assay (for example, HPLC, thin-layer chromatography [TLC], liquid chromatography-mass spectometry [LC-MS], and perhaps involving comparison with a standard), or (2) bioactivity assay.

It is not within the scope of this book to discuss at length biological screening and the rapid developments that are being made in this field, but some typical bioactivity screens are listed in **Table 1**.

There are a number of basic points that should be kept in mind when assaying fractions:

1. Samples dissolved or suspended in a solvent different from the original extraction solvent should be filtered or centrifuged to remove any insoluble matter. Assay samples that include a volatile solvent, or different solvents, are usually best dried and redissolved in the original extraction solvent, water, or other solvent in which the compound is known to be soluble. For example, an aliquot of a methanol extract of a broth may be dried, then resuspended and partitioned between water and chloroform. The two phases, or part thereof, can then be redried, redissolved in equal volumes of methanol, and assayed. This may make subsequent assay easier for two reasons:
   a. The test solvent may not be compatible with the assay.
   b. Redissolving the two phases back into the same solvent makes quantitative and qualitative comparisons much easier, particularly if one of the test solvents is very volatile, creating problems with evaporation and differences in concentration.
2. Samples acidified or basified should be readjusted to their original pH to prevent them from interfering with the assay. If volatile acids/bases are present, they may be removed by evaporation.
3. Controls consisting of the solvents and/or buffers, acids, and so on, without sample, should always be carried out to ensure that observed assay results are in

Table 1
Typical Bioactivity Screens

Activity	Common assay form
Antibacterial	Seeded agar diffusion, turbidometric
Antifungal	Seeded agar diffusion, turbidometric
Enzyme inhibitory	UV, colorimetric, radiolabeled, scintillation proximity assay (SPA)
Antitumor	Cell line
Toxicity	Whole organism, e.g., brine shrimp lethality
Antiparasitic	Whole organism, e.g., insect larvae, antihelminth
Receptor binding	Enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), SPA, chemiluminescence, fluorescence
Transcription-based	Chemiluminescence, fluorescence

fact caused by the natural product. The separation may result in fractions that do not have homogeneous "backgrounds" and this may affect the assay. For example, a gradient chromatography system may well result in fractions with increasing organic solvent concentration that might itself affect the assay. In order to allow for the effect of this discrepancy, either a series of control samples should be tested–in this case, fractions from a blank gradient run with no sample–and these results subtracted from the assay results, or, all of the fraction aliquots must be treated in a way that allows them to be presented to the assay in the same form. This might mean drying the samples and redissolving them in the same solvent. Care must be taken to redissolve in a solvent compatible with the assay (e.g., methanol, dimethylsufoxide [DMSO]), and that will solubilize compounds eluting from both the polar and nonpolar ends of a gradient elution. Additionally, for practical reasons, it is often preferable not to take samples to complete dryness as it is sometimes difficult to resolubilize all the components. Samples can be partially dried by evaporation or vacuum centrifugation, such that the more volatile organic solvent is removed leaving only the residual aqueous extract; then volumes can be adjusted to give the same relative concentration. Alternatively, samples may be adsorbed each on its own solid phase extractant (*see* **Subheading 2.3.2.**) and then eluted in a small volume of suitable solvent. This can serve both to concentrate and to further clean the sample by "desalting"–separating the compounds from more polar materials or inorganic components that may have been introduced into the mobile phase to improve chromatography, which may affect the assay.

4. Ideally, the assay should be at least semiquantitative, and/or samples should be assayed at a series of dilutions in order to determine where the majority of the target compound resides. It may well be that the separation process, e.g., the chromatography column, dilutes the activity in a way such that it is not detectable in the assay without concentration, and so the nonappearance of an active fraction may

not mean that the activity is lost but that the assay is insufficiently sensitive for unconcentrated fractions. For this reason, it is always wise to quantify approximately the recovery of compound at each stage.

Such matters may sound obvious and trivial, but preparing fractionation-samples for assay in a suitable way can be a time-consuming and surprisingly troublesome process, often representing a major portion of the work in a bioassay-guided extraction.

1.5.1. Overlay Assay

Sometimes it is possible to combine more closely the separation and the bioassay, as in the case of TLC overlay assays. In this case, the sample may be separated by TLC, the TLC plate dried to remove traces of solvent, and the assay performed *in situ*, on top of the plate. This usually takes the form of the reactants immobilized in a gel poured or sprayed over the plate and the results visualized. The most commonly used form of this assay is an antimicrobial assay in which the plate is covered with agar seeded with microorganism and then incubated, after which microbial growth is seen throughout the agar except over those regions of the chromatogram that contain the antimicrobial components.

As long as the assay can be visualized, either by obvious microbial growth or by the use of a colored reaction product, this principle can be applied to a wide range of assays, including enzyme and receptor-based assays. This principle of immobilizing, or spotting, a small amount of sample onto a TLC plate is one of the quickest and most convenient means of assaying a large number of samples, and this method of overlay assay is widely used for assaying fractions from all types of separation.

1.6. Quantification

During the isolation of a natural product, it is necessary to track the compound and, if possible, obtain some estimate of the recovery at each stage. This can often be done by routine analytical techniques that may involve the use of a standard.

During the isolation of an unknown bioactive compound, the compound is monitored by following the bioactivity at each stage. It is also useful to quantify, at least approximately, this bioactivity at each stage. Approximate quantification is generally carried out by assaying a set of serial dilutions of each fraction at each stage of the separation. To detect the peaks of activity, it is often necessary to assay fractions at a range of dilutions, which serves to indicate the relative amounts of activity/compound present in each fraction. It can then be seen in which fraction(s) the bulk of the active components lie and also allows for some estimation of the total amount of activity recovered, relative to the starting material. Accounting for all the initial activity can be helpful in avoiding potential problems.

For example, one may produce column fractions that obviously contain active compound but which a quick calculation reveals, represent only approx 5% of the activity that went on to the column. There are many possible explanations for such "disappearance" of activity, but essentially, quantification can act as a warning that there is more to look for. Likely explanations may include:

1. There is more than one active component and the major component has not been eluted.
2. Most of the active component has been degraded or modified by the separation process.
3. The starting sample was not prepared so as to be fully compatible with the mobile phase, so that a large proportion of the active component precipitated when loading on to the top of the column.
4. Most of the active component(s) spread across a wide range of fractions in a concentration too low to be detected by the assay

Quantification also helps to avoid the temptation to assign all the activity of an extract to a particular peak on a chromatogram, when in fact, much of the activity may be represented by a very minor peak or a potent compound present in very low concentrations almost insignificant apart from its bioactivity. These are often more interesting than abundant compounds as they are more bioactive and are less likely to have been previously described.

For similar reasons, it is prudent to retain a reference sample of the mixture at each stage of the process so that it can be assayed alongside the fractions and serve as a record of material recovered at each stage of the process.

2. Where to Start?

How to begin the isolation of a natural product? First, something about the nature of the compound needs to be known so that the approach to take can be determined.

2.1. Determination of the Nature of the Compound

How much needs to be discovered depends on how much is already known and what our aim is. The general features of a molecule that are useful to ascertain at this early stage might include: solubility (hydrophobicity/hydrophilicity), acid/base properties, charge, stability, and size.

1. If the aim is to isolate all of the secondary metabolites of an organism and not to focus on a specific molecule, this information may be less important but still can be useful in getting an idea of the range of compounds being worked with.
2. If the aim is to isolate a known compound(s), much of this information will already be established, or will probably be apparent from the structure. (It may even be that a physical assay exists for the compound and this may provide the basis for an isolation.)
3. If the target is an unknown molecule, it is probable that little is known about the nature of the compound.

At this early stage, a small portion of the mixture can be examined in a series of small batch-wise experiments.

2.1.1. Hydrophobicity/Hydrophilicity

An indication of the polarity of the compound in question can be determined by drying a portion of the mixture and attempting to redissolve it in a few, perhaps three or four, solvents covering the range of polarities. Suitable solvents include water, methanol, acetonitrile, ethyl acetate, dichloromethane, chloroform, petroleum ether, and hexane.

It is usually fairly obvious if everything redissolves, but if the mixture is a complex one, the sample can be centrifuged or filtered and the supernatant tested to determine solubility of the desired natural product (**Note 1**).

The same information can be obtained by carrying out a range of solvent-partitioning experiments, typically between water and ethyl acetate, chloroform/dichloromethane, or hexane, followed by assay to determine how the compound distributes itself (**Note 2**).

2.1.2. pK_a (Acid/Base Properties)

Further information can be gleaned by carrying out the partitioning experiments mentioned above at a range of pH values–typically 3, 7, and 10. Adjust the aqueous solution/suspension with a drop or two of acid or alkali (or preferably buffer), then add the solvent, mix, and assay the two phases.

$$HA \rightleftharpoons H^+ + A^-$$

Organic Phase Aqueous Phase

As well as providing information on the pK_a of the target compounds, these tests can also be useful in ascertaining their stability at different pH values (**Note 3**).

2.1.3. Charge

Information about the charge properties of the compound can be obtained by testing under batch conditions, the effect of adding a range of ion-exchangers to the mixture.

To aliquots of the aqueous mixture (ion-exchangers will, of course, only act as such in water), portions of different ion-exchange resins are added. When dealing with an unknown quantity of a compound, it is not possible to know how much to add–if ambiguous results are obtained, e.g., apparent binding to all forms of exchanger, an order of magnitude more or less should be tried. The important thing is that approximately the same amount in terms of binding capacity is added to each to make the results comparable. An approximate starting concentration might be 100 mg resin/1 mL microbial broth.

The supernatant of samples should be tested after mixing with strong and weak anion exchangers and strong and weak cation exchangers, each at a range of pH values, e.g., pH 3.0, 7.0, and 10.0. From this can be deduced whether the compound is a strong or weak acid or base. This may then suggest an extraction approach, perhaps involving ion exchange or adsorption chromatography (**Note 4**). A guide to the various types of ion-exchange resins is given in Chapter 5.

2.1.4. Heat Stability

If a biologically active natural product, or any product from a natural source, is not stable to heat, there is a likelihood that it is a protein. If this is the case, the observed biological activity may be as a result of enzyme activity or simply to nonspecific binding by the protein to components of the biological assay, giving rise to interference or "false" activity. Most natural product chemists, particularly those guided by bioactivity for the purposes of finding potential therapeutic agents or those with chemotaxonomic interests, are generally more interested in small nonprotein secondary metabolites where proteins are an unwanted distraction.

A typical heat-stability test would involve incubation of the sample at 80/90°C for 10 min in a water bath (taking into account loss of volume or any physical changes in the sample, e.g., clotting, aggregation), then assay for the unaffected compound. This is most appropriate for biological assays as it may be difficult to detect breakdown of a compound in a mixture by physical means.

A positive result may mean that the extraction comes to a halt because of lack of further interest or that it can proceed unhindered by the interference of associated protein. However, it should be remembered that heat can also denature or modify other natural products.

Many biological samples prior to assay are extracted with a water-miscible solvent such as methanol. Proteins can be excluded from the extract by ensuring that the samples (and the methanol) are completely dry prior to extraction. Proteins will rarely dissolve in most solvents in the absence of water. This can be carried out by extracting the sample in methanol, drying the extract, then re-extracting the extract with methanol, thus ensuring that no water is carried over into the final extract.

2.1.5. Size

Proteins can also be detected and/or eliminated by the use of ultrafiltration membranes. These come in a variety of forms through which a sample may be passed by pressure, by vacuum, or by centrifugal force in the case of ultrafiltration cones (filters contained in sample tubes that are centrifuged). The filters have a cutoff at a given molecular weight and can be used to separate proteins from small molecules. They are not generally useful for the fractionation of

mixtures of small molecules as the lowest cutoff values are approx 2000 amu. Dialysis tubing can be used in the same way; small molecules (less than a few thousand amu) can pass through dialysis tubing into the surrounding medium, whereas proteins will be retained within the tubing.

2.2. Localization of Activity

At the early stages of the extraction, one of the first and the most obvious questions to ask is whether the natural product of interest is localized to one part of the organism.

2.2.1. Microbial Broths–Extracellular or Intracellular?

If the compound is in the free medium, already separated from the bulk of the biomass, this is likely to make the extraction easier, particularly in a liquid culture grown in a minimal medium (such as that generally used for a plant cell or microalgal culture) and which is therefore fairly "clean." If the material is associated solely with the cells, it may be possible to separate immediately the extracellular material and concentrate on extracting the cell mass. In this way, the sample is at least concentrated in a single swift step, virtually free of media constituents. If the material is divided between the cells and the supernatant, it is desirable to shift this balance one way or the other so that the compound is associated entirely with either the cells or the supernatant. This may be possible by altering the pH or by adding surfactants. It may be that the natural product is actually excreted by the cells but remains associated with the cell surface for reasons of hydrophobicity, adsorption, or biological affinity.

