Isolation of Natural Products

by
Y. Tsuda
H.E.J. Research Institute of Chemistry
University of Karachi, Karachi 75270, Pakistan

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PREFACE

Yielding a single compound from natural source is an inevitable problem from the old age. Once the compound isolated in pure, the structure elucidation is rather routine work. This owes to amazing development of modern spectroscopic techniques. However, isolation problem is still a hurdle to clear in natural product chemistry. There is no routine method to solve it, though recent progress of high performance liquid chromatography (HPLC), in its techniques and column-packing material, brought great aid in this field. But, "isolation of natural product" is still the slowest developing field in natural product chemistry, because all isolation work differs from the previous one, in the source of material, quantity in hands, and particularly in the person who is performing this isolation, even though it is repeating the known procedure. None of them is identical with the previous ones.

This booklet will give some ideas on how to isolate natural compounds with the aid of preparative HPLC. But it is only schematic manual. Actual isolation and purification of natural products must not be stereotyped; it requires critical spirit, creativity and originality. In this sense, isolation is an "art" in natural product chemistry.

April 21, 2004. Y. Tsuda (the author)

High performance Liquid Chromatography (HPLC), particularly preparative Recycling HPLC, is a powerful tool for natural product chemists in the separation of very close structurally related compounds.

On the other hand, the system is so delicate and expensive that pre- and post-cautions are necessary. All the students who are going to use preparative HPLC are requested to read this booklet in order to increase the life of your machine. It doesn't describe how to handle it, but all the issues which may rise in the mind of students, have been covered in it. For example, selection of column, restricted/allowed solvent system for a particular column, sample preparation before loading onto the column and many relevant questions have been answered in this booklet. I strongly recommended to the students for reading this prior to plan for using Recycling Preparative HPLC. I hope this booklet would help to enhance your knowledge and life of your machine.

M. Shaiq Ali

Contents

1. General Concept of Natural Product Isolation 4
- Historical Survey
- Modern Isolation Method

2. Types of Isolation 7
- Activity Directing Isolation
  - Preliminary Extraction
- Structure Directing Isolation
  - Preliminary Work
- Isolation for Chemotaxonomical Study

3. Bioassays for Screening of Natural Products 11
- Bioassays for Activity Directing Isolation
- Requirements for Bioassays in Screening of Natural Products
- Enzyme-inhibitory Assay

4. Extraction and Fractionation 12
- Extraction (Important Methods of Separation)
- Choice of Solvent
- Direct Extraction from Raw Materials
- Extraction from Wet Sample
- Two-step Extraction
- Questions Regarding Extraction
  - Scheme 4-1 Example of Two-step Extraction (solid-liquid extraction)

5. Chromatography 17
- Types of Chromatography
- True or False?
- Types of Column Packing
- How Long You Need for Chromatography
- Separation by Changing Solvent
- Flash Chromatography
- Selection of Column and Solvent
- Alkaloids
- Other Methods of Separation
  - Scheme 5-1. Counter-Current Distribution

6. MPLC and Pretreatment for HPLC 22
- Necessity of MPLC
- HPLC and MPLC

Necessary Kits for MPLC
Choice of Column and Solvent
Procedure of MPLC

7. Preparative (Recycling) HPLC 24
Analytical and Preparative HPLC
Preparative (Recycling) HPLC
Is Your Sample Appropriate for Recycling HPLC?
Profile
Profiling Procedure
Separation by Recycling Preparative HPLC
Fig. 7-1. Necessity of Appropriate Amount of Sampling
Fig. 7-2. Example of Overstepping Peaks
Practice of Separation (or purification) by Recycling Technique

8. Selection of Columns 28
Difference of HPLC and MPLC Columns
Basic Idea for Selection of Columns
What HPLC Columns Are Available at HEJ?
Table 8-1 Pre-packed columns for HPLC (examples)

9. Identification and Characterization 31
When Identification Problem Occurs?
Difference in Identification and Structure Determination Problems
Identification
Consult to the Original Paper (primary data)
Reliability of the Data
Structure Determination and Spectral Data
Chart 9-1 Structure Determination and Spectral Data

10. Manual (Shematic) for Isolation of Natural Products 34
Further treatment of Extracted Material
10-1 Pretreatment for Preparative (Recycling) HPLC
Step 1 (Chromatograph)
Step 2 (MPLC)
Step 3 (Preparative Recycling HPLC and Procedure)
10-2 Schematic Procedure for Isolation and Purification
Scheme 10-1 General Scheme of Pretreatment for HPLC
Scheme 10-2 Schematic procedure for Isolation and purification
Case 1. Small scale extraction (5 - 500 g)
Case 2. Extraction of 0.5 - 1 kg Scale (Standard Example)
Case 3. Large Scale Extraction (More than 3 - 5 kg)

1. General Concept of Natural Product Isolation

Historical Survey

Plant or animal tissues always contain several classes of compounds with markedly different structures. Each class usually contains several or a lot of compounds closely related in the structure. Natural product chemistry usually begins from the separation and isolation of single pure compound from such many similarly related ingredients.

The compounds essential for living body, which exist beyond species, such as glycogen, proteins, nucleic acids, some enzymes, and etc. are called “primary metabolite”. The other compounds present in relatively narrow class as specific to each species of the plants are called “secondary metabolite”. The role of these compounds in living body is still obscure. Target of natural product chemistry is usually such secondary metabolite.

Historically, isolation of secondary metabolites from the target plant begins by extraction with solvents. For example, alkaloids are extracted by acidic water, and after basification of the extract, they are again extracted with organic solvent such as chloroform. Sometimes, the methanol extract was suspended in water, and re-extracted with successive increase of solvent polarity, from hexane to chloroform. Afterward, each extract is subjected to separation or purification by the other methods such as chromatography.

By the above method, many of water-soluble compounds, such as saponins, tannins, and carbohydrates are elusive. In view point of this, and by the development of carrier material in chromatography, the isolation method has been changed into the following manner. The methanol extract is made first. After concentration of the extract, the residue is put on the top of silica gel column, then eluted with solvent gradually increasing its polarity, starting from hexane to chloroform-methanol. By this method, the range of compounds for isolation was widened from non-polar hydrocarbons to some water-soluble compounds such as saponins. However, isolation of very polar compounds was still difficult.

Later, variety of new packing material for column chromatography such as Amberite XAD-2, Diaion HP-20, MCI gel, and Sephadex LH-20 were introduced. Those new materials effected to fractionate highly polar compounds with greater efficacy and improved reproducibility. For example, chromatography on Sephadex LH-20 effected isolation of highly polar tannins.

Modern Isolation Methods

Recent introduction of HPLC technique, particularly Recycling HPLC technique, made to allow the separation of structurally very close compounds, those which were inseparable or

hardly separated by usual column chromatography. Thus, general strategy of isolation of natural products must be revised from classical simple (stereotype) chromatography to modern multi-conceptual method. But, the weak issue of HPLC is in its non-applicability to the sample of large quantity. Usually, one treatment is about 10 to 30 mg, at most 0.1 g even if a preparative column is used. Therefore, modern method of isolation adopts multi-concept approach; combination of all separation and fractionation methods and techniques.

The following general strategy is recommended.

1) Preliminary fractionation of the extract has to be made by using various solvents depending on difference of solubility (*i.e.* fractionation by solubility class). After preliminary fractionation, water-soluble (hydrophilic) fraction and water-insoluble (hydrophobic) fraction must be treated by different concepts.

2) Each of water-insoluble (organic solvent-soluble) fractions is subjected to column chromatography on silica gel or ODS to separate the mixture as possible as one may can. This is Step 1 for isolation, which is the same with classical chromatographic separation.

3) Water-soluble fraction must be treated differently. Recommendable treatment is as follows. Firstly separate the mixture by the degree of hydrophobic (lipophylic) nature of the compounds shaking with n-butanol to divide into butanol layer (more lipophylic) and water (hydrophilic) layer. Then each layer is concentrated, dissolved in water, and passed through a column of Diaion HP-20 or Amberite XAD-2 resin. This is a mode of separation with the aid of p-p interaction between resin and compound. Compounds containing double bond(s) and/or aromatic group are held back by the resin and those without such group are eluted through the column. Carbohydrates are separated by this procedure. By increasing percentage of MeOH, more lipophylic compounds are eluted. Column chromatography on Sephadex LH-20 and/or polyamide is used as an option of sub-fractionation. Particularly, polyamide is effective for separation of phenolic compounds. Special care must be taken for tannins, which are hard to elute from the column. The above procedure is Step 1 toward separation of water-soluble fraction.

4) The procedure adopted in Step 1 not only fractionates the mixture into the compounds of similar chromatographic behavior, but also reduces the amount of each resulting fraction making them easier and convenient for handling during purification stages. When each fraction amounts to 0.1 to 1 g, it is further separated by MPLC (medium pressure HPLC) to yield compounds of very close chromatographic behavior. Sometimes you can obtain single compound in this stage. This is Step 2 of pretreatment.

5) Finally, each of above semi-pure fractions (usually less than 0.1 g) is subjected to a Recycling HPLC to separate or to purify into single pure compound. This is Step 3 of isolation.

This booklet describes the general strategy how to isolate natural products in single and pure form. It is written for the readers who already have general knowledge of natural product chemistry. However, before getting into actual manual of isolation, the readers are requested to have some preliminary and fundamental knowledge for isolation works, extraction, bioassay (when bio-activity is concerned), chromatography, nature of columns, MPLC, HPLC and Recycling HPLC, and how to identify the isolated compounds.

2. Types of Isolation

Any of isolation work should be clarified in ones mind mined should be concisely answered to the question, if asked, “Why you carry this isolation work? It is essential and core question.

Purpose of isolation of natural products is broadly classified into one of the following three categories.

