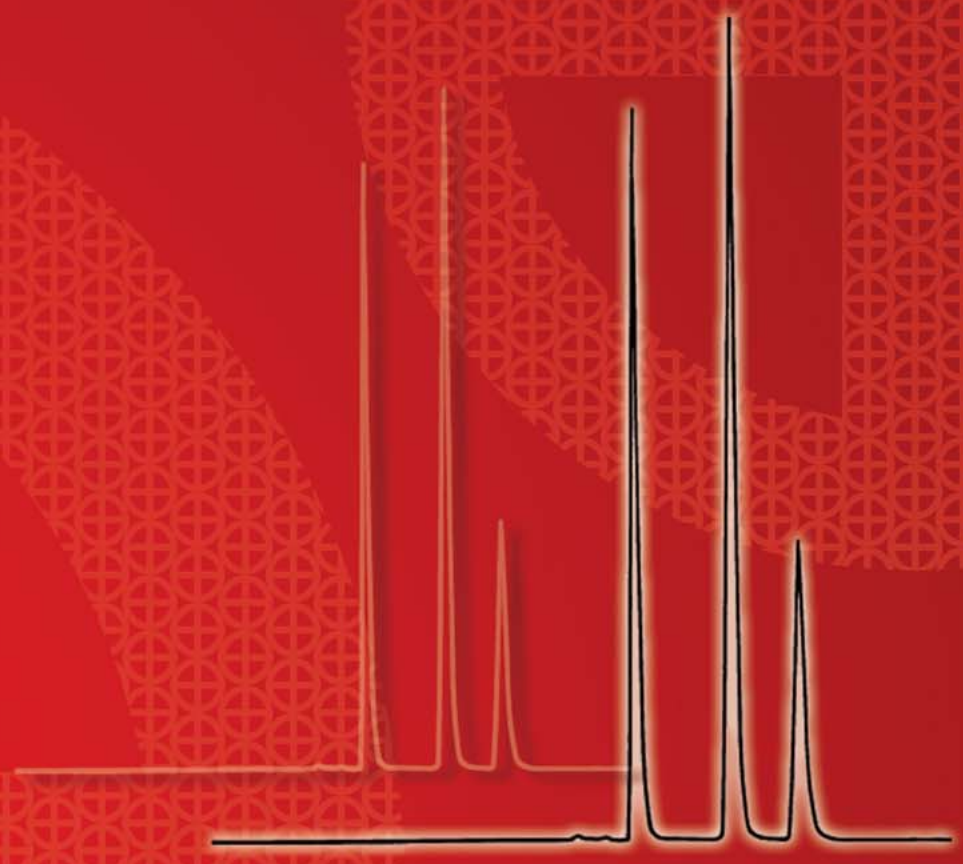
C190-E093

Access to your success
SHIMADZU

Introduction to HPLC

Shimadzu
LC World Talk Special Issue Volume1



This is an compilation of introductory section of past issues of the "LCtalk", Shimadzu's newsletter for HPLC users, Japanese version.

Reversed-phase Chromatography

Reversed-phase chromatography is the most commonly used HPLC separation mode. It is far superior to the other modes in the variety of target compounds it can handle. The dominant phenomenon retaining the sample in the column in reversed-phase chromatography is the hydrophobic interaction between the solid phase and sample. Two types of reversed-phase chromatography column packing are used: one type is a silica gel matrix with chemically bonded alkyl chains and the other is resin-based packing. Except in special circumstances, the silica gel matrix type is used due to its high number of theoretical plates. A resin-based packing must be used if the pH of the mobile phase used for separation is set outside the range that can be used with silica gel or if unreacted silanol groups remaining on the silica gel surface have a detrimental effect on separation and this problem cannot be resolved by changing the composition of the mobile phase. However, these are comparatively rare cases. Typical alkyl groups that are chemically bonded to the silica gel include the octadecyl group, the octyl group, and the trimethyl group (see Fig. 1). The longer the alkyl chain, the greater the retaining force. The column is selected according to the hydrophobic retaining force on the target compounds. Use a column with weaker retaining force if the target compounds do not elute within an appropriate time under mobile phase conditions providing the strongest elution force (100% organic solvent). Conversely, use a column with stronger retaining force if target compounds are not retained for an appropriate time under mobile phase conditions providing the weakest elution force (100% buffer).

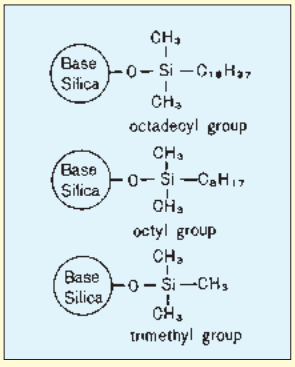


Fig. 1 Types of Solid Phase

The mobile phase composition is determined after the column has been selected. It is basically a mixed solvent comprising water or buffer mixed with an organic solvent. The three major factors that influence separation are (1) type of organic solvent, (2) proportion of organic solvent, and (3) pH of the buffer solution. Acetonitrile and methanol are the most commonly used organic solvents (point (1)). Acetonitrile is the most desirable organic solvent for HPLC analysis. Acetonitrile offers the dual benefits of low noise effects due to its low UV absorbance and extended column life, as it permits analysis at low pressure. However, in exceptional cases, methanol may be chosen due to its high selectivity. Methanol is selected if peaks A and B can be separated using methanol but not using acetonitrile, or if peaks A and B separate easily in both solvents but the a value (k' value ratio) of A and B is larger for acetonitrile, such that analysis can be completed in a shorter time using methanol.

The proportion of organic solvent (point (2)) is selected as the proportion that achieves the required separation and most rapidly achieves elution. (Increasing the proportion of organic solvent achieves more rapid elution. Decreasing the proportion results in slower elution but better separation.) When only neutral substances are analyzed, the retaining time is not affected by the mobile phase pH, so that analysis is normally conducted using water and organic solvents. In this case, only conditions (1) and (2) need to be set. However, condition (3) must also be set when analyzing acidic or basic substances.

For example, benzoic acid exists in the solution in the state of equilibrium shown in Fig. 2. Dropping the pH of the solution pushes the equilibrium toward the right, increasing the proportion of the uncharged state. Conversely, increasing the solution pH increases the dissociated state (the left side). The uncharged state is better retained during reversed-phase chromatography: lowering the pH increases the retaining force; increasing the pH achieves faster elution. Conversely, for basic substances such as amines, increasing the pH increases the retaining force. The method of setting the pH based on awareness of this pH dependency to increase the retaining force while suppressing dissociation is called the "ion suppression method." As acids and bases are affected by the pH of the mobile phase, a buffer must be used instead of water to ensure analysis repeatability. To control separation, the mobile phase pH can be changed to achieve selective separation of acids and bases from other substances.

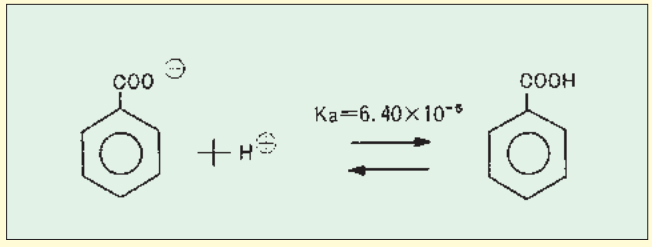


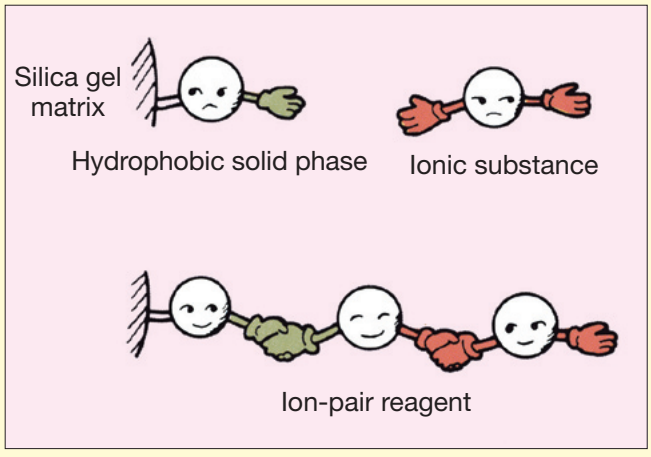
Fig. 2 Benzoic Acid

The buffer is normally prepared by dissolving a weakly acidic or weakly basic salt in water. Commonly used buffers are phosphoric acid, acetic acid, boric acid, citric acid, and ammonium. The buffer is selected according to its pKa, as a buffer exhibits the strongest buffer capacity at the same pH as the weak acid pKa used (or pKa of the conjugate acid for a weak base group). Assume that the target buffer pH is 4.8, for example. As the 4.8 pKa of acetic acid is extremely close to the target buffer pH, an acetic acid buffer is desirable. However, an acetic acid or citric acid buffer is not suitable for measurements with a UV absorbance detector at short wavelengths near 210 nm, as the background increases due to the absorbance of the carboxyl group if acetic acid or citric acid is used. Setting condition (3) requires information on the properties of chemical compounds and attention to the points described above to set a pH that achieves separation from other substances in the minimum possible time.

Reversed-phase Ion-pair Chromatography

In this article, we discuss reversed-phase ion-pair chromatography (RP-IPC), which extends the application range of RPC.
IPC was originally developed from a solvent extraction method called ion-pair extraction. The ion-pair extraction method involves extraction to an organic solvent layer after applying counter ions with the opposite charge to an ionic substance in aqueous solution to form ion pairs and neutralize the overall charge. Silica gel was used in the initial application of IPC to HPLC but now most IPC is conducted using RPC. The basic feature of RPC is that retention increases as the sample polarity decreases. As discussed in the previous issue, when analyzing an ionic substance using RPC, the retention depends on the dissociation state: retention is enhanced in the undissociated state.