2.2.2. Plants

Provided that the whole plant is not already milled to a homogeneous powder, it should be fairly straightforward to determine whether the compounds of interest are localized to certain parts of the plant, e.g., leaves, root, stem, bark, root bark, and so on. If so, this may allow for disposal of perhaps three-quarters of the plant before the actual work even begins, thus rendering the mixture less complex and possibly leading to avoidance of problems brought about by the presence of other parts of the plant.

2.3. Selecting General Separation Conditions

2.3.1. Literature

It obviously makes sense to find out whether the extraction of the natural product has been reported in the literature. However, there is no correct purification method for each natural product and no compulsion to follow such a method. It may be easier to develop a new process, particularly if the biological matrix is

different, e.g., the same compound from a different organism or a different medium, or if facilities for the reported separation are not readily available.

In the isolation of unknown compounds, this approach is, of course, not possible, but the literature may be used to facilitate and limit the amount of necessary work. This is discussed further in Chapter 10.

2.3.2. Solid Phase Extraction

This involves sorption of solutes from a liquid medium onto a solid adsorbent (like a flypaper removing flies from a room by retaining them when they land on the sticky paper) by the same mechanisms by which molecules are retained on chromatography stationary phases. These adsorbents, like chromatography media, come in the form of beads or resins that can be used in column or in batch form. They are often used in the commercially available form of syringes packed with medium (typically a few hundred milligrams to a few grams) through which the sample can be gently forced with the plunger or by vacuum. Solid phase extraction media include reverse phase, normal phase, and ion-exchange media.

If an aqueous extract is passed down a column containing reverse phase packing material, everything that is fairly nonpolar will bind, whereas everything polar will pass through. The nonpolar material can then be eluted with a nonpolar solvent to give a sample that has been partially purified.

There are a number of uses of solid phase extraction:

1. Determination of the nature of the unknown compound: Determination of the resins that bind the desired natural product provides information about the nature of the compound. For example, if the compound is retained by a reverse phase medium, it must have some degree of hydrophobicity.
2. Selection of separation conditions: By the same reasoning, it is possible to get some idea of a suitable starting point for a purification. By eluting bound material using a stepwise series of solvents with increasing eluting power, rather than by a single elution step, it may be possible to find chromatographic conditions that selectively bind and elute a particular compound.
3. Dereplication and characterization: The characteristic binding profile of a compound on a number of solid phase extraction resins can be used for comparative and dereplication purposes. Compounds that bind differently to the same media must be different, and if a series of extracts is suspected of containing the same unknown natural product (e.g., all the extracts possess the same biological activity), the fact that they all exhibit the same binding profile might lead to a decision to first isolate the component from one of the extracts, then use that as a standard with which to examine the other extracts.
4. Preparative purification: As well as functioning as a means of developing separation conditions, solid phase extraction is widely used as a purification step in its own right and in this sense can be viewed as a form of two-phase partition. This

is commonly used at the early stages of an extraction as a fairly crude "clean-up" step, to separate the target natural products from the bulk of the contaminants, or at the final stages of a purification to get the isolated natural product in pure, concentrated solution. In both cases, this usually involves the removal of large amounts of polar contaminants (e g , buffer salts, media components) by extracting target compounds using some form of nonionic binding medium, washing the resin with water to remove nonbinding contaminants, and then eluting the compounds with solvent.

5 Concentration· Compounds at low concentrations in relatively large volumes can be concentrated by extraction onto a solid phase extraction medium and then eluted in a small volume of a strong eluent. This is typically used for concentrating analytes that are present in only trace amounts, such as drug metabolites in serum samples, or environmental contaminants in seawater.

2.3.3. Gradient HPLC

Running a sample of the natural product extract on analytical gradient HPLC (**Fig. 1**) using a mobile phase gradient of wide ranging polarity should serve to separate the mixture and elute all the components. On the basis of retention time, it should be possible to select general chromatographic conditions (either HPLC or low-pressure columns) for further preparative purification.

2.3.4. TLC

TLC separations can also be used to select column chromatography conditions. TLC conditions that give a useful R_f value, i.e., compound separates from the majority of other components without staying at the origin or with the solvent front, can be approximately transferred to column chromatography. Identification of the target compound or on the TLC plate can be carried out by comparison with a standard, by chemical staining, or by an overlay assay carried out on top of the developed plate in the case of an unknown biologically active component, or by scraping off, extracting, and assaying portions of the adsorbent.

However, it is not always straightforward to translate TLC systems to column systems. This is partly because TLC cannot be seen simply as a two-dimensional column. Unlike column chromatography, TLC is a nonequilibrium technique, which means that the conditions of the mobile and stationary phases are not constant throughout the plate but vary during the run and according to the position on the plate. This can lead to difficulties in trying to reproduce TLC separations in a column form. As a general rule, it is advisable to use a slightly less polar mobile phase for normal phase column chromatography (and slightly more polar for reverse phase columns) than that used to obtain a reasonable R_f value on TLC.

Armed with some knowledge of the nature of the compound and some idea of suitable general separation approaches, one can usually establish a successful isolation method much more quickly than if none of these preliminary tests is carried out.

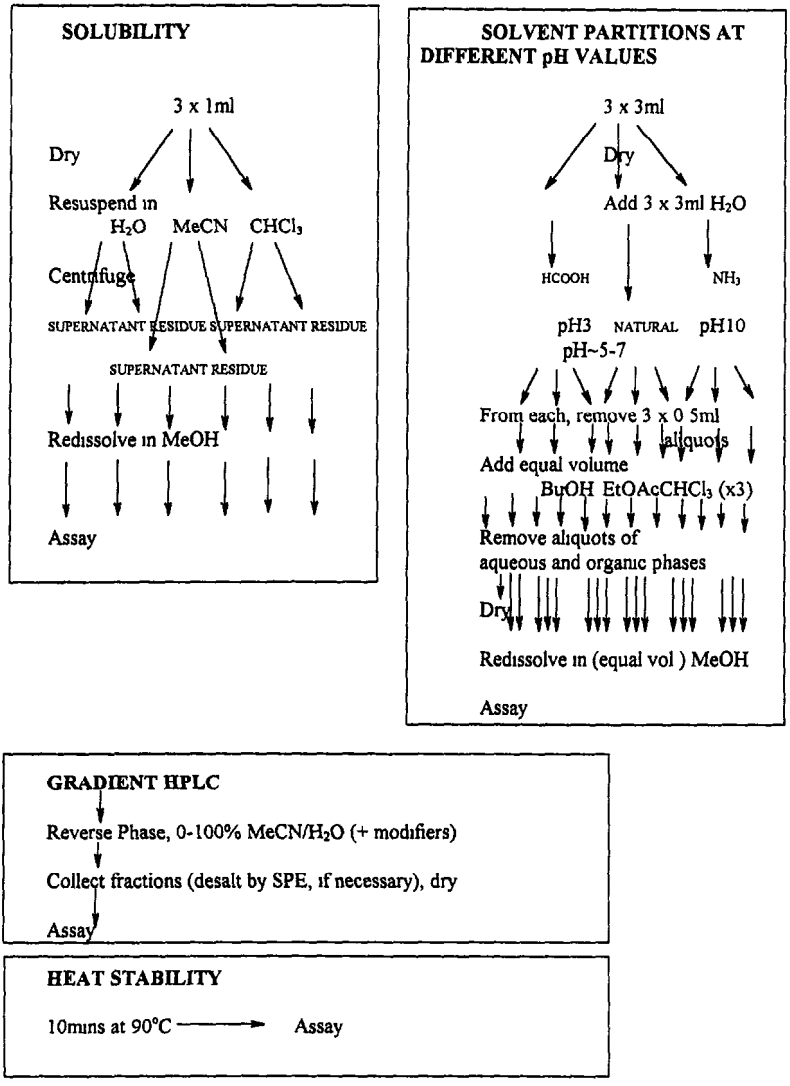


Fig. 1. Example of some simple tests that might be carried out on a crude methanol extract to determine the nature of compound for selection for general separation methods.

3. Chromatography

Many of the separation processes described in later chapters are forms of chromatography. It might therefore be of value to have some understanding of what chromatography is. Chromatography involves the distribution of a compound between two phases–a moving, *mobile phase* that is passed over an

immobile *stationary phase.* Separation is based on the characteristic way in which compounds distribute themselves between these two phases.

For Compound X this can be described in terms of its distribution coefficient:

$$K_D = \frac{[X]_{\text{stationary phase}}}{[X]_{\text{mobile phase}}}$$

This is characteristic for a molecule independent of the amount of solute.

So, as one phase carrying the solute passes over the stationary phase, the solutes are in constant, dynamic equilibrium between the two phases. For any given compound, the position of this equilibrium is determined by the strength of interaction of the compound with the stationary phase and the competition for the stationary phase between the compound and the mobile phase. The stationary phase may be a solid or a liquid and the fluid mobile phase may be liquid or gas, to give liquid chromatography and gas chromatography, respectively.

Gas chromatography, particularly gas-liquid chromatography (GLC) is a widely used analytical technique but cannot be used for preparative isolation of natural products and will not be discussed in this book. Liquid chromatography however, is widely used and comes in many different forms.

3.1. Classification of Liquid Chromatography

Chromatography can be classified in a number of ways (**Fig. 2**):

1 Classification on the basis of the physical arrangement of the system. Chromatography can be carried out in the form of a column (column chromatography) (**Fig. 3**) or on a flat surface (planar chromatography). The latter includes various forms of TLC and paper chromatography, amongst others and is the subject of Chapter 7. Countercurrent chromatography involves two liquid phases and is the subject of Chapter 8. Most chromatography is carried out in the form of a column of stationary phase with a moving liquid mobile phase and comprises everything from capillary columns, to HPLC, to large-scale gravity-fed columns.

2. Classification according to the mode of separation. It is perhaps more useful to divide chromatographic forms according to the mode of separation on which each is based. These basic forms of molecular interaction, which determine chromatographic behavior, are listed below but are discussed at greater length in the relevant chapters.

3.1.1. Adsorption

This involves partitioning of molecules between the surface of a solid stationary phase and a liquid mobile phase. The dynamic equilibrium of solutes as they switch between the stationary and mobile phases (the processes of sorption and desorption, respectively) is specific for each molecule and is affected by competition that exists between solutes and solvent for sites on the stationary phase. This is a purely physical process involving the formation of no

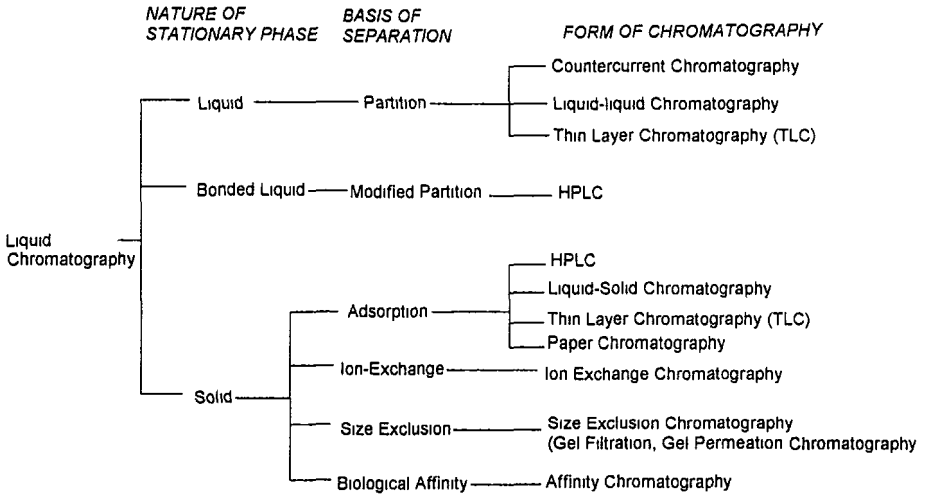


Fig. 2. Classification of liquid chromatography (LC) systems.

chemical bonds, but only the relatively weak forces of hydrogen bonds, Van der Waals forces, and dipole–dipole interactions. For this reason, almost any inert material can in theory be used as an adsorbent, the only proviso being that it does not react either with the sample or the mobile phase and that it is insoluble in the mobile phase. Common examples include silica (as a column or as a TLC stationary phase), cellulose, styrene divinylbenzene, alumina, and carbon.

3.1.2. Partition

Partition chromatography employs the separation principle of liquid–liquid extraction. When one of the liquids is coated onto a solid support, such as a column of cellulose coated with water, or a silica TLC plate coated with adsorbed water, a stationary phase is created on which separation can be carried out with an immiscible/organic mobile phase, employing the principles of liquid–liquid extraction with the advantages of chromatography. However, this method suffers from the disadvantages that the liquid stationary phase tends to be stripped (leached) from the column as a result of shear forces acting on it from the movement of the mobile phase and by the solubility of the liquid stationary phase in the mobile phase. For this reason, partition chromatography in this simplest form is rarely used these days.

