APPENDIX A
INTRODUCTION

Vitamins

As its well known, vitamins are a broad group of organic compounds that are minor, but essential, constituents of food required for the normal growth, self-maintenance and functioning of human and animal bodies. These compounds can be classified in two groups: water-soluble and fat-soluble vitamins. Among water-soluble vitamins, the B group including B_1, B_2, B_6 and B_{12} are the most important. They play different specific and vital functions in metabolism, and their lack or excess produces specific diseases [183]. Increasing interest in good eating habits in human and also animals, has meant greater awareness of the vital role that vitamins play in growth and health. In addition, the presence of fruits and vegetables in the daily diet and the consume of vitamin-supplemented and preserved foods, has substantially increased [183].

Fat soluble vitamins are compounds essential for the human body, as they regulate various processes. For example, deficiency of vitamin A may give rise to serious disorders like night blindness and xerophthalmia [184], and lack of vitamin E may lead to breakage of cell membranes, possibly leading to heart diseases and certain cancers [185]. Vitamin A is a group of vitamins containing retinoids (all-*trans*-retinol, 13-*cis*-retinol and others), and carotenoids (such as β-carotene) [186]. Normally (and also here), vitamin A refers to all-*trans*-retinol, which is the most active from of the vitamin. Vitamin E is a family including tocols (tocopherols) and trienols, of which α-tocopherol as the highest vitamin E activity [187].

Vitamins A and E are not synthesized within the body, and must be supplied in the diet [6]. Since fat-soluble vitamins are sensitive towards oxygen, light, heat and extreme pH values the fortification of food is commonly achieved with the more stable vitamin esters, such as retinol acetate, retinol palmitate and tocopherol acetate.

Vitamin A (Retinol)

Vitamin A is a fat-soluble vitamin, an unsaturated cyclic alcohol, which tends to accumulate in the liver (it is excreted slowly). Toxicity reports have recently indicated 50,000 I.U. daily to be toxic. This toxicity has put limits on the use of the reported selective anti-tumor activity of vitamin A at high doses.

Vitamin A is obtained as such from animal sources and from β-carotene in plant sources, begin converted in the intestine to vitamin A. Its absorption depends on the presence of bile and fats in the intestine.

Vitamin A is rapidly degraded by UV light, whether it be high-intensity UV light (bilirubin light) used in phototherapy, or sunlight [188]. Vitamin A is the generic descriptor for all β-ionine derivatives, other than the provitamin A carotenoids, with the qualitative biological activity of all-*trans*-retinol (vitamin A alcohol). Provitamin A carotenoid is the generic descriptor of all carotenoids with qualitative of β-carotene. The formula for retinol is shown in Fig. A-1.

There is on indication that dietary supplementation is needed for the normal adult on an average American diet. Special conditions requiring supplementation are: pregnancy, lactation, infections, fevers, poor vision and hepatic insufficiency.

Physical and chemical properties [189, 196, 197]

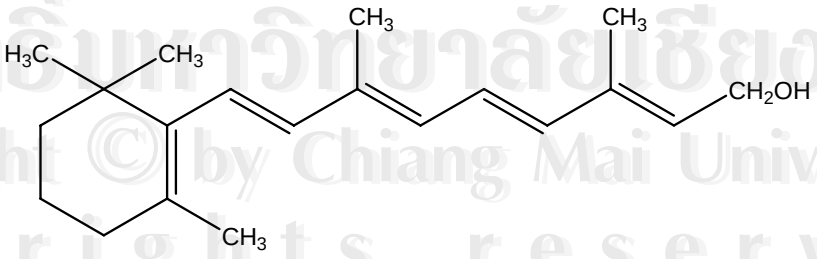

Figure A-1 Structure of retinol (Vitamin A)

Chemical name	:	3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl) -2,4,6,8-nonatetraen-1-ol
Common name	:	Retinol, vitamin A
Empirical formula	:	$C_{20}H_{30}O$
Molecular weight	:	286.4
Solubility	:	soluble in Alcohol, Ethanol, and Chloroform
Melting point	:	62.64°C
Appearance	:	Yellow oil

Vitamin E

Vitamin E is today perhaps the most mysterious of all known major vitamins, although recently some of its functions have become clearer. With the demonstration of the essentiality of Se (selenium) and its synergism with vitamin E, the antioxidant function of vitamin E seems to be the major one, although others will most likely be discovered. The cooperative action of Se and vitamin E preserves membranes from destruction by oxidation products and especially retards hemolysis of red blood cells. Vitamin E alone prevents fetal reabsorption in rats (not in humans), a reproductive function. A major deficiency disease or condition is red cell hemolysis.

Vitamin E is a fat-soluble vitamin, a complex alcohol (quinoid), which slowly accumulates in liver and fatty tissues and is excreted fairly rapidly. Toxicity reports indicate certain individuals are susceptible to high blood pressure, allergies, and Fe^{2+} metabolic derangements in the presence of excess vitamin E. Proponents of the lipid peroxidation or free-radical theory of aging claim vitamin E involvement in aging. Confirmation with controlled experiments in needed. Many claims have been made for its use in megadoses by athletes and others; these claims require confirmation because of high individual variability of responses. Vitamin A and Se act synergistically with vitamin E, where as Fe^{2+} acts antagonistically [189].

There is on indication that dietary supplementation is needed for the normal adult on an average American diet of a normal adult individual, especially in view of the removal or inactivation of vitamin E during food processing and its fairly rapid

excretion. Special conditions requiring supplementation are: pregnancy, newborn infancy, air pollution, diet of processed foods, and high-polyunsaturated-fat diets.

Physical and chemical properties [189, 196, 197]

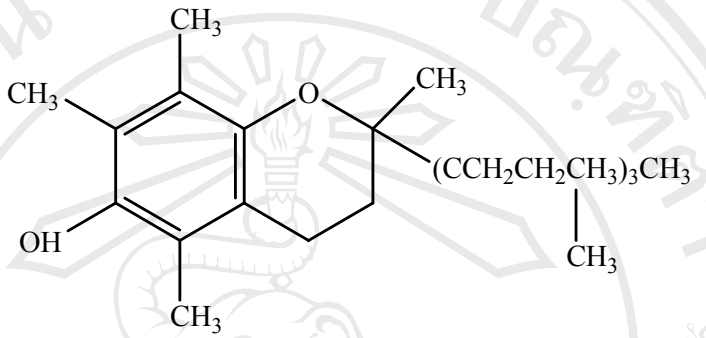

Figure A-2 Structure of α-tocopherol (Vitamin E)

Chemical name	:	2,5,7,8-tetramethyl-2-(4',8',12'-trimethyl tridecyl)-6-chromanol.
Common name	:	α-Tocopherol
Empirical formula	:	$C_{29}H_{50}O$
Molecular weight	:	430.7
Solubility	:	soluble in Alcohol, Ethanol, Chloroform, Benzene
Melting point	:	2.5-3.5°C
Appearance	:	Yellow oil

Beta-carotene

Carotenoids such as beta carotene, sometimes called pro-vitamin A, are water-soluble precursors which are made into Vitamin A by the body. While you can overdose on fat-soluble Vitamin A, large doses of water-soluble beta carotene, found in carrots, broccoli, spinach, cabbage, orange and yellow fruits, are non-toxic and constitute an extremely potent source of antioxidant activity.

Many are proving to be very important for health. Beta-carotene is the most widely studied carotenoid, but others are proving to be of great interest. As with some, but not all, carotenoids, beta-carotene is known as a provitamin A because it converts to the vitamin in the body.

In addition to begin a precursor for vitamin A, beta-carotene is an important antioxidant that helps to prevent harmful free radical damage in the body. These effects are believed to be helpful in such diseases as high cholesterol. Another study evaluated beta-carotene with vitamin C and vitamin E in people with cataracts. One hundred and fifty eight patients were followed for three years and used beta-carotene, vitamin C, and vitamin E daily. The use for these antioxidants delayed the progression of age-related cataracts [190].

The continues to be a great deal of controversy concerning the role of beta-carotene and different types of cancer [191, 192]. There is a long past history of an inverse relationship between cancer and beta-carotene [193].

Beta-carotene may also enhance immunity by boosting the activity of certain immune fighting agents in the body [194]. It may also offer relief to people who suffer from a genetic disorder, erythropoientic protoporphyria (EPP), that makes them abnormally sensitive to sunlight [195].

Physical and chemical properties [196, 197]

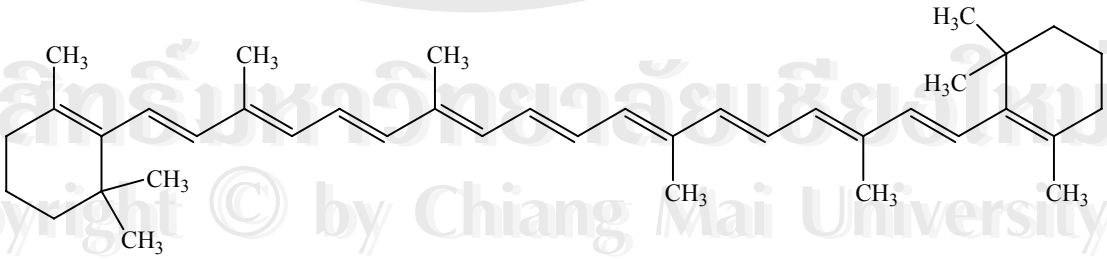

Figure A-3 Structure of Beta-carotene

Chemical name	:	3,7,12,16-tetramethyl-1,18-bis(2,6,6-trimethyl-1-cyclohexenyl)-octadeca-1,3,5,7,9,11,13,15,17-nonaene
Common name	:	Procatene, Solatene
Empirical formula	:	$C_{40}H_{56}$
Molecular weight	:	536.88
Solubility	:	Benzene
Melting point	:	183 °C
Appearance	:	Deep-purple

Vitamin C (Ascorbic acid)

L-ascorbic acid is essential for the prevention and cure of scurvy, a disease that has been known since ancient times. In the first part of the sixteenth century, Jacques Cartier, during his exploration of Canada, found that the natives prevented and cured scurvy by drinking extracts from the bark and needles of pine trees, which we know provided L-ascorbic acid. In the eighteenth century, scurvy was traced to a lack of fresh fruits and vegetables in the diet. Some of the characteristics of scurvy are loosening of teeth, swollen joints, petechial hemorrhages from venules, and subcutaneous and intestinal hemorrhages, which can be attributed to defects in collagen synthesis [198]. During the process of collagen synthesis, ascorbic acid participates in the hydroxylation of particular prolyl and lysyl residues previously incorporated into peptides linkages [199].