A. Activity directing isolation (What is the origin of that activity?)
B. Structure directing isolation (Searching new or novel structure)
C. Chemotaxonomical study (Relationship between histo- and chemo-type)

Each of the above categories consists of different idea and distinct isolation strategy. Particularly, the isolation work directing to bio-activity and that to new compound are different in scale (quantity) and methodology of fractionation, because the fractions under interest in these two methods are usually different. So don't be avaricious, or otherwise, you will fall into troubles between two stools.

Activity Directing Isolation

The work of this type is done only when the plant is known as bio-active (such as traditional medicines) or an activity under investigation is shown by the crude extract. The work is done to disclose what ingredient(s) is responsible to the relevant activity. In any of the above two cases, one needs the method of bioassay, or to find out the method to evaluate the fractionated products. For the bioassay to screen active natural product the following criteria are necessary: simplicity (small quantity), rapidity, comparability (clear-cut result), and reproducibility. Those will be discussed in next session. Without such bioassay method, one cannot start activity directing isolation. When such method and co-worker are available, then proceed into the followings:

Preliminary Extraction

Start with 100 g of the plant material or 10 g of extract. Extract the pulverized material successively with each 200 ml of hexane (3 times), chloroform or AcOEt (2 times), and MeOH (2 times) under reflux for 3-4 h. Each fraction is concentrated (check quantity), and a part of residue is supplied to bioassay.

Compare TLC and activity of above three extracts, and judge which spot(s) may be responsible to the activity. Is the activity increased when compared to the original extract?

Make chromatography of the strongest active fraction. Elute with hexane, hexane-AcOEt (1:1), AcOEt, and MeOH (check quantity of each fraction). Supply a portion of them to bioassay.

You need not necessary to obtain single or a pure compound at this stage. But the above procedure is essentially necessary to judge what type of compound is responsible to the activity.

After this stage, make chromatography carefully to obtain pure compounds. Choose the solvent system according to the hitherto obtained knowledge. Detailed isolation will be done on the basis of the preliminary extraction. You may increase the amount of initial plant material or the extract.

Important! Do not use all samples. You have to keep small amount of each sample (fraction). They are valuable standard and reference for comparisons in future isolation.

Structure-directing Isolation

You are searching new compound or hopefully novel structure. Why did you choose this plant? Does it have a possibility to yield novel structure? Can you confidently defend if asked why you choose this plant? Even if it is not chosen with yourself, make literature search as possible as much as possible: family, genus, species, local name and local uses. Search all references and compounds which has been isolated previously from the target plant. Are the structures known or unknown? Search literatures for plants of the same Genus of a similar use?

If there are some work on this plant, read that work carefully and critically. This literature survey will very much help your, or otherwise, your work will be of little fruits. When you start your work with poor knowledge, collect all information during the work. The more the knowledge is acquired more it promise the fruits in work.

Once getting the sufficient information, start the work as follows.

Preliminary Work

B-1 Start your work with 100–200 g of the material repeating the previous investigator's work, if it is present. Or if it is not, follow to the analogous isolation, with considering why they took such procedures. And observe how easy it is or how difficult it is. At the same time what compound(s) were neglected or discarded. Then you will find out, if your work is the second one, what work is remained to you and which part of work is unexplored.

B-2 At this stage, check to specify the chemical class of your compound in hand! Check the presence of alkaloid by Dragendorf reagent. Are you going to work low polar,

medium polar, or high polar compound? By the class of compound, strategy of isolation is different and varies significantly.

B-3 **[Low–medium polar (water-insoluble) compounds]** Prepare MeOH extract. Mix it with celite (if you do not have it, use material of low adsorption capability such as coarse silica gel) and dry. Extract it successively with hexane, EtOAc, and MeOH. Compare TLC of each extract (see also **Extraction**).
Hexane extract (least polar fraction) is subjected to chromatography.

EtOAc extract (medium polar fraction): Filter if there is a precipitate, and shake the filtrate with water to remove water-soluble material from this fraction, then subject to chromatography as follows. Add the same volume of hexane to the above AcOEt fraction, and filter if any precipitate is present. Pour the solution onto silica gel column and make elution with hexane-EtOAc (1:1), EtOAc, $CHCl_3$, acetone, and $CHCl_3$-MeOH (1:1). Compare TLC of each fraction.
MeOH extract and water washing of EtOAc extract contains high polar compounds. For treatment of this part, see **B-6**.

B-4 When you get single compound (that usually means single spot on TLC), try to crystallize it from appropriate solvent, describe crystalline forms (prisms, needles, leaflets), and measure mp. Consult with the literature (the same plant, the same mp), if it is known or not?
Take UV, IR, MS, ^{1}H-NMR, ^{13}C-NMR spectra, if necessary, to know possible structures. Is it identical to any of the reported compound? If it is possible to be a known compound, compare the all reported physical and spectral data. Do not compare the structure (it is sometimes wrong), simply compare the reported data. If it is unknown (new) compound, go into structure determination (see **Identification and Structure Determination**).

B-5 **[High polar (water-soluble) compound]** The MeOH extract and water-layer from **B-3** contain high polar compound(s). They are usually glycosides, carbohydrates, amino acids, and tannins. These must be treated by different concept from low to medium polar compounds. Each class of compounds is separated by some special techniques, for example, chromatography over Sephadex LH-20 is useful for separation of tannins. Before going into such special separation, I recommend the following general method.

B-6 Combine the high polar fractions, dissolve it in water, and extract with *n*-butanol to divide into butanol-soluble and insoluble (water-soluble) fractions. Each of them is treated separately.

B-7 BuOH extract is concentrated and subjected to chromatography on Diaion HP-20 column, and treated as shown in **B-8**. Usually glycosides containing saponins come

into this fraction. For Direct chromatography choice of the solvent is the most important factor.

B-8 Water layer (after removal of all organic solvent contaminated in this fraction under reduced pressure) is passed though Diaion HP-20 column. Wash the column with water. This will give simple carbohydrate (non-lipophilic molecule). Following elutes with water-MeOH (2:1), water-MeOH (1:1), water-MeOH (1:2), and MeOH (100%) give the fractions where lipophylicity is increasing in this order. Tannins are difficult to be eluted. They are eluted with the use of much powerful solvent, such as acetone containing acetic acid. Usually such solvent must be avoided because of damage of the column.

B-9 On the basis of information obtained above, get into detailed isolation work, if necessary, increasing amount of the material.

Isolation for Chemotaxonomical Study

This work is, more or less, the same with type B. But, particularly in this work, do not bother or stick to new compound: the known and new compounds are treated in equal weight, and you have to clarify all constituents together with (preferably) relative existing amount.

3. Bioassays for Screening of Natural Products

Bioassays for Activity Directing Isolation

Any of the methods and procedures can be taken for bioassay except for ethical problems: they are biological, pharmacological, and/or biochemical. The ideal bioassay is the method to explain the original activity or uses of that material (compound or plant). However, when assay for screening and isolation of bio-active natural compound is planned, it has to meet the following requirements: that is, simplicity, rapidity, reproducibility, and comparability. Theoretical strictness, if it really explains that activity, is not important in this moment. It has to somehow concern to the activity in question. This is particularly important for activity directing isolation. Does it hopefully explain or related to the use or the activity of the plant? However, this is not always the case. In such cases, choose any other method related to the observed activity.

Requirements for Bioassays in Screening of Natural Products

Simplicity: Small scale sample (1-5 mg or less) for each assay. If >100 mg is required, different method pertaining to the activity in hand has to be considered. Assay procedure must be easy to carry on.

Rapidity: The result must be available within a week, preferably within two days, or otherwise another method must be considered. In most cases this is a neck of bioassay.

Reproducibility: The same or similar data have to be obtained by the assay at different time and of different person. Or otherwise, the data are not reliable.

Comparability: The data must be clear-cut and quantitatively (numerically) expressed. They are compared to the other data. Or otherwise, you can not know if the activity was actually concentrated or not by your purification procedure.

Enzyme-inhibitory Assay

Bioassay using **enzyme-inhibiting activity** is one of the ideal methods satisfying the above requirement. However, you always have to keep in mind that enzyme inhibitory assay is the assay of biochemical level and is not identical to biological or pharmacological phenomena of whole body.

Screening of active ingredients is done by the following procedure: First screening, then go to second screening (usually 1/10 volume of the first screening), and third screening (1/10 of the second).

4. Extraction and Fractionation

Extraction (Important Methods of Separation)

Extraction is the first step for isolation of the compound. So the question arises; what is an ideal extraction?

It depends on what compound(s) you are going to obtain. Are you isolating a special compound or special class of compound, or are you isolating all compounds in the material? Method and procedure are different in these two cases.

As a particular case, if you wish to obtain a special compound, **super critical extraction** is recommended. It is the extraction with liquid carbon dioxide (super critical liquid) with or without additives (usually ethanol). It is already used industrially (*ex*. Essential oils).

Choice of Solvent

You can use any kind of solvent. However, it has to be selected by consideration of the following two factors.

1. Polarity: There is a rule in solubility "similar polarity between solvents and compounds produces the best fit".

Hexane (pet. ether)	lowest polarity	hydrocarbons
Benzene (toluene)		aromatics
Ether		
Chloroform	modest polar	many classes
Ethyl acetate		
Acetone		
Methanol		
Water	highest polarity	amino acids, carbohydrates, tannins
Acidic water	salt formation	alkaloids

DMF is also used as a special case.

Polarity of the compound depends on the ratio of a polar functional group (hydrophilic part) to C-H moiety (lipophylic part) of the molecule. For example, compare isopropanol vs. cholesterol. The latter is more lipophylic.

A simpler relationship is hold for **chromatography**. More polar solvents make flow out more polar compounds.