Consequently, adding counter ions to an ionic substance to form ion pairs reduces the polarity and increases the retaining force. Consider the example of the analysis of thiamine (vitamin B_1). Thiamine is a strong basic substance that contains quaternary nitrogen in its molecule and normally carries a positive charge in a mobile phase. Therefore, it passes directly through the hydrocarbon solid phase of the RPC packing without undergoing a hydrophobic mutual interaction. Negatively charged octanesulfonic acid ions added to the mobile phase form ion pairs with the thiamine, thereby reducing its polarity such that it is retained by the solid phase. That is, the ion-pair reagent (counter ions) mediates between the nonpolar solid phase and ionic samples.



The actual RP-IPC retention mechanism cannot in practice be fully explained using the simple model above. Three models are available, the first of which is the "ion-pair model" that was explained above. Next is the "ion-replacement model," in which the nonpolar parts of the ion-pair reagent are initially adsorbed onto the packing to form an ion-replacement surface that retains the sample by ion replacement. Finally, we have the "ion interaction model," which has a broader range of application than the "ion-pair model" and "ion-replacement model." This model considers the dynamic equilibrium; including the effects of not only electrostatic forces but also mobile phase attractive and repulsive forces and solid phase attractive and repulsive forces. Although some phenomena are observed that cannot be explained by these models and other models are also available for consideration, the models will not be discussed further here.

An alkyl sulfonate salt such as sodium 1-pentane sulfonate or sodium octane sulfonate is generally used as the ion-pair reagent for basic substances, as described in the thiamine analysis example. Tetraalkyl ammonium ions such as tetrabutyl ammonium hydroxide are often used as the ion-pair reagent for acidic substances. All of these reagents are readily obtainable from reagent manufacturers. Recently, highly refined high-purity ion-pair reagents for HPLC applications have appeared on the market. As with normal RPC, RP-IPC sample retention is affected by the amount of organic solvent in the mobile phase, the concentration of the buffer, and the pH. However, RP-IPC sample retention is also affected by the type and concentration of the ion-pair reagent. Generally, the longer the alkyl chains of the ion-pair reagent and the higher the concentration (up to a certain level), the greater the sample retaining force. A reduction in retaining force (foldover) is observed when the ion-pair reagent concentration reaches a certain limit.

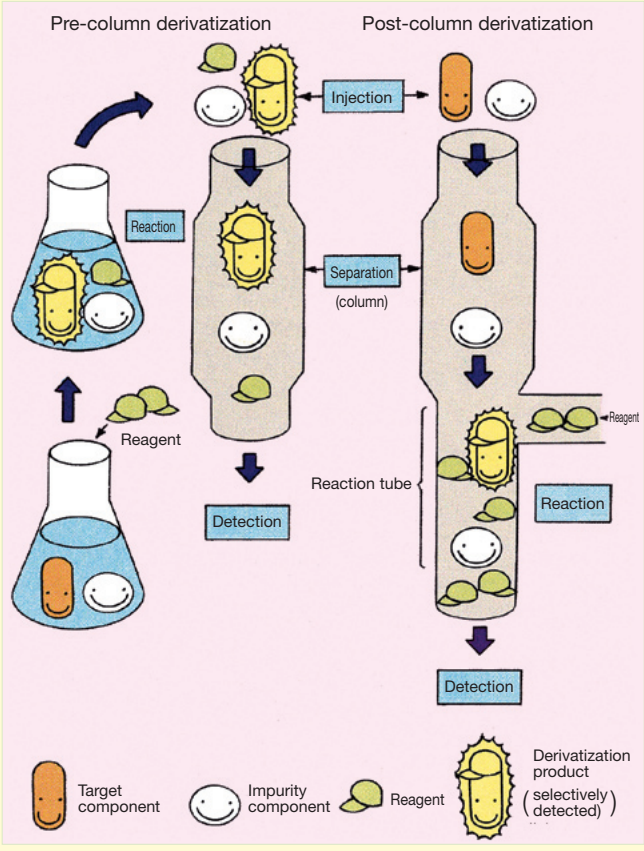
Two other points must be considered when using RP-IPC. Firstly, it takes some time for the column to completely stabilize from the time that the ion-pair reagent starts to flow into the column. Sometimes, the retention time will not stabilize if a sample is injected after the time required to achieve equilibrium with a normal RPC mobile phase. It is important to wait patiently for the column to fully stabilize. Secondly, water should not be used immediately to flush the ion-pair reagent out of the column (especially a basic ion-pair reagent such as tetraalkyl ammonium ions). If the column is flushed with water instead of buffer solution, the tetrabutyl ammonium ions, which have hydrophobic regions, can remain adsorbed on the packing and cause deterioration of the silica gel. In this case, it is important to flush out the ion-pair reagent using a mixture of acidic buffer and organic solvent with a proportion of between 1:1 and 1:2.

RP-IPC is a powerful technique that greatly expands the range of applications of RPC and, consequently, HPLC. We expect that new applications for it will be developed in the future.

Derivatization in HPLC

Advances in HPLC and its widespread application bring demands for the separation and quantitation of ever-smaller traces of target components in increasingly complex samples. However, special methods are required for this type of analysis, as it can be difficult to conduct using a normal HPLC detector. One approach is derivatization, which enhances the detection sensitivity and selectivity for the target component. Many special reagents and techniques have been developed to improve derivatization. In this article, we will discuss the general derivatization methods used in HPLC.

Two methods of derivatization are used in HPLC: pre-column derivatization and post-column derivatization. The difference between these methods is that the derivatization reaction occurs before the column with the pre-column method and after the column with the post-column method. The features of each method are discussed below.



a) Pre-column derivatization

Pre-column derivatization is often conducted off-line. As the derivatization reaction takes place outside the chromatography system, the pre-column method offers the following advantages over the post-column method:

1) simple equipment configuration;
2) no restrictions on the reaction conditions: reaction time, reaction temperature, number of reagents, etc.;
3) small volumes of derivatization reagents used, permitting the use of expensive reagents;
4) the derivatization reagent can be separated from the derivatization product and have no influence on the quantitation accuracy or detection limits in cases where the derivatization regent could be detected (for example, the reaction reagent has photoabsorbance at 400 nm and the reaction product also has photoabsorbance at 400 nm); and
5) derivatization often permits easier selection of the separation conditions than the original components.

While the pre-column method offers significant freedom in selecting the reaction, the following conditions must be met to achieve accurate quantitation:

1) excellent reaction reproducibility;
2) reaction products are stable over time; and
3) byproduct peaks do not interfere with the target peaks.

Reagent manufacturers market numerous reagents for pre-column derivatization that react with groups such as the carboxyl group, amino group, and hydroxyl group.

b) Post-column derivatization

As the post-column method conducts derivatization online, it offers the following advantages over the pre-column method:

1) automated reaction process permits unattended operation;
2) handles time-unstable reaction products, as the reaction time can be accurately controlled by the flowrates of the mobile phase pump and reaction reagent pump; and
3) permits partial reaction.

However, the post-column method must satisfy the following conditions:

1) to avoid increased background, the reaction reagent itself must be undetectable - it changes to a detectable substance only after reacting with the target component;
2) separation must not be sacrificed due to the restrictions on mobile phase factors (pH, organic solvent concentration, etc.) required to improve the reaction conditions; and
3) spreading of the component bands must not impair separation (in other words, short reaction time).

Examples of the post-column method include the analysis of amino acids using o-phthalaldehyde (OPA) and reducing sugar analysis using arginine.

The derivatization method must be selected according to the goal of the analysis, based on a good understanding of the features outlined above.

Demands on HPLC are likely to become more extensive and more severe in the future. Meeting these demands will require the development of novel HPLC systems that incorporate derivatization.

Reversed-phase Chromatography and Hydrophobic Interaction Chromatography

The separation of proteins and nucleic acids was conventionally handled by gel filtration chromatography or ion-exchange chromatography. Recently, research has been conducted into separation using reversed-phase chromatography and hydrophobic interaction chromatography. Thanks to the many new hard gels based on chemically bonded silica gels that have been developed recently to dramatically accelerate the separation of bio-macromolecules, the role of HPLC in this field will become increasingly important.

Separation methods exploiting the hydrophobic interaction between proteins and the solid phase include reversed-phase chromatography (RPC) and hydrophobic interaction chromatography (HIC). The features of these two methods are described below.

RPC has conventionally been the main stream of HPLC that was widely used for the analysis of low molecular weight substances and has been recently applied to the analysis of nucleic acids and proteins. A chemically bonded silica gel with a large pore size is used as the column packing when analyzing proteins. The gradient elution method is used for the mobile phase conditions, with the amount of organic solvent increasing at pH 2 to 3 or near neutral pH. As the protein sample is often denatured during this process, this method should more correctly be known as polypeptide analysis.

Conversely, HIC employs packing that is less hydrophobic than RPC packing and uses gradient elution in which the salt concentrations gradually decreases. HIC often permits the analysis of proteins without destroying their higher-order structure, as no organic solvent is used in the mobile phase, unlike RPC. Consequently, this method should be applicable to preparative separation.

Let us now compare the two packings used in these methods. The main packing type for RPC is chemically bonded silica gel with a large pore size of at least 30 nm (compared to the conventional 8 to 10 nm small pore size). This packing that has an embedded alkyl group is said to have a good sample recovery rate. The residual silanol group in the chemically bonded silica gel affects the recovery of protein samples and the amount of residual silanol group differs according to the manufacturer and production lot, such that quantitative considerations are currently difficult to make.

Silica generally contains approximately 8μmol/m^2 silanol groups. During synthesis of normal RPC packing, a silanol-coupling agent containing alkyl groups reacts with the silanol groups. The amount of the silanol group that reacts with the agent is 3 to 4μmol/m^2, although it depends on the length of the alkyl-group chain.

To reduce the effects of residual silanol groups during chromatography, secondary silylation is generally conducted using a reagent such as trimethylchlorosilane. Problems with denaturing and recovery rate occur when such RPC packing is used for protein analysis because an organic solvent must be used as the mobile phase, as described above. Consequently, RPC is mainly used to analyze polypeptides with comparatively low molecular weights. A known example is the analysis of polypeptides obtained by the digestion of proteins by an enzyme such as trypsin.