Ascorbic acid (Vitamin C) is a white crystalline solid, very soluble in water. It was two ionisable groups, with pKa values of 4.25 and 11.8, respectively. Hence the mono-anion (ascorbate) is the predominant form in all body compartments except the gastric juice. The importance of ascorbate to humans is illustrated both by the evolution of efficient carrier proteins to take it up from the gastrointestinal tract [200] and by the lethal nature of prolonged Vitamin C deprivation, which causes scurvy [201].

Ascorbic acid is the least stable vitamin in TPN mixtures and is in compatible with many drugs, degrades and darkens on exposure to light of all kinds

and is rapidly oxidized in air and alkaline media. The most important mechanism of degradation is that of oxidation to inactive products. The amount of ascorbic acid degraded depends on the dissolved oxygen content of the TPN mixture, the amount of residual air in the bag after filling, the permeability of the plastic to oxygen and storage time [202].

Vitamin C is a water soluble vitamin, very unstable in water solution, chemically a modified sugar, which is stored to a small extent in the body. Its use in megadoses for the abovementioned dysfunctions has not yet found general clinical acceptance; ongoing clinical tests may revise some negative aspects. Its toxicity in pharmacological (mega-) doses has been noted: *possible* (oxalic aciduria, reproductive failure, vitamin C dependency, inactivation B_{12}, loss of folic acid in urine, allergies, diarrhea, abortion, and thrombosis). Its chief synergists are vitamins A and E, Zn^{2+}, Fe^{2+} and citric acid. Chief antagonists are deoxycorticosterone, estrogens, and Cu^{2+}.

Vitamin C is obtained mainly in citrus fruits, green peppers, rose hips, acerola berries, guavas, black currants, kale, horseradish, collards papayas strawberries, and green vegetables. It is synthesized in most mammals (except humans) and rapidly although normal healthy adults on an average American diet are obtains minimum vitamin C for antiscorbutic protection. Dietary supplementation may be necessary because of its instability and because exact requirements for vitamin C have not been determined. Apparently vitamin C fulfills unique new functions at progressively higher concentration levels starting form the lowest antiscorbutic level. Because of the manifold functions of vitamin C it is likely that the present RDA (45 mg/day) is too low. Special conditions requiring definite supplementation of the diet are: scurvy, pregnancy, lactation, heavy mental intoxication, stress, trauma, allergies, old age, high protein diet, and infections [189].

Physical and chemical properties [189, 196, 197]

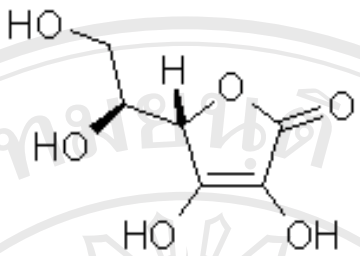

Figure A-4 Structure of Ascorbic acid (vitamin C)

Chemical name	: 3-oxo-L-gulofuranolactone
Common name	: Vitamin C, antiscorbutic vitamin
Empirical formula	: $C_6H_6O_6$
Molecular weight	: 176.12
Solubility	: soluble in Water, slightly sol. in Alcohol
Melting point	: 190-192°C
Appearance	: White powder

Vitamin B_1 (Thiamine hydrochloride)

Thiamine hydrochloride, also known as vitamin B_1, thiamine chloride, thiamine, aneurin, antineuritic vitamin, has the structural formula shown in Fig. A-5.

Vitamin B_1 is a water-soluble vitamin, a pyrimidine combined to an organic base, thiazone; it is not accumulated in the body and is excreted rapidly. Vitamin B_1 may by toxic in high concentrations. Megadoses have been administered for individual cases of depression, tumbago, sciatica, facial paralysis, and so on, but their effectiveness needs to be confirmed by controlled testing. Synergists include Mg^{2+}, Mn^{2+}, MoO_4, vitamin B_2, and vitamin B_6. Antagonists are oxythiamine and pyrithiamine.

Vitamin B_1 is obtained from plant and animal sources, but is not stored to and extent. Up to one-fourth of the human requirement may be available from intestinal bacteria. Human requirements are proportional to the amount of calories

ingested. There is some indication that dietary supplementation of Vitamin B_1 may be required for the normal healthy adult on an average American diet because of cooking losses and refining of cereals and grains. Special conditions requiring supplementation are: pregnancy, lactation, heavy exercise, alcoholism, high carbohydrate intake, processed food diets, deficiency diseases (beriberi, polyneuritis, etc.), old age, gastrointestinal disturbances and antibiotics [189].

Physical and chemical properties [189, 196, 197]

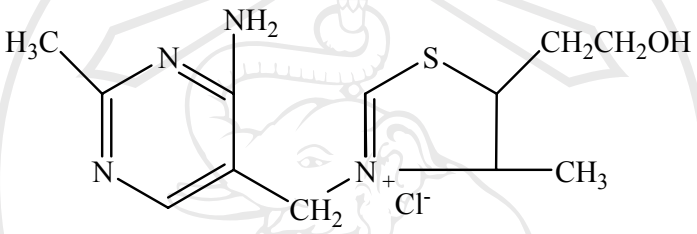

Figure A-5 Structure of Thiamine hydrochloride (vitamin B_1)

Chemical name	:	3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-Hydroxyethyl)-4-methylthiazolium
Common name	:	Vitamin B_1, aneurin
Empirical formula	:	$C_2H_{17}OSCl.HCl$
Molecular weight	:	337.3 (as HCl)
Solubility	:	soluble in Water, slightly sol. in Alcohol
Melting point	:	244°C
Appearance	:	White crystals

Vitamin B_2 (Riboflavin)

Riboflavin (synonymous with vitamin B_2, lactoflavin, vitamin G, hepatoflavin is a yellow-green, fluorescent, water-soluble pigment widely distributed in plant and animal cells [203-207]. It has the following structural formula in Fig.A-6.

Vitamin B_2 functions principally in various coenzyme components of cellular respiration, i.e., the mitochondrial energy system. It is also concerned with

fetal development, as well as maintenance of ectodermal tissues, including the eyes and skin. Its major importance stems from the fact that energy production by the mitochondria cannot proceed without those coenzymes containing vitamin B_2 (FAD, FMN, etc.) needed by the respiratory proteins. There is no major deficiency disease associated with B_2 lack as there is with B_1: a minor disease is cheilosis.

Vitamin B_2 is a slightly water-soluble vitamin, a nucleotide and substituted purine that is not stored in the body. No toxicity has been reported. There are no valid reports of any benefits derived from megadose treatment with vitamin B_2. It is synergistic with vitamin B_1, B_6, Fe^{2+}, Cu^{2+}, and PO_4^{3-}, and antagonized by boric acid and various flavins.

Riboflavin is relatively stable in dry form under normal lighting. Riboflavin is stable in strong mineral acid even at elevated temperatures, and toward most oxidizing agents (H_2O_2, Br_2H_2O, concentrated HNO_3), but it is oxidized by chromic acid. It is also destroyed by $KMnO_4$ in 0.1 N acetic acid in 10 min at room temperature; but at pH 4.5 there is less than 10% destruction by $KMnO_4$ in 10 min [208]. It is unstable in alkaline solutions.

It is very sensitive to both visible and ultraviolet light [208, 209]. One of the distinguishing properties of riboflavin is its yellow-green fluorescence in neutral solutions which reaches a minimum at pH 6.7-6.8.

Vitamin B_2 is obtained from plant and animal sources, but is not stored to any extent in the body and is excreted rapidly. Although it is synthesized by the intestinal bacteria, a very small amount of this form is available. Human requirements are proportional to energy expenditure. There is some evidence the dietary supplementation of riboflavin may be required for a normal, healthy adult on the average American diet, because of its low concentration in the common foods, its low intestinal synthesis, its low solubility (poor absorption), and its destruction by ultraviolet light and alkaline conditions. Special conditions definitely requiring dietary supplementation are: pregnancy, lactation, liver malfunctions, high energy expenditure, antibiotic treatment, fevers, hyperthyroidism, and traumatic stress.

Physical and chemical properties [189, 196, 197]

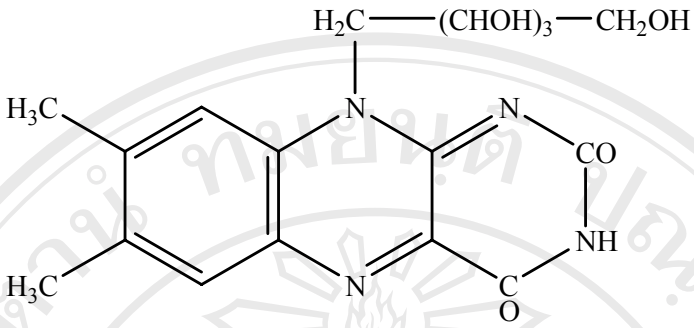

Figure A-6 Structure of Riboflavin (vitamin B_2)

Chemical name	:	1-deoxy-1-[3,4-dihydro-7,8-dimethyl-2,4-Dioxobenzo[g]pteridin-10(2H)-yl]-D-ribitol
Common name	:	Vitamin B_2, vitamin G
Empirical formula	:	$C_{17}H_{20}N_4O_6$
Molecular weight	:	376.4
Solubility	:	soluble in Water, Ethanol
Melting point	:	282°C
Appearance	:	Orange-yellow powder

Vitamin B_3 (Nicotinamide)

Niacin (nicotinic acid or nicotinamide) was one of the first water-soluble vitamins to be isolated and characterized. Chemical, nicotinic acid is pyridine-3-carboxylic acid and nicotinamide is pydine-3-carboxylic acid amide. Nicotinamide functions metabolically as a component of the coenzymes, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes act in many oxidation-reduction reactions and function as hydrogen acceptors of donors. Structure of nicotinamide is presented in Fig. A-7.

Nicotinamide occur as white, needle-shaped crystals or as white crystalline powders. Nicotinamide is more water soluble and stable in the dry from and in

aqueous solutions. They are unaffected by light or pH extremes. Nicotinamide hydrolyzes easily in acid or alkali media.