2) Boiling point: Solvents can be removed by evaporation under reduced pressure. It is recommendable to use the solvents of moderate bp 60-100˚C (in small scale) or 70-120˚C

(in large scale), for concentration purpose. Water is not recommendable because of its low vapor pressure. Notice! Ether is very volatile, usually too volatile to use as a solvent of large scale extraction.

Direct Extraction from Raw Materials:

For separation and isolation of natural products, the first step is isolation from raw material (usually solid). Therefore, it is solid-liquid extraction. Extract the pulverized material (do not make it to fine powder because feasibility of filtration) successively with hexane, benzene, chloroform, ethyl acetate, acetone, methanol, and water.

Extraction from Wet-sample

Fresh sample (particularly from animal origin) contains a lot of water. Therefore, extract first with acetone or methanol (2-3 times) to remove water, then extract with usual way (for example, firstly with hexane to remove fat).

Two-step Extraction (Extraction from Methanol Extract)

Since fresh plant tissues usually contain a considerable amount of water which prevents dissolving the compound in hexane, sometimes two step extraction procedure is adopted: first extraction is made with methanol to yield the "MeOH extract", then extract from this extract successively by various solvent.

a) Wrong procedure: Many of you adopt the followings. Add water to the MeOH extract (after concentration), then extract successively with hexane, chloroform or ethyl acetate, and chloroform-methanol. This is in the category of liquid-liquid extraction. It looks theoretically good, but practically it is a bad procedure. MeOH extract, when added in water, usually produces a lot of hexane insoluble mass that emulsifies the solution and prevents two-layer separation. If you add chloroform, it is not hexane extract any more. It is usually troublesome to recover materials from the final water-methanol residue (you have to evaporate water).

If you stick to liquid-liquid extraction by hexane, do not evaporate MeOH completely. Hexane and MeOH containing water are separable into two phases. In this extraction, very low polar compounds which are hardly soluble in MeOH (such as hydrocarbons) come into hexane fraction and MeOH-soluble compounds remain in MeOH layer.

b) Recommended procedure: Mix the concentrated methanol extract with celite. If you do not have celite, any material of low capability of adsorption can be used. If you have neither of them, use coarse silica gel. Dry and extract with the above suggested solvent

successively, preferably with use Sohxlet apparatus; finally with MeOH, MeOH-chloroform, and then with water. This is solid-liquid extraction.

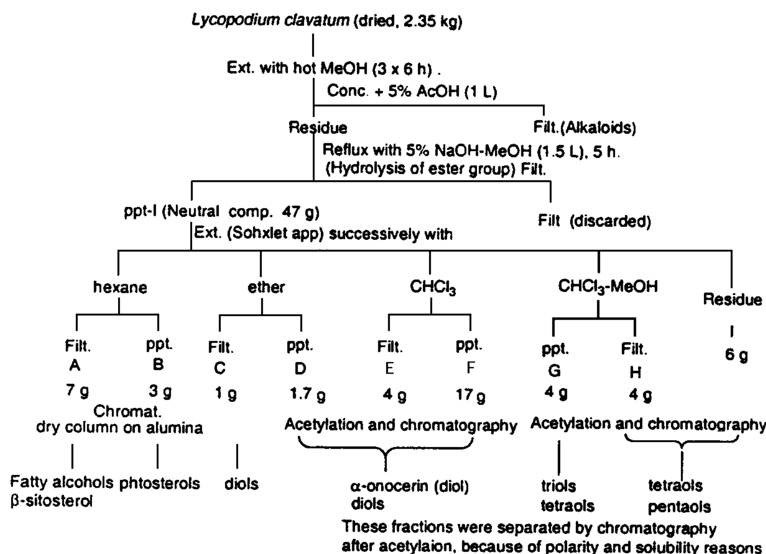
(Scheme 4-1. Example of Two-step Extraction, Solid-liquid Extraction)

Questions for Extraction

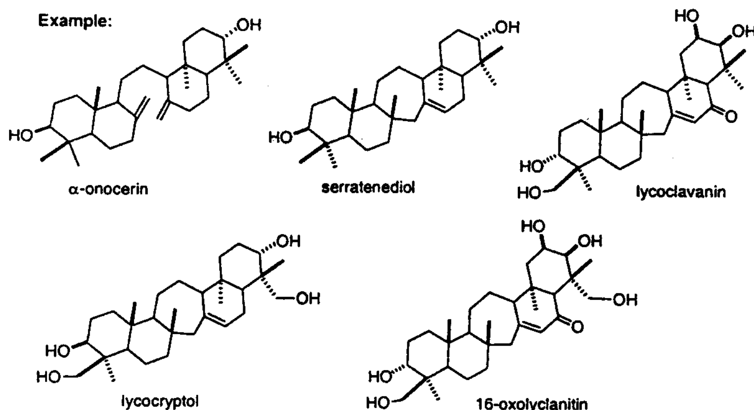
Extraction from water layer with an organic solvent often occurs. Efficacy of extraction depends on the partition coefficient (a) of the solvent and times of extraction.

If a=0.7, b=0.3, you get 70% by 1 ext., 91% by 2 ext. and 97% by 3 extractions.

Scheme 4-1. Example of Two-step Extraction (Solid-liquid Extraction)
***Lycopodium* Triterpenoids**



Solvent extraction roughly separate compounds by the numbers of hydroxyl group in the molecule.
Sixteen compounds of serratane group were isolated from the bove fractions



If a=0.4, b=0.6, you get 40% by 1 ext., 64% by 2 ext. and 78% by 3 extractions.

Where b is partition coefficient to water

Question-1 (for liquid-liquid extraction)

Which procedure is more effective? a) Extract two times with an equal volume of the solvent, or b) extract a solution with a double volume of the above solvent. For these two, total volume of the solvent is the same. (Simple calculation!)

If a=0.4, b=0.6, you get 40% by 1 ext., 64% by 2 ext. and 78% by 3 extractions.
Where b is partition coefficient to water

Question 1 (for liquid-liquid extraction)

Which procedure is more effective? a) Extract two times with an equal volume of the solvent, or b) extract a solution with a double volume of the above solvent. For these two, total volume of the solvent is the same. (Simple calculation!)

Question 2
Is the following idea correct? "If the quantity is available, it is better to use more material (for example, use 4 kg than to use 2 kg), because we can obtain more of the products."

Suggestion: Use smaller amount and keep a half of the material for the next extraction. Even if you used double quantity you may obtain almost the same amount of the products. When larger amount is used, you need larger solvent, larger chemicals, and longer time! Those increase the factors of deterioration of the compounds and mechanical loss during procedures. If the amount is too much, keep half the material for storing in freezer. When you need a more amount of compounds (it often occurs), use this. You already have enough knowledge how to get this compound. So you can get it quickly by using a revised procedure.

Other method of Fractionation (see also other methods of separation in next Chapter)

Stream Distillation

Essential oils are distilled with vapor of water (definition of essential oil). Therefore, when you treat essential oils stream distillation is the first choice. Make it from MeOH extract or directly from raw materials.

Fractionation by Hardly-soluble Salt Formation

Some water-soluble compounds (saponins, poly-alcohol etc.) form insoluble precipitate when basic lead acetate solution was added to their water solution, thus separating these compounds. Lead can be removed by treatment of their water-suspension with H_2S.

Alkaroids from hardly-soluble precipitate by so-called "alkaloid precipitating reagent" such as picric acid, tannic acids, I_2-KI solution, or Reinecke salt. Reinecke salt is particularly important to isolate quaternary (water-soluble) alkaroids. The salt can be removed by treatment with silver reagent.

The following will be discussed in next Chapter.
Counter current distribution
Affinity chromatography

5. Chromatography

Types of Chromatography

Chromatography is one of the powerful method of separation and purification of natural products.

The method of chromatography is classified by the following concepts/principles.

Separation mechanism: Adsorption, partition, ion exchange, gel-filtration
Supporter and carrier: Paper, thin-layer, column
Mobile phase: Gas, liquid

Practically, the following analytical method is used. Thus, detection of ng to pg scale (by GC or HPLC) became possible. They are used for checking purity of isolated products.

Gas chromatography: GC or GLC. Detector: TCD, FID, ECD. ng〜pg.
Paper chromatography: PC. Filter paper (partition). Reproducible for amino-acids.
Thin-layer chromatography: TLC. Rf is variable. Use reference!
High performance liquid chromatography. HPLC. Good reproducibility,

For isolation of natural product, chromatography of preparative scale is used, which provides separation of milligrams to grams of the sample. They are usual column chromatography and preparative HPLC. In this section, let us discuss usual column chromatography. HPLC will be discussed in a separate section

Types of Column Chromatography

Adsorption: Silica gel (normal phase), ODS (reversed phase), alumina, floridil, etc.
Partition: Cellulose powder, celite, silica gel, charcoal, starch, etc.
Ion exchange: Ion exchange resins (Amberite, Dowex, etc)
Non-ion exchange interaction: Diaion HP-20, MCI gel; Sephadex LH-20, polyamide,
etc.
Gel-filtration, Size exclusion chromatography (SEC): Sephadex, JA Igel, etc.

.

True or false (in the practical sense, for example, in your chromatography on silica gel)?

1. Longer column has better separation than shorter column.
2. Column must be packed appropriately loose, or otherwise, it stuck.
3. Slow elution is better for separation than fast elution.
4. Avoid air-bubbles in the column. Always you have to put stopper when interrupt chromatography.

Practically, all are wrong in most cases! Efficacy of separation depends on theoretical plate number (n) of the column (cf. HPLC). For the column packed without care, n is 0 or 1. Such column does not improve separation, even if it is long, instead only increase troubles. The most important is the homogeneity of the column packing and homogeneity of particle size

and shape. Pack the column tightly as possible as you can to obtain good deal of plates that will assist the separation.