HIC generally uses packing created by embedding hydrophobic groups, such as alkyl groups and phenol groups, onto a hydrophilic gel used for gel filtration chromatography. However, the amount of embedded hydrophobic group is significantly lower than for RPC. Silica-based and polymer-based commercial HIC packings are currently available. The silica-based packings are designed by the manufacturers to eliminate the effects of residual silanol groups. The RPC and HIC packings exhibit a large difference in the degree of hydrophobia, which results in the significant difference in elution conditions described above.

RPC is superior to HIC in sample separation, whereas HIC results in lower sample denaturing. As both HIC and RPC are expected to play an important role for the analysis of proteins and nucleic acids in the future, the careful selection of the appropriate technique will be essential.

Silica Gel Based Packing

Octadecylsilylated (ODS) and other chemically bonded, porous, spherical silica gels are currently widely used as the packing in HPLC columns. Many people who have conducted HPLC analysis have probably experienced problems with differences between column production lots or have questions about the differences with ODS columns produced by different manufacturers. This article is intended to clarify some of these queries about silica gel-based packings.

General-purpose ODS silica gel is typically produced by reacting spherical, porous silica gel that has 5μm average particle size and 6 to 10 nm average pore size with a silanol-coupling agent such as dimethyl-octadecylchlorosilane. Approximately 8μmol/m^2 silanol groups remain on the silica gel surface but the reaction with the silanol-coupling agent embeds octadecyl groups via siloxane bonds. However, when using a bulky substituted group such as the dimethyl-octadecylsilyl group, unreacted silanol groups always remain due to steric hindrance. The amount of the unreacted silanol groups depends on the reaction conditions, but is around 5μmol/m^2 for normal ODS silica. Consequently, attempts were made to conduct silylation of the residual silanol groups using a silanol-coupling agent with a substituted group with lower steric hindrance. This is the so-called "secondary silylation", which uses trimethylchlorosilane (TMS-CI) or other substances. Modern commercial ODS silica gels are treated with TMS in this way (Fig. 1). The residual silanol groups affect the adsorption of basic samples but the amount of residual silanol groups differs from manufacturer to manufacturer. Therefore, special attention is required during the analysis of basic samples.

Let us now take a closer look at ODS silica gels. When "spherical silica gel" is observed under a microscope, broken particles of silica gel are often observed mixed in with it. Also, despite the nominal 5μm particle size, many fine particles below 2μm and large particles of about 10μm are also included. This disparity in particle size varies according to the manufacturer, and it has an adverse effect on the state of the column packing. Most customers probably purchase packed columns. If a gap occurs at the column inlet after a short period of use, there are probably problems with the column packing due to particle size discrepancies.

A silica gel with a nominal 10 nm average pore size has a certain pore-size distribution and the size of the pore-size distribution varies from manufacturer to manufacturer. A bulky silanol coupling agent, such as ODS, does not disperse in a fine-pored silica gel, such that the amount of embedded ODS decreases and in some cases remains as residual silanol

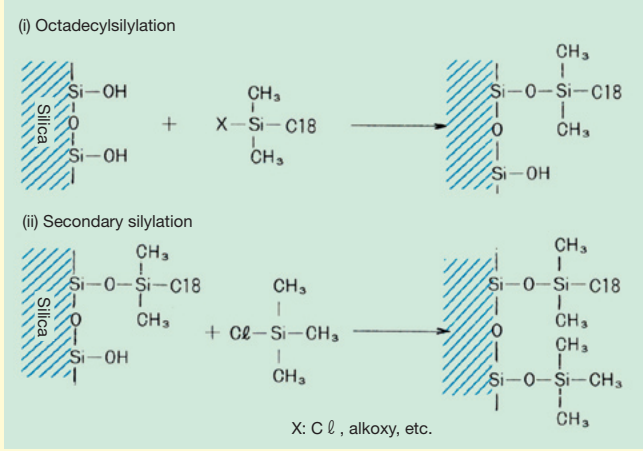


Fig. 1 Silylation Reaction on Silica Surface

groups without undergoing secondary silylation. Consequently, pore size distribution control is essential to eliminate differences between production lots in packing manufacture. In general, as the average pore size increases, the silica gel specific surface area decreases, resulting in a decrease in the apparent amount of embedded ODS and weaker retention of low molecular weight substances. So-called "wide-pore" silica gels with a pore size of 30 nm or more have recently been applied to the analysis of macromolecules, especially proteins and nucleic acids. This is because larger packing pore sizes result in more rapid dispersion of a macromolecular sample in and around the pores.

Using different types of silylation agent permits the manufacture of octyl, phenyl, cyanopropyl (CN), and aminopropyl (NH_2) types of chemically bonded silica-gel packing, in addition to ODS. The packing must be selected according to the aim of the analysis. These packings can be used in a pH range from 2 to 8; the silica dissolves in the alkaline range and the chemically bonded solid phase separates at a pH below 2 due to cleavage of the Si-C bonds. Columns with polymer-coated silica gel and columns with octadecyl groups embedded onto a synthetic polymer surface are used to improve alkali resistance. However, chemically bonded silica gel columns are these days widely used as cheap, durable, high-performance packing.

Absorptiometric Detection

Absorptiometry is a detection method that measures the absorbance based on excitation of the valence electrons in the molecules. It is the most widely used detection method for HPLC as it offers great generality, superb selectivity, and good ease-of-handling.

The absorbance (A) is defined as the negative logarithm of the ratio of the transmitted light intensity (I) to the incident light intensity (Io) when monochromatic light passes through a light path length ℓ in a solvent. It bears the following relationship with the analyte concentration C:

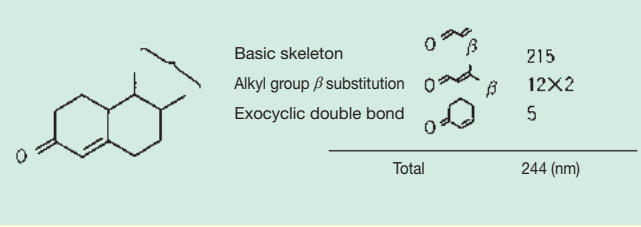
$$A = \varepsilon C \ell$$

Where, ε is the molar absorptivity of the analyte.

This relationship, where absorbance is proportional to the concentration, is known as Beer's Law. It applies when the interactions between analytes can be ignored and the analyte chemical status is not dependent on the concentration. With HPLC, the analysis target components that elute from the column have a dilute concentration, such that Beer's Law applies and is used as a linear function for component concentration during quantitation.

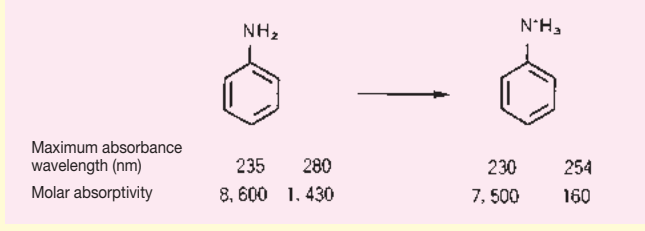
So, let us consider what substances can be analyzed by absorptiometry and what the measurement conditions are. When the σ electrons, π electrons, and *n* electrons (which are non-bonding) in a molecule are excited by light, they undergo transition from bonding or nonbonding orbitals to antibonding orbitals. (An antibonding orbital is denoted by an asterisk *.) As the σ -> σ* transition requires significantly higher energy than the other transitions, compounds with only single bonds, such as saturated hydrocarbons, cannot be detected in the 190 to 700 nm wavelength range by this detection method. Compounds with *n* electrons and isolated π bonds exhibit absorbance in the ultraviolet range due to *n* -> π*, *n* -> σ*, or π -> π* transition but the molar absorptivity is generally small. Consequently, in practice, target substances are limited to conjugated systems. However, as many substances handled by HPLC are conjugated systems, absorption photometry detection is commonly used.

The measurement wavelength setting is an important point that determines the detection sensitivity and selectivity. If many impurity components exist, the wavelength can be selected to reduce their influence; but the measurement wavelength is normally set to the maximum absorbance wavelength of the analysis target component. This is not just to obtain the highest sensitivity but also to eliminate effects of wavelength-setting errors on the sensitivity. The maximum absorbance wavelength is determined by measuring the absorbance spectrum with a spectrophotometer. For a simple polyene structure or enone structure, the maximum absorbance wavelength can be calculated using the Woodward and Fieser rules. This calculation involves adding values applied to the basic skeleton and the bonded functional groups. An example of this calculation for a steroid enone, such as a mineral steroid, is shown below.



This method is always described in organic chemistry textbooks that deal with spectroscopy. Refer to such a textbook for more detailed information.

The absorbance spectrum and molar absorptivity are governed by the chemical structure but in solution they change due to the solvation, interactions with the third substance, and dissociated state. These changes are particularly large when the dissociated groups are in conjugated systems. As an example, the maximum absorbance wavelength of aniline and the molar absorptivity at this wavelength change as follows.



Consequently, the optimal mobile phase conditions must be investigated for detection as well as for separation. For aniline, setting the mobile phase pH higher than the aniline pKa is superior from the point of view of both sensitivity and selectivity. For this type of substance that exhibits significant changes in absorbance due to the dissociated state, good repeatability and linearity can be obtained only by using a buffer to maintain a constant degree of dissociation.

The mobile phase is an important factor for this type of detection, as it affects the chemical status of the target component. In addition, it is important to consider the absorbance of the mobile phase itself. For example, the noise increases and system peaks may appear if the mobile phase contains a substance with high absorbance. This may also cause baseline fluctuations during gradient elution. Ideally, a mobile phase with low absorbance at the measurement wavelength should be selected.

With today's dramatic increase in instrument performance, absorptiometry can detect some components at pmol levels if the optimal conditions are set. It is a powerful tool for trace element analysis if the absorbance of the substance is known.

Affinity Chromatography

Biological substances, such as enzymes and substrates or antigens and antibodies, have the property of selectively recognizing each other to form complexes. Affinity chromatography is a separation and purification method that exploits this biological affinity. For example, a specific enzyme can be extracted from a matrix containing an extremely high number of components by using a substance as the ligand that exhibits specific affinity for the target enzyme, such as its corresponding substrate or inhibitor.