More usually, the liquid stationary phase is chemically bound to the inert support to give a "bonded phase." This involves the formation of a (hydrolytically) stable bond, often, for example, between the surface silanol group of a silica support and a chlorosilane. The silane usually carries a hydrocar-

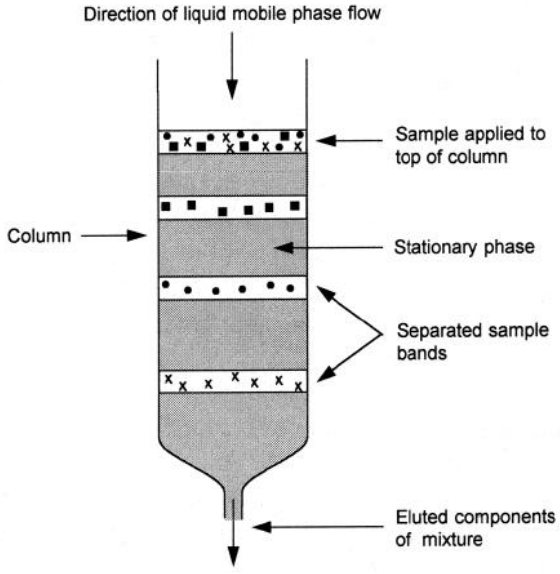


Fig. 3. Much preparative chromatography is carried out as a form of liquid chromatography with a column of solid packing material (stationary phase) over which is passed a liquid (mobile phase) containing the sample.

bon chain (usually of 1, 2, 6, 8, or 18 carbon length) and this is in effect, the liquid stationary phase. Bonded phase chromatography (also known as modified partition chromatography) of this sort is really a combination of partition and adsorption chromatography, and the distinction between these two forms is generally somewhat blurred. A liquid can also act as a stationary phase even when it is not bound to a support, as in the case of countercurrent chromatography.

Normal phase/reverse phase: If the stationary phase is more polar than the mobile phase, this is normal phase chromatography. An example of this is a silica column with its polar silanol groups and a mobile phase of an organic solvent. When the stationary phase is less polar than the mobile phase, this is reverse phase chromatography, exemplified by the hydrocarbons bound to the silica support and a water/acetonitrile mobile phase. Reverse phase chromatography is very widely used as a form of HPLC (*see* Chapter 6) and most natural products have a region of hydrophobicity that leads to their retention to some extent on a reverse phase column.

3.1.3. Charge

Many natural products exist as ionic species or are ionizable at a given pH and this property can be used as a "handle" for isolating these molecules.

3.1.3.1. Ion-Exchange Chromatography

This involves a stationary phase that consists of an insoluble matrix, the surface of which carries a charged group, either negative or positive. These charged groups are associated with a counter-ion of the opposite charge, and as the name suggests, the principle of separation lies in the ability of the sample ions to exchange with the counter-ions and bind to the stationary phase. A system in which the stationary phase carries a negative charge and the counter-ions are positively charged is cation-exchange chromatography, the opposite is anion-exchange chromatography. Separation occurs because of the differences between sample molecules in their degree and strength of interaction with the exchange sites. Once the sample molecules are bound, they can be eluted selectively from the binding site by altering the pH of the mobile phase, thus altering the dissociation characteristics of the charged species, or by increasing the ionic concentration of the mobile phase, thus increasing competition for the exchange sites and forcing off the sample ions. The degree of interaction obviously depends on the nature of the sample ions and of the functional groups on the ion-exchange resin. Sample ions that react strongly with the stationary phase ions are strongly retained and will elute more slowly, whereas weakly binding solute ions will be eluted more rapidly. This process is discussed at greater length in Chapter 5.

3.1.3.2. Ion-Pair Chromatography/Ion-Suppression Chromatography

Ions can also be separated on a nonpolar stationary phase such as a reverse-phase modified partition column by altering the pH of the mobile phase to suppress the ionization of a molecule so that it will be retained as a neutral species and hence will interact with the stationary phase. Essentially, the same principle is applied for ion-pair chromatography, in which the mobile phase contains a relatively large organic molecule with an ionizable group and lipophilic region and which acts as a counter-ion to form a reversible ion-pair with a sample ion. This ionic modifier acts either by pairing with the sample ion in free solution to form an uncharged species that can then partition into the stationary phase and/or by interacting with the stationary phase via its lipophilic region to give a stationary phase with charged groups that can form an ion-pair with sample ions. The advantage of this technique is that it allows polar and nonpolar samples to be separated in the same system.

3.1.4. Size

Chromatography based on differences in the size of molecules is somewhat different from other forms of chromatography as there is no direct sorption between sample and stationary phase. Size exclusion chromatography (also known as gel permeation chromatography or gel filtration) is based around a

column of packing material formed from beads of a polymer such as polyacrylamide, agarose, or silica. The degree of polymer crosslinking is controlled and gives rise to beads with a certain porosity. This pore structure is such that the largest solute molecules cannot enter the pores—they are excluded because they are too big, whereas the smaller molecules with a diameter less than that of the pore diameter are able to diffuse into the beads, and the smallest molecules are able to diffuse into the smallest pores. It is these spaces in the crosslinked polymer that act as the stationary phase. The largest molecules will pass rapidly through the column, following the most direct route between the beads. Smaller molecules with a greater diffusible volume accessible to them will take a more circuitous route through the column as they diffuse into the pores and will take a longer time to reach the bottom of the column. The smallest molecules can penetrate the smallest pores, travel further, and hence will be eluted last (**Fig. 4**).

The interstitial spaces of the beads act as eddies where solutes are out of the full flow of the mobile phase, and this serves to increase retention time.

Even though there is no physical interaction between the sample molecules and the stationary phase, it is still possible to describe the behavior of a sample molecule/solute in terms of a distribution coefficient (K_D) where the stationary phase is represented by the interstitial spaces of the polymer bead.

$$K_D = (V_e - V_o)/V_i$$

where V_e is the elution volume of the solute, V_o is the void volume–the elution volume of compounds that are completely excluded from the gel pores, and V_i is the volume of liquid inside the gel pores available to the very smallest solutes.

Although size exclusion chromatography is a very simple and nondestructive technique, it is best suited for the separation of biological molecules over a very wide size range, such as proteins, for which it is frequently used. As most secondary metabolites are fairly small molecules without marked differences in size, size exclusion chromatography is rarely used for the separation of these molecules. The exception to this is Sephadex LH20, which is widely used because of its advantages of being usable in nonaqueous systems and that gel filtration is not the only factor at work in the separation process; adsorption also plays a major role.

3.1.5. Biological Specificity

Affinity chromatography depends on specific interactions of biological molecules such as an antibody–antigen interaction, enzyme-inhibitor interaction, DNA–DNA binding, DNA–protein interaction, or a receptor–agonist/antagonist interaction. The ligand (or receptor) is covalently bound to the packing mate-

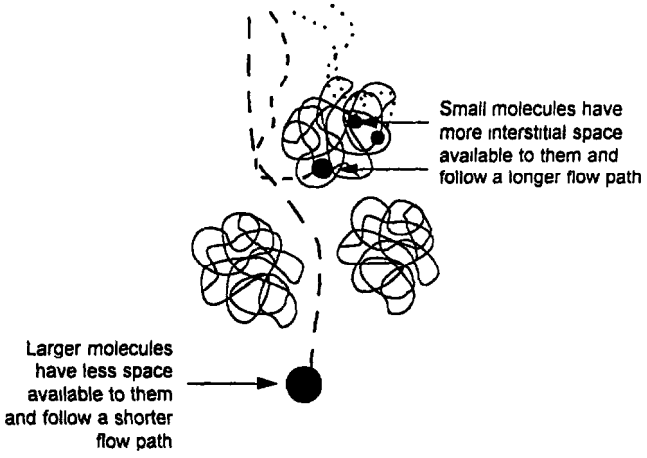


Fig. 4. Behavior of solutes in a size exclusion chromatography system

rial and acts as the stationary phase. The mixture is then passed through the column, and those molecules with a specific affinity for the ligand are retained while the remainder pass through. The material might then be eluted by altering the pH and/or the buffer composition in order to weaken the interaction between the samples and the ligand (**Fig. 5**).

As natural product extraction often involves the isolation of compounds with specific biological activity, this might seem the ideal way to isolate such molecules. Indeed, biological specificity is very often the basis by which the natural product is originally detected within the mixture and is the means by which the compounds or fractions can be assayed.

However, the preparative isolation of secondary metabolites is not often carried out by affinity chromatography, mainly because it is often a rather laborious and time-consuming process to obtain sufficient quantities of a suitable ligand and to carry out the reactions necessary to form a stable stationary phase. Also, if the starting mixture is complex, as is often the case with microbial or plant extracts, there are likely to be other components present that interfere and/or disrupt the receptor-ligand interaction or themselves adsorb nonspecifically to the stationary phase. Moreover, recovery of the product depends on the interaction being reversible.

The use of affinity chromatography for the preparative isolation of natural products is not discussed at length in this book though its role may increase in the future (as purified recombinant proteins become more available). However, the use of biological specificity as the basis for screening organism extracts as part of the overall discovery process of bioactive natural products is extremely important and is increasing as the range of biological targets for which a bioactive molecule is aimed, increases.

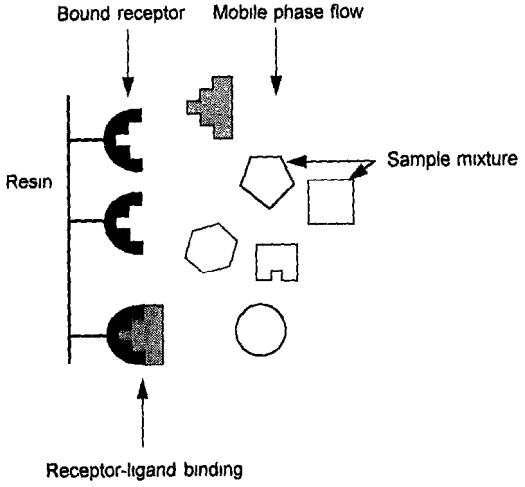


Fig. 5. Principle of affinity chromatography.

3.2. Detection

Chromatography is usually monitored by changes in UV absorbance. As each compound has a characteristic absorption coefficient, this absorbance may be quite different for different compounds, particularly at longer wavelengths. For this reason, monitoring unknown compounds by UV detection is best carried out using short wavelengths, near "end absorbance," typically between 200 and 220 nm. Almost all organic compounds will exhibit some absorbance in this range; wavelengths any shorter tend to cause the absorbance of the mobile phase solvents to interfere. Ideally, a UV diode array detector is used; this measures absorbance over the entire UV wave range so that a UV spectrum can be obtained at any point on the chromatogram. As every compound has a characteristic UV spectrum, this can provide useful information about the compounds in the mixture. Compounds with no significant UV absorption relative to the mobile phase can be monitored by changes in refractive index or by electrochemical detection (rarely used for preparative work).

3.3. Principles of Chromatography

Detailed knowledge of chromatography theory is not necessary in order to perform effective separations, but a knowledge of the principles underlying the chromatographic separation is helpful in understanding how to monitor and improve separations.

As has been described, separation occurs because in the dynamic equilibrium of solute molecules transferring between the two phases, different molecules spend different proportions of time in the mobile and stationary phases. Solute molecules

migrate only when they are in the mobile phase (and all solutes in the mobile phase migrate at the same speed). The speed at which a solute moves through the column is directly related to the proportion—the mole fraction—of a particular group of molecules in the mobile phase. If R_x = mole fraction of X in the mobile phase.

$$R_x = \mu x/\mu m$$

or

$$\mu x = \mu m \cdot R_x$$

where μx = rate of movement of solute band and μm = rate of movement of mobile phase.

This is essentially the same as the distribution coefficient and the chromatographic process consists essentially of thousands of dynamic equilibria. However, there is not sufficient time for the sample to reach fully its equilibrium distribution between the two phases. The sample remaining in the mobile phase is carried down to a fresh portion of the column where it moves onto the stationary phase from the mobile phase. As concentration of the solute in the mobile phase from this first portion decreases, solute in this region of the column moves back from stationary phase to mobile phase, in keeping with the distribution coefficient, and is carried down to the second portion of the column where once again equilibrium is (nearly) achieved. This does not take place as a series of discrete steps but as a continuous dynamic process (**Fig. 6**).

3.3.1. Retention

Solutes only move down the column when they are in the mobile phase, moving at the same speed as the mobile phase. The rate of migration of a solute, therefore, is inversely proportional to its distribution coefficient.