Nicotinamide is obtained from plant and (mainly) animal sources, and is excreted fairly rapidly. It is not available from intestinal synthesis, but a small amount is available from dietary protein-tryptophan conversion. Human requirements are based on caloric intake. There is no evidence that dietary supplementation is required for a normal, healthy adult on an average American diet. However, many special conditions exist requiring additional supplementation: pregnancy, lactation, high-caloric diets, pellagra, malnutrition, dermatosis, high serum cholesterol, high corn intakes, sprue, and golssitis.

Physical and chemical properties [189, 196, 197]

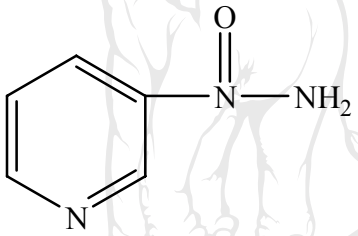

Figure A-7 Structure of Nicotinamide (vitamin B_3)

Chemical name	:	pydine-3-carboxylic acid amide
Common name	:	Vitamin B_3
Empirical formula	:	$C_6H_6ON_2$
Molecular weight	:	122.12
Solubility	:	soluble in Water, Ethyl alcohol
Melting point	:	150-160°C
Appearance	:	White crystalline

Vitamin B_6 (Pyridoxine hydrochloride)

The major functions of vitamin B_6 include participation in the direct line of protein, carbohydrate, and lipid metabolism, as well as its being a coenzyme constituent in amino acid metabolism and in erythrocyte formation. Its major importance stems from the fact that B_6 as pyridoxal phosphate coenzyme controls the various types of amino acid transformations and hence the formation of specialized proteins. There is no major deficiency disease associated with B_6 lack although minor disease of the skin, microcytic hypochromic anemia, and acrodynia are well known.

Vitamin B_6 is a water-soluble vitamin found in all foods of both animal and plant origin. Toxicity has been reported at higher levels (1000 mg/day). The need for vitamin B_6 is well established in all living systems. Plant and microorganisms can synthesize it, but most animals require a dietary source of the vitamin. Structure of pyridoxine are presented in Fig. A-8.

Vitamin B_6 is obtained from plant and animal sources, but is not stored to any extent in the body and is excreted rapidly. Although it is synthesized by the intestinal bacteria only a very small amount of it is available. Human requirements are proportional to the protein content of food. There is some evidence that dietary supplementation of vitamin B_6 may be required for normal, healthy adult on the average American diet because of its low concentrations in common foods, losses on refining of foods, its low intestinal availability, its rapid excretion, its instability to light and oxidation (cooking losses), its synergism with most vitamins as well as minerals, and its central location in the metabolism of major nutrients. Special condition requiring defining supplementation include: pregnancy, lactation, and irradiation, inborn errors of metabolism, high protein diets, and stress.

Physical and chemical properties [189, 196, 197]

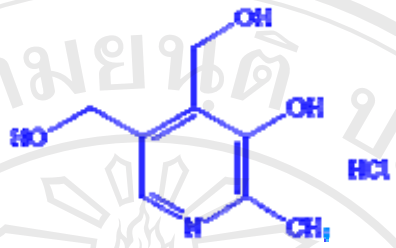

Figure A-8 Structure of Pyridoxine (vitamin B_6)

Chemical name	:	5-Hydroxy-6-methyl-3,4-pyridinedimethanol
Common name	:	Vitamin B_6
Empirical formula	:	$C_8H_{12}ClNO_3$
Molecular weight	:	205.64
Solubility	:	soluble in Water
Melting point	:	150-160°C
Appearance	:	White crystalline

APPENDIX B

Method Choice and Development

The field of vitamin determination is undergoing rapid change. No longer are analysis limited to a few slow biological assays or to chemical methods that are of limited usefulness due to their lack of sensitivity and selectivity. The analyst today is faced with a dazzling array of methods for the determination of vitamins. There are method using liquid chromatograph, gas chromatography, mass spectrometry, infrared, visible, and ultraviolet spectroscopy, enzymes, flow injection analysis, and many others. Recently, there have been an increasing number of methods that use more than one technique, the so-called hyphenated methods, for example, the combination of gas chromatography and mass spectrometry. The problem is that the choice of the appropriate method can become very difficult since most analysts do not have the expertise to evaluate all the available techniques. It is often difficult to assess the appropriateness of any one method even if no others are available. Since most method development studies are not done under the conditions associated with the particular problem of the individual analyst, it is common that analysts will be required to do some method development or modification to solve their current problems.

Successful selection of the appropriate method, the successful development of a new method, or the successful modification of an existing method requires considerable insight into the nature of the problems and a careful use of the available resources. There are very few overviews that suggest the appropriate strategy for the selection and development of methods. Most of these are a few line or pages in general textbooks on analytical chemistry [210]. Yet obviously such strategies are needed for those who do vitamin determinations. It is quite likely that the recent surge in new method development will continue for some years, and that the analysts of the future will be faced with an even more perplexing array of assay methods.

Biological Assay

Biological assays with animals have been indispensable in the development of vitaminology for the isolation, purification, and identification of individual vitamins from natural sources. Physical and chemical assays determine accurately the total amounts of vitamins in food or feed samples but are valid only if the results can be related to biological activity. Traditional biological assays measure the effect on an animal's physiological processes such as reproduction, growth storage in liver, and so on, of adding supplements to a diet lacking in only one vitamin. Other different criteria may now be used, based on the specific roles of these vitamins in metabolic reactions such as energy transfer, regulation of structural units, and so on. Biological assays are inevitably expensive and imprecise and are directly relevant only to the species used and to the dietary conditions in the assay procedure, but they do provide positive evaluations of vitamin potency and bioavailability. The bioassay is still used as a check against all other methods.

Animal Assay

In the growth rate assay for vitamin A in rats, the weanling rats may have enough tissue of vitamin A; so curative assay is done after feeding the animals a vitamin A free diet for a depletion period. The index of depletion is either cessation of weight gain for a certain period or more than a 1 g gain offer a period of 4 days.

Vitamin E deficiency in rats is characterized by a failure of females to produce live young, and the correction of this has proved a more useful measure of vitamin E activity than has weight gain [211].

Weight gain is not satisfactory response in the biological assay of vitamin C using guinea pigs. Most assays are based on changes in the growth rate of other characteristics of their teeth. The preferred method is based on sectioning and staining incisor teeth and measuring the length of the odontoblast cells [212].

Methods now used in the biological assay of these vitamins are listed in Table B-1 with references. It should be easy to modify the procedure to test linearity of response and for variability.

In the early days of vitamin assay, the methods used were mostly biological in nature. Growth rates or the lack of a pathological response were common assay techniques. The elucidation of the chemical structures and the metabolic pathways of the vitamins led to the discovery that given biological response could be stimulated by several chemical compounds. As a result, the general concept was established that a given vitamin activity was associated with a number of chemically closely related compounds. The class of chemically similar compounds that elicited the same qualitative biological response has been called vitamers; for example, the vitamers of vitamin B_6 are pyridoxine, pyridoxal, pyridoxamine, and their respective phosphate esters. The discovery that sometimes even if the vitamers were present, the biological response was absent or was limited, led to the development of the concept of biological availability. Furthermore, it soon became apparent that while vitamers elicit the same qualitative biological response, often the quantitative responses differed with the animal species with the different chemical isomer of a vitamin. Measurement of a given vitamin activity with one species did not necessarily measure its activity in another. Obviously, more effective assay systems were needed. Fortunately, the potential for such systems was available.

Modern bioanalytical chemistry can be said to have started with the development of gas chromatography by Martin and James [213] and the amino acid analyzer by Spackman [214]. Since the invention of these powerful new techniques in the 1950s, the analystical chemistry of the vitamins has expanded explosively. There is now a large literature of new technique for the assay of different vitamins and vitamers. No attempt to review of the current literature will be made since tit has been will covered by the other chapters in this book. Today many analysts use modern separation techniques and are determining the concentration of each separate vitamer in a sample. When the vitamin activity is needed, the quantity of each vitamer is multiplied by its biological potency for the species in question, and then the total activity is obtained by summing the individual activities as exemplified by the work of Slober [215] with vitamin E assay.

Table B-1 Animal Assay Procedures to Measure Vitamin Activity of Foods and Pharmaceuticals

Vitamin	Species: Response Measured	Reference
Vitamin A	Deplated rats: weight gain	[216, 217]
Vitamin D	Deplated rats: bond calcification Normal rats: bone ash Chicks: bone ash, alkaline phosphatase	[218] [219] [220, 221, 222]
Vitamin E	Rats: fetal resorption	[212, 223]
Vitamin K	Chicks: prothrombin times	[224]
Ascorbic acid (vitamin C)	Guinea pigs: length of odontoblast cells In incisor teeth	[212]
Thiamine	Rats: weight gain and enzyme activity	[225]
Riboflavin	Depleted rats: weight gain Chicks: weight gain	[226] [227]
Pantothenic acid	Rats: weight gain Chicks: weight gain	[228, 229] [230]
Pyridoxine (vitamin B_6 complex)	Rats: weight gain and enzyme activity Chicks: weight gain	[231, 232] [227]
Niacin	Rats: weight gain	[233]
Biotin (vitamin H)	Rats: weight gain Chicks: ordinary weight gain assay	[234] [235, 236, 237]
Folacin (Pterylglutamic acid)	Rats: weight gain Rats: levels of vitamin in liver Chicks: weight	[238, 239] [240] [227]
Cobalamin (vitamin B_{12})	Depleted mice: weight gain Depleted chicks: weight gain	[241] [242]

Human Assays

Despite satisfactory results from small-animal assays, it would be wrong to presume that the results must also apply to human. Bioassay with humans is limited are we cannot deliberately induce a vitamin deficiency in subjects just to measure the relative curative effect of dietary supplements, although such experiments have been done in the past and provide unique information [243, 244].

Currently, a combination of physicochemical methods is used to measure the urinary excretion of vitamins and their metabolites, and/or blood vitamin levels to establish the bioavailability [244].