Types of Column Packing

a) Wet-column (usual) Soak silica gel in solvent. Pour onto the column portion-wise manner (stopper must be open to escape air), during which give vibration to the column. Pack the column tightly as possible as you can. Tap the column until no solvent appears on the top and is just about to dry the top surface of silica gel. Such column does not stuck, even if it is longer or shorter, and also would give appropriate speed of elution. You can keep the column without stopping for a while.

There are three methods for application of a sample.

1. Dissolve the sample in a small amount of easily soluble solvent and apply it on the top. Then elute with the solvent for chromatography.
2. If you know the major part of the sample is not eluting with this solvent, dissolve it in this solvent (do not mind for the volume), and pour onto the column. Then elute.
3. Mix the sample with silica gel and dry (slurry method). Place the slurry on the top of the column. Then elute.

In either of the above cases, if elution is too slow, make some pressure on the top either by a weak pump or keeping enough amount o0f solvent at the top. Don't make suction – why (consider)?

b) Dry Column The column is packed with silica gel portion-wise without solvent by giving constant vibration. The column must not be too long. The mixture for separation is placed on the top of the column. Then the solvent is poured slowly and continuously onto the column, and stop elution when the solvent begins to elute from the column. Give pressure if necessary. The bottom stopper must be opened all the time to escape air. Take it out and cut the zone.

How Long You Need for Chromatography (for 100 mg sample)?

a) Product by synthesis or by transformation: Column of 1.5-2 x 20 cm size. Complete it within 1-3 hrs (standard).
b) Natural product: Use a column of the same size. Complete it within 3-5 h.

If you do not achieve good separation, repeat chromatography of some combined fractions under knowledge available from the previous chromatography.

Separation by Changing Solvent

In many cases, long column does not produce any of merit instead bring much of troubles. You may have an experience that any of fractions gave almost the same mixture. However,

separation is sometimes incidentally available when solvent is changed. Yes, changing solvent is a powerful method particularly when your column is not perfectly packed. Elute compound by changing the solvent according to the order of polarity (gradient elution).

Flash Chromatography

The standard method of flash chromatography is based on the report by Still (*J. Org. Chem.* 1978, 43, 2923-2925). It will give you an idea that rapid elution of the column does not interfere the separation, instead and contrarily, it helps separation, because short time chromatography minimizes dispersion of the compound in solution. Slow elution by inappropriate solvent system will produce mixing of compound. Avoid band expansion, which often observed unnecessarily less polar solvent is used. Bear in mind to obtain sharp (peak shape) separation, usually by rapid elution.

Selection of Column and Solvent

Silica gel: Normal phase chromatography is used for fraction insoluble in water but soluble in the following organic solvents: hexane, AcOEt, acetone, chloroform, or chloroform-MeOH.

Low polar compounds (such as hydrocarbons) are eluted with low polar solvent (such as hexane). If the compound eluted only with high polar solvent system (such as chloroform-MeOH of 50% or more or 100%MeOH), it is not appropriate to silica gel chromatography. Try reversed phase (ODS) chromatography for such compounds.

For chromatography of acids, addition of an amount (1-3%) of formic or acetic acid is sometimes effective to yield good separation. Acid-washed alumina or silica gel is also used. However, chromatography of the derived methyl ester is essentially recommended.

For chromatography of alkaloids (basic compounds), the use of solvent (usually chloroform or chloroform-MeOH) containing an amount (1-3%) of diethylamine is often effective for good separation.

Alumina: (basic) is also used for chromatography of alkaloids.

Sometimes, silica gel column is used for separation of saponins (water-soluble compound) with use of a polar solvent system, BuOH-AcOH-water. This is a “partition chromatography” which is different in the mechanism from the above discussed “adsorption chromatography”.

ODS: Reversed phase chromatography is applied to water-soluble compounds by using MeOH-water or acetonitrile-water (the former is recommended for economical reasons). Generally, water-soluble fraction must be pretreated with Diaion HP-20 column preferably followed by Sephadex LH-20 column before ODS chromatography. This technique will be

discussed in Chapter 10-1 (Pretreatment for HPLC, Step 1). ODS column chromatography is used as Step 2 (MPLC).

Alkaloids

Total alkaloid mixture in the plant is obtained by one of the following methods.

1) Direct extraction (room temperature) of the plant material by acidic water, such as 3-5% HCl or tartaric acid solution. Basification of the extract with ammonia and re-extraction with chloroform gives water-insoluble alkaloid fraction. Addition of Reinecke solution will make precipitate of water-soluble (quaternary) alkaloids.

2) If you have methanol extract (concentrated and dried), treated this as above to yield alkaloids.

3) If you have methanol extract as a solution, pass it through a column of Amberite IRA-120 (acid form). Wash the column with methanol and water, then elute with 5%NH_3-MeOH to yield total alkaloids.

The alkaloid mixture thus obtained was further separated by chromatography by one of following methods as discussed above.

a) Chromatography on silica gel column with chloroform containing diethylamine.
b) Chromatography on basic alumina.
c) Chromatography on ODS column by the use of buffer solution. This is usually used for Step 2 (MPLC) and/or Step 3 (HPLC) separation.

Other Method of Separation

Counter-current Distribution

Sometimes, I was asked as follows: "a mixture of my compounds was obtained as BuOH-soluble and water-eluting fraction of Diaion HP-20 column. How I can separate this mixture further?" Useally they are compounds of short retention time for ODS column. Provided your observation is correct, your mixture means that it has good solubility to the both BuOH and water, indicating that it is a good example to apply **counter-current distribution** technique. Try to separate them by four-plates distribution.

If there is an indication of separation, apply **droplet counter-current distribution**. The machine is available at HEJ (it is also commercially available).

Affinity Chromatography

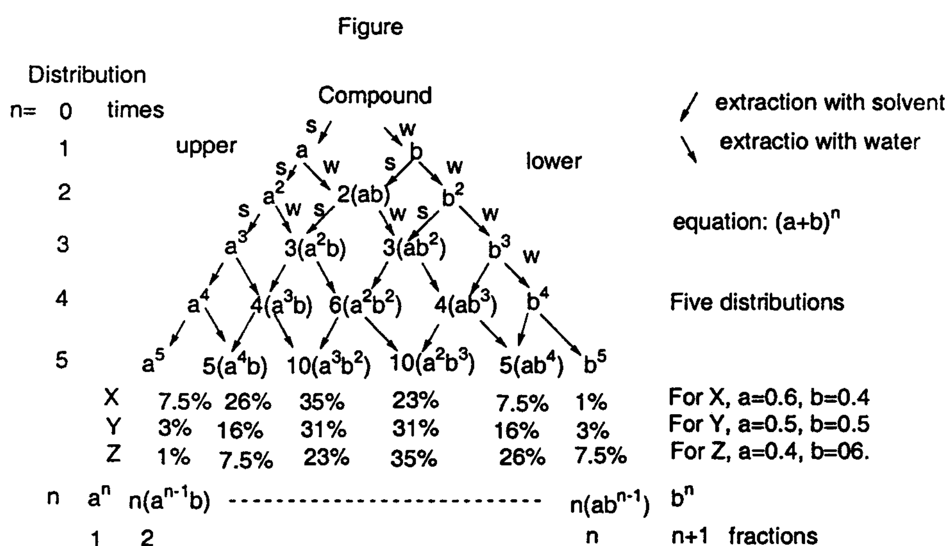
If your sample is biologically active and has some affinity to combine with enzyme or tissue, affinity chromatography is sometimes dramatically effective. For this consult with biochemist or biologist.

Change mode of chromatography

Another method to separate the above fraction is to change the column.
Try a column of Daiso ODS BP-120 for HPLC. This column can be used with 100% water.
Size exclusion chromatography (SEC) on GS-columns (for methanol-soluble compounds) or on Jaigel H-series columns (for chloroform-soluble compounds) is another choice.

Scheme 5-1. Counter-Current Distribution

Shake with equal amount of solvent and water.
Shake the solvent layer with water . Shake ater layer is shaken an equal amount of solvent.
This procedure is repeated n times as shown in the Scheme.



Partition Coeficient (k)of two solvents A, B is defined as $k=C_A/C_B$,
where C_A and C_B are sample concentration in unit volume of A and B.

When you extract ω (g) of sample with v_A ml of A and v_B ml of B,
the quantity (x) of the sample extracted in A is

$$x = \frac{k\omega v_A}{v_B + kv_A}$$

If $k=2$, $v_A=v_B$, then $x=2/3 \cdot \omega$

If you extract 2 times with a half vilume of solvent ($v_A=1/2v_B$), you obtain

$$x=1/2\omega + 1/4\omega = 3/4 \cdot \omega$$

by extraction of n times

$$x= \omega(1/2+1/4+\cdot \cdot \cdot +1/2^n)$$

Extract several times with small volume, than one extraction of large volume.

(Scheme 5-1. Counter-current Distribution)

6. MPLC and Pretreatment for HPLC

Necessity of MPLC

The fraction appropriately separated by usual chromatography (Step 1) is further fractionated or purified by HPLC (high performance liquid chromatography). However, it is strongly recommended to insert one more step (Step 2) before HPLC using MPLC technique. It saves time and money. It also protects the expensive HPLC preparative column from damaging. Usually, a fraction obtained by Step 1 is still in a good amount (0.1 –1 g). It is not appropriate to preparative Recycling HPLC treatment (10 mg –0.1g). If you subject it directly to HPLC with dividing several portions, that is too much time and solvent consuming. MPLC of this fraction is the first choice. This is HPLC at low pressure and applicable to a sample of larger scale.

HPLC and MPLC

The word "HPLC" originally meant "high pressure liquid chromatography". Usually 50–150 kg/cm^2. Now, it is changed to indicate "high performance liquid chromatography", also including medium to low pressure (up to 20 kg/cm^2) high performance chromatography. Sometimes, the latter is distinguished from the former HPLC as MPLC. This came to be possible by recent development of carrier materials. The merit of MPLC is that it made possible to use glass column, which contrarily to metal column not only reduced the price but also made possible large-scale treatment.