The degree of retention of a solute can be described by three retention parameters: retention time (t_R), retention volume (V_R), and capacity factor (k').

The retention time (or elution time) is the time between injection and elution (measured at peak maximum) of a solute. As this is directly related to the mobile phase flow rate, which may vary between systems, it is sometimes more appropriate to express this value in terms of retention volume, V_R.

$$V_R = F \quad t_R$$

where F = flow rate of mobile phase.

The retention time is the time that a solute spends in the stationary phase plus the time it spends in the mobile phase (t_M). t_M is the same for all solutes and includes time spent in the dead volume of the column, and so it is sometimes preferable to use the adjusted retention time (t'_R), which consists only of the time a solute spends in the stationary phase.

$$t'_R = t_R - t_M$$

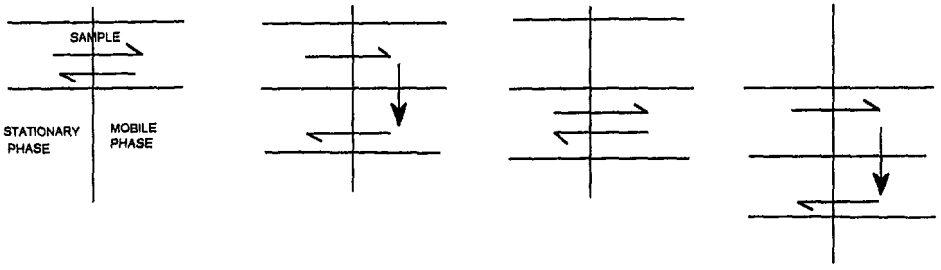


Fig. 6. The sample is continuously exchanging between the mobile and stationary phases as the mobile phase flow carries it down the column.

The phase capacity factor (k') is the net retention time relative to the nonsorbed time (t_0) and is therefore directly related to the distribution coefficient of a solute between two phases:

$$k' = \frac{t_R - t_0}{t_0}$$

or in terms of retention volumes:

$$k' = \frac{V_R - V_M}{V_M}$$

where V_M is the volume of mobile phase in the column.

$$k' = \frac{K \cdot V_S}{V_M}$$

where V_S is the volume of the stationary phase.

The elution time of a nonsorbed compound (t_0) is equal to the column length (L) divided by the mobile-phase velocity (v). Therefore, the retention time of a sorbed compound can be described as:

$$t_R = L/v\,(1 + k')$$

This gives the overall relationship between solute retention, distribution of a solute across stationary and mobile phases, column length, and mobile phase flow rate.

It can be seen from this that doubling the length of the column, for example, will double the retention time. By substituting a term for k' ($k' = [V_R - V_M]/V_M$) into this expression, it can be seen that, aside from column length and flow rate (variables that will be the same for all the solutes of the same chromatographic separation), the important factor affecting the retention time of a solute is K_D, or its distribution between the stationary and mobile phases. This in turn is dependent on the relative volumes of mobile phase and stationary phase (or

more correctly, in the case of adsorption chromatography, the adsorbent surface area). Ultimately therefore, it can be seen once again that the relative retention time depends on the distribution coefficient of a particular molecule, and the larger this is, the greater will be the retention time. It is the differences in this value between different molecules, that we can exploit to bring about their separation by chromatography.

3.3.2. Column Efficiency

So, the process of sorption and desorption of solute molecules onto the stationary phase is similar to samples partitioning between two phases in a separating funnel, or it is analogous to a series of steps in fractional distillation, except that the process of a group of solute molecules reaching equilibrium in a particular part of the column is not a series of discrete steps but is a continuous, dynamic process. We can picture the chromatographic system as comprising a number of regions in which equilibrium is assumed to be achieved or, effectively, to be a series of very short transverse columns.

The number of these theoretical transverse slices of column is known as the theoretical plate number (N) and reflects the number of times a solute partitions between the two phases. N is a measure of the efficiency of the column and will determine how broad the chromatogram peaks will be. A column with a high number of theoretical plates will be efficient and will produce narrow peaks.

$$N = (t_R/\sigma)^2$$

where σ^2 is the band variance.

N can be measured from the peak profile (**Fig. 7**). Assuming the peak to be Gaussian, the baseline width of the peak as measured by drawing tangents from the curve to the baseline is equivalent to four standard deviations. Therefore:

$$\sigma = \omega_b/4 \text{ and } N = 16(t_R/w_b)^2$$

In practice, it is often preferable to use the value for the peak width at half-peak height, particularly when the peak is not symmetrical. This results in the expression:

$$N = 5.54\ (t_1/w_{1/2})^2$$

N calculated this way using t_R is a measure of the efficiency of the whole system that includes the dead volume. In order to calculate and compare efficiencies of columns alone, it is necessary to use t'_R (adjusted retention time) in place of t_R in the above expression, to obtain the effective plate number.

As N is a measure of the number of partitions a solute undergoes, it is directly proportional to column length; i.e., the longer the column length, the greater the separation. It is sometimes more useful to express the column efficiency in

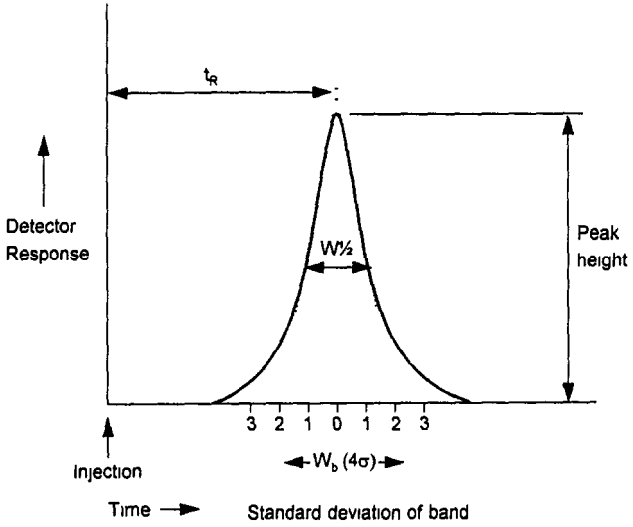


Fig. 7. Profile of a solute peak and measurement of N.

terms of the distance a solute travels in the mobile phase when undergoing one partition. This is the height equivalent to a theoretical plate or plate height (H).

$$H = L/N$$

where L is the column length, and H is independent of the column length.

3.3.3. Dispersion

Obviously, the aim is for a chromatographic separation in which peak width is narrow relative to the time of elution (w_t/t_R is minimized), i.e., the number of theoretical plates is maximized. There are three main factors that give rise to band broadening: (1) multiple path effect (eddy diffusion), (2) axial (longitudinal) diffusion, and (3) mass transfer—slow transfer/equilibration between mobile and stationary zones.

1 The multiple path effect refers to the fact that the flow through a packed bed of particulate matter is very tortuous The total distances traveled, and the velocity at any given time, of individual particles will vary widely due to the heterogeneous and random nature of the flow paths through the bed. As particles can diffuse laterally between two flow paths (a process known as coupling), the overall effects of this Eddy diffusion are not as significant as they would be if every particle remained in its own "individual" flow path, as this lateral diffusion tends to average out the flow paths. The effects of this can be minimized by the use of spherical particles of regular size.

This effect of Eddy diffusion on the overall plate height can be expressed as the A term

$$A = 2 \lambda d_p$$

where d_p is the particle diameter and λ is a packing constant.

2. Within a column, there will also be *longitudinal* (or *axial*) *diffusion* of solutes. This is most significant at low flow rates when the band is resident in the column for relatively long periods of time This can be expressed as the B term.

$$B = 2\gamma D_M$$

where D_M is the diffusion coefficient of the solute in the mobile phase and γ is an obstruction factor.

3. Mass transfer relates to the rates at which equilibration of the chromatographic process is achieved, and is governed by the diffusion of the solutes within the mobile phase and a liquid stationary phase, and governed by the kinetics of sorption-desorption. When equilibration between the mobile and stationary phases is slow, band dispersion will be relatively large due to the fact that molecules in the stationary zone get "left behind" as the main band passes over. This dispersion will increase with increase in flow rate (and with increase in equilibration time). The effects of mass transfer can be expressed as two C terms; C_m describes mass transfer in the mobile phase, and C_s describes mass transfer in the stationary phase

The overall effect of these factors can be combined in the van Deemter Equation, which relates plate height (column efficiency) to flow rate (μ).

$$H = A + B/\mu + C_s\mu + C_m\mu$$

An inverse plot of the van Deemter Equation (**Fig. 8**) illustrates the relationship between column efficiency and flow rate.

Other band-broadening effects derive from the fact that solute species in the same flow path will not all move with the same velocity (those in the center will move faster than those at the edge) and that the particle structure may contain pores or eddies of nonmoving mobile phase out of which solute particles must diffuse in order to return to the main flow of mobile phase.

3.3.4. Sorption Isotherms

Sorption is the general term that refers to the interaction of a solute with the stationary phase, whether that interaction involves adsorption, ion-exchange, or gel permeation (size exclusion). When the relative concentrations of solute in the stationary and mobile phases are the same, independent of concentration (as is normally expected by reference to the K_D of the solute), the peak shape will remain basically the same and will be symmetrical. This relationship, which describes amount of solute sorbed relative to concentration in mobile phase (at constant temperature), is the *sorption isotherm.*

When describing or theorizing about chromatography, the assumption is made that the sample load is a flat-ended plug that, through the processes of

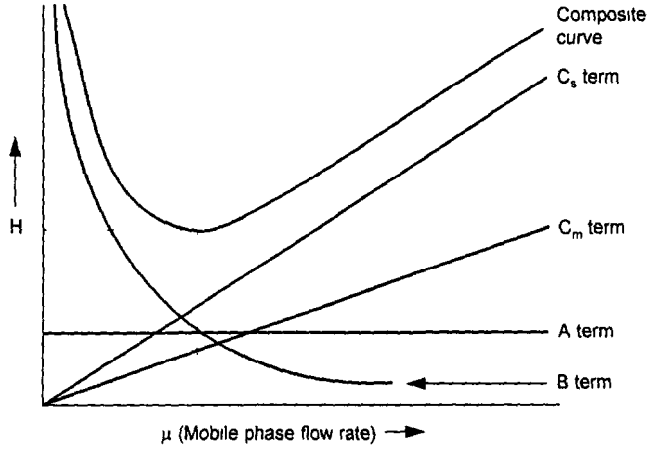


Fig. 8. Typical Van Deemter Plot showing optimal flow rate.

diffusion, soon broadens with a Gaussian distribution. In this case, the sorption isotherm is linear—the value of K_D will be constant across the peak.

However, not all systems exhibit a linear isotherm; when relatively strong interactions exist between solute and stationary phase, and relatively weak interactions exist between solutes themselves, there will initially be rapid sorption of solute onto the stationary phase until the stationary phase is "covered" by solute, at which point the uptake of solute will decrease. This means that the K_D of the solute is not constant across the peak, at low concentrations, K_D will be large and this results in a peak shape that is not symmetrical but that "tails."

Less often, the interactions between solute molecules may be strong relative to those between solute and stationary phase, in which case initial uptake of solute molecules by the stationary phase is slow but increases as the first solute molecules to be adsorbed draw up additional ones. In such a case, the peak has a shallow front and a sharp tail and is said to be a "fronting" peak (**Fig. 9**). Fronting and tailing can be a problem because they tend to lead to overlap of peaks. Dealing with the sorts of problems associated with such phenomena is discussed more fully in Chapter 6.

3.3.5. Separation and Selectivity

The preceding sections should show, if nothing else, that separation of compounds occurs because the compounds have different distribution coefficients. The column shows selectivity toward the compounds or, put another way, the compounds are selectively retained as is shown by their different retention times. This selectivity (α) can be measured by the separation factor and the relative retention and can be described in a number of ways:

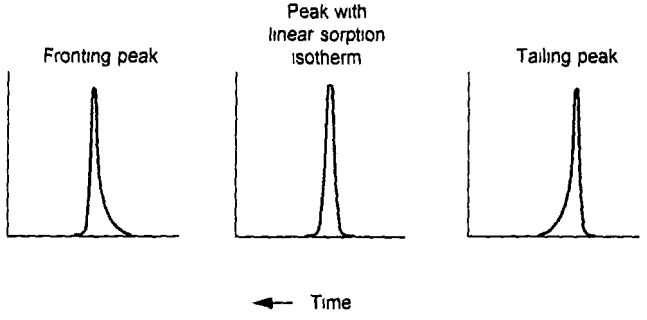


Fig 9 Fronting and tailing peaks.

$$\alpha = K_B/K_A = k'_B/k'_A = t_RB/t_RA$$

Once again, all of these are expressions simply describing the relative values of the distribution coefficients of A and B, that is, just a measure of the way in which the equilibrium of A lies between the mobile and stationary phases compared to the way in which B is distributed between the two phases. If $\alpha = 1$, A and B will partition between the two phases in exactly the same way and there will be no separation (even on a very efficient column).