For many water-soluble vitamins, urinary excretion of the vitamin or its metabolites reflects the subject's nutrient status with regard to that vitamin [246]. In other words, a depleted subject shows little excretion but this increases with dosing of the vitamin or any of its precursors, such as tryptophan in the case of niacin. If the vitamin in given in a form that cannot be a absorbed or metabolized, there will not be any corresponding increase in urinary excretion. The response to a test dose is so rapid that the same subject can be used for a succession of tests.

Microbiological Assays

Microbiological methods of vitamin determination are based on the nutritional requirement of a microorganism for a certain vitamin. This allows the formulation of a basal medium that provides all of the growth requirements for the organism except for the vitamin to be assayed. When aliquots of the sample containing the vitamin begin quantitated are added to the initially translucent medium, followed by inoculation with the test organism, the organism reproduces in proportion to the vitamin content, which can be measured photometrically, or the metabolic products can be directly proportional to the amount of vitamin present, and within this range, the sample and reference solutions can be compared accurately.

Compared with biological assay methods using animals, microbiological techniques possess the advantages of minimal requirements of space, labor, materials, and time.

Tetrahymena themophila, previously *T. pyrifomis* WH_{14}, ATCC 3088, is a heterotrophic protozoan that requires thiamin, riboflavin, vitamin B_6, niacin, pantothenic acid, and lipoic acid [247]. Generally, *T. termophila* is as capable as the rat or chick in using conjugates of the water-soluble vitamins [248]. *Tetrahymena* does not require vitamin B_{12}, ascorbic acid, and the fat-soluble vitamins [249]. A few clones require biotin and choline. The inhibitory effect of visible light on the growth of *Tetrahymena* is due to the photodecomposition of thiamin, riboflavin, vitamin B_6, and folic acid. *Tetrahymena* obtains nutrients by diffusion through the cell membrane, pinocytosis, and phagocytosis [247]. Ingested food particles collect in food vacuoles which contain glycosylases, deoxyribomuclease, ribonuclease, proteases, and conjugase.

APPENDIX C

High Performance Liquid Chromatography

Introduction

"Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system [250]." Liquid chromatography (LC), which is one of the forms of chromatography, is an analytical technique that is used to separate a mixture *in solution* into its individual components. As indicated by Tswett, the separation relies on the use of two phases differently relative to the other components in the mixture. High performance liquid chromatography (HPLC) is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column and a detector.

Basic Concepts of HPLC

Chromatography is described and measured in terms of four major concepts: capacity, efficiency, selectivity and resolution. The capacity and selectivity of the column are variables that are controlled largely by the column manufacturer, where as efficiency and resolution can be controlled, to some extent, by chromatographer. To obtain to best possible separation, the efficiency of the chromatographic system must be optimized in order to minimize band broadening (Figure C-1)[251]. The column should have the capacity to retain the solutes, and it should have the appropriate selectivity to resolve the analytes of interest.

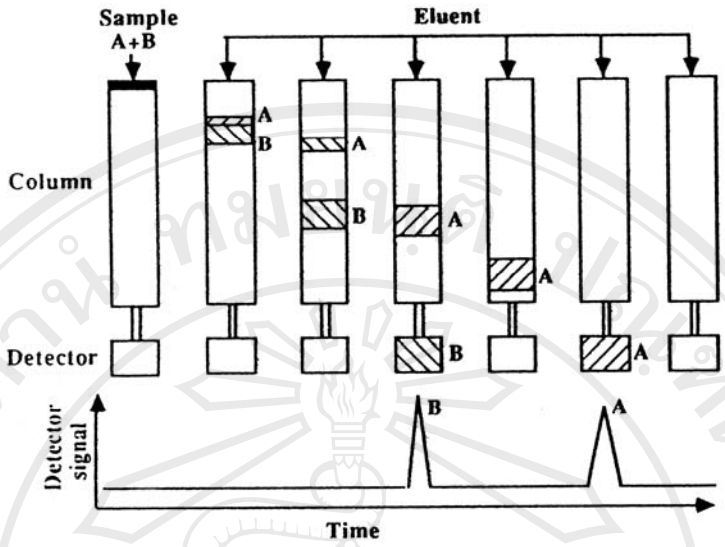

Figure C-1 Separation of two components, A and B. (Reprinted from Ref. [251] with permission.)

Capacity factor

For efficiency liquid chromatographic separation, a column must have the capacity to retain samples and the ability to separate sample components, efficiently. The capacity factor, k'_R, of a column is a direct measure of the strength of the interaction of the sample with the packing material and is defined by the expression.

$$k'_R = \frac{t_R - t_0}{t_0} = \frac{V_R - V_0}{V_0} \qquad (2.1)$$

Where t_R is the time taken for a specific solute to reach the detector (retention time) and t_o is the term taken for non-retained species to reach the detector (holdup time). These terms are illustrated in Figure C-2. The same value for k' is obtained if volumes are used instead of time: V_R is the volume of solution that is pumped through the detector before a specific peak is eluted (retention volume), and V_o is the volume of solvent pumped through the detector between the time of injection and the appearance of the non-retained species (void volume). The void volume is

equal to the volume of the column not occupied by packing material. The capacity factor of a column is mostly a function of the packing material but can be manipulated to a degree by varying the solvent strength. The higher the capacity factor of the column, the greater is its ability to retain solutes. Using a column with a higher capacity factor is often the best way to improve the resolution of a separation. Because a higher capacity factor will also result in longer analysis times, a compromise between resolution and analysis time must be reached. Typically, a *k'* value between 2 and 5 represents a good balance between analysis time and resolution, however, *k'* value between 1 and 10 are usually acceptable.

Selectivity

The selectivity of the chromatographic system is a measure of the difference in retention times (or volumes) between two given peaks and describes how effectively a chromatographic system can separate two compounds (Figure C-3). Selectivity is usually defined in terms of α, where,

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0} = \frac{V_2 - V_0}{V_1 - V_0} = \frac{k'_1}{k'_2} \qquad (2.2)$$

The selectivity of a column is primarily a function of the packing material, although the chromatographer has some control using the mobile phase or temperature. The value for α can range from unity, when the retention times of the two component of interest is eluted in the void volume. If α is approaching, then regardless of the number of theoretical plates or the length of time the components stay on the column, there will be no separation.

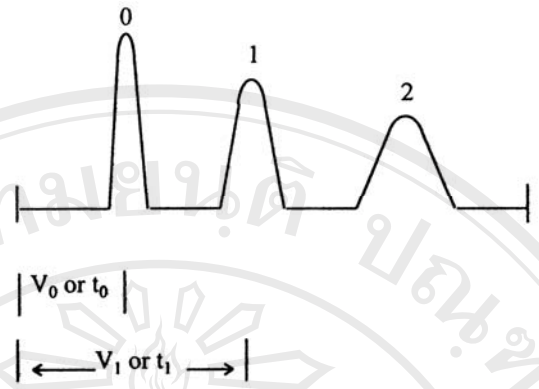

Figure C-2 Chromatographic terms used to calculate column capacity.

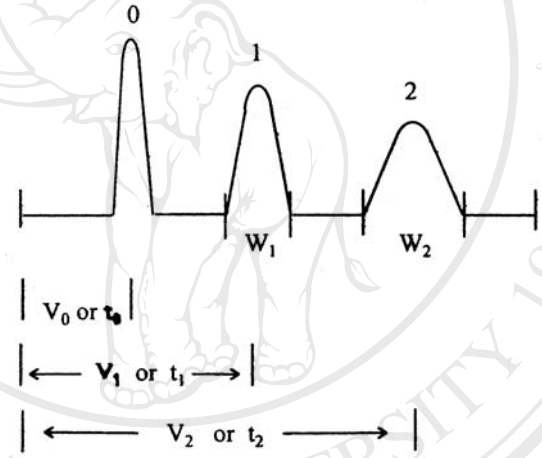

Figure C-3 Chromatographic terms used to calculate selectivity.

The most powerful approach to increasing α is to change the composition of the mobile phase. If changing the concentration of the components in the mobile phase provides insufficient change, altering the nature of one of the components will often be sufficient. Figure C-4 [252] shows the effect on the separation of acetonaphthalene and dinitronaphthalene of changing the mobile phase from 23% dichlorometane / 77% pentane to 5% pyridine / 95% pentane. The α value changes from 1.05 when using dichloromethane to 2.04 when using pyridine.

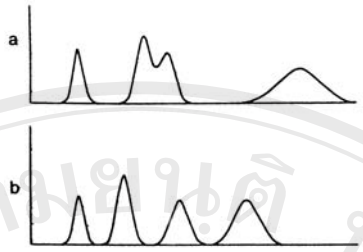

Figure C-4 Effect on selectivity of changing the composition of the mobile phase: (a) 23% dichlorometane / 77% pentane and (b) 5% pyridine / 95% pentane. (Reprinted from Ref. [252] with permission.)

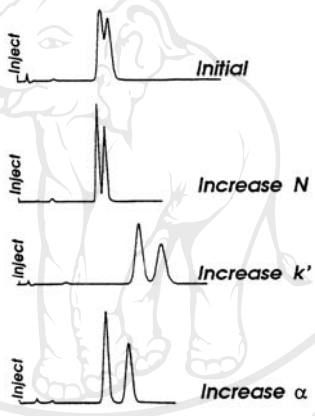

Figure C-5 Effect of selectivity, capacity factor, and efficiency on resolution.

Resolution

Resolution is a term used to describe the degree of separation between neighboring solute bands or peaks. It is affected by the selectivity (α), efficiency (N) and capacity (k') of the column. The resolution equation [Eq. (2.3)] describes the relationship between those factors and indicates how they can be manipulated in order to improve the resolution between two peaks.

$$R = \frac{1}{4}\frac{\alpha - 1}{\alpha}\left(N^{1/2}\right)\frac{k'}{1 + k'} \qquad (2.3)$$

The effect of selectivity, capacity factor, and efficiency on resolution is illustrated in Figure C-5. Typically, an *R* value greater than 0.8 is required for accurate quantification of two peaks. A value of 1, for two equally sized peaks, indicates an overlap of about 2% for one band over the other [253]. Chromatograms of two peaks of unequal sized for given resolution values are shown in Figure C-6 [254].

The most effective way to alter resolution is to change the selectivity or the capacity factor of the column.