However, there is still merit in ordinary HPLC. Thus, let us use MPLC for pretreatment in HPLC (Step 2). In many cases, single compound will be obtained in pure form by MPLC. In such cases, further HPLC is not necessary.

Necessary Kits for MPLC

The simplest MPLC system consists of 1) liquid driving pump, 2) de-gasser, 3) sample injector, 4) UV detector, 5) recorder, 6) fraction collector with 3 way valve, collecting tubes, 7) glass column, 8) column holder, 9) system holder, and 10) connecting tubes. In addition, you need carrier material in the column (silica gel or ODS). It is not so expensive.
The above is enough for low-medium pressure HPLC (MPLC). You can perform good separation of more than 0.1 g scale of a mixture by that system.

However, if you use this system for pretreatment of preparative Recycling HPLC, de-gasser (2), UV detector (4), and recorder (5) are not necessary. All eluted fractions are collected in tubes by a fraction collector, checked by TLC, and appropriately combined. Such combined fraction is supplied for next step (Step 3, Recycling HPLC).

Choice of Column and Solvent

The following technique is advisable. Check TLC of the compound by the plate of the same type of adsorbent in the column (silica gel or ODS), and select the solvent system which give Rf ca. 0.5. This is the best choice of solvent. Usual choice of column and solvent combination is as follows:

Silica gel column: EtOAc-hexane or chloroform-methanol. For alkaloids, appropriate addition of diethylamine in the solvent is recommended.

ODS column: MeOH-water, acetonitrile-water. ODS column is also used for MPLC of alkaloid with buffer solution, because of solubility reasons.

Of course, you can use MCI gel, Sephadex LH-20, polyamide and any other type of column, if necessary, as an alternate option.

Procedure of MPLC

1. Dissolve the sample in appropriate solvent (5 -15 ml). If you have larger amount, divide it into several portions, because of depending on loop capacity (5 -15 ml). Make it sure before operation.
2. Inject the sample solution into the loop.
3. Drive the solvent.
4. Collect in tubes (5-15 ml) by fraction collector. Usually within 100 fractions.
5. Check TLC of each tube, combine appropriately.
6. Concentrate the combined fractions

The sample prepared in this way is enough pure (frequently single compound) or appropriate for further separation by Recycling HPLC.

7. Preparative (Recycling) HPLC

Analytical and Preparative HPLC

The major difference of HPLC between analytical and preparative purpose is in column size and liquid delivery unit. Usually

	Sample	Column	Flow	Solvent	Time
Analytical	g- ng	<4x150 mm)	<1 ml/min	<50 ml	0.5-1h
Preparative	>mg	>20x200 mm	<10 ml	0.5-2 L	hrs

Preparative (Recycling) HPLC

The important factors in separation of a natural product fraction, which was already pretreated by MPLC (Step 2), are as follows. 1) Choose the best fit of column and the compound, 2) choose the best fit of solvent system and the compound, and 3) inject correct amount of the sample to the column. These three are the most important and will also be discussed in Chapter 8.

Is Your Sample Appropriate for Recycling HPLC?

Your sample must be soluble in the solvent (usually in 3 ml) which you are going to use. In many cases, this is the most severe problem. If not, you have to seek different kind of solvent-column fit.

Secondarily, your sample must be appropriately pretreated for preparative (Recycling) HPLC. The sample appropriately pretreated by Step 1 and Step 2 is good for Recycling HPLC, because it is a mixture of very close Rf's (retention time). Recycling HPLC is powerful tool for separation of such mixture.

If not (not rigorously pretreated), or it is a mixture of compounds of so much different retention time (for example 10 min and 70 min, it is usual for crude fractions), do not apply this sample to Recycling HPLC. Those samples need pretreatment of Step 1 or Step 2.

In order to know if your sample is appropriate or not to Recycling HPLC, firstly taking "profile" is recommended. Profile is essentially the same with analytical HPLC. It will tell many things such as, is it good fit to Recycling HPLC and if so, how to separate it practically.

Profile

Following question is often asked. "How we can transform the analytical data to the preparative separation, there is so much difference between them in retention times and

pattern”. This happens due to the difference of column. To avoid such trouble, I suggest to use a preparative column for analytical purpose, the procedure which we call taking “profile”.

Profiling Procedure

Dissolve 0.5 –1 mg of the sample in 3 ml of the solvent. Inject and run HPLC with siphon or fractionation mode recording under appropriately high sensitivity (RI=20-50. UV=0.2-0.1). For example, Column: ODS H/M/L-80 (reverse phase). Solvent: methanol-water (1:1). Flow rate: 4 ml/min

The data obtained here can be directly and perfectly transferable to the preparative HPLC by the same column, thus very useful for setting recycling condition.

When your sample is good enough for Recycling HPLC, consider the strategy of separation taking account of the followings in mind.

Separation by Recycling Preparative HPLC

The followings are the most importance to be considered

1) Selection of **column**: SEC, reverse (ODS) or normal (silica gel) phase Column is the key for separation: For lipophylic compounds, normal columns and for water-soluble compounds, ODS columns are recommended. Size exclusion chromatography (SEC) is useful for discrimination of the compounds with different molecular size and sometimes for stereoisomers.

2) **Solvent** and Flow rate: for ODS, MeOH-water (1:1), 4 ml/min. (standard). If the retention time is two short, reduce flow rate or increase the ratio of water in the solvent. If the retention time is too long (usually more than 60 min, increase flow rate or preferably change the solvent (increase the ratio of MeOH).

3) **Quantity** of injection: Theoretically, bigger column has capacity of the separation of larger amount. However, there is a limitation of loading amount to one column, because of ability of column and recorder. The peaks must be well separated on the recorder. When several peaks merged they are not separated. Therefore, the quantity recommendable in one loading is 50-100 mg, when the sample is a mixture of 3-5 components (approximately 10 mg for each component). If you load too much on the column, good separation would not be expected.

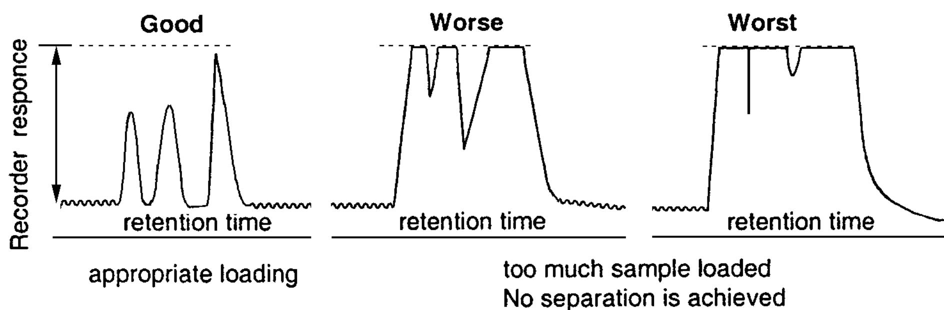


Fig. 7-1 Necessity of Appropriate Amount of Sampling

4) **Overstepping peaks**: This is the major problem when you carry Recycling HPLC. Supposing your compound has retention time *a*, the impurity which has retention time *a/2* will give the peak at the same position with your target compound on its second recycling. Another impurity which gives the peak at *2a/3* will present overlapping for its third recycle with the second recycle of *a*. These phenomena called "overstepping" prevent separation and purification of *a*. Therefore, the following technique is recommended, if you find overstepping peaks in your sample. This is why the profile is necessary.

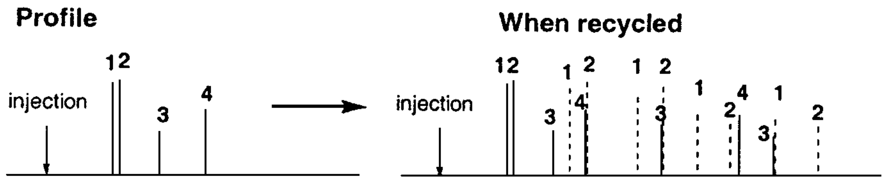
Practice of Separation (or purification) by Recycling Technique

There are several methods in practice for yielding single compound by recycling technique.

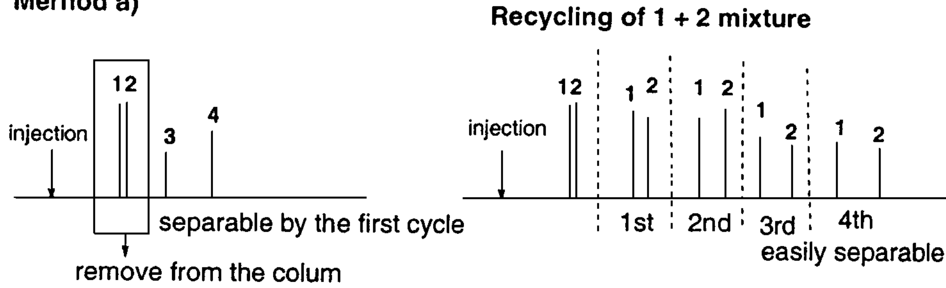
Two typical example are as follows:

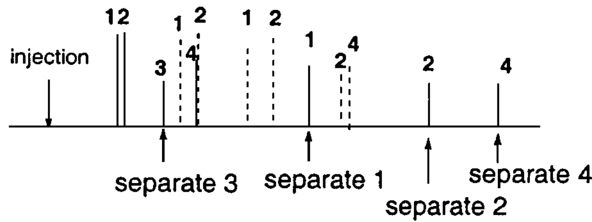
Method a) At the first cycle (without recycling), you divide the sample peaks into several zones noticing to the retention time. Repeat this procedure (if necessary) and combine the same zones (fractions). Then make recycling each of them as follows.