3.3.6. Resolution

Figure 10 shows a typical chromatogram. It is clear that the peaks appearing on the chromatogram after a short time are sharp and symmetrical whereas compounds that elute later give rise to broader peaks.

The degree of separation of two compounds, or degree of resolution, is determined by two factors: how far apart the tops of the peaks are and how broad the base of the peaks are. As compounds separate, the individual bands will tend to disperse and broaden, and to obtain the best resolution, peaks should be as narrow as possible. So, two compounds may have very different retention times—they may be well-separated—but if the column gives rise to very broad dispersed bands, they will not be well-resolved. Similarly, two compounds may not be well-separated, but if they move through the system as tight bands, they may be well-resolved.

The resolution (R_s) of two compounds can be defined as the peak separation divided by mean peak width.

$$R_s = t_RB - t_RA$$
$$1/2(wA + wB)$$

where t_RB and t_RA are the retention times of compounds B and A respectively and wA and wB are the basal peak widths of peaks A and B.

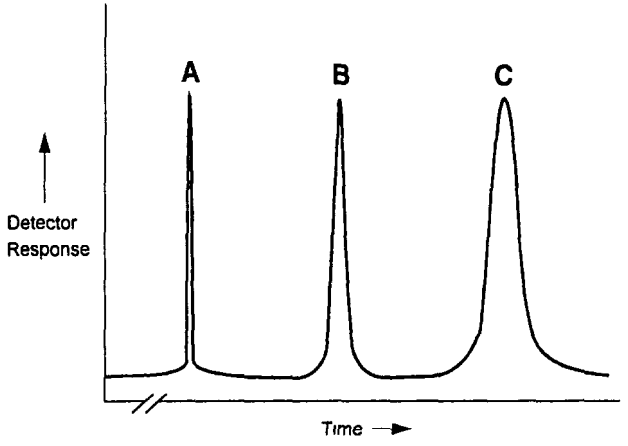


Fig 10. Peak broadening with time.

Figure 11A demonstrates that when two triangular peaks are just touching on the baseline; i.e., when they are just resolved, $R_s = 1$. However, this assumes that the peaks are triangular, whereas in reality they are Gaussian and as shown in **Fig. 11B**, a value of $R_s = 1$ corresponds to a separation of only approx 94%. In order to achieve baseline resolution (**Fig. 11C**), an R_s value of about 1.5 is required. Strictly speaking, these figures are only valid when the peaks are of equal height.

Certainly, the most important factor in resolution is the selectivity of the system, but it is not the only factor; the efficiency of the column is also important, as this determines the degree of peak broadening.

To describe resolution in terms of experimental variables that can be used to optimize resolutions, the expression is:

$$R_s = 1/4N[\alpha - (1/\alpha)][k'/(k' + 1)]$$

The application of this expression is described in **Subheading 3.5.**

It is rarely necessary to carry out any calculations in the process of purifying materials by chromatography, but making some estimates of resolution can help to give an idea of how pure a sample one can expect to obtain from a given separation. In practice, one rarely finds it necessary even to make estimates but for the purpose of this exercise, applying some R_s values to various peak profiles will help to give an idea of the type of results to be expected from a given separation (**Fig. 12**). This is the kind of information that can usefully be elicited from an analytical chromatogram prior to commiting a large amount of sample onto a large-scale preparative separation.

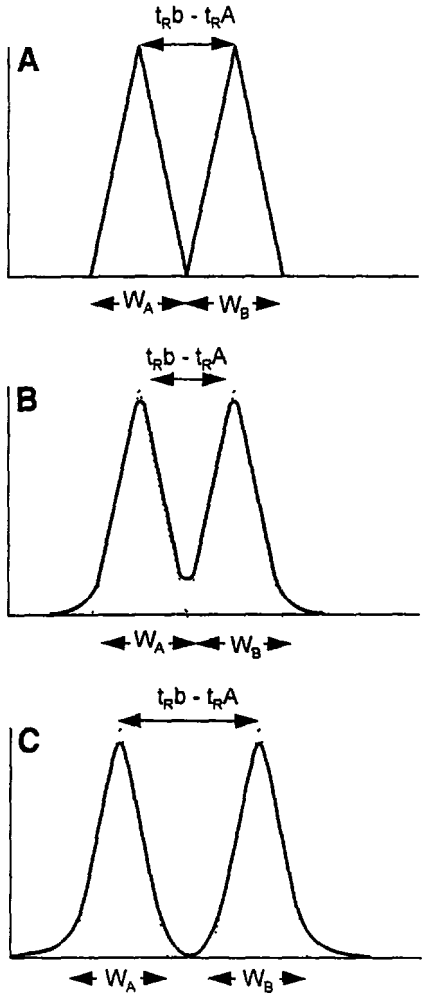


Fig. 11. Measurement of peak resolution. **(A)** $R_s = 1$. **(B)** $R_s = 1$. **(C)** $R_s > 1$.

3.4. How To Use a Chromatogram

Simply by looking at a chromatogram, one can obtain a good deal of information that is useful in the preparative isolation of a natural product.

3.4.1. Identity

The single most useful piece of information that can be obtained from a chromatogram is the retention time (t_R). A compound will always have the same retention time under a given set of conditions, and if two samples have

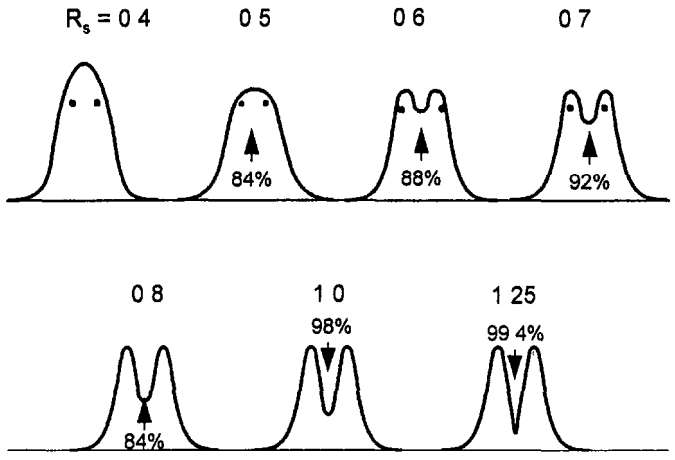


Fig. 12. Retention values for two components of equal peak width with corresponding purity percentages (. represents point of true band center).

the same retention time, they are very possibly the same compound. To be of use, the process requires being able to measure t_R with reasonable accuracy (one of the strengths of HPLC). In practice, other compounds may have the same, or at least indistinguishably similar, retention times, so one should not rely on retention time as a sole means of identifying a peak/compound with absolute certainty.

The surest means of determining whether two samples are the same compound by reference to their retention time is to inject them together in the same sample, thus avoiding problems associated with slight variations in retention times in different runs. If two samples coinjected produce a single peak, then it is probable that they are the same compound (with the degree of certainty dependent on the efficiency of the system and the "quality" of the chromatography). If, however, the two samples give rise to two peaks, it is certain that they cannot be the same compound. The use of diode array detectors also allows for comparison of UV spectra.

The retention time of a sample in a particular chromatographic system also reveals more general information about that compound.

3.4.2. Physical–Chemical Nature

Generally, nonpolar compounds will tend to elute more slowly from a reverse phase system, whereas the more polar a compound, the later it will elute from a normal phase system.

When starting with an unknown compound, a useful generic method for discovering something about the nature of its polarity is to run it on an HPLC (or

TLC) system with gradient elution, followed by some form of assay to determine the compound's chromatographic behavior. By using a gradient mobile phase covering a wide polarity range, it should be possible to ensure that almost every compound is eluted. Some idea of the polarity of the compound and useful starting conditions for the development of a system to separate the compound(s) from the rest of the mixture can be inferred from the time of elution.

3.4.3. Amount

For a given compound, the area under a chromatographic peak is directly proportional to the amount of compound. By using standards of known concentration, it is possible to calibrate a chromatographic system and to use it to establish the amount of a known compound in a sample. Of course, in the isolation of an unknown compound, no standard is available, and as each compound has a characteristic extinction coefficient (absorptivity), the degree of UV absorbance is specific to individual compounds. It is possible to quantify material corresponding to a particular peak only in relative terms.

Means of integration: Most modern HPLC apparatuses have an associated integration system that integrates the peaks on a trace as a set of values or relative percentage values that can then be translated into real values with standards of known concentration. If no built-in integration system is available, the most common practice is to measure the peak height and to use this value as a measure of quantity. On a chromatogram with sharp peaks, the relationship between peak height and concentration bears a close approximation to that of peak area and concentration. Although the relationship between peak height and concentration deviates more for broader peaks, peak height is often sufficient to provide an approximate value of the proportion of natural product recovered at each stage of the purification.

Alternatively, it is possible to calculate peak area by multiplying the peak height by half-peak width at half-height, or even by cutting out peaks from a paper chromatogram and weighing them to get a relative measure of compound concentration. (Measurement of peak area is less convenient than that of peak height and may sometimes even be less accurate due to the practical difficulties of measuring peak width.) Where two or more peaks overlap, the best means of roughly estimating peak area is to draw a line from the bottom of the valley between two peaks to the baseline and calling this the division between the two compounds. However, the accuracy of this method decreases as the difference in size of the peaks increases. However, in the case of overlapping peaks, the center of the chromatographic band is not the same as the top of the peak when the two bands are displaced toward each other (**Fig. 13**). This means that the

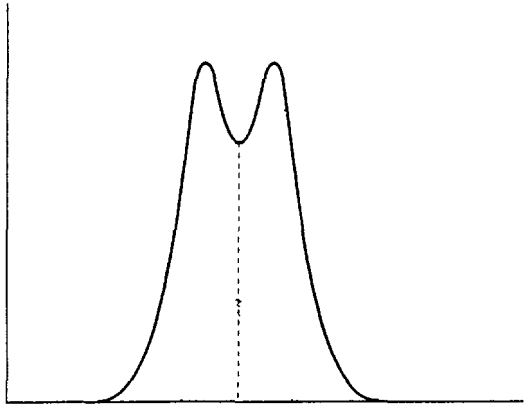


Fig. 13. Apparent and true position of overlapping peaks.

peak-height measurement in such cases is an overestimation of concentration. It also means that apparent retention times are shifted, so that obtaining an accurate t_R value for identification purposes is made much more difficult in cases of incomplete resolution. Ideally, to get both quantitative and retention time data with reasonable accuracy, it is best to have a R_s value in the region of at least 0.8–1.0.

Quantitation is generally more important in analytical chromatography than in preparative isolation work. In natural product extraction, it is generally used to monitor approximate levels of recovery following different stages of extraction, or to get a feel for the amounts of material being examined. However, by examination of peak shape, it is also possible to get a feel for the degree of purity that can be expected.

When collecting fractions from chromatographic separations in which peaks overlap, a judgment has to be made between quality and quantity; that is, taking a cut at *A* (**Fig. 14**) will result in recovery of the main compound that is purer than that obtained if the cut were taken at *B*, but will mean a lower recovery.

If the aim is to extract only some material of high purity, it is more sensible to sacrifice some of the compound of interest and take a cut that contains little or none of the peak overlap. If, on the other hand, the emphasis is on isolating the maximum yield, a broader cut should be taken with a view to subsequent purification steps to remove minor contaminants. Additionally, of course, it is often prudent to collect material in a number of fractions so that a majority of compound will be contained in a fairly pure form, and only a small proportion will need to be further purified. (All of these estimations are based on the assumption that both compounds give the same detector response per unit of concentration.)

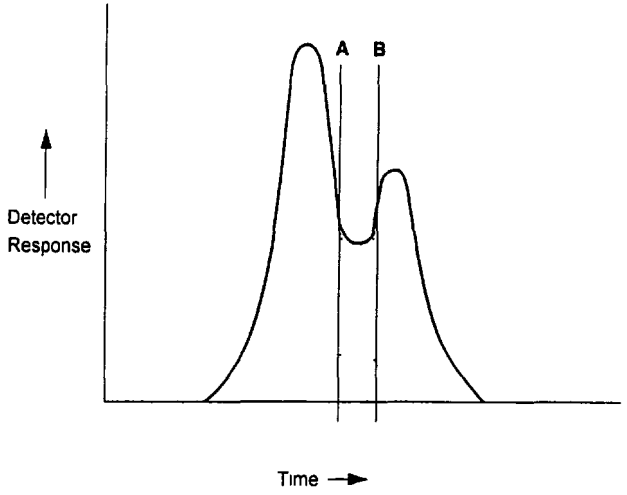


Fig. 14. Overlapping peaks.