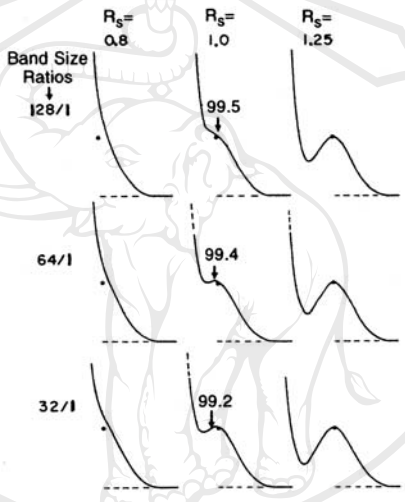

Figure C-6 Standard resolution curves for band-size ratios of 32/1, 64/1 and 128/1 and R_S values of 0.8-1.25. ((Reprinted from Ref. [254] with permission.)

The effect of increasing the efficiency of the column by increasing the column length or flow-rate velocity is less significant, as resolution increases proportionally as the square root of the number theoretical plates. Thus, doubling the number of theoretical plates by adding a second column increases resolution by only a factor of 1.4. If increased resolution is required, a column with a higher capacity factor is often the best choice. However, increasing the capacity factor will increase the analysis time, so a compromise must be reached between resolution and analysis time.

Band broadening and column efficiency

When a sample mixture is fist applied to the head of a column, as illustrated in Figure C-1, the width of the sample is very narrow. By the time the components are eluted from the end of the column, however, the band widths have broadened. This phenomenon occurs because, as the sample mixture moves down the column, the various sample components interact with, and are retained to various degrees by, the stationary phase. This interaction, along with the torturous path of the sample components through the packing material, causes the increase in band width, a process known as band broadening. The amount of band broadening determines, to an extent, the degree to which two components can be separated; thus, band broadening should be kept to a minimum.

The efficiency of a column is a number that describes peak broadening as a function of retention, and it is described in term of the number of theoretical plates, *N*. Two major theories have been developed to describe column efficiency, both of which are used in modern chromatography. The plate theory, proposed by Martin and Synge [255], provides a simple and convenient way to measure column performance and efficiency, whereas the rate theory developed by van Deemter *et al* [256], provides a means to measure the contributions to band broadening and thereby optimize the efficiency.

Plate Theory

The empirical expressions derived in the plate theory are generally applicable to all types of column chromatography. Although the relationships are valid only for Gaussian peak shapes, for convenience they are also generally applied to nonsymmetrical peaks. The major assumption in the plate theory is that there is an instantaneous equilibrium set up for the solute between the stationary and mobile phases. The main criticisms of the plate theory are that it does not consider the effects of band broadening on separation, nor does it consider the influence of

chromatographic variables such as particle size, stationary phase loading, eluent viscosity, and flow rate on column performance [257].

In the chromatographic model proposed in the plate theory, the chromatographic column is considered to consist of a number of thin sections or "plates," each of which allows a solute to equilibrate between the stationary and mobile phases. The greater the number of theoretical plates (N), the more efficient the column is considered to be. The movement of a solute along the column is viewed as a stepwise transfer from one theoretical plate to the next. The thinner the theoretical plates, the greater the number that can be envisaged within a given length of column. These terms are related as follow:

$$H = \frac{L}{N} \tag{2.4}$$

where L is the length of the column (millimeters). Thus, the smaller the height equivalent to a theoretical plate (HETP, or H), the greater is the efficiency of the column. In general, the H value is smaller for small stationary phase particle sizes, low mobile phase flow rates, less viscous mobile phases, higher separation temperatures, and smaller solute molecule sizes.

Efficiency, N, is defined in terms of the retention time (t_R) of the solute, measured at the peak apex, and the standard deviation, σ, of the solute population in the peak measured as the peak width:

$$\mathrm{N} = \left(\frac{t_R}{\sigma}\right)^2 = \left(\frac{t^2{}_R}{\sigma^2}\right) \tag{2.5}$$

where σ for a Gaussian peak is given by

$$\sigma = \frac{W_{50}}{2.345} = \frac{W_T}{4} = \frac{W_{4.4}}{5} \tag{2.6}$$

as shown in Figure C-7, and W is the peak width at different heights on the curve. From the 5σ table, N can be calculated in a number of ways, depending on where the

width is measured. The most commonly used method for the calculation of N is the tangent method, owing to its relative simplicity, but the 5σ method provides the greatest sensitivity to peak tailing. Because of the relative insensitivity to peak tailing, the inflection, peak at half-height, and 3σ method should not be used.

Peak half-height: $$N = 5.54\left(\frac{t_R}{W_{50}}\right)^2 \quad (2.7)$$

Tangent method $$N = 16\left(\frac{t_R}{W_T}\right)^2 \quad (2.8)$$

5σ $$N = 25\left(\frac{t_R}{W_{4.4}}\right)^2 \quad (2.9)$$

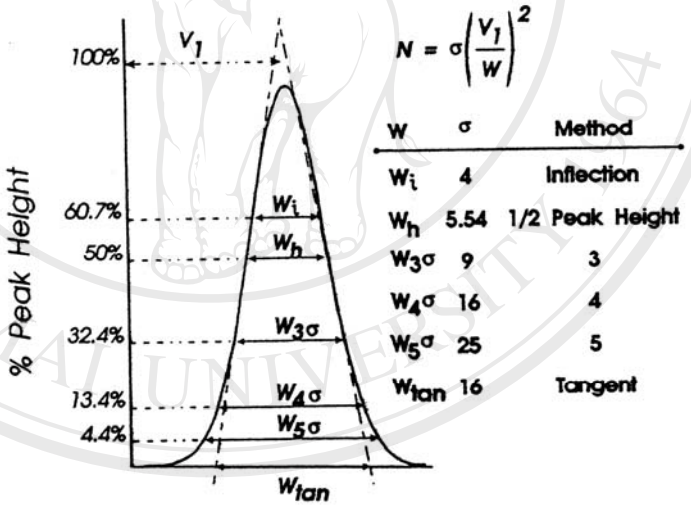

Figure C-7 Methods for estimating the standard deviation (σ), and 5σ table. (Reprinted from Ref. [258] with permission.)

The efficiency can be varied by changing physical column parameters such as the length, diameter, and construction material of the container of the column. It can also be varied by changing chemical parameters such as the size of the particles constituting the packing material or the mobile phase velocity.

Rate theory and band broadening

There are three predominant mechanisms for transport of a solute through a chromatographic column: (1) convective transport in the mobile phase as it flows between the particles in the column, (2) diffusive transport through stagnant pools of liquid in the column packing, and (3) perfusive transport through the porous particles. The rate theory developed by van Deemter *et al* [256], and later modified by others [259, 260, 261], considers the diffusional factor that contribute to band broadening in the column (column variance, σ^2_{col}) and avoids the assumption of an instantaneous equilibrium inherent in the plate theory.

In its most general form, the van Deemter equation may be written

$$H = A + \frac{B}{\mu} + C\mu \qquad (2.10)$$

where H represents the efficiency of the column and μ represents the average linear velocity of the mobile phase. The A term represents the contribution to band broadening by eddy diffusion, the B term represents the contribution from longitudinal diffusion, and the C term represents the contribution from resistance to mass transfer. Diffusion is not restricted to transport of solute particles through stagnant pools of liquid in the stationary phase, however, but also occurs as the solute is carried by convective transport between the particles in the column. Huber [262] found that there were at least four terms that should be considered to describe column efficiency adequately, and the contribution of these factors to efficiency is described in the modified van Deemter equation:

$$H = A + \frac{B}{\mu} + C_s\mu + C_m\mu \qquad (2.11)$$

In Eq. (2.11) the C term represents the contributions to zone broadening from resistance to mass transfer in the stationary phase and the mobile phase, respectively.

Because H represents the column variance or band broadening, the value for H should be kept to a minimum. One way to determine the experimental conditions that will give minimum zone dispersion and maximize efficiency is provided by the used of a van Deemter plot. A van Deemter plot, as shown in Figure C-8, is a graph of plate height versus the average linear velocity of the mobile phase. The data are determined experimentally using measured values for retention time, void volume or

dead time, and peak width to determine *N* and hence *H* at various flow rates. According to the plot, at flow rates below the optimum the overall efficiency is dependent on diffusion effects (the *B* term). At higher flow rates the efficiency decreases because the mass transfer, or *C*, terms become more important. In the plot shown in Figure C-8, the *A* term is a constant, independent of flow rate. Despite the reduced efficiency at higher flow rates, it is common to operate the system at high flow rates to save time and operating costs; thus, the plot may also be used to determine the best conditions to minimize the analysis time consistent with an acceptable value for *H*.

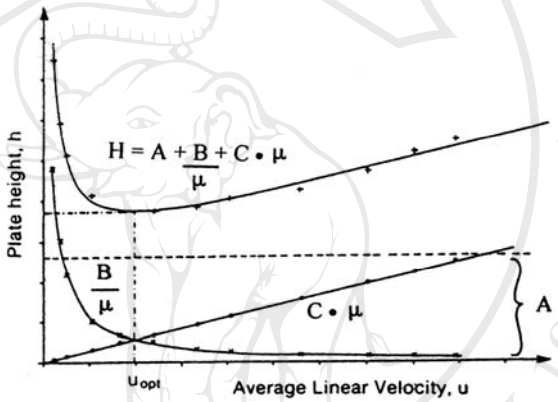

Figure C-8 Hypothetical van Deemter plot showing the relationship between efficiency and average linear velocity of the mobile phase.

A. Eddy Diffusion As a solute molecule passes through the column, it can follow a variety of different paths around the stationary-phase particles, as illustrated in Figure C-9. Each of the paths will be of a different length, so that as solute molecules of the same species follow different paths, they will arrive at the outlet of the column at different times. This form of diffusion is known as eddy diffusion and is represented by the *A* term of the van Deemter equation.

In practice, the solute molecules are not fixed in a single path but can diffuse laterally into other channels, thus decreasing the contribution to band broadening from eddy diffusion. This band broadening process is dependent completely on the stationary phase and is independent of the flow rate of the mobile phase. It can be minimized if the column is packed uniformly with particles of constant size.

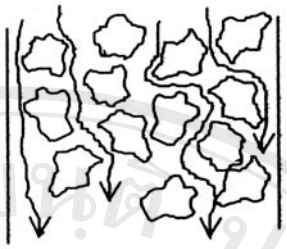

Figure C-9 Eddy diffusion.