Method b) At the first cycle you will cut off (remove) shorter retention time peaks (using siphon or fractionation). Leave the target peak in the column. After the target peak appeared, you will carefully cut off (remove) all impurity peaks until the target peak again appears at the second cycle. Then recycle the target peak(s) until expected separation is obtained.



Isolation of each component





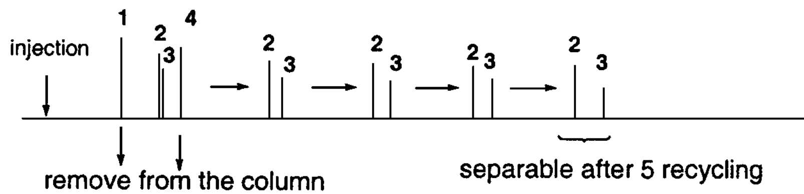


Fig. 7-2 Example of Overstepping Peaks and Practice of Separation

8. Selection of Columns

Difference of HPLC and MPLC Columns

The difference between MPLC and HPLC columns lie in the difference of pressure applied to the column. MPLC columns are made to withstand up to 20 kg/cm^2. Therefore they are made of glass or plastic (insoluble in organic solvent). Usual HPLC columns made of steel withstand up to 200 kg/cm^2. For the both, pre-packed columns are available. For example, Merck Si-60 (silica gel), RP-8 (reverse phase, C8), RP-18 (reverse phase, C18, ODS) are famous for MPLC columns. But for large-scale treatment, packing of MPLC column by the use of column packing apparatus is recommended. For high pressure HPLC column, use of pre-packed column is recommended. Examples of these pre-packed columns (available in Japan) are shown in **Table 8-1.**

Basic Idea for Selection of Columns

You will select the column by the criteria, if the mixture in hand is soluble in organic solvent (hexane, AcOEt, $CHCl_3$) or it is soluble in water or methanol-water.

For water-insoluble mixture:

MPLC column: silica gel with EtOAc-(hexane) or $CHCl_3$-(i-PrOH).
HPLC column: silica gel or polyamine column for adsorption chromatography.
Solvent, as above. MeOH has to be avoided.
Jaigel H columns for SEC. Solvent, chloroform only.

For water-soluble mixture:

MPLC column: ODS with MeOH-water, CH_3CN-water
MCI gel with MeOH-water, MPLC version of Diaion HP-20.
Charcoal: water increasing EtOH, good for carbohydrate
Diaion HP-20, Sephadex LH-20, and polyamide can also be used.
However, the particles size of these resins are too coarse to use as MPLC material.
They are recommended to use for Step 1 chromatography.

HPLC column: ODS or polyamine, many types with MeOH-water.

GS columns for SEC: solvent, water-MeOH

HPLC Columns Available at HEJ

The columns available at HEJ at the moment is as follows:
Size exclusion chromatography

Jaigel H: 1H (good for MW<1000) and 2H (good for MW<5000). Chloroform only
GS columns: water-MeOH use will be available in near future.

Reverse phase column: MeOH-water for preparative, or CH_3CN-water

YMC Co. ODS-H80: MeOH-water. Useful for low-medium polar compounds.
YMC Co. ODS-L80: MeOH-water. Useful for high polar compounds.
YMC Co. ODS-M80: MeOH-water. Middle of the above two.

Daiso Co. SP-ODS-120-BP: Useful for the compounds only soluble in water.

Normal phase column

Silica gel: Water-insoluble comp, Normal phase. EtOAc-hexane, chloroform..

Polyamine column: As above. This column can also be used in hydrophilic solvents. Good for oligosaccharide.

Table 8-1. Pre-packed Columns for HPLC (examples)

Columns	Supplier	Packing material	Nature	Mobile phase	Application
SEC columns (1) GS Column GS-220 GS-320 GS-520	Asahi Chem. Co.	polyvinyl alcohol (polymeric column)	70% SEC 30% reverse phase	water, methanol	amino acid, peptide, proteins, oligosaccharides, nucleotides
(2) JAIGEL Jaigel-1H Jaigel-2H	JAI	polystyrene	SEC (size exclusion)	$CHCl_3$, toluene, THF	syntetic and natural comp. (hydrophobic)
NORMAL Phase Column (3) SILICA R&D-SIL-10-A R&D-SIL-10-A-06 SIL-S-043-15	YMC Daiso	silica gel silica gel	normal phase normal phase	Hexane, EtOAc, $CHCl_3$ as above	usual organic comp. (non-hydrophlyic) as above
(4). Polyamine II R&D-PBMN-5-A	YMC	silica gel modified by polyamine	normal phase weak anion exchange power	water, CH_3CN, hexane, EtOAc (wide use)	widely used for acid, base, neutral comp. oligo-saccharide
REVERSE Phase Column (5). J'sphere ODS ODS-H80 ODS-M80 ODS-L80	YMC	ODS	reverse phase	water, MeOH, CH_3CN, buffer	widely applicable for water-soluble comp.
(6). BP Columns BP-120	Daiso	ODS	reverse phase	can be used with 100% water	useful for trannins and polysaccharide
(7). Polymer C18 R&D-P18-10	YMC	Meta-acrylate polymer capped with OD	reverse phase	MeOH, CH_3CN, water	
(8). C4 Column SH-843-10	YMC	silica gel modified by C4 alkyl group	reverse phase	MeOH, CH_3CN, water	

(Table 8-1) cont.....

Table 8-1. Pre-packed Columns for HPLC (example)

9. Identification and Characterization

When Identification Problem Occurs?

When you have a pure compound in your hand, next you face at “identification” problem if it is known or at “structure determination” problem if it is new. How do you know it is new or known?

Sometimes, structure determination is done without taking through identification process, and the deduced structure is compared with the reported one to judge whether they are identical or not. However, this is not a correct procedure, since sometimes the compound is reported without knowing the structure and the reported structure is not always correct.

Difference in Identification and Structure Determination Problems

Morphine was discovered (firstly isolated) by Sertuerner in 1805. Since then, a number of structures were presented (including Pschorr 1907, Knorr 1907, Robinson 1923), and finally established by the total synthesis of Gates in 1952. Its *O*-methyl derivative, codeine, also has an analogous history. Various structures were proposed time to time, 1907 by Knorr, 1923 by Wieland, 1923 by Robinson, and 1925 again by Robinson, and finally settled by the synthesis of morphine. Certainly, morphine was known compound since 1805, though the structure was not settled until 1952.

Identification

Identification is made by comparison of two samples. What is comparison?

When **reference compound is availabl**e: mixed mp.(different compound often makes depression of mp when mixed), mixed TLC (Co-TLC), and others (comparison of spectral data)**.** The spectra of both compounds must be superimposed.

When **reference compound is not available**, compare the physical and spectral data with reported ones. Physical and spectral data: Mp, $[a]_D$, UV, IR, CD, MS, ^{1}H-NMR, ^{13}C-NMR. Among those reliability of the data is important.
For identification, I recommend to make data-table of comparison. It is so much helpful for identification.

Consult to the Original Paper (primary data)

The source of data must be considered. There are two sources: one is original paper itself (primary data) and the other is those from so-called data-base, CA, handbooks and review books (terpenoids, alkaloids, carbohydrates etc), dictionary (Dictionary of Natural Product) and internet connection (SciFinder) etc. Whenever you find your target compound in a

secondary data, try to contact to the original reference (primary data) if possible, because secondary data sometimes contain error in the transfer process. Read reference critically

Reliability of the Data:

Mp: depends on apparatus. You have to consider 10˚C allowance. It also depends on the crystalline forms. Remember the same compound sometimes gives different mp depending on difference of crystalline forms.

TLC: Compare on the same plate, or you can not say identity. Use the reference sample. Rf is not reliable, which is largely affected by solvent and nature of TLC plates. Co-TLC is one of the powerful tools for identification.

$[a]_D$: This is so much affected by solvent and a little by concentration and temperature. It may include 10% error even if measured in the same solvent system.

UV: <0.1 mg. Calculate e or loge. The same UV tells the same conjugate system, but not identity of the compounds.

IR: Functional groups will be clarified. Compare **finger-print region**. If two spectra in chloroform solution are superimposed (in all region including finger-print), this is the **proof of identity** except optical isomers (enantiomers gave the same IR). However, the spectra in solid (KBr, Nujol) are sometimes different because of the difference of crystalline lattice. The *R, S*, and racemic compounds give different spectra in solid, but sometimes they give the same spectra in KBr (conglomerate or racemic mixture).

MS: EI, CI, FAB, FD, etc. Fragmentation pattern is dependent on eV and peculiarity of machine. It is not good for identification purpose. Stereoisomers usually give the same MS.

^{1}H-NMR: It represents functional group. The same ^{1}H-NMR shows the close similarity for H-functional groups, thus strongly suggests the identity, but not conclusive. The chemical shifts depend on the magnetic field, but coupling constants are not. Therefore the spectral patterns in 100 and 500 MHz are very much different. Do not compare only chemical shift values.

^{13}C-NMR: It is also a powerful tool for identification. It is not affected so much by solvents. Usually 1-3 ppm difference is plausible (due to solvent, instrument, and measurement condition) for comparison. This is almost conclusive, but not almighty. In some cases, 3 ppm becomes to be crucial difference.

X-Ray Analysis: When you get a full structure of the compound, it is a rigid structure determination. But, keep it sure, it is one of the data (for comparison) in identification problem.

Structure Determination and Spectral Data

If the compound is new (unknown), you get into structure determination. For this purpose, you need to isolate some quantity of the compound, mainly for taking spectral data of the compound. It may be helpful to know the relationship between spectroscopy and quantity.