3.5. How To Improve a Separation

As described in a previous section, the resolution factor R_s can be expressed as:

$$R_s = 1/4N[\alpha - (1/\alpha)][k'/(k' + 1)]$$

This shows that the resolution of a number of components can be related to three main functions: the capacity factor (k'), number of theoretical plates (N), and selectivity (α).

3.5.1. Capacity Factor (k')

k' is a measure of the number of additional volumes of mobile phase in the system required to elute a solute following the elution of a nonsorbed component and hence is a direct measure of solute retention. In practice, often the easiest way of improving the resolution of a separation is to ensure that a system is used in which the compounds to be separated have a reasonable value of k'. A value of $k' = 0$ means that the solute is not retained at all—it elutes at the solvent "front." In most cases, a useful value of k' is between 1 and 10, and in attempting to resolve two close peaks, it is rarely of any value to increase retention so that $k' > 20$. k' values of greater than 20 represent inordinately long retention times; from the above equation, it can be seen that as k' increases, $k'/k'+1$ tends toward 1, and further increases in k' have little effect in improving resolution.

3.5.1.1. Solvent Selection:

In practice, the means by which k' is optimized in most chromatographic separations is by the selection of a solvent of suitable polarity, or solvent

strength. There are a number of different measures of polarity of a solvent, the most commonly used being the Snyder solvent strength parameter, E^0. This is based on the adsorption energy of the mobile phase on alumina. A list of solvents in order of increasing eluent strength, an eluotropic series, is given in **Table 2**. Descending the table, the values of E^0 increase, resulting in a higher value of k' in a reverse phase system. Generally, increasing solvent strength E^0 by 0.05 will decrease k' by a factor of 2–4 (**Note 5**).

There are a few basic rules of thumb that can usefully be followed in the selection of a suitable mobile phase solvent.

1. The first basic starting point is to find a solvent in which the sample is soluble. However, high solubility in the solvent at the expense of nonsolubility/noninteraction in the stationary phase is not desirable
2. When using an aqueous-organic solvent mixture for partition/adsorption chromatography, the 10% rule is a good approximate guide. This states that a 10% change in the organic solvent content of the mobile phase results in a two- to threefold change in k'
3 As most detection is based on UV absorbance, it is usually necessary to use solvents that have low absorbance at fairly low wavelengths so that the absorbance of the solutes is detectable against the background, even at wavelengths as low as 210 nm. This is one of the reasons why methanol, acetonitrile, tetrahydrofuran, and water have become such widely used mobile phases for HPLC.

The selection of particular solvents for individual forms of chromatography is discussed further in later chapters.

3.5.1.2. Gradient Elution

In circumstances where not all of the components of a mixture are eluted from the column within a reasonable time using a single, unchanging solvent system (isocratic elution), it may be necessary to use a system in which the mobile phase composition changes during the separation (gradient elution). By using two solvents, the proportions of which change during a run, it is possible to separate solutes with widely different retention times. These mobile phase changes may occur at a number of set intervals (step gradient) or continuously throughout the run (continuous gradient). This is commonly used, for example, in reverse phase chromatography, in which the polarity of the mobile phase is gradually decreased (and vice versa in normal phase) or in ion-exchange chromatography, in which the ionic strength of the mobile phase is increased. Gradients also have the advantage that not only do they allow for the separation of components with widely different properties in a single separation, but they often result in better separations of closely eluting solutes because the gradient serves to focus and sharpen the chromatographic bands. The disadvantages lie in the extra apparatus required, as well as the greater inconvenience—repeat

Table 2
Eluotropic Series of Solvents

Solvent	$E^0(Al_2O_3)$	Boiling pt., °C	Viscosity, mN $s.m^{-2}$(20°C)	UV Cutoff, nm
Pentane	0	36	0.24	210
Cyclohexane	0 04	69	0 98	210
CCl_4	0 18	77	0.97	265
Toluene	0.29	111	0.59	286
Diethyl ether	0.38	35	0 25	218
Chloroform	0.40	62	0.57	245
Dichloromethane	0 42	40	0 44	235
Tetrahydrofuran	0.45	66	0.55	220
2-Butanone	0.51	80	0.32	330
Acetone	0.56	56	0.32	330
1,4-Dioxane	0.56	107	1 44	215
Ethyl acetate	0.58	77	0.45	255
Diethylamine	0.63	115	0.33	275
Acetonitrile	0 65	82	0.37	190
2-Propanol	0.82	82	2.50	210
Ethanol	0.88	78	1.20	210
Methanol	0 95	64	0.59	210
Water	1 00	100	1 0	–

gradient separations are less amenable to automation and generally require more time than the equivalent number of isocratic separations, as it is necessary to equilibrate the column between runs (**Note 6**; **Fig. 15**). Examples of gradient elutions relating to various forms of separation are given in the relevant chapters.

In developing separations of natural products, obtaining a useful value of k' is so fundamental that we generally do it instinctively, and it is obvious that a primary objective is to find stationary and mobile phases that retain the solutes but from which the solutes are eluted within a reasonable time.

3.5.2. Efficiency (N)

Improving the column efficiency, i.e., increasing N, will make the peaks sharper and narrower and hence will reduce the overlap between peaks, but it will not affect the fundamental basis of the separation. The relative retention times of a mixture of components will remain the same. This factor in the above expression involves the square root of N which means that in reality, an increase in N has to be fairly large to make any significant difference. This sort of improvement in separation is seen most dramatically in the difference between classical gravity-fed, large particle columns with plate values of hun-

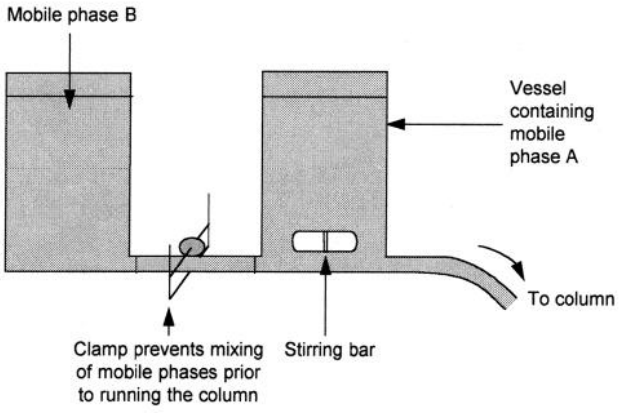


Fig. 15. A simple gradient former (**Note 6**).

dreds or thousands, and HPLC columns with their small regular particles and their typically high plate numbers of tens of thousands. This is why HPLC has become such a valuable technique—the increase in theoretical plates has made it possible to resolve components for which classical columns are just too inefficient.

3.5.2.1. Particle Size

In order to increase the number of theoretical plates, it is necessary to increase the opportunity for equilibrium of solutes between the mobile and stationary phases to occur, i.e., it is necessary to maximize the surface area of the stationary phase. This can usually be done by reducing the particle size—at least in the case of a stationary phase in which the chromatography takes place on the surface of the column packing material. This has led to common use of columns consisting of regular particles 5 μm (or even 3 μm) in diameter. This also means that high pressures are required to force mobile phase through such tightly packed material, which is why HPLC columns, pumps, and associated hardware are so widely used.

When discussing column efficiency, it is sometimes more valuable to express this in relation to particle size. This is known as reduced plate height (h):

$$h = H/d_p$$

where d_p is the mean particle diameter. This describes the number of particles corresponding to H and is a dimensionless parameter that can be used to directly compare columns of different particle size.

Increasing the column length will increase the number of theoretical plates; but, as has been shown, this will also proportionately increase retention time and, hence, band-broadening. In practice, there is not a great deal that can be

done to alter N significantly in order to improve resolution, except by radically changing the form of column used, e.g., switching from open column to HPLC or to a column with a smaller, more regular particle size.

3.5.3. Selectivity (α)

In practice, once the basic type of chromatography and mode of separation have been established, the factor that tends to occupy most of the extractor's time during the process of obtaining a separation is selectivity (α).

Even if two compounds have the same K_D under a given set of conditions, it should be remembered that liquid chromatography involves three sets of molecular interactions (*see* **Fig. 16**)

These interactions are all combinations of the various electrostatic forces, namely:

1 Ionic interactions.
2. Dipole–dipole interactions.
3. Van der Waal's forces
4. Hydrogen bonding

By changing the chemical nature of the mobile phase or stationary phase, it is possible to alter the balance of all three sets of interactions. For example, if a strongly nonpolar stationary phase is used with a very polar mobile phase to separate lipids, the principle of "like has an affinity for like" applies and the solutes will tend to spend a much greater proportion of time on the stationary phase and hence will have a very long retention time.

The process of selecting a mobile phase solvent to give a suitable value of k' is mentioned above. However, in order to optimize *relative* retention (α) of solutes, the commonest approach is to modify the mobile phase more subtly. There are a number of basic approaches that can be tried in order to optimize selectivity:

1. In systems involving an organic solvent, whether alone or in an aqueous mixture, changing the solvent to a different one that gives about the same value of k' may lead to changes in selectivity. This may require a slight alteration of the organic solvent concentration.
2. The aqueous content of the mobile phase can be modified by addition of modifiers such as acids (or less commonly, bases) or inorganic buffer salts that can give rise to changes in the overall sets of interactions.

Solvents can be grouped on the basis of their properties as proton donors (acidic), proton acceptors (basic), and dipole interactions. Solvents can be positioned within the Solvent Selectivity Triangle (**Fig. 17**) on the basis of the relative involvement of each of these three factors as parameters of solubility. Mobile phases consisting of a mixture of three solvents can be optimized by

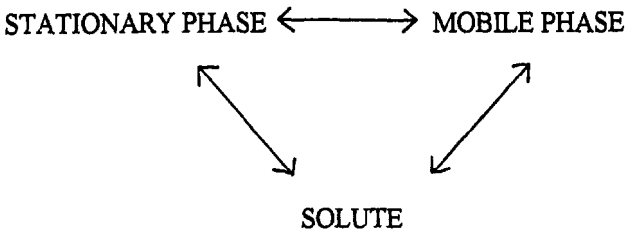


Fig. 16.

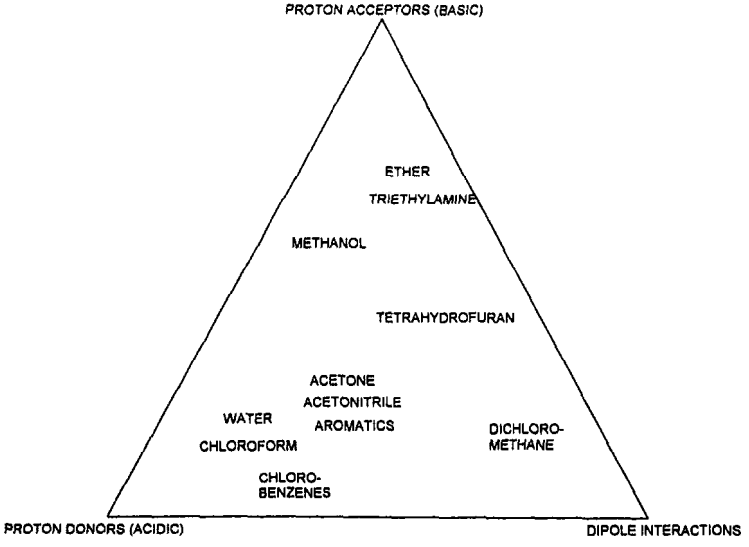


Fig. 17. Solvent selectivity triangle.

selecting solvents from as far as possible from each other in the Solvent Selectivity Triangle (as long as they are miscible). In practice, it is rarely necessary to go to such lengths to obtain a separation, but in problematic cases and as a general underlying model of how to compare solvents, it may be of some use.

The interaction between mobile phase and stationary phase should not be overlooked. For example, many of the very widely used HPLC columns consist of silica particles coated with chemically bonded hydrocarbons to give a nonpolar reverse phase stationary phase. However, there are generally a number of free silanol (-Si-OH) groups that can play a significant role in the adsorption of solutes. The addition of various mobile phase modifiers (e.g., inorganic buffer ions) can lead to interaction with these groups and alter the nature of the solutes' adsorption. This is particularly important in the elution of

basic compounds, which, at the pH values generally used on silica-based columns, tend to interact strongly with silanol groups and in some cases prove very difficult to elute. This is often the stage at which ion-pair modifiers may be added. This may take the form of pH adjustment to ensure, for example, that weak acids are protonated and so are retained satisfactorily on a reverse phase column, or it may involve the addition of larger counter-ions (e.g., tetrabutylammonium ion as counter-ions for anionic species, and alkyl sulfonates as counter-ions for cationic solutes). When separating ionic and nonionic solutes in the same sample, it is generally best to optimize the separation of the nonionic solutes first and then add counter-ions to improve the chromatography of the ionic solutes.