■ Procedure for Affinity Chromatography

The procedure comprises the following four steps:

(1) Column equilibration
The column is filled with a packing onto which a suitable ligand is immobilized. The column is then equilibrated with the solvent that will be used for sample adsorption.

(2) Sample addition and adsorption of target substance (Fig. a)
The sample is introduced into the column and the target component adsorbed onto the packing.

(3) Column washing (Fig. b)
Washing is conducted to eliminate components not adsorbed onto the packing from the column.

(4) Elution of target components (Fig. c)
A solvent is introduced into the column to elute the target component adsorbed on the packing to recover the target component. The procedure then reverts to step (1).

In principle, affinity chromatography can effectively extract a target component from a complex matrix that contains multiple components just using simple operations.

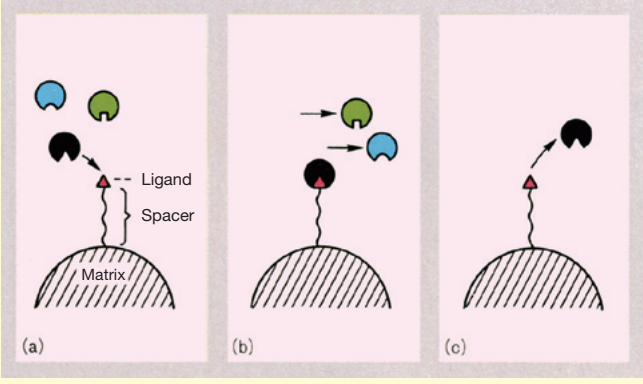


Fig. Principle of Affinity Chromatography

■ Ligand Selection and Immobilization on the Packing

The selection of the ligand and packing matrix and method to immobilize the ligand must be carefully considered before conducting actual affinity chromatography.

• Selecting the Ligand

Elution from the column may be difficult if the affinity between the substance used as the ligand and the target component is too strong. If multiple ligands can be considered, select the substance which offers the greatest selectivity for the target compound and which permits elution from the column under the most moderate conditions.

• Selecting the Matrix

Commonly used matrices are agarose and hydrophilic synthesized macromolecules (polyvinyl alcohol, polyacrylate, etc.) beads. In principle, a matrix is selected that is inactive with respect to the sample and is physically and chemically stable under the conditions used. It must also be noted that if the beads are porous, their pore distribution and specific surface area affect the amount of immobilized ligand and the effective amount of ligand on the packing surface.

• Immobilizing the Ligand

The CNBr method and many other techniques to immobilize the ligand have been reported. A ligand immobilization method must be selected that permits the effective function of the regions exhibiting affinity for the target component. The length of the spacer between the ligand and matrix is also important, especially if the target component is a macromolecule such as a protein. Many types of active matrix that permit easy ligand immobilization are now commercially available.

■ Application Examples

Many cases of separation and purification by affinity chromatography have been reported. Separation of lectin with sugar as the ligand and separation of hormone receptors using a hormone-bound matrix have been reported as examples of the separation of proteins with an immobilized low molecular weight ligand. Examples using a macromolecular substance as the ligand include the separation of calmodulin-bound proteins using calmodulin immobilization matrix and the separation of fibronectin using collagen as the ligand.

Many cases of enzyme purification have been reported. However, if the substrate is used as a ligand, careful control of the chromatography conditions is required to prevent the enzyme breaking down the ligand.

Antigens or antibodies are widely used as the ligand to isolate a specific antibody or antigen. In this case, the extremely strong affinity between the antigen and antibody requires measures such as lowering the eluate pH or the use of a denaturing agent or chaotropic ions.

Affinity chromatography often presents the disadvantage that the user has to make the adsorbent. Even so, this technique is likely to continue to be used as a convenient and effective way to isolate target components.

Normal-phase Chromatography

Reversed-phase chromatography is currently the most commonly used HPLC separation mode, whereas normal-phase chromatography is used in just a limited range of applications. The difference results from the many advantages of reversed-phase chromatography, including its expanded application to previously difficult to analyze compounds such as ionic components, thanks to the development of reversed-phase ion-pair chromatography, and its ease of operation, as water-based mobile phases can be used. However, normal-phase chromatography offers different properties from other separation modes, including reversed-phase chromatography, and can be extremely effective for some purposes.

What is Normal-phase Chromatography?

The original definition of normal-phase chromatography was that it is a type of partition chromatography in which the polarity of the solid phase is higher than the polarity of the mobile phase. In addition, adsorption chromatography, which was classified as a separation mode called "solid-liquid chromatography," is now considered to be normal-phase chromatography. In most cases, the solid phase used for normal-phase chromatography is an untreated porous silica-gel column (SIL column) or a column containing silica gel chemically bonded at the surface to polar functional groups, such as the aminopropyl group (NH_2 column) or cyanopropyl group (CN column). The mobile phase used is generally ethanol or another polar solvent added to a nonpolar solvent such as n-hexane. However, a mobile phase containing water is sometimes used for the analysis of highly polar components. The separation of each component differs according to the distribution ratio between the solid phase and mobile phase. The interaction between the solid phase and target components during normal-phase chromatography are mainly hydrophilic interactions, such as hydrogen bond interactions and electrostatic interactions. Consequently, normal-phase chromatography generally offers different separation selectivity to reversed-phase chromatography, which mainly involves hydrophobic interactions.

What is Normal-phase Chromatography Used For?

Normal-phase chromatography can easily separate tocopherol isomers that are difficult to separate by reversed-phase chromatography and sugars that are difficult to retain by reversed-phase chromatography. It can elute together all components with different alkyl chain lengths and branches during the analysis of alkyl benzene sulfonate. These properties arise as the regions involved in retaining compounds differ from those in reversed-phase chromatography, as described above. In addition, as the mobile phase used for normal-phase chromatography generally contains no water, this technique is ideal for the separation of easily hydrolyzed compounds, such as acid anhydride; concentration after fractioning; or preparative separation and purification that requires drying. Normal-phase chromatography can also be advantageous from the viewpoints of quantum yield for fluorescence detection, molar absorptivity and detection wavelength in absorption detection.

Investigations into Separation using Normal-phase Chromatography

With normal-phase chromatography, increasing the mobile phase polarity generally accelerates elution. For example, if an n-hexane/ethanol mixture is used as the mobile phase, elution occurs faster if the proportion of ethanol, which has higher polarity, is increased. Care is required, as this is the reverse relationship to reversed-phase chromatography, whereby the rate of elution increases when the mobile phase polarity is decreased. Using a low-viscosity solvent in the mobile phase sometimes permits high flow rates and rapid column equilibration. If an NH_2 column or CN column is used, some mobile phase compositions result in reversed polarity of the mobile phase and solid phase, such that the column functions as a reversed-phase chromatography column. Therefore, it is important to be aware of the possibility that the elution behavior may fluctuate wildly, especially if a mobile phase with a high proportion of water is used.

Conclusions

Normal-phase chromatography is an important separation technique for some analyses. It will probably continue to be used for a limited range of purposes.

Ligand-exchange Chromatography

In this article, we will discuss ligand-exchange chromatography: a technique that is now commonly used for the separation of sugars and optical isomers.

We will first consider the separation mechanism. Substances (molecules or ions) have the property of accepting or donating electrons. For example, metal ions have the property of accepting electrons and are called "electron acceptors." If a substance that donates electrons (an "electron donor") exists near a metal ion, a complex is created by coordinate bonds resulting from electron transfer between the two. Such electron donors are called "ligands." An example of this phenomenon is that most metal ions in aqueous solution form complexes (aqua complexes) of coordinated water molecules. When other ligands coexist in an aqueous solution of metal ions, these ligands compete with the water molecules and try to form complexes with the metal ions. This reaction is represented by the expression below:

$$M(H_2O)_n + L \rightleftarrows (H_2O)_{n-m}L + mH_2O$$

Where, M is a metal ion with the coordination number n and L is a ligand with the coordination number m. This exchange reaction is known as a "ligand-exchange reaction." Ligand-exchange chromatography uses this reaction to separate components using the different bonding force to metal ions of each component (ligand) (that is, the difference in stability of the complex) in a column with immobilized metal ions.

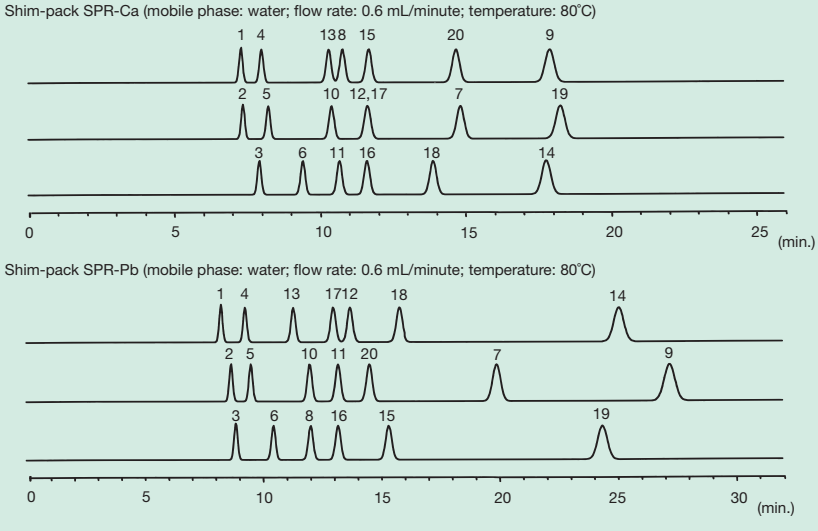
The metal ions are immobilized by ion bonds or coordinate bonds to a resin with embedded functional groups, such as sulfone groups, carboxyl groups, or amino groups. In some cases appropriate ligands are embedded onto the resin. A solvent containing a suitable competing ligand is used as the mobile phase. Elution of target components is controlled by adjusting the concentration of the competing ligand and pH.

The stability of the complex depends on the basicity, coordination number, and steric factors of the ligand. In a polydentate ligand, the size and number of chelate rings also have an effect. The stability of the complex is also related to the diameter and charge of the metal ions.