Chart 9-1. Structure Determination and Spectral Data

Spectral Data	Quantity	Information
UV	<1 mg	conjugation
IR	1-5 mg	Functional groups. CO, OH, NH etc.
MS	<0.1 mg	Fragmentation (Strengh of bonds). MW.
^{1}H NMR	1-10 mg	H and its surroundings. δ, int., J.
^{13}C NMR	10-20 mg	C and its surroundings. CH_3, CH_2, CH, C.

Advanced 2D-NMR

Connectivity

H-H COSY	→	Sequence of H arrangement. **H**-C-C-**H** connectivity
C-H COSY (HMQC)	→	**H-C** (one bond) connectivity
HMBC, COLOC	→	H and C long range connectivity. **H**-C-C-**C** (3 bonds), strongest, most common **H**-C-**C** (2 bonds), usually weak **H**-C-C-C-**C** (4 bonds), usually very weak Connectivity with quat. C is also observable.

Spacial relationship

NOE (nOe)	→ %.	NOE enhancement, Quantitative
Difference NOE	→ %.	Quantitative
NOESY	→	Qualitrative (NOE + H-H COSY)

10. Manual for Isolation of Natural Products

The first stage of isolation is separation by extraction (solid-liquid or liquid-liquid). Each fraction thus prepared has to be further separated useally by repeating several types of chromatography (usual chromatography and MPLC), and finally subjected to preparative (Recycling) HPLC. The “Preteatment for Recycling Preparative HPLC” separation and final purification consisted of the following three steps.
schmatically, seeGeneral Scheme of preteatment for HPLC).

10-1. Further Treatment of Extracted Material

Step 1. Usual chromatographic separation and Fractionation

Step 2. Preliminary HPLC separation (MPLC)

Step 3. Separation by Rcycling HPLC

Step 1 (Chromatography)

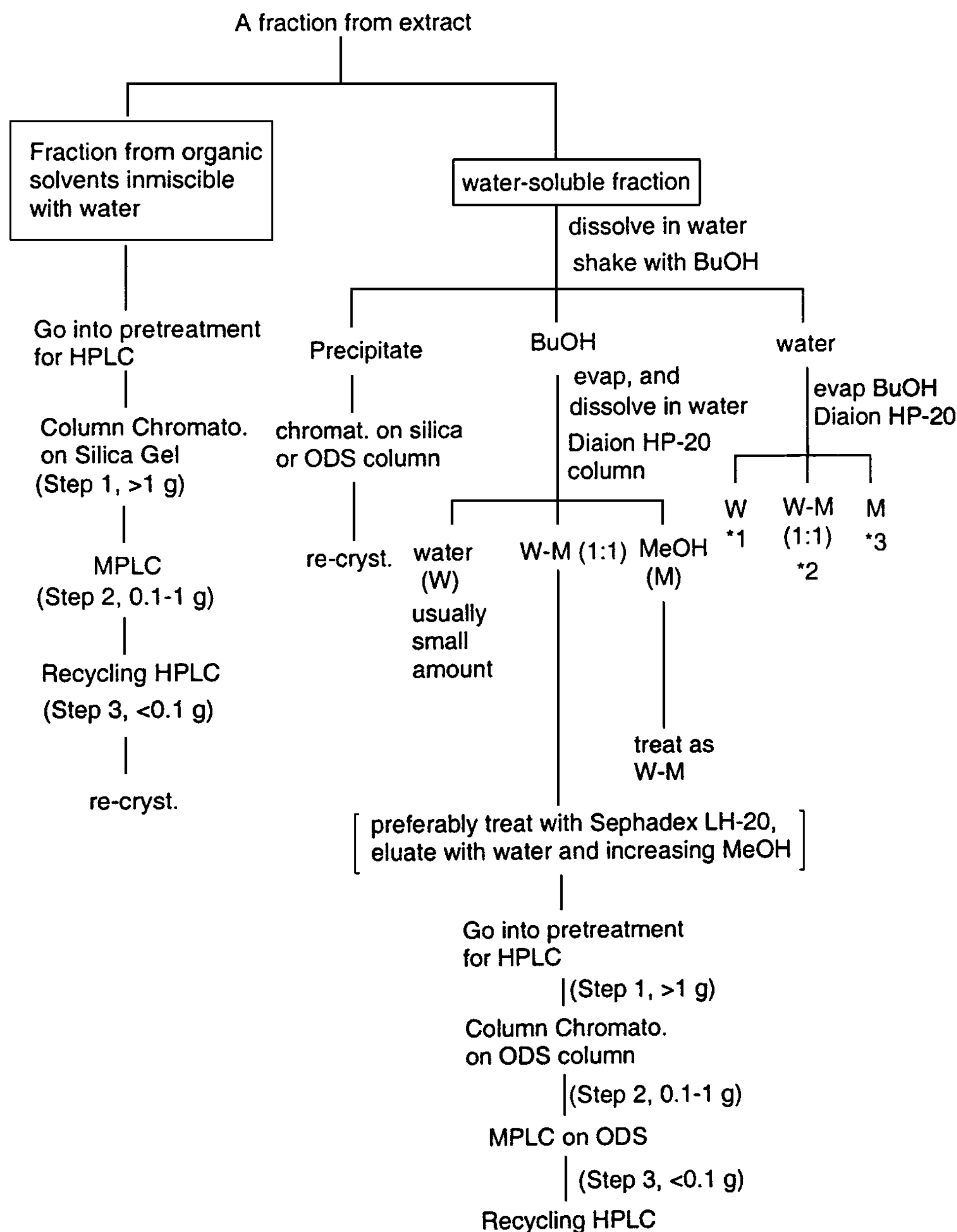
Water–soluble and water-insoluble fractions must be treated with different concept. The latter fraction has to be separated by chromatography on silica gel as discussed already. The former fraction has to be separated as follows.

First, shake the water-soluble fraction with BuOH to divide into BuOH layer and water layer. This is the separation with aid of lipophilicity –hydrophylicity balance. Relatively less polar compounds (saponins of small number of sugars) come into BuOH layer and highly polar compounds (such as carbohydrates, polar saponins, and most of tannins) remain in the water layer. On concentration of the BuOH layer, sometimes precipitate will appear which is collected by filtration and treated as one of the fractions.

Second, each fraction is dissolved in water and passed through a column of Diaion HP-20, and wash the column successively with water (W), 50% MeOH-water (M-W), and MeOH (M). This is the separation with the aid of ˜ interaction between compound and the Diaion resin. As easily being anticipated, fraction W (carbohydrates and related polyols) from BuOH layer is negligible amount and that from water layer is in good amount. Contrarily, fraction M (mostly aromatic glycosides) from BuOH layer is in good amount and from water layer is in negligible amount. Fraction M-W consists of compounds of various kind of lipophylicity. When his fraction is found to be a large amount, further separation by dividing into the fractions of M-W ratio 1:4 (25%), 1:2 (33%), 1:1 (50%), 2:1 (67%), and 4:1 (75%) is recommended. Further separation by Sephadex LH-20 and･or polyamide column is another option.. Sephadex LH-20 separates the fraction by lipophylicity –hydrophylicity factor, when water with gradual increase of MeOH was used as a solvent. Polyamide separates the mixture by the degree of hydrogen bonding *number of phenolic hydroxyls).

The fraction of less than 1 g prepared by the above treatment is appropriate for one treatment of Step 2 (MPLC).

10-1. General Scheme of Pretreatment for HPLC



Step 2 (MPLC)

Based on the knowledge available in Step 1, each of the fractions is subjected to MPLC for separation. Choice of solvent and column was already discussed.

When you reduce the sample amount to less than 0.1 g, it is appropriate amount of Step 3 (purification by preparative Recycling HPLC) in one treatment.

Step 3 (Preparative Recycling HPLC)

Each fraction is concentrated and subject to HPLC. Take "profile" of each fraction. From the above pretreatment and profile you can find "a suitable solvent system" and the practical quantity at one HPLC.

For water-soluble compounds, reversed phase column is useful. At this moment, we have the following columns (all usable at pH 2-11): YMC ODS-H80/M80/L80: and Daiso ODS BP-120. For water-insoluble compounds, use JAI gel 1H+2H column, with chloroform as a solvent.

Procedure (for Recycling Preparative HPLC, LC-980)

Take a mixture, which is supposed to contain 10-20 mg of each component. Sometimes you can take more. Dissolve the sample in 3 ml of the solvent. Inject and run HPLC recording under lowest sensitivity (RI=2000. UV=2) (this can be increased if necessary). For example, Column: ODS H/M/L-80 (reverse phase). Solvent: methanol-water (1:1). Flow rate: 4 ml/min.