In practice, in many separation systems, these processes are at work to some extent even if the separations are not explicitly characterized as such (e.g., much practical reverse phase HPLC involves the use of modifiers and pH adjustment that often exploits the processes of ion-pairing and ion suppression, in order to achieve separations).

3.5.4. Summary

It is worth remembering that most forms of chromatography involve more than one form of interaction. Most systems tend to be classified neatly as adsorption, partition, ion-exchange, or other single form of chromatography. In practice, however, a separation mechanism is almost always very complex and multifarious because of the array of interactions that involve the sample molecules, and the various components of the mobile phase and the stationary phase, including the "inert" stationary phase material (e.g., the cellulose on which an ion-exchanger may be supported or the free silanol groups of silica coated with a reverse phase stationary phase).

This is perhaps both the beauty and the bane of chromatography. There is an infinite number of overall interactions so that chromatography is an amazingly versatile tool that can separate an infinite variety of mixtures, but working out how this might be done in each individual case is not always easy or straightforward

These same basic principles apply for all forms of chromatography. Although at first sight HPLC, centrifugal TLC, countercurrent chromatography, and size exclusion chromatography are physically very different and the basis of separation may or may not be different, the underlying principles governing the chromatography are the same in all cases.

4. General Extraction Strategy

The purification of a natural product can often be broken down into three main stages:

1. Release of compound from intracellular milieu/cell mass and removal of bulk of biomass. Most of the bulk of the biomass (plants or microbes) exists as fairly inert, insoluble, and often polymeric material, such as the cellulose of plants or fungi and the microbial cell wall. The first step of the extraction is to release and

solubilize the smaller secondary metabolites by a thorough solvent or aqueous extraction This can be done by a series of stepwise extractions, using solvents of varying polarity, which acts as the first fractionation step, or by using a single "all-purpose" solvent such as methanol, which should dissolve most natural products at the same time as enhancing their release from the cellular matrix/cell surface by permeabilizing the physical barrier of the cell walls. The bulk of the insoluble material can then be removed by filtration or centrifugation.

2 Having made the initial extract, one is usually still faced with a pretty complex mixture. Much of this material will be grossly different from the target compound in that much of it may be inorganic or very polar organic material, whereas the target compound is fairly nonpolar, and the aim of the second step is often to try and strip away a large proportion of the unwanted material in a fairly low-resolution separation step. Such a step may involve an open silica column or a series of liquid–liquid extractions, with the aim of a mixture containing all the natural product of interest but comprising only a small proportion of the initial extract, i.e., a relatively small volume of sample that is amenable to the final high-resolution step that will follow.

3 The third general stage is often a high-resolution separation to separate those components still remaining and which must, by reason of still being associated with the target compound after two main fractionation procedures, bear at least some similarity with it. Whereas the second stage might involve a general fractionation with subsequent analysis and work-up of the fractions, this third stage tends to involve preliminary work modifying and altering conditions to achieve the desired separation before preparative work is carried out. This final stage is often, but not always, achieved by HPLC or TLC.

This breakdown of an extraction into three well-defined stages is certainly a simplification. Many purifications do not divide neatly into routine steps, and short cuts are more than welcome. Sometimes the extractor is lucky and a desired compound crystallizes out of a crude broth extract, but unfortunately this does not happen very often!

Schemes 1–5 are typical examples of extractions taken fairly randomly from the *Journal of Antibiotics* and the *Journal of Natural Products*. The general process outlined above is exemplified in the isolation of spirocardins A and B (**Scheme 1**) *(3)*. The compounds were found to be in the broth filtrate, which was extracted twice with ethyl acetate (half volume of supernatant). The ethyl acetate phase containing the compounds was concentrated by evaporation under reduced pressure, then washed with an equal volume of water saturated with sodium chloride to completely remove water and very polar materials, then further reduced to give an oil. This was then redissolved in a minimal volume of ethyl acetate and chromatographed on a silica column developed with hexane containing increasing amounts of acetone. This resulted in two fractions, containing spirocardins A and B, respectively, as the main compo-

nents. These two fractions were then chromatographed separately again on silica gel (the more polar spirocardin B requiring a more polar eluent). The compounds were finally chromatographed on a reverse phase HPLC system to remove minor impurities.

A similar type of procedure is shown in **Scheme 2** for the isolation of glucopiericidinols from a *Streptomyces* sp. fermentation, the main difference being that this extraction incorporates chromatography on Sephadex LH20. This purification is also notable in that the final stage involved a chiral HPLC separation of two diastereoisomers *(4)*.

The isolation of antifungal and antimolluscicidal saponins from *Serjania salzmanniana* also involved the use of a silica column, but it then followed this with separation by countercurrent chromatography (**Scheme 3**). An interesting feature of the final preparative TLC stage was the use of water as a nondestructive visualization "stain." As these compounds are so hydrophobic, this region of the plate remained white (dry), and the remainder of the plate turned dark (wet) *(5)*.

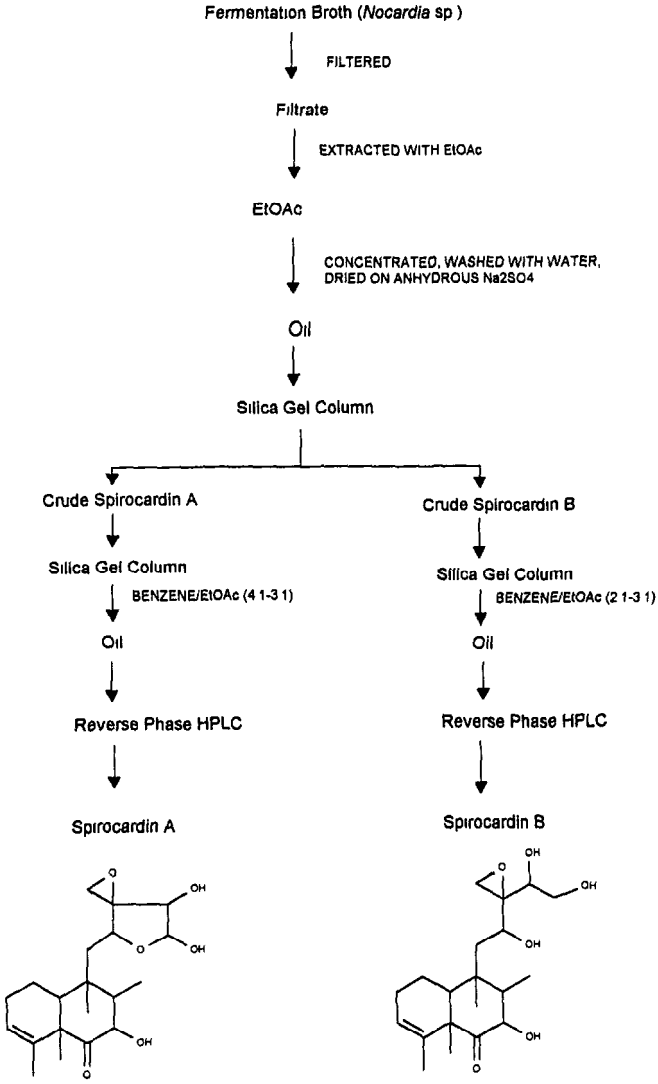
For the more polar fungal metabolite epipentenomycin I (**Scheme 4**), an aqueous extract of the fungus was purified by two forms of ion-exchange chromatography. A final reverse phase HPLC step was employed, but the very polar mobile phase suggests that the compound was hardly retained by this stationary phase, and that this technique was only just within the limits of its usefulness for such a polar compound *(6)*.

Cispentacin, shown in **Scheme 5**, bears some structural similarity to epipentenomycin and was also isolated using two ion-exchange chromatography systems. The broth supernatant was loaded directly onto the ion-exchange column without any prior treatment, and unlike the epipentenomycin I isolation, the final step employed chromatography on a column of activated charcoal to afford a solid of 96% purity. The compound was then purified even further by recrystallization from acetone-ethanol-water *(7)*.

An unusual extraction procedure is shown in **Scheme 6** for the purification of soraphen $A_{1\alpha}$ from a myxobacterium. An adsorbent resin was added to a large-scale fermentation vessel prior to inoculation so that the metabolite was continuously adsorbed as it was produced. Not only did this simplify the isolation, but it also resulted in increased production of the compound by the organism, possibly because adsorbent effectively removed metabolite from the system, thus reducing any feedback inhibition. The eluate from the resin was sufficiently clean that it required only solvent extraction prior to a crystallization step that yielded reasonably pure soraphen $A_{1\alpha}$ *(8)*.

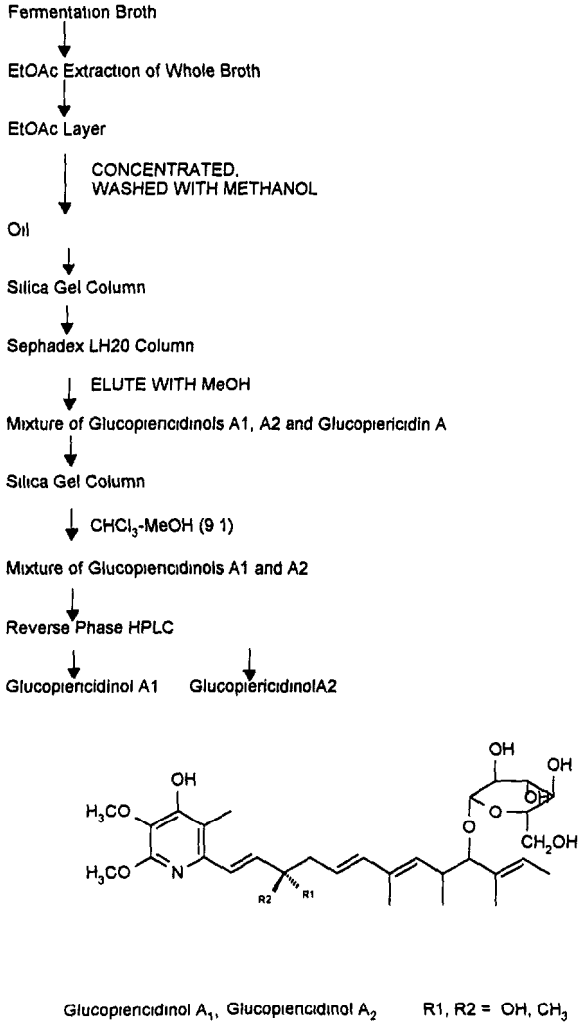
5. Conclusions

Once some information about the nature of the compound has been established, a strategy for the purification can be planned. There is no "correct way"



Scheme 1. Isolation of Spirocardins A and B.

to extract a natural product; there are a myriad of effective methods. In practice—and quite sensibly—people tend to turn to a method or technique that they are familiar with or that is routinely used in their lab (and the experience that comes with this



Scheme 2. Isolation of Glucopiericidinols

obviously makes a succesful isolation more likely). There is a danger, however, in becoming blinded or narrow-minded and not considering fully the worth or ease of other techniques or being reluctant to try other approaches because they are unfamiliar.