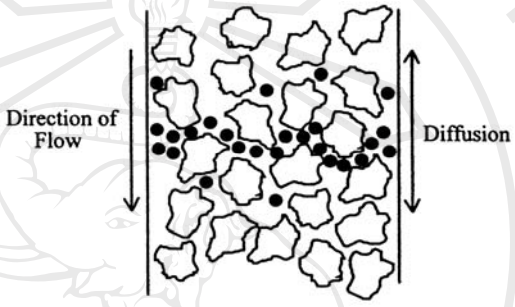

Figure C-10 Longitudinal diffusion.

B. Longitudinal Diffusion In chromatography, the sample mixture ideally travels through the column as tight zones of individual sample components separated by zones of mobile phase, or as regions of high solute concentration separated by regions of high solvent concentration. Whenever a concentration gradient exists, however, diffusion of molecules will occur, from a region of high concentration to a region of low concentration (Figure C-10). This form of diffusion is known as longitudinal diffusion and is represented by the *B* term of the van Deemter equation. It is related only to the mobile phase and is independent of the stationary phase.

Longitudinal diffusion occurs in all directions; the molecules at the front of the zone will move forward into the next zone, the molecules at the end of the zone will fall back into the previous zone, and diffusion will also occur toward the column walls. As diffusion is a time-dependent process, the longitudinal diffusion effect increases at low mobile-phase flow rates.

C. Resistance to Mass Transfer In the plate theory, it was assumed that the transfer of solute molecules between the mobile phase and the stationary phase was instantaneous. In the rate theory, it is accepted that there is a finite rate of mass

transfer. In addition, molecules of the same species may spend different lengths of time in the stationary and mobile phases (Figure C-11). Resistance to mass transfer is represented by the *C* term of the van Deemter equation.

If the time required for mass transfer is much greater than the time required for the solute molecules to flow over the surface of the packing material, some molecules in the convective stream can move down and out of the column before diffusion of others into and out of the centers of the particles can be completed. Thus, with conventional porous packing materials, increasing the flow rate reduces resolution and capacity.

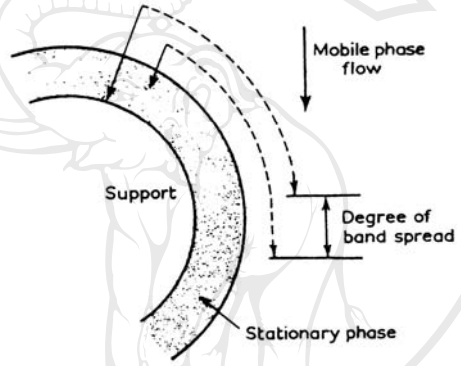

Figure C-11 Stationary-phase mass transfer.

D. Stagnant Mobile Phase Stagnant mobile-phase mass transfer has been identified as one of the major contributors to peak dispersion in liquid chromatography [252, 263, 264] and is also represented by the *C* term of the van Deemter equation. As shown in Figure C-12, the presence of immobile solvent trapped either between particles of packing material or in the pores within the particles provides the means for solute particles to diffuse right into the stagnant pool in the pore, effectively becoming trapped. Thus, column variance may be reduced by using short columns, uniformly packed with small-diameter particles of constant size.

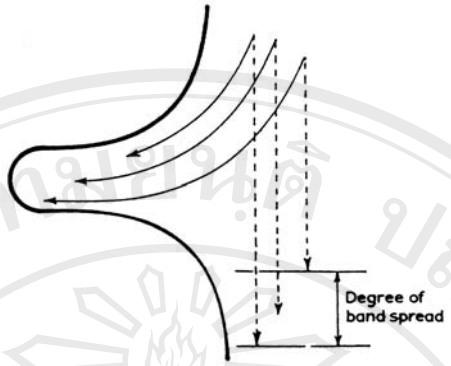

Figure C-12 Stagnant mobile-phase mass transfer.

E. Extracolumn Band Broadening or Variance To maximize the effective number of theoretical plates, the contribution of the entire chromatographic system to band broadening (system variance, σ^2_{sys}) must be minimized. The system variance may be broken down into contributions from the column variance, σ^2_{col}, as described above, and extracolumn diffusion and mixing processes, σ^2_{ex}. As with the case of the column variance, extracolumn variance is an additive property and may be broken down into the major components:

$$\sigma^2_{ex} = \sigma^2_{inj} + \sigma^2_{det} + \sigma^2_{t} \qquad (2.12)$$

where σ^2_{inj} is the injection volume variance, σ^2_{det} is the detector variance, and σ^2_{t} is the variance contribution from the tubing in the chromatographic system. The maximum injection volume has been calculated by Guiochon and Colin [265] and is dependent on the column length and the packing material diameter.

The detector variance is a sum of the variances due to the detector cell volume and the detector time constant. The detector cell volume is dependent on the column length and the diameter of the packing material [265] and should be no greater than 0.1 times the peak band volume (4σ) [266]. As a detector cell volume becomes larger, the efficiency of the column, *N*, decreases. This effect is most dramatic for the early eluting peaks. Because the solute passes through the detector cell quickly, the detector time constant must be fast enough to allow the detector to respond to the brief presence of the solute in the flow cell. If the time constant is too

high, resolution and sensitivity are compromised [267], as illustrated in Figure C-13. The setting of the time constant is dependent on the length of the column and the diameter of the packing material [268].

The final contribution to extra-column variance, namely, the contribution from the tubing in the chromatographic system, is a function of both the length and more importantly the radius of the tubing [269]. At high flow rates and with short lengths of narrow-bore tubing, less band broadening is seen than would have been expected [270, 271]; thus in practice, when a well constructed chromatographic system is used with a column containing uniformly packed, small-diameter particles, the most significant contribution to band broadening comes from the variance arising from mass transfer effects.

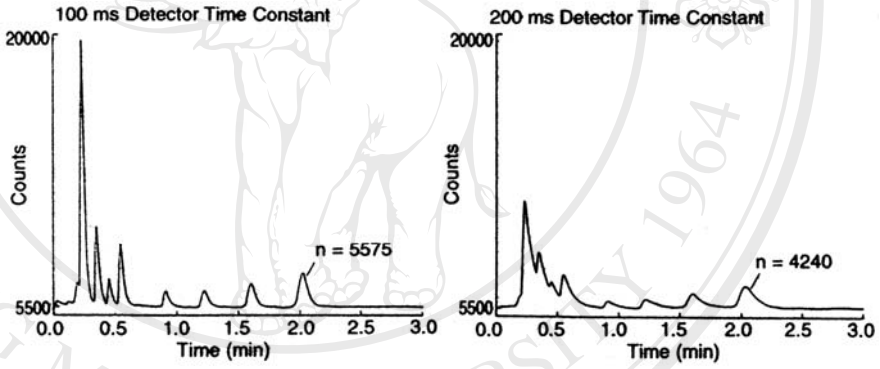

Figure C-13 Effect of detector time constant on resolution, system efficiency, and sensitivity: (a) 100-msec, (b) 200-msec. Flow cell volume was 2.4 mL, and both chromatograms were recorded at the same sensitivity. (Reprinted from Ref. [267] with permission.)

High Performance Liquid Chromatography [272, 273]

Type of chromatography

Liquid chromatographic methods may be classified according to either the mechanism by which analytes are retained on the column or the mechanism by which they are removed from it. The mechanism of retention classification is the most popular scheme, and five major retention mechanisms have been identified:

adsorption,
partition,
ion exchange,
affinity, and
size exclusion.

Chiral Chromatography

In “chiral” separations enantiomers are separated on the basis of stereoselectivity. Chiral chromatography has developed with the need for racemically pure drugs. Many chiral drugs have been used as their racemates because of difficulties in stereoselective synthesis and purification. As only one of the enantiomers may exhibit the pharmacological effect and the other may even show side effects, the development of analytical methods for the separation and determination of enentiomers for drug use and in biological samples, such as serum and plasma, is an area of increasing interest and practical utility.

Ion-exchange Chromatography

In ion-exchange chromatography (IEC), species are separated on the basis of differences in electric charge. The primary mechanism of retention is the electrostatic attraction of ionic solutes in solution to “fixed ions” of opposite charge on the stationary phase support. The stationary phase or ion exchanger is classified as an anion-exchange material when the fixed ion carries a positive charge and as a cation exchanger when it carries a negative charge.

A specialized form of IEC is ion chromatography (IC), which is the name applied to the analysis of inorganic anions, cations, and low molecular weight, water-soluble organic acids and bases. Although any HPPL technique used to separate the above species can be termed ion chromatography, in general IC involves the use of ion-exchange columns and a conductivity detector. Ion chromatography itself can be subclassified. Suppressed IC involves the use of a membrane device, known as a suppressor, between the column and the detector to lower the response of the eluent

and thereby enhance the signal from the solute; nonsuppressed or “single-column” IC does not contain a suppressor.

Mechanism of Retention

As an ionic solute passes through the column, it distributes itself between the mobile phase and the stationary phase by exchangeing with the counterions associated with the stationary phase. As the electroneutrality of the solution must be maintained during the ion-exchange process, the exchange is stoichiometric: a single monovalent solute ion displaces a single monovalent counterion. Separation occurs as a consequence of differences in the size, charge density, and structure of the difference ionic solutes.

The equibrium constant for the exchange reaction is known as the selectivity coefficient and provides a rough means for predicting the elution order of the various ions. In general, the selectivity coefficients increase with increasing polarizing power of the solute ions. Thus, ions with high charge and a small radius of hydration should have the greatest affinity for the ion exchanger. Although this tends to be true, occasionally ion exchange is not the only operative mechanism of retention.

Stationary Phases for Ion-Exchange Chromatography

Ion exchangers are characterized both by the type of support and by the functional group providing the charge. Functionalized silica and synthetic polymeric resins are the most common supports, although some inorganic materials are sometimes used. Synthetic polymeric resins are typically styrene-divinylbenzene or methacrylic acid-divinylbenzene copolymers treated with an appropriate reagent to produce the desired functional group. The major drawback to the use of a silica support is the pH limitation imposed by the instability of silica at high and low pH (i.e., above pH 8 and below pH 2). Synthetic polymers may be used over a much wider pH range and are often used for the analysis of carbohydrates at pH 12 or above. Synthetic polymers, however, may suffer from a degree of swelling when in contract with aqueous mobile phases, depending on the composition of the eluent.