For example, a column packed with sulfonated polystyrene gel with immobilized Ca^{2+} or Pb^{2+} ions is used for ligand-exchange chromatography of monosaccharides. Water is used as the mobile phase. The water molecules provide the competing ligand. Fig. 1 shows the elution positions of each monosaccharide obtained by Ca and Pb ligand-exchange chromatography. It shows that the retention of some components differs.

Complexes of sugars and metal ions are formed by coordinate bonds between the metal ions and the two hydroxyl groups bonded to the adjacent carbon of the sugar. A complex forms when these two hydroxyl groups adopt an eclipsed or gauche conformation; however, no complex results if they adopt the anti conformation. Consequently, the stability of the metal complex with each monosaccharide is determined by how readily it adopts these conformations. Sugar alcohols such as mannitol or sorbitol are retained strongly, as they readily adopt a desirable conformation due to the many hydroxyl groups involved in forming the complexes. The possible conformations for ring-formed cyclohexitol or pyranose are restricted, such that complexes form less readily with them than with the sugar alcohols. However, the stability of these sugars increases as the three hydroxyl groups take a form of aq-aq-aq.

In ligand-exchange chromatography, the conformation of a substance also contributes to its separation, as described above. In some cases, ligand-exchange chromatography permits the analysis of components that cannot be separated in a normal mode. Naturally, the target components must have coordination capacity. To apply this type of chromatography, the coordination of the component must be considered in order to design an appropriate solid phase and mobile phase.



■Analysis Conditions
Mobile phase: water
Flow rate: 0.6 mL/minute
Temperature: 80°C

■Peak Components

1. Raffinose
2. Maltotriose
3. Sucrose
4. Maltose
5. Lactose
6. Glycose
7. Mannitol
8. Rhamnose
9. Sorbitol
10. Galactose
11. Mannose
12. Fructose
13. Xylose
14. Xylitol
15. Inositol
16. Fucose
17. Arabinose
18. Glycerol
19. Ribose
20. Ethanol

Fig. 1 Elution Positions of Monosaccharides by Ca and Pb Ligand-exchange Chromatography

Explaining GLP/GMP Terminology: RSD (C.V.)

The importance of quality control is growing internationally, as typified by the ISO-9000 Series and GLP/GMP for pharmaceuticals. The validation of instruments and analytical measures is now required as a method to objectively verify analytical instruments and data reliability in a variety of industries. A check of the precision, i.e., the degree of discrepancy in the analysis results, is one item in the method validation and system suitability tests that are conducted during validation. This article explores RSD (C.V.), which is used to express precision.

When recording or displaying analysis data, if only one data point is collected, that value is used directly; if multiple data points are collected, the mean value or median value is often used as statistical characteristics. However, when discrepancies do exist in the data, the exact status of the data cannot be accurately expressed by these statistical characteristics alone. In practice, data never matches perfectly from analysis to analysis and a discrepancy results. RSD (C.V.) is often used as an objective indicator in the statistical analysis of such discrepancies. In Table 1 below, the mean value is 100 for both Results (1) and Results (2), but the discrepancies in Results (2) are clearly larger. The RSD calculation yields RSD = 2.92% for Results (1) and RSD = 29.2% for Results (2).

Table 1 Mean Values and RSD

Data	Results (1)	Results (2)
x_1	101	110
x_2	96	60
x_3	103	130
x_4	102	120
x_5	98	80
Mean ($\bar{x}$)	100	100
RSD (C.V.)	2.92%	29.2%

Relative Standard Deviation (RSD) and Coefficient of Variation (C.V.) are synonymous. These values are used to objectively express the data discrepancy (precision). It is defined as the standard deviation (SD) divided by the mean ($\bar{x}$), as shown in the expression below. For the degree of freedom of the standard deviation (the numerator inside the square root), (n-1) is normally used instead of n (number of data). This is because a statistical characteristic to estimate the population is used instead of the entire data population.
The standard deviation (SD) is normally calculated using expression (2) or (3).
Table 2 shows an example of an RSD (C.V.) calculation. This calculation is easily handled using a calculator or spreadsheet.

$$\text{RSD (C.V.)} = \frac{\text{SD}}{\bar{x}} \quad \cdots\cdots ①$$

$$\text{SD} = \sqrt{\frac{\Sigma (x_i - \bar{x})^2}{n-1}} \quad \cdots\cdots ②$$

$$= \sqrt{\frac{\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}}{n-1}} \quad \cdots\cdots ③$$

$$\bar{x} = \frac{\Sigma x_i}{n} \quad \cdots\cdots ④$$

Table 2 Sample RSD (C.V.) Calculation

Data (n=5)	Retention time RT [min]	Area value AREA [μVsec]	Concentration CONC.[ppm]
x^1	9.234	513513	100.38
x^2	9.252	514403	100.55
x_3	9.243	513612	100.40
x^4	9.233	512712	100.22
x^5	9.242	513403	100.36
Σx^1	46.204	2567643	501.91
$\Sigma (x^{1^2})$	426.962162	1318559569475	50382.7849
$\bar{x}$	9.241	513528.6	100.382
SD	0.00773	603.0	0.1176
RSD (C.V.)	0.084%	0.12%	0.12%

The number of data n (number of repeated analyses) is determined according to the degree of data discrepancy and the difficulty in acquiring the data. The International Conference on Harmonization (ICH) guideline entitled "Text on Validation of Analytical Procedures" (1997) recommends the use of one of the following two procedures to evaluate precision:

a) repeat all operations required for the analysis method at least nine times covering the specified concentration range (for example, repeat all operations three times each at three concentrations); or
b) repeat all operations required for the analysis method at least six times at 100% the test concentration.

The Shimadzu LCsolution Workstation for HPLC automatically calculates and displays the standard deviation and RSD of the retention times and area values.

Generally, a retention-time precision check on an HPLC system yields information to evaluate the (1) flow rate stability (pumping rate stability; check valve operation stability and leaks), (2) column performance, (3) gradient accuracy, (4) mobile phase stability, (5) ambient temperature fluctuations, and (6) blockage of the flow lines. A check on the area value or concentration precision provides information to evaluate (1) injection accuracy, (2) detection stability, and (3) reaction stability (for a pre- or post-column reaction system).

Explaining GLP/GMP Terminology: Tailing Factor, Resolution

This article discusses the tailing factor and resolution that appear in the specificity item in the method validation and system suitability tests.

1. Tailing Factor (Tf)

The Tailing Factor (Tf) is an indicator that expresses the symmetry of the individual eluted peaks. It is sometimes called the "Symmetry Factor." Ideally, chromatography peaks should be symmetrical (Tf=1) but may be asymmetrical due to a number of factors, the majority of which are as follows:

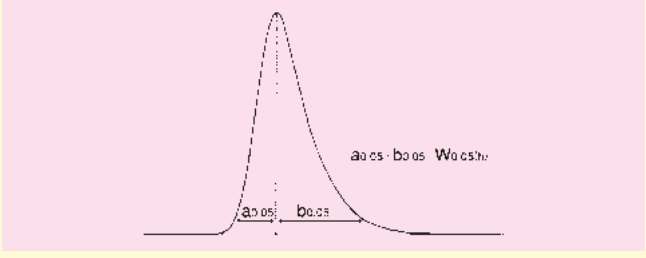
(1) secondary adsorption of sample components in the column;
(2) status (non-uniformity, contamination) of the column surface (solid phase);
(3) excessive concentration or viscosity of the sample components;
(4) excessive flow rate or other inappropriate analysis condition;
(5) non-uniform column packing;
(6) dead space or channeling in column;
(7) dead space in flow lines such as injectors or pipes; and
(8) temperature irregularities or gradients in the column.

One method to determine whether the asymmetricality of peaks originates in the system or is inherent in the components (analysis conditions) is to analyze a component with low interaction and compare the tailing factors. For example, naphthalene or benzoate esters are used for reversed-phase chromatography.

The Tailing Factor (Tf) is defined by the following expression:

$$Tf = \frac{W_{0.05h}}{2 \times a_{0.05}}$$

Where,
$a_{0.05}$ is the width of the front of the peak (start point to apex) at 5% peak-height position; and $W_{0.05h}$ is the peak width at 5% peak-height position.



Generally, if Tf<1, the peak is described as "leading" or "fronting"; if Tf>1, the peak is described as "tailing". A range of values from 0.5 to 1.5 is normally appropriate. If Tf lies outside this range, the cause should be investigated. By way of reference, the System Suitability item in the FDA Reviewer Guidance [3]) states that Tf<2 is desirable.

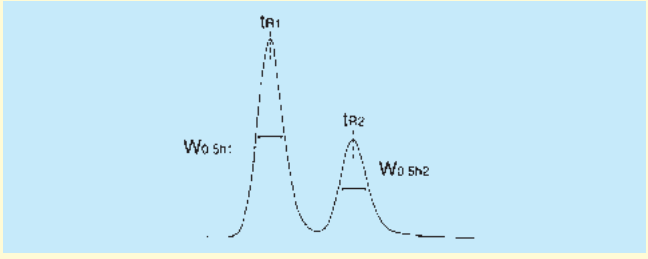
2. Resolution

Resolution (Rs) is an indicator of the degree of separation between two components. It is defined as the difference in retention time between two peaks, divided by the mean peak width. Like the number of theoretical plates, resolution is defined slightly differently in the Japanese Pharmacopoeia[1]) and U.S. Pharmacopoeia[2]), which adopt the peak width at half height method and peak width tangent method, respectively.