MeOH Extract of Powdered Stems of *Serjania salzmanniana*

↓ EVAPORATED TO DRYNESS

MeOH-H_2O (1 2) Suspension of Residue Extracted x3 with $CHCl_3$

↓ COMBINED $CHCl_3$ EXTRACT EVAPORATED TO DRYNESS

Column Chromatography of Residue on Silica Gel

↓

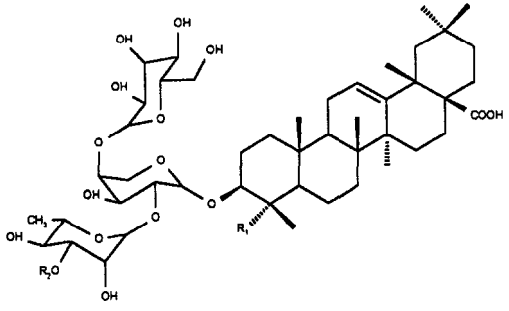
Fractions Monitored by TLC (CHCl3-MeOH-H2O [7 3 1])
Fractions with R_f Values 0 04-0 14 Pooled

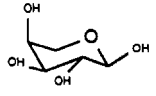
↓

Countercurrent Chromatography ($CHCl_3$-MeOH-H_2O [7 3 1])

↓

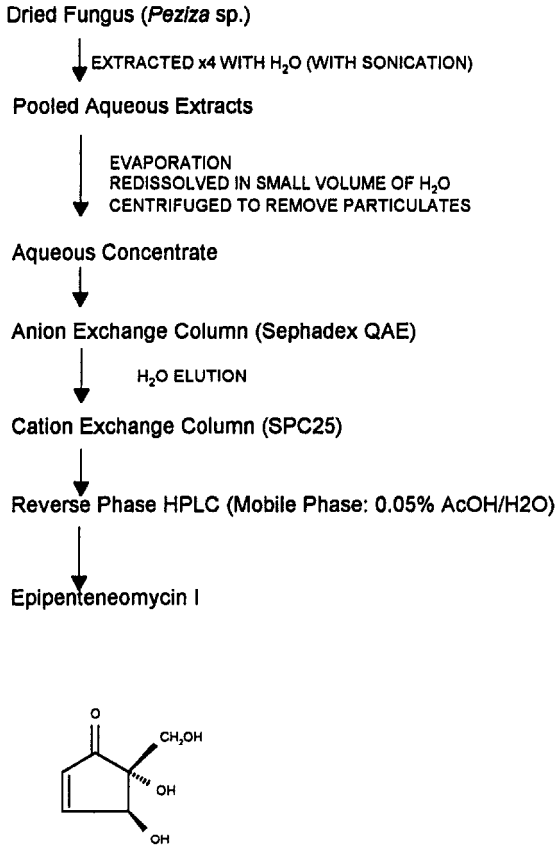
Silica Gel Column Chromatography OR Preparative TLC (EtOAc-MeOH-H_2O [18 7 10])
of Fractions Containing Saponins **1-4**



	R1	R2
1	CH_2OH	H
2	CH_3	H
3	CHO	H
4	CH_2OH	

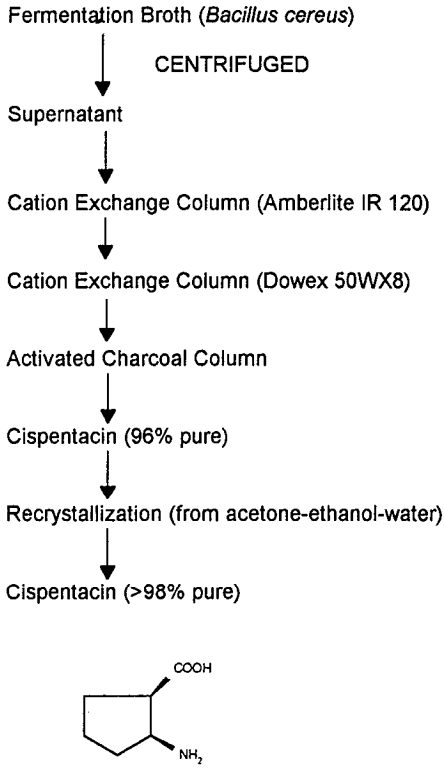
Scheme 3. Isolation of Saponins from *Serjania salzmanniana*.

It is perhaps slightly artificial to discuss purification apart from the context of structure determination. We rarely isolate compounds without attempting at



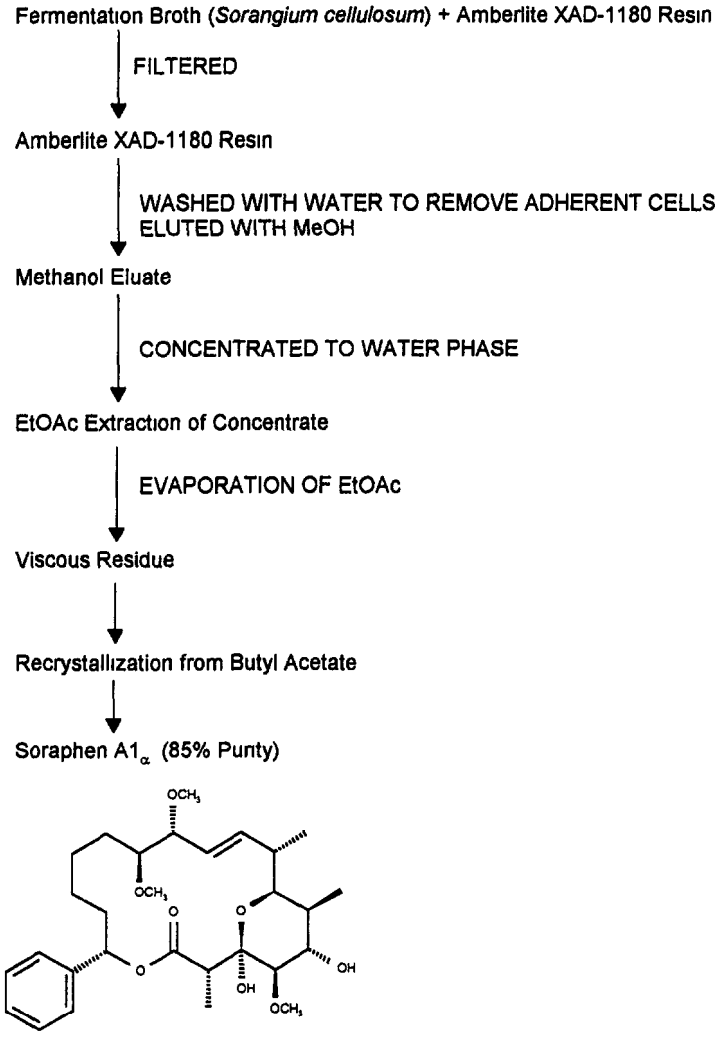
Scheme 4. Isolation of Epipentenomycin I.

some stage to determine exactly what they are. In many instances, the aim of isolating a natural product is to determine its structure and whereas it has generally been the case that in order to elucidate the structure of a natural product, that natural product has first to be isolated, technological developments mean that this necessity is decreasing. Coupled, or "hyphenated," techniques, such as LC-MS and LC-NMR, that unite separation and structural analysis into a



Scheme 5. Isolation of Cispentacin.

single system, mean that structures can be fully or partly determined as a separation is carried out, without first preparing some pure material. Developments in two-dimensional NMR techniques also mean that it is becoming increasingly possible to determine structures of components of complex mixtures such as microbial broths. Additionally, the increasing power of physical methods exemplified by the increasing field strength of NMR spectrometers, and improving ionization techniques of mass spectrometry, mean that now it is



Scheme 6 Isolation of Soraphen A1α.

often possible to elucidate structures from very small amounts of material thus adding to the blurring of the boundaries between analytical and preparative methods.

6. Notes

1. Care should be taken to ensure that the material is fully resuspended. If, for example, the sample is dried for several hours in a heated vacuum centrifuge, it may settle on the bottom of a tube as a hard crust that may be very difficult to fully resuspend. There is also a danger of a soluble compound appearing to be insoluble because it is physically associated with insoluble material and not properly accessible to the solvent In this case, ultrasonication, or mechanical mixing of samples is useful
2. If the compound does not appear to be in either phase, check that it does not form part of the insoluble layer, or emulsion, that sometimes forms at the partition interface.
3. These tests, essentially very simple and straightforward, present their own practical problems. If a small portion of what may be a fairly limited sample is being used, one may be forced to work with small volumes, which can lead to errors and problems. It is difficult, for example, to carry out solvent–solvent extractions using very small volumes. A small amount of solvent may easily evaporate in a short time or "creep" up the side of the vessel, and the loss of these small amounts may represent a large proportion of the total volume and may lead to large errors in the results of subsequent assays With small volumes, the formation of some insoluble material/emulsion at the phase interface can lead to distortions in the relative volumes of the phases, and sampling from, or separating, the phases without significant carryover from the other phase can be tricky. These problems can be countered by using larger volumes where possible.

   Many of the solvents used (particularly water-immiscible solvents) will need to be evaporated before bioassay in aqueous systems The resulting residue can be redissolved in a more suitable solvent, such as methanol or DMSO, then diluted to an appropriate tolerable level. Similarly, it is sometimes wise to adjust the pH with volatile acids, bases, or buffers so that they can be readily removed if it is necessary to dry a sample prior to assay, thus reducing the chance of interference with the assay or the compound.
4. The importance of testing controls (i.e., resins treated with solvents/solution but containing no sample) should be emphasized, particularly when using bioactivity assays. It is not uncommon for assay-interfering materials to be washed from resins or for traces of contaminants in solvents to be concentrated during the process, giving rise to misleading bioactivity results or even spurious chromatogram peaks
5. Although adsorption energy on alumina is used as the standard parameter, the values for silica are very similar.

   The other main measures of solvent strength, the Hildebrand solubility parameter (δ) and the solvent polarity parameter (P'), are calculated by addition of the various intermolecular forces and addition of figures related to the solubility of the solvent in ethanol, dioxane, and nitromethane, respectively. They result in similar eluotropic series, though there are significant differences that reflect the fact that there are several properties of the solvent (dipole moment, solvation properties, and so on) that determine the degree of interaction between the solvent and the stationary phase.

6. Stepwise elution has the advantage that it doesn't require complex equipment. However, it is possible to make a simple gradient former suitable for some circumstances, as shown in **Fig. 15**.

References

1. Zähner, H., Drautz, H., and Weber, W. (1982) Novel approaches to metabolite screening, in *Bioactive Secondary Metabolites. Search and Discovery* (Bu'Lock, J. D., Nisbet, L. J., Winstanley, D J , eds.), Academic, London, pp. 51–70.
2. Ghisalberti, E. L. (1993) Detection and Isolation of Bioactive Natural Products, in *Bioactive Natural Products: Detection, Isolation and Structural Determination* (Colegate, S. M. and Molyneux, R. J , eds.), CRC, Boca Raton, FL.
3. Nakajima, M., Okazaki, T., Iwado, S., Kinoshita, T., and Haneishi, T. (1989) New diterpenoid antibiotics, spirocardins A and B. *J Antibiot* **42,** 1741–1748.
4. Funayama, S., Ishibashi, M., Anraku, Y., Miyauchi, M., Mori, H., Komiyama K , and Omura, S. (1989) Novel cytocidal antibiotics, glucopiericidinols A1 and A2. Taxonomy, fermentation, isolation, structure elucidation and biological characteristics. *J. Antibiot.* **42,** 1734–1740.
5. Ekabo, O. A., Farnsworth, N. R , Henderson, T. O , Mao, G., and Mukherjee R (1996) Antifungal and molluscicidal saponins from *Serjania salzmanniana J Nat Prod.* **59,** 431–435.
6. Bernillon, J., Favre-Bonvin, J., Pommier, M., and Arpin, N. (1989) First isolation of (+)-epipentenomycin I from *Peziza* sp. carpophores *J. Antibiot.* **42,** 1430–1432
7. Konishi, M., Nishio, M., Saitoh, K., Miyaki, T., Oki, T., and Kawaguchi, H. (1989) Cispentacin, a new antifungal antibiotic I. Production, isolation, physico-chemical properties and structure. *J Antibiot.* **42,** 1749–1755.
8. Gerth, K., Bedorf, N., Irschik, H., Hofle, G., and Reichenbach, H. (1994) The soraphens· a family of novel antifungal compounds from *Sorangium cellulosum* (Myxobacteria) I. Soraphen $A_{1\alpha}$ fermentation, isolation, biological properties. *J. Antibiot* **47,** 23–31

Selected References

Sewell, P. A. and Clarke, B. (1987) *Chromatographic Separations.* Wiley, Chichester, U.K

Bristow, P. A. (1976) *LC In Practice* hetp, Cheshire, UK.

Poole, C. K. and Poole, S. K. (1991) *Chromatography Today* Elsevier, Amsterdam

Colegate, S. M. and Molyneux, R. J. (eds.) (1993) *Bioactive Natural Products: Detection, Isolation and Structural Determination.* CRC, Boca Raton, FL.

Weinstein, M. J. and Wagman, G. H. (eds.) (1978) *Antibiotics: Isolation, Separation, Purification,* Journal of Chromatography Library, vol. 15. Elsevier, Amsterdam.

Natori, S., Ikekawa, N., and Suzuki, M. (1981) *Advances in Natural Products Chemistry. Extraction and Isolation of Biologically Active Compounds* Halstead, Wiley, New York

Wagman, G. H. and Cooper, R. (eds.) (1989) *Natural Products Isolation. Separation Methods for Antimicrobials, Antiviral and Enzyme Inhibitors,* Journal of Chromatography Library, vol. 43. Elsevier, Amsterdam.

Verrall, M. S. and Hudson, M. J. (eds.) (1987) *Separations for Biotechnology.* Ellis Horwood, Chicester, UK.

Kennedy, J F. and Cabral, J. M. S. (eds.) (1993) *Recovery Processes for Biological Materials* Wiley, Chicester, UK

Marston, A. and Hostettmann, K. (1991) Modern separation methods. *Nat Prod Rep.* **8,** 391–413.

Verrall, M S. (1996) *Downstream Processing of Natural Products* Wiley, Chichester, U.K.

Ikan, R. (1991) *Natural Products A Laboratory Guide.* Academic, San Diego, CA.

Belter, P. A , Cussler, E L , and Hu, W. S. (1988) *Bioseparations* Wiley, New York