Mobile Phases for Ion-Exchange Chromatography

Mobile phase, or eluents, in IEC are aqueous solutions of a salt or mixture of salts, often with a small percentage of an organic solvent added. The salt mixture may be a buffer, or a buffer may be added if required. The main component of the eluent is the competing ion that causes the solute ions to be eluted.

In nonsuppressed IC, eluent competing ions of low limiting equivalent ionic conductance, such as carboxylate, are required. In suppressed IC, the mechanism of suppression dictates the choice of an eluent. In the case of suppressed anion-exchange separations, cations in the eluent are replaced by hydronium ions from the anion suppressor. These hydronium ions react with the eluent anion to form an undissociated weak acid (e.g., bicarbonate forming carbonic acid), thereby reducing the conductivity of the eluent. In suppressed cation-exchange chromatography anions from the cation seppressor are replaced by hydroxide ions which react with the eluent cations to form an undissociated weak base (e.g., with hydrogen ions to form water). In suppressed IC, therefore, eluent competing ions that can be easily neutralized in an acid-base reaction, such as carbonate or mineral acids, are used.

Selectivity in the separation of ionic solutes may be varied by changing either the pH of the mobile phase or the nature or concentration of the displacing ions. The pH of the eluent affects not only the ionic form in group on the ion-exchange resin. The nature and concentration of the displacing ion will determine the ease with which solute ions are displaced; the more concentrated the competing ion in the eluent, the more effectively it will displace soluted ions from the stationary phase.

Ion-Pair Chromatography

The analysis of strong acids of strong bases using reversed-phase columns is typically accomplished by the technique known as ion-pair chromatography (also commonly called paired-ion or ion-interaction chromatography). In this technique, the pH of the eluent is adjusted in order to encourage ionization of the sample; for acids pH 7.5 used, and for bases pH 3.5 is common. Retention is then altered by including in the mobile phase a bulky organic molecule having a charge opposite

from that of the ion to be analyzed. The counterion is the ion-pairing reagent. Three basic models have been proposed to describe the ion-pair mechanism: the ion-pair model, the dynamic ion-exchange model, and the ion-interaction model.

The ion-pair model postulates that because the ion-pairing reagent contains bulky organic substituents the ion pair, which is formed in the mobile phase between the solute and the ion-pairing reagent, is hydrophobic in character and will therefore adsorb onto the hydrocarbon stationary phase. The longer the alkyl chain on the pairing agent, the less polar is the ion pair, the greater is the affinity of the ion pair for the stationary phase, and the longer is its retention. The dynamic ion-exchange model proposes that it is the unpaired organic counterion that adsorbs to the surface of the nonpolar stationary phase, forming a dynamic equilibrium between ion-pairing reagent in the mobile phase and ion-pairing reagent adsorbed to the surface of the stationary phase. This interaction cause the column to behave as an ion exchanger, and sample ions are therefore separated on the basis of conventional ion-exchange mechanisms.

Normal-Phase Chromatography

The term “normal phase” is used to denote a chromatographic system in which a polar stationary phase is used for elution of the analytes. In the normal-phase mode, neutral solutes in solution are separated on the basis of their polarity; the more polar the solute, the greater is its retention on the column. Since the mobile phase is less polar than the stationary phase, increasing the polarity of the mobile phase results in decreased solute retention.

Although normal-phase chromatography can be performed using either partition or adsorption mechanisms, the dominant retention mechanism is adsorption. As a consequence, normal-phase chromatography is also known in the literature as adsorption chromatography or liquid-solid chromatography. The stationary phase is polar, typically as a result of hydroxyl groups (-OH); thus, if a neutral solute molecule

has a permanent dipole, or if a dipole can be induced on it, then it will be attracted by dipole-dipole interaction the stationary phase surface. In adsorption chromatography, sample retention is directly proportional to the surface area of the stationary phase. The surface area should be kept below 400 m^2/g, however, because higher surface areas can be achieved only at the expense of smaller pores. Pores that are too small lead to poor mass transfer and lower column efficiencies [273].

Mechanism of retention

Two models have been developed to describe the adsorption process. The fist model, known as the competition model, assumes that the entire surface of the stationary phase is covered by mobile phase molecules and that adsorption occurs as a result of competition for the adsorption sites between the solute molecule and the mobile-phase molecules [273]. The solvent interaction model, on the other hand, suggests that a bilayer of solvent molecules is formed around the stationary phase particles, which depends on the concentration of polar solvent in the mobile phase. In the latter model, retention results from interaction of the solute molecule with the secondary layer of adsorbed mobile phase molecules [274].

Stationary phases for Normal-Phase Chromatography

The typical stationary phases employed in normal-phase or adsorption chromatography are common porous adsorbents, such as silica and alumina, that have polar hydroxyl groups on the surface. Silica is the preferred stationary phase owing to ready availability, low cost, and known performance. For basic compounds such as amines, which are very strongly retained on silica, however, it may be advantageous to used alumina. In addition to the porous adsorbents, a variety of polar bonded phases exist in which functional groups, such as cyano [$-(CH_2)_5C{\equiv}N$], diol[$-((CH_2)_5OCH_2CH(OH)CH_2OH$] and amino groups [$-(CH_2)_nNH_2$, where *n* is 3 or 4] are chemically bonded to the silica stationary phase. These functional groups are

significantly less polar than the silanol group (-SiOH) and therefore result in less retention than is seen with the silica and alumina columns.

Mobile phase for Normal-Phase Chromatography

The mobile phase used in normal-phase chromatography are based on nonpolar hydrocarbons, such as hexane, heptane, or octane, to which is added a small amount of a more polar solvent, such as 2-propanol [275]. Solvent selectivity is controlled by the nature of the added solvent. Additives with large dipole moments, such as methylene chloride and 1,2-dichloroethane, interaction preferentially with solutes that have large dipole moments, such as nitro- compounds, nitriles, amines, and sulfoxides. Good proton donors such as chloroform, *m*-cresol, and water interact preferentially with basic solutes such as amines and sulfoxides, whereas good proton acceptors such as alcohols, ethers, and amines tend to interact best with hydroxylated molecules such as acids and phenols.

Reversed-Phase Chromatography

Reversed-phase chromatography, the most widely used chromatographic mode [273], is used to separate neutral molecules in solution on the basic of their hydrophobicity [276]. As the name suggests, reversed-phase chromatography (often referred to as RP chromatography) is the reverse of normal-phase chromatography in the sense that it involves the use of a non-polar stationary phase and a polar mobile phase. As a result, decreases in the polarity of the mobile phase result in a decrease in solute retention. Modern reversed-phase chromatography typically refers to the use of chemically bonded stationary phases, where a functional group is bonded to silica, as illustrated in Figure C-14. For this reason, reversed-phase chromatography is often referred to in the literature as boned-phase chromatography. Occasionally, however,

polymeric stationary phases such as polymethacrylate or polystyrene, or solid stationary phases such as porous graphitic carbon, are used.

Figure C-14 Reaction of silica gel with a functional group to produce a reversed-phase stationary phase.

Mechanism of retention

Two main theories, the so-called solvophobic and partitioning theories, have been developed to explain the separation mechanism on chemically bonded, non-polar phase, as illustrated in Figure C-15 In the solvophobic theory the stationary phase is thought to behave more like a solid than a liquid, and retention is considered to be related primarily to hydrophobic interactions between the solutes and the mobile phase [277, 278, 279] (solvophobic effects). Because of the solvophobic effects, the solute binds to the surface of the stationary phase, thereby reducing the surface area of analyte exposed to the mobile phase, adsorption increases as the surface tension of the mobile phase increases [280]. Hence, solutes are retained more as a result of solvophobic interaction with the mobile phase than through specific interactions with the stationary phase.

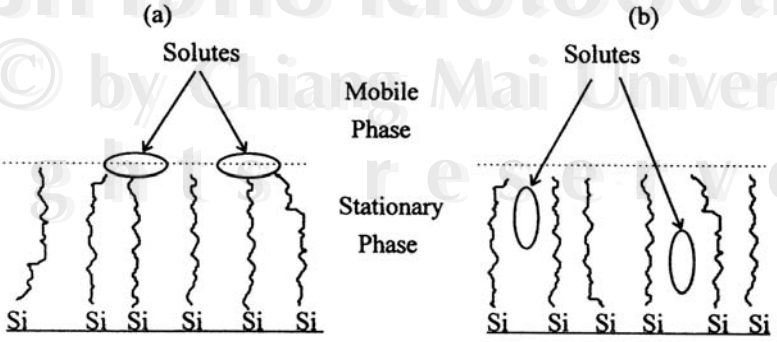

Figure C-15 (a) Solvophobic and (b) partitioning models of solute retention.

In the partitioning model of retention, the stationary phase plays a more important role in the retention process [281, 282]. The solute is thought to be fully embedded in the stationary phase chains, rather than adsorbed on the surface, and therefore is considered to be partitioned between the mobile phase and a "liquid-like" stationary phase. Although the exact mechanism of retention on chemical bonded, non-polar phases is still a matter of debate, there is general agreement that as the chain length of the bonded material increases, the retention mechanism approaches a partitioning mechanism; with shorter chain lengths, the retention mechanism becomes more similar to the adsorption mechanism [283]. Needless to say, depending on the nature of the bonded phase, the mechanism is probably a combination of both those described above.

Stationary phase for Reversed-Phase Chromatography

The most common stationary phases in reversed-phase chromatography are those in which a functional group is chemically attached to a silica support (boned phases). The most popular boned phases are the alkyl groups, such as $-CH_3$, $-C_4H_9$, $-C_8H_{17}$, and $-C_{18}H_{37}$, phenyl ($-C_6H_5$) groups, cyano [$(-CH_2)_3CN$] groups, and amino [$(-CH_2)_3NH_2$] groups, with retention increasing exponentially with chain length. The performance of the bonded phases is determined by four factors: (1) the base silica and its pretreatment, (2) the choice of functional group, (3) the amount of material bonded to the silica (carbon load), and (4) secondary bonding reactions (end capping).