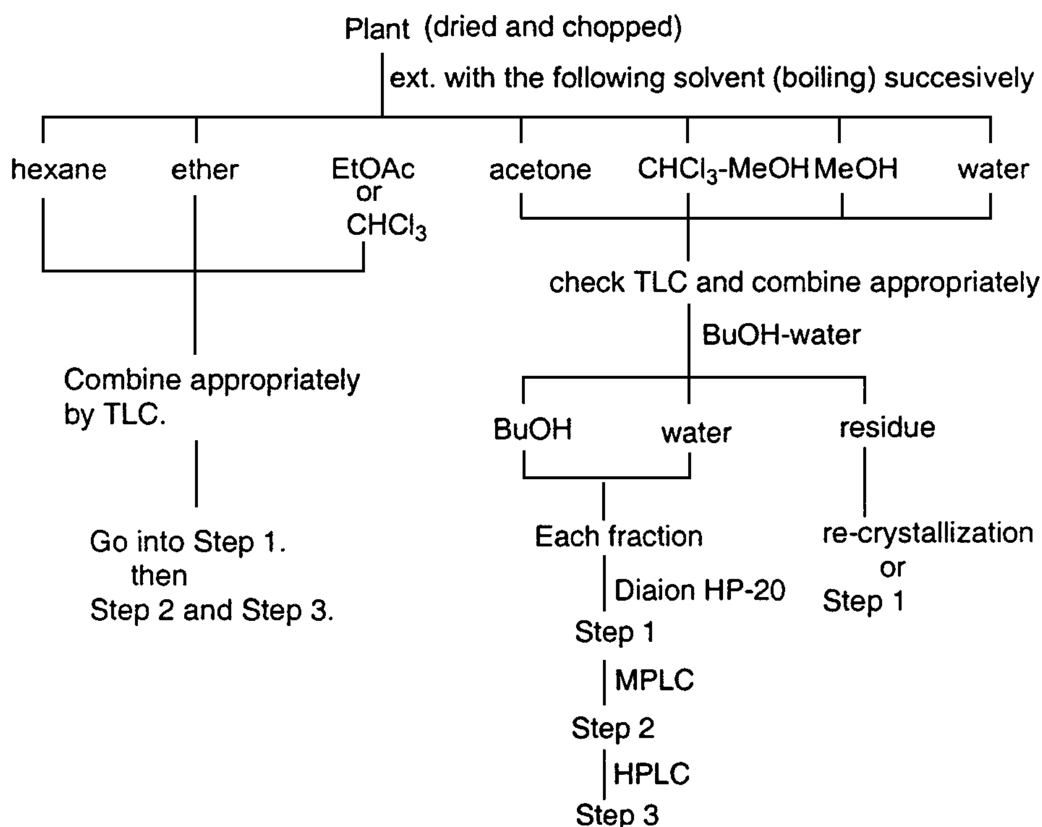
Recycle if necessary. Collect the necessary components. Repeat this procedure several times until you obtain sufficient quantity for spectral measurement.

Case 1. Small Scale Extraction (5 - 500 g)

Direct extraction is recommended.

Treat each fraction as shown in general scheme (Scheme 10-1).

If it is the activity directing work, supply each fraction to the assay without combining.

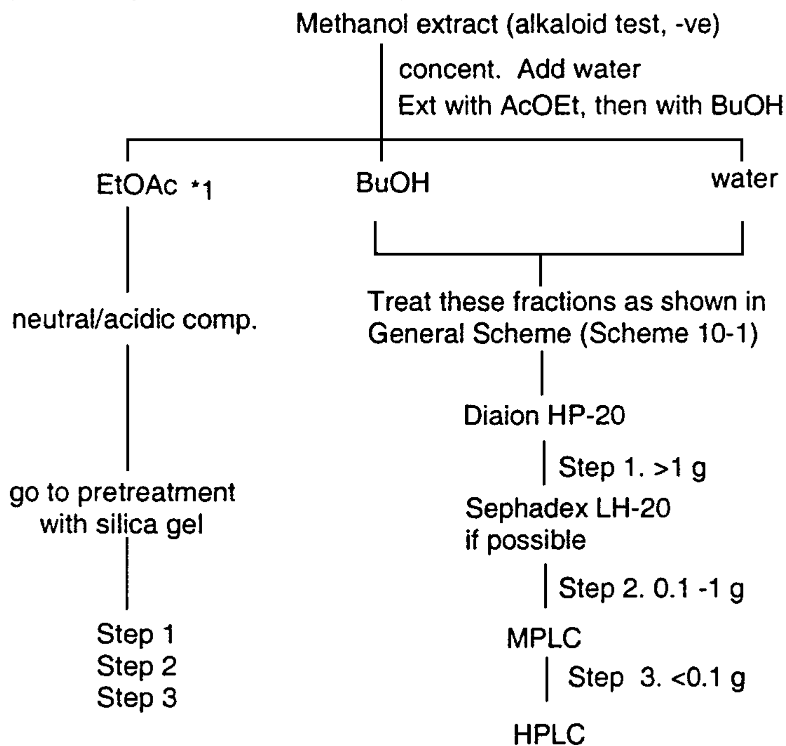


* If the plant contains alkaloid, first extract with acidic water (such as 3-5% HCl or tartaric acid solution), then dry and treat it as above.

* Two step extraction is also used. In this case, the MeOH extract is concentrated, mixed with Celite, dried, and treated as above supposing the Celite-mixture is the original plant.

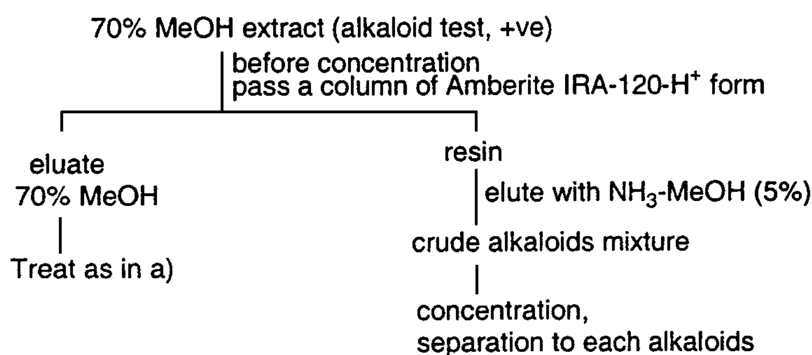
Case 2. Extraction of 0.5 -2 kg Scale (Standard Example)

a) Two step extraction focusing water-soluble fraction (no alkaloid)



*1 Wash with water. The water is combined to water-soluble fraction.

b) When the plant contains alkaloids



Separation of alkaloids:

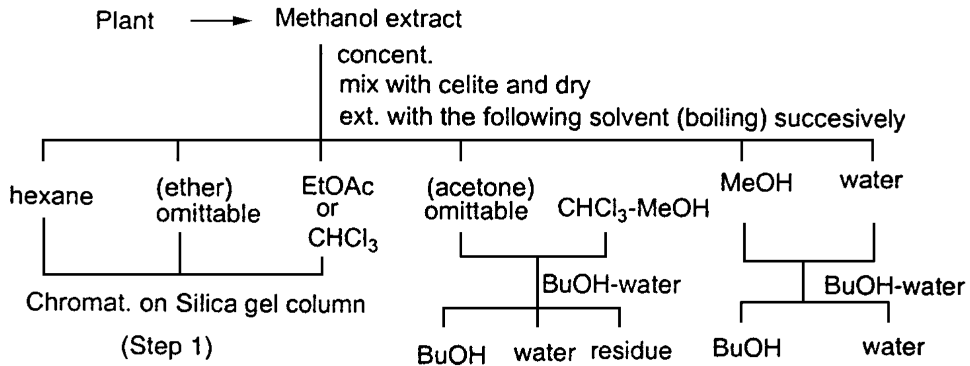
Chromatography: Silica gel column with chloroform containing diethylamine, or alumina (basic) column chromatography.

HPLC: Use of buffer solution is recommended, because of mainly solubility reasons

Case 3. Large Scale Extraction (3 kg or more)

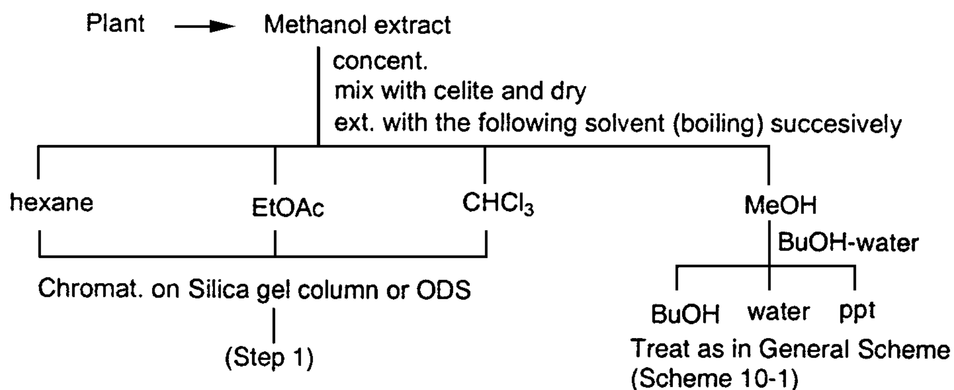
Two step extraction is recommended.

a) The plant does not contain alkaloids

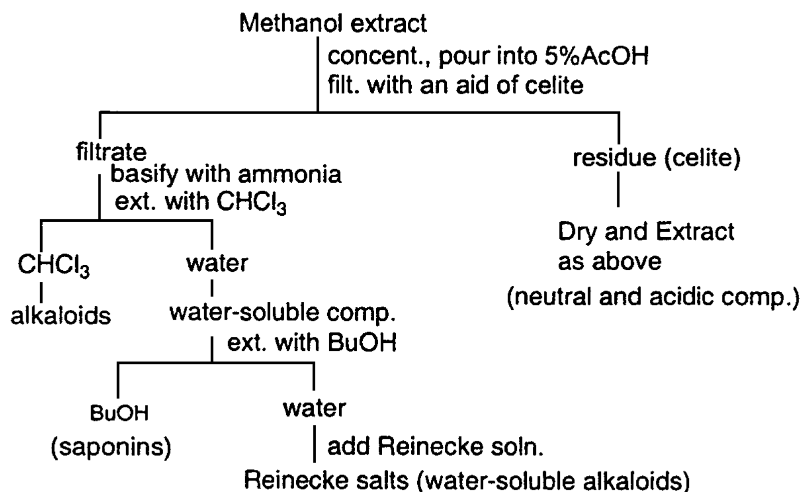


Sohxlet apparatus is covenient when the quantity is not so large.
The fractions can be combined appropriately.

Simplified Procedure



b) When the extract contains alkaloid



The indicated above is only schematic. Actual procedure differs in detail as the case may be depending on the nature and quantity of the extract in your hand. You need your own devise every case to find out the most effective method. Perform your isolation work critically. Thus, isolation and purification of natural product must not be stereotyped. It is an "art" of chemistry.

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