1) Japanese Pharmacopoeia [1)]

$$Rs = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{0.5\,h2} + W_{0.5\,h1}}$$

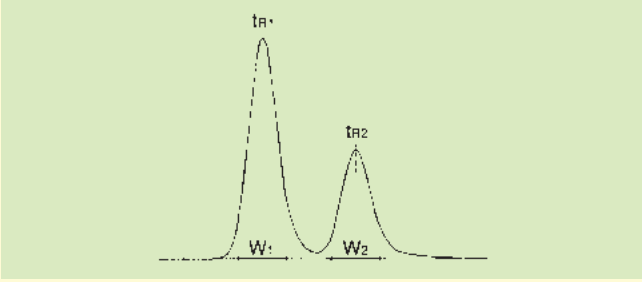
Where,
t_{R1} is the retention time of the front peak;
t_{R2} is the retention time of the rear peak;
$W_{0.5h1}$ is the front peak width at 50% peak height position; and
$W_{0.5h2}$ is the rear peak width at 50% peak height position.



2) USP [2)]

$$Rs = 2 \times \frac{t_{R2} - t_{R1}}{W_2 + W_1}$$

Where,
W_1 is the peak width of the front peak *;
W_2 is the peak width of the rear peak *;
(* Note: The peak width is taken as the time amplitude between the two points of intersection of the baseline and the tangents to the left and right peak inflection points.)



Normally, Rs >= 1.5 indicates that separation has occurred (baseline separation), thereby providing desirable conditions for reliable quantitation. This value is particularly important for chromatography, as one characteristic of chromatography is its ability to conduct the simultaneous analysis of multiple components.

By way of reference, the System Suitability item in the FDA Reviewer Guidance [3] states that "Rs >2 is desirable between the target peak and the nearest adjacent peak (impurity, diluting agent, separated component, internal standard, etc.)."

In some cases, the separation coefficient (α, relative retention) is also used. The separation coefficient is the ratio of the capacity factors (k') of two peaks. It is defined as follows:

$$\alpha = \frac{k'_2}{k'_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

Where,

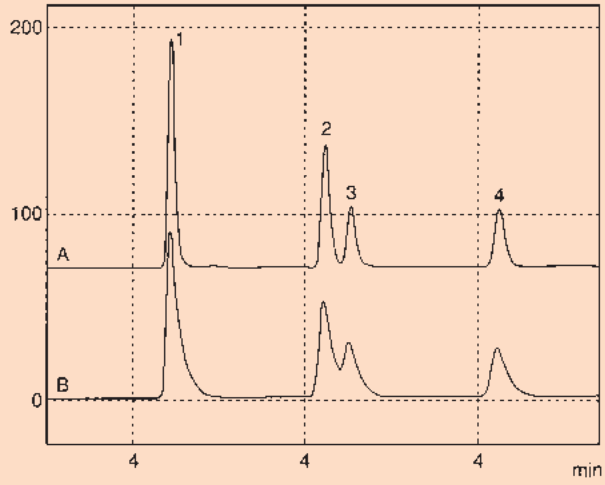
k'_1 is the k' value (capacity factor) of the front peak;
k'_2 is the k' value (capacity factor) of the rear peak;
t_0 is the unretained peak time (dead time);
t_{R1} is the retention time of the front peak; and
t_{R2} is the retention time of the rear peak.

The column performance validation function of the Shimadzu LCsolution Workstation for HPLC can automatically calculate the tailing factor, resolution, and separation coefficient using the Japanese Pharmacopoeia, USP, and other calculation methods. If the calculation results for these parameters do not meet the pass criteria, pass/fail evaluation using QA/QC functions can take actions such as reinjection or stopping analysis.

References

1. Japanese Pharmacopoeia 12th Edition, General Test Methods (Liquid Chromatography)
2. USP (United States Pharmacopeia) XXIII-5, <621> Chromatography
3. Center for Drug Evaluation and Research (CDER, FDA), Reviewer Guidance, Nov. (1994) "Validation of Chromatographic Methods"
4. LCtalk Vol. 34, TEC "Calculation of Number of Theoretical Plates."

Sample Calculations of Tailing Factor and Resolution



	Peak	Japanese Pharmacopoeia, DB			USP		
		Tailing factor	Number of theoretical	Separation	Tailing factor	Number of theoretical	Separation
A	1	1.41	15649		1.41	12701	
	2	1.28	20444	11.34	1.28	17558	10.34
	3	—	20389	1.65	—	17718	1.53
	4	1.20	22245	8.47	1.20	20233	7.97
B	1	2.49	5972		2.49	5426	
	2	—	7917	7.02	—	7310	6.70
	3	—	—	—	—	5371	0.90
	4	1.71	9957	—	1.71	9316	4.91

Detection Limit

Often, the word “sensitivity” is used to express the ability of an analytical method or analytical instrument when discussing the detection ability with respect to minute quantities of substances. Sensitivity is actually a term indicating the magnitude of the detection response with respect to an amount of a component in a sample, and to be correct, “detection limit” or “quantitation limit” is an index of detection ability.
Detection limit is defined as the amount of a substance that generates a signal which differs significantly from a blank. However, “differ significantly” is not actually prescribed in ISO or in JIS, and the lack of a uniform interpretation causes confusion in the handling of the “detection limit”.
Here we will explain the definition of detection limit and its measurement method as set forth by the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use), organized for the purpose of harmonizing interpretation among Japan, the United States and the European Union.

According to ICH, detection limit is defined as “the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value.” The phrase “not necessarily quantified as an exact value” means that there is no necessity for an accompanying permissible degree of truth and accuracy, differing from quantitation limit in this respect.
The method for determining the detection limit differs depending on whether or not the analytical method is an instrument analytical method, however, it can be categorized broadly into the following 3 methods.

(1) Method based on visual evaluation

This method is used when the analytical method is not dependent on an instrument analytical method. Here, a sample of known concentration is gradually diluted, and the greatest dilution stage at which the sample can be distinguished from the blank is taken as the detection limit.

(2) Method based on signal-to-noise

This method primarily used in chromatography. The analyte signal and the baseline noise are measured, and the analyte concentration (mass) at which the ratio is 2:1 or 3:1 is taken as the detection limit. First the sample concentration is adjusted so that the peak height is at least 10 times that of the baseline noise, and then measurement is conducted to obtain the peak height h. (European Pharmacopeia-1987)
For noise, the maximum fluctuation h_N is measured over a time period 20 times that of the peak width at half height, and 1/2 of that value is taken as noise N (Fig. 1, European Pharmacopeia- 1987), or the baseline recorded for 15 minutes is divided into 0.5 - 1 minute segments (X_1, X_2, X_3....), parallel lines are drawn in the horizontal axial direction separated by the minimum width required to include the noise within each segment, the vertical distance between the parallel lines is calculated (Y_1, Y_2, Y_3....) along the segment division lines, and the average value is obtained and taken as noise N (Fig. 2, JAIMA S 0005-1984, ASTM E 1657-1994).

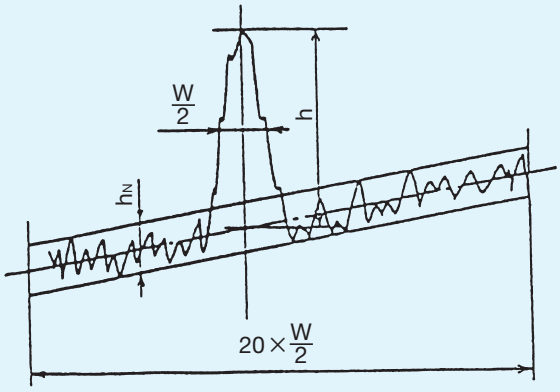


Figure 1

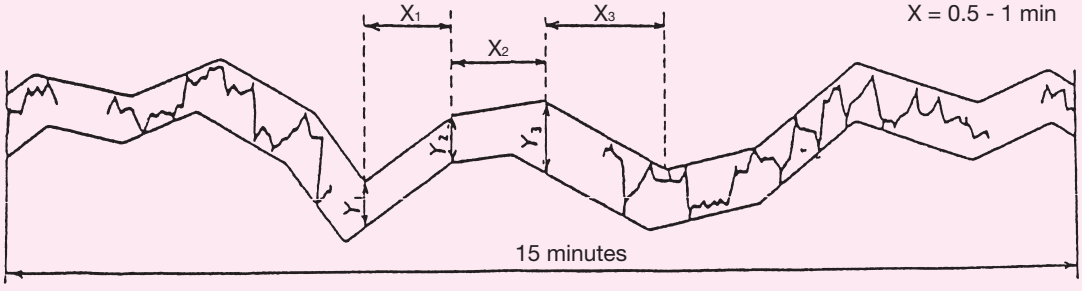


Figure 2

(3) Based on standard deviation of response and slope of calibration curve

This is a method in which a blank sample is analyzed and its standard deviation is obtained, or several samples containing an analyte with concentrations (mass) close to that of the detection limit are analyzed, the residual standard deviation of the regression line or the standard deviation of the y-intercept is determined, and the detection limit is calculated using the following equation.

$DL = 3.3\sigma/a$

(σ : standard deviation of response
a : slope of calibration curve)

This is called the Currie detection limit (L.A. Currie : IUPAC Provisional Draft, 1994), in which the detection limit is the quantity of substance corresponding to the signal at the position $\mu_B + 3.29\ \sigma_B$ obtained from the average μ_B of the blank signal distribution (μ_B, σ_B). In other words, signal distribution N (μ_s, σ_B) when the probability of an error of the first kind occurring (probability of falsely deciding that a signal is present when it is not) and the probability of an error of the second kind occurring (probability of falsely deciding that a signal is not present when it is) are both 5%.

In HPLC, the determination method normally used is either that based on the signal-to-noise ratio or that based on the standard deviation of response and slope of the calibration curve.
The determination method based on signal-to-noise is easy and is the most commonly used, however, when there is baseline fluctuation such as in gradient elution analysis, or when there are nearby impurity peaks, this method is not suitable. Although the method based on the standard deviation of response and slope of the calibration curve requires abundant data for the calculation, it has the advantage of being applicable to any case.
The method based on the standard deviation of response and slope of the calibration curve is shown below. However, since the standard deviation of the blank cannot be obtained in HPLC, it is obtained from the standard deviation of the regression line (residual or *y*-intercept).