The amount of carbon introduced into the stationary phase by the functional group is referred to as the carbon load, and it is measured as a weight percentage of the bulk silica packing. The carbon load is altered by changing the functional group; the higher the carbon load, the greater is the reversed-phase retention. For steric reasons, as illustrated in Figure C-16, it is not possible for all the silanol groups on the silica surface to react with the functional groups, and usually only about 45% of the silanols will be bonded [284]. Residual, unreacted, acidic silanol groups can cause

tailing of basic solutes such as amines, owing to a mixed adsorption / partition retention mechanism. Thus, unreacted silanols are ofetn removed by treatment with a small silating agent, such as trimethychlorosilane ($Si(CH_3)_3Cl$), a process known as end-capping.

The functional group affects not only the carbon load, but also column selectivity and efficiency; the nature of the functional group controls selectivity, while the chain length controls column efficiency. Although shorter chains result in more efficient columns, the sample capacity decreases with decreasing chain length. Thus a good general purpose RP column is one packed with 3-5 µm spherical silica and a pore size of 60-120 A^o, which has C_{18} functional groups, has a carbon load of 7-10%, and is end-capped.

A few polymeric reversed-phase stationary phases are available which provide the advantage that they can be operated over a wider pH range than the silica-based columns. Polymeric columns, however, tend to be less efficient than silica-based ones and are often less retentive.

Mobile phase for Reversed-Phase Chromatography

The mobile phases used in reversed-phase chromatography are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. Solvent selectivity is controlled by the nature of the added solvent in the same way as was described for normal-phase chromatography; solvents with large dipole moments, such as methylene chloride and 1,2-dichloroethane, interact preferentially with solutes that have large dipole moments, such as nitro- compounds, nitriles, amines, and sulfoxides. Solvents that are good proton donors, such as chloroform, *m*-cresol, and water, interact preferentially with basic solutes such as amines and sulfoxides, and solvents that are good proton acceptors, such as alcohols, ethers, and amines, tend to interact best with hydroxylated molecules such as acids and phenols.

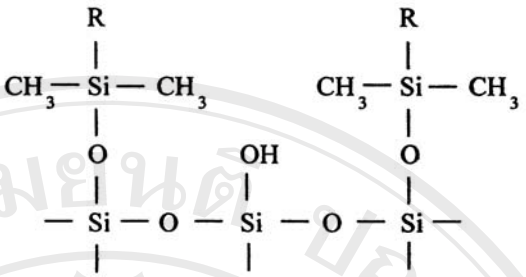

Figure C-16 Silica surface following reaction with a functional group but prior to end capping.

Size Exclusion Chromatography

Size-exclusion chromatography (SEC) is a convenient and highly predictable method for separating simple mixtures whose components are sufficiently different in molecular weight. For small molecules, a size difference of more than about 10% is required for acceptable resolution; for macromolecules a twofold difference in molecular weight is necessary. Size-exclusion chromatography can be used to indicate the complexity of a sample mixture and to provide approximate molecular weight values for the components. It is an easy technique to understand, and SEC can be applied to the separation of delicate biomacromolecules as well as to the separation of synthetic organic polymers.

In SEC species in solution are separated on the basis of their molecular size, which in turn is related to the logarithm of the molecular weight. There are two modes of size-exclusion chromatography: gel-filtration chromatography (GFC) and gel-exclusion chromatography (GPC). In GFC, aqueous mobile phase and hydrophilic packing are used to separate and identify biological macromolecules. On the other hand, GPC is usually performed using hydrophobic stationary phases and organic mobile phases to obtain molecular weight distribution information on polymers. Because the solutes are all eluted within a small retention volume, peaks in SEC are generally narrow, thereby enhancing sensitivity and allowing the use of relatively insensitive detection methods such as refractive index (RI) detection.

Refractive index detection is particularly suitable for GPC, as many polymers have no chromophores or other detectable properties.

Machanism of Retention

The stationary phase in SEC is a highly porous substrate whose pores are penetrated best by small solute molecules. Because larger solute molecules are unable to enter as deeply into the pores, they will travel further down the column in the same time. The largest molecules, which are totally excluded from the pores, are eluted first from the column. Because the solvent molecules are usually the smallest, they are normally the last to be eluted. The rest of the solute molecules are eluted between these two extremes, at a time dependent on their ability to penetrate into the pores.

Stationary Phases for Size-Exclusion Chromatography

There are two classes of stationary phases in SEC, one type for GFC and the other for GPC. Stationary phases for GFC are hydrophilic and include polydextrans, polyvinyl alcohol gel, and silica gel; those for GPC are hydrophobic, typically cross-linked, rigid polystyrene-divinylbenzene gels. Generally, columns of 15 to 50 cm length are used, packed with 7- to 10- μm particles and with an internal diameter between 0.6 and 0.8 cm. In SEC, unlike in other chromatographic modes, the stationary phase is the primary factor controlling retention.

Small molecules (molecular weight < 5000) are separated using columns with the smallest pore size (60 – 100 Å). A column containing a packing material with a single nominal pore size is capable of separating a molecular weight range of about two orders of magnitude. By placing two columns of the same pore size in series, peak resolution can be improved; by placing columns with different pore size in series, the range of molecular weights that can be separated may be extended.

Mobile Phases for Size-Exclusion Chromatography

Mobile phases for SEC fall into two broad categories: aqueous buffer for GFC and organic solvents for GPC. In SEC, the mobile phase is selected not to control selectivity but for its ability to dissolve the sample. In addition, the mobile phase should have a low viscosity and be compatible with the detector and column packing. For example, polar solvents such as methanol and ethanol should not be used with polystyrene packings since they cause excessive shrinkage which will result in permanent damage to the column; instead, solvents such as toluene or chloroform should be chosen. Silica gels can be used with a wide range of solvents, including water, but they are limited to an operating range of pH 2-8.

Instrumentation

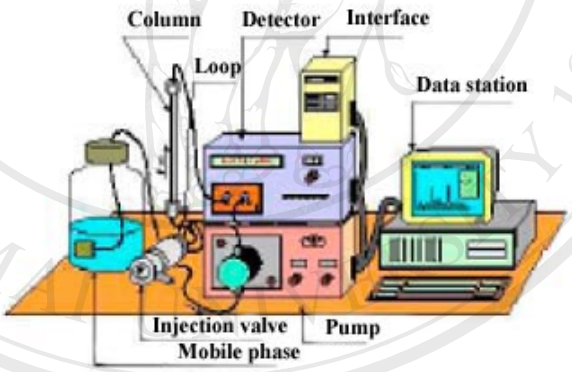

Figure C-17 HPLC instrumentation.

HPLC instrumentation includes:

- Pump
- Injection
- Column
- Detector
- Recorder or data system

Mobile phase reservoir, filtering

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with the special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium was found not to be sufficient for degassing of aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere.

Pump

High pressure pumps are needed to force solvents through packed stationary phase beds. However, many separation problems can be resolved with larger particle packing that requires less pressure. Flow rate stability is another important pump feature that distinguishes pumps. For most types of separation stable flow rate is not very important. However, for size exclusion chromatography the flow rate has to be extremely stable. External electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. Modern pumps have the following parameters:

- Flow rate range: 0.01 to 10 $mL.min^{-1}$
- Flow rate stability: not more than 1% (short term)
- For size exclusion chromatography flow rate stability should be less than 0.2%
- Maximum pressure: up to 5000 psi (345 bar, 340 atm)

It is desirable to have an integrated degassing system, either helium purging, or better vacuum degassing.

Column

Typical analytical columns are 10, 15 and 25 cm in length and are fitted with extremely small diameter (3, 5 or 10 µm) particles. The internal diameter of the columns is usually 4 or 4.6 mm; this is considered the best compromise among sample capacity, mobile phase consumption, speed and resolution. Preparative columns are of larger diameter. Packing of the column tubing with the small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase repacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment. In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new, and to retain the chromatogram. If questionable results are obtained later the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.

Detector

Optical detectors are most frequently used. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~10 mL) flow cell. The most commonly used detector in LC is the ultraviolet absorption detector. A variable wavelength detector of this type, capable of monitoring from 190 to 460-600 nm, will be found suitable for the detection of the majority samples. Other types of detectors:

Fluorimetric detectors are generally available and offer the advantages of higher sensitivity and greater selectivity than UV detection. The high sensitivity of

this detector, which is typically on the order of 10^{-11} g, has resulted in its extensive use in environmental and biomedical analysis.

Refractive index detectors are very sensitive to changes in temperature and solvent composition. This type of detection is limited strictly to isocratic solvent systems. There are very few HPLC procedures for pharmaceutical analysis, which utilize the refractive index detector. It is often a detection device of choice for use in preparative HPLC system.

Mass spectrometry (MS) is the ideal detector for chromatography because of its high sensitivity and its ability to provide structural information. MS is especially useful for analyzing the molecular weight and composition of oligomers, for identifying chain-end groups and for determining the composition and molecular weight of unknown or multicomponent polymeric materials.

Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where sample introduction is done with the help of autosamplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, loss in efficiency or all of these. It is always best to remove particles from the sample by filtering, or centrifuging since continuous injections of particulate material will eventually cause blockage of injection devices or columns. Sample sizes, typical sample mass with 4.6 mm I.D. columns range from the nanogram level up to about 2 mg diluted in 20 ml of solvent. In general, it will be noted that much less sample preparation is required in LC than in GC since unwanted or interfering compounds, or both, may often be extracted, or eliminated, by selective detection.

Data system

The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention. In routine analysis, where no automation (in terms of data management or process control) is needed, a preprogrammed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs:

- Additional automation options become easier to implement;
- Complex data analysis becomes more feasible;
- Software safeguards can be designed to reduce accidental misuse of the system.

For example, the controller can be set to limit the rate of solvent switching. This acts to extend column life by reducing thermal and chemical shocks. In general, these standalone, user programmable systems are becoming less expensive and increasingly practical. Other more advanced features can also be applied to a chromatographic system. These features include computer controlled automatic injectors, multi-pump gradient controllers and sample fraction collectors. These added features are not found on many systems, but they do exist, and can save much time and effort for the chromatographer.

VITA

Name: Mr. Chonlayut Raweewan

Date of Birth: September 21, 1975

Academic Status:

-High school certificate, Donmuangthahanargardbumrung School, Bangkok.

- B.S. (Chemistry), Pibulsongkam Rajabhat University, Phitsanulok, 1999.

Work Experience: General Hospital Product., Ltd. Co. Bangkok, 1999 – 2001.