Theoretical Conc.	0.04%	0.06%	0.08%	0.10%	0.12%
1st run	3551	4446	6182	7963	9405
2nd run	3282	5089	6294	8154	9226
3rd run	3013	5050	6418	8078	9084
4th run	3635	4907	6793	7668	9780
5th run	3119	4686	6109	7525	9591

Table 1 Data of Samples Containing Analyte at Known Concentrations

- Detection Limit : $DL = 3.3s_{y/x}/a = 0.011$ (%)
  (Residual standard deviation :
  $s_{y/x} = \{\Sigma\{y_i - (ax_i+b)\}^2/(n-2)\}^{1/2} = 252.857$)
  or
- Detection Limit : $DL = 3.3s_y/a = 0.013$ (%)
  (*y*-intercept standard deviation :
  $s_y = s_{y/x}\{1+1/n+ (\Sigma x_i/n)^2/S\ (x_i-\Sigma x_i/n)^2\}^{1/2} = 294.879$)
  (a = 76182 : regression line slope,
  b = 267.36 : *y*-intercept
  n = 25 : total analysis repetitions)

Note that the y-intercept standard deviation calculation formula shown above represents the distribution of the regression-dependant variable (extrapolation predicted value) when the independent variable (theoretical concentration) is 0. There is a separate equation for calculating the y-intercept standard deviation, however, it should be pointed out that when that equation is used, a smaller value is obtained compared to when using other methods (calculation by S/N ratio or residual standard deviation). For that reason, it is preferable to use this equation.

LC-MS Discussion (part 1)

• Introduction

Currently, liquid chromatograph-mass spectrometry (LC-MS) is spreading widely throughout the pharmaceutical, environmental, food, industrial materials and other fields. From this issue forward we will offer commentary on the topic of LC-MS over several issues.

• What Are the Merits of LC-MS?

In general, in LC, various constituents of a sample are separated due to differing affinity (retention force) for the stationary phase (column) and mobile phase, and depending upon the properties of the components, they are detected using UV, fluorescence, electrical conductivity, etc. Using these detectors, qualitative analysis of the substances is conducted primarily on the basis of retention time, and quantitation is conducted using peak height and area. Chromatography can provide excellent separation, however, reliable identification and quantitation are difficult in cases where many constituents elute from the column at about the same time, such as when conducting simultaneous analysis of many components.

On the other hand, MS is a high sensitivity detection method in which analyte species are first ionized using various techniques, then generated ions are separated in a vacuum based on the ratio of their mass and electrical charge (m/z), and finally each ion intensity is measured. The obtained mass spectrum shows the extent to which an ion with a given mass number is present, thereby greatly assisting in qualitative analysis. The mass number is information that is specific to the molecule, and this information is obtained directly from the MS. However, this is the situation in which constituents are analyzed individually. If multiple analytes are injected simultaneously, spectral analysis becomes extremely difficult.

LC-MS is an instrument system that combines the excellent separation ability of LC with the excellent qualitative ability of the MS. A mass spectrum obtained using scan analysis can provide molecular weight and structural information, supplementing the retention time given by other LC detectors for conducting qualitative analysis (Fig. 1). Moreover, in SIM (Selected Ion Monitoring) analysis, detection is conducted using the mass number, a parameter providing high selectivity. Even in the event of inadequate LC separation, quantitative analysis can be conducted to circumvent the influence of impurities. The combination of wide analyte accommodation and selectivity afforded by the MS make it a powerful LC detector.

• Comparison with GC-MS

Mass spectrometers were used as detectors in gas chromatography (GC) relatively early, and their benefits are widely recognized. From the viewpoint of separation and qualitative analysis of substances, GCMS is an effective method. However, the samples that can be analyzed are limited to relatively low molecular weight gases or volatile compounds, and compounds that are very thermally stable. In LC, on the other hand, if the sample can just be dissolved in the mobile phase (liquid), substances that are not suitable for GCMS analysis, such as those that are not easily volatilized or are thermally unstable, can be analyzed. In other words, LC-MS has the advantage of being suitable for analysis of a wide range of samples.

• LC-MS Instrument Composition

The mass spectrometer anaytical system consists of the sample introduction component (HPLC, GC, etc.), the interface between the sample injection and the MS sections, the ion source which ionizes the sample, the electrostatic lenses which efficiently guide the generated ions, the mass spectrometer which separates the ions according to m/z, and the detector which detects the separated ions.

There are various types of mass spectrometers depending on the ion separation method. Shown here (Fig. 2) is the composition of an atmospheric pressure ionization type quadrupole MS which is generally used as an LCMS detector. Atmospheric pressure ionizers include the electrospray ionizer (ESI) and the atmospheric pressure chemical ionizer (APCI), etc., and they serve as an interface with the HPLC and as an ion source. Following desolvation, ions which are generated here are directed by the octapole, to the quadrupole mass analyzer. In the quadrupole mass analyzer, combined direct current and RF alternating current potentials are impressed on the rods so that only ions with the selected m/z can pass through. The number of ions that reach the detector are converted to a signal, which is acquired at the PC.

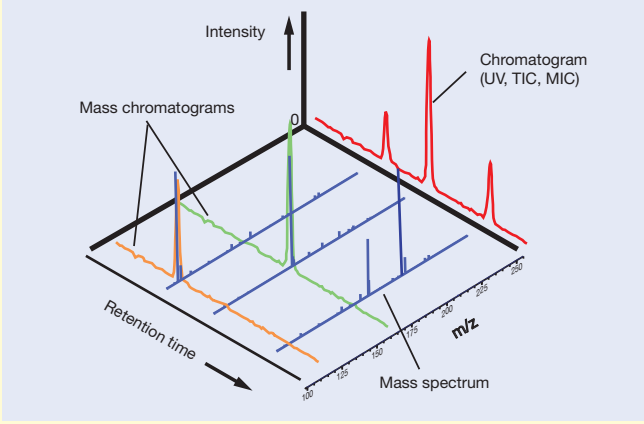


Fig. 1 Chromatogram and Mass Spectrum

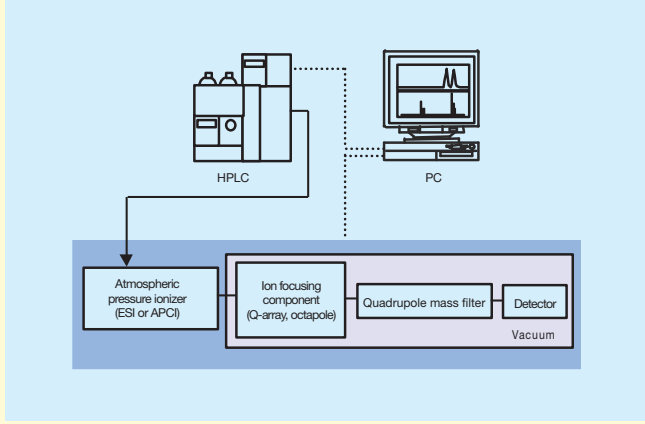


Fig. 2 LC-MS System Composition

LC-MS Discussion (part 2)

In this article, we discuss the ionization methods used in LC-MS.

• Combination of LC and MS

The GC-MS has been a success as a 'combination' analytical instrument, and although the expectation has been high for online LC and MS, general purpose use of LCMS has begun only recently.

A mass spectrometer is an instrument in which analytes are taken to the vapor phase as ions, which are then detected under high vacuum. In the case of GC-MS, the analytes are already volatilized inside the GC, so they can be introduced directly to the MS. In LC-MS, however, merely connecting the LC to the MS would result in volatilization of the liquid mobile phase, and the large amounts of gas introduced into the MS would reduce the degree of vacuum to the point that the target ions could not reach the detector. Even with eluate flowing from the LC at a mere 1mL/min, depending on the solvent, volatilization of this would increase the volume 1000 times, resulting in a huge amount of gas. In LC-MS, the amount of mobile phase that can be eliminated is critical. Various types of interfaces were developed to deal with this problem, however, problems of sensitivity, stability and convenience remained.

• Atmospheric Pressure Ionization

Recently, the interface has been greatly improved with the appearance of the atmospheric pressure ionization (API) method, which allows stable ionization. As its name suggests, the characteristic of this method is that ionization is conducted under atmospheric pressure, and the point that the solvent is prevented from entering the vacuum using this method makes it extremely effective. Currently, there are mainly two main types of API interfaces.

One is the electrospray ionization (ESI), which is effective for ionic and polar compounds (Fig. 1). In ESI, the sample solution is conducted through a capillary, at the tip of which 3 - 5kV high voltage is applied. A nebulizer gas flows in a sheath external to the capillary, and nebulizes the eluent, producing charged droplets with the same sign as that of the applied voltage. As the charged droplets proceed, solvent evaporation continues while the charges on the droplets increase, and when the repulsion force of the charges exceeds the surface tension of the droplet, the droplet divides. This repeated evaporation and fission results in very fine droplets, so that finally the sample ions are expelled in the gas phase (ion evaporation). ESI is the softest ionization method, and accordingly, it is applied with high polarity, difficult to volatilize, and thermally unstable compounds. For the most part, the produced ions are protonated (or deprotonated) molecules, and since complex fragment ions are not produced, it is easy to obtain the molecular weights of compounds. Moreover, since multivalent ions are generated depending on compound, for example even if there is a compound with a molecular weight of 10,000, it will become a 20 valent ion with m/z 501, and a 10 valent ion with m/z 1,001, which can be detected even with a small mass spectrometer. From these multivalent ions, it is possible to estimate the molecular weight by computer calculation. ESI is widely used for analysis of natural substances, biological macromolecules, and drugs, etc.

Another type of API interface is the atmospheric pressure chemical ionizer (APCI), and similar to the CI of GC-MS, it is another chemical ionization method (Fig. 2). The construction of the interface is similar to that of the ESI, but the ionization principle is different, and is suitable mainly for low and moderately polar compounds. In APCI, the sample solution (LC

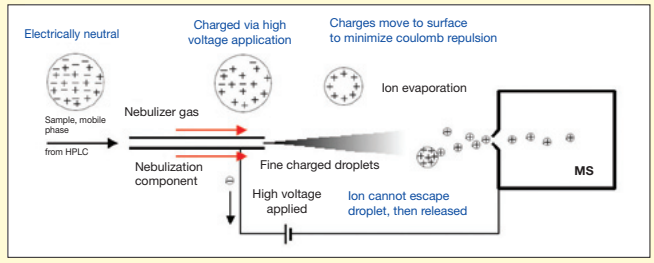


Fig. 1 Electrospray Ionization (ESI): Ion Evaporation

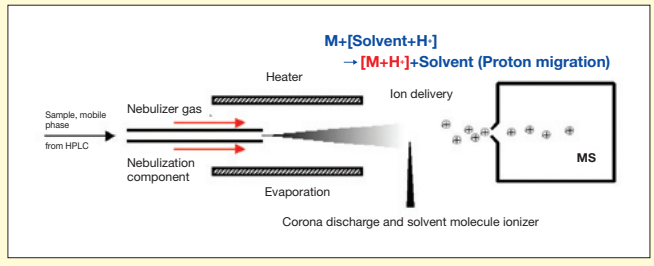


Fig. 2 Atmospheric Pressure Chemical Ionization (APCI): Ion-Molecule Reaction

eluate) is nebulized with N_2 or another nebulizer gas and directed into the heater (heated to about 400°C), to evaporate the solvent and sample molecules. The solvent molecules are ionized via corona discharge to produce stable reaction ions. Protons are given and received between these reaction ions and the sample molecules (ion-molecule reaction), and the sample molecules become protonated or deprotonated ions. Several patterns of this ion-molecule reaction are known, including the proton migration, electrophilic addition, etc. As with the ESI, for the most part, protonated (or deprotonated) molecules are detected. This method is used for analysis of highly lipophilic compounds and compounds that are not ionized in solution.

Fig. 3 shows the relation between ionization methods and analyte substances. HPLC itself can be used for analysis of an extremely wide range of compounds, and by selectively using the ESI and APCI ionization methods, a wider range of organic compounds can be handled.

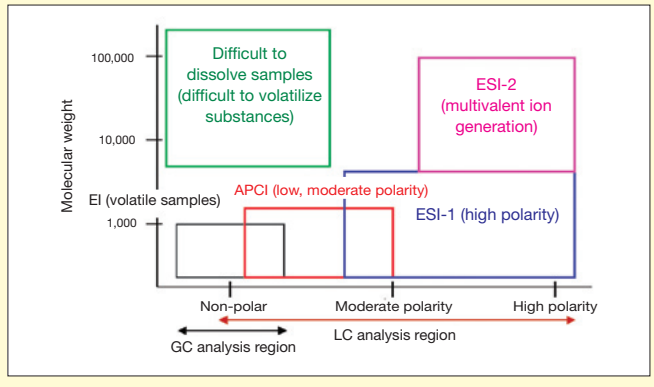


Fig. 3 Ionization Methods and Analyte Compounds

LC-MS Discussion (part 3)

With the development of atmospheric pressure ionization (API) methods, a wider range of organic compounds can be ionized, expanding the range of applicability of LC-MS. In this article, we discuss what type of mass spectrum can be obtained using API.

• Mass Spectrum using API

In both ESI and APCI, protonated molecules, in addition to ions with attached metal or solvent, are mainly detected. Here we compare ESI and APCI spectra with the EI (electron ionization) spectrum generally obtained in GC-MS. Fig. 1 shows the mass spectra of vitamin B2 (riboflavin) obtained using EI and ESI. The vertical axis indicates ion intensity, and the horizontal axis the mass-to-charge ratio (m/z).

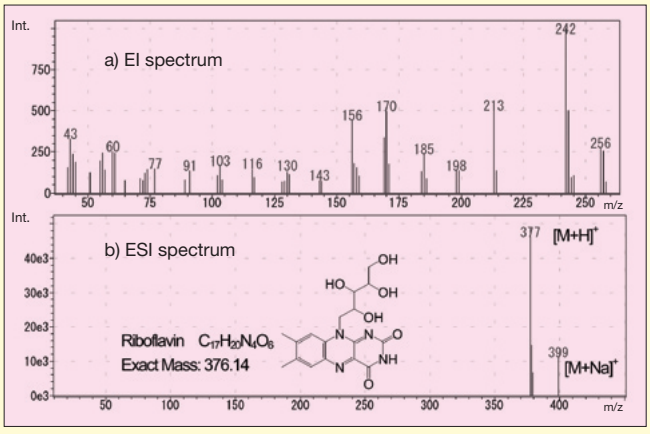


Fig. 1 Vitamin B2 Mass Spectra

In EI, one electron is removed from a gaseous molecule by an electron beam to form a molecular ion (radical cation). This divides instantaneously to produce a group of fragment ions. Structural information can be obtained based on the pattern of these fragment ions. However, there are cases where molecular ion peaks cannot be detected. In Fig. 1 (a), actually, only fragment ions are detected, while no molecular ions are detected. It is difficult to obtain molecular weight information using EI, requiring complementary analysis using CI (chemical ionization), etc.

In the case of ESI, a softer ionization method, on the other hand, a simple spectrum is displayed, indicating detection of a protonated molecule at m/z 377 and a sodium-added ion at m/z 399, while almost no fragment ions are seen. In this way, molecular weight information can be obtained easily using API, enabling deduction of the structure of unknown compounds. (The positive ion mode is used in this example because vitamin B2 possesses a basic functional group.) However, the fact that fragment ions do not easily occur suggests that it is difficult to obtain structural information such as functional groups.

As a technique for obtaining structural information using API, a method called collision induced dissociation (CID) can be employed to produce fragment ions, which are then measured. CID occurs in the electrostatic lens unit (Fig. 2) or in a tandem mass spectrometer with a collision chamber. Figure 2 shows an example of fragment ions of the antibiotic substance erythromycin, which were generated by increasing the voltage applied to the electrostatic lenses. Using these ions, it is possible to identify minor constituents which appear on the chromatogram (Shimadzu Application News, LCMS No. C21).

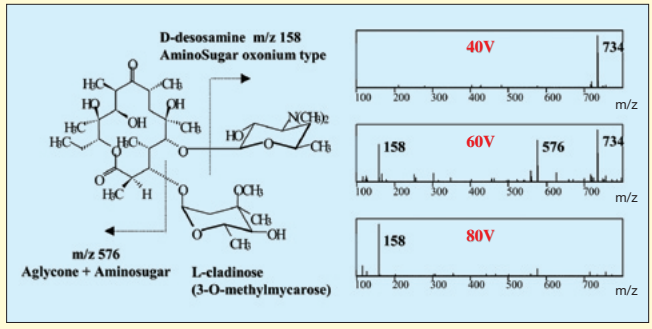


Fig. 2 Erythromycin Collision Induced Dissociation Spectrum

• Multivalent Ion Measurement

With the API methods, especially the ESI method, molecular ions of compounds possessing multiple ionization sites might have multiple charges (in a positively charged ion, multiple protons are added, $[M+nH]^{n+}$). They show a consecutive range of charged state in the m/z 500 - 2000 range. The degree of proton addition is strongly influenced by the pKa of the compound and pH of the solution. When these kinds of multivalent ions are observed, molecular weight information can be obtained even for compounds with molecular weights exceeding the measurement range of the mass spectrometer. This is used in measurement of biological macromolecules like proteins and nucleic acids that have extremely large molecular weights and high polarity.

Fig. 3 shows an ESI spectrum of horse myoglobin as a protein example. Here, ions with valences 9 - 20 are detected, and the molecular weight can be calculated to be 16951.3 using deconvolution computation. Comparing this to the theoretical molecular weight of 16951.5 calculated from the amino acid composition, the difference is less than 0.002%, indicating that a very accurate value was obtained.

A big difference from GC-MS can be seen here, where in almost all cases, only peaks with z=1 can be obtained.

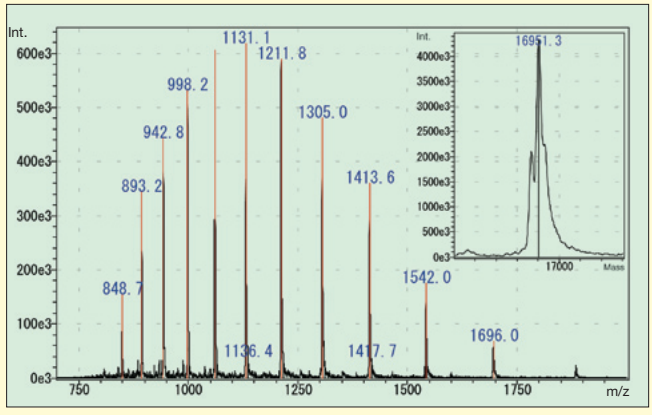


Fig. 3 ESI Spectrum of Horse Cardiac Muscle Myoglobin and Deconvolution-Computed Molecular Weight

Separation of Sugars

Sugars belong to one of the organic compound groups that exist most widely and abundantly in the natural world, with the types extending across many categories, from monosaccharides and oligosaccharides to polysaccharides, neutral sugars, acidic sugars, amino sugars, sugar alcohols, and further, their isomers. HPLC is widely used for separation analysis of these sugars, however, it is necessary to select suitable separation and detection methods according to the objective. Here we will talk about the separation methods (mainly for neutral sugars).

• Types of Separation Methods

Separation methods for sugars can be broadly classified into five representative modes, in which separation occurs based on different mechanisms.

- Size exclusion …… separation by molecule size
- Ligand exchange … complex formation with metal ions
- Normal phase …… distribution due to hydrophilicity
- Anion exchange … ion interaction
- Borate complex anion exchange

…… anion exchange of complex with borate

(More than one of these modes may be involved)

• Size Exclusion

This is used for separating sugars according to molecular weight. The molecular weight distribution can be obtained in a range from hundreds to millions. Since the separation is fundamentally based on the molecule size, molecules having the same molecular size cannot be separated. A hydrophilic polymer is used as a packing material, and water is normally used as the mobile phase. However, if ionic constituents are being separated, salts may be added to the mobile phase to alleviate interaction with the packing material.

• Ligand Exchange

This method uses a packing material consisting of sulfonated polystyrene gel with a metal counter ion, such as a sodium (Na type), calcium (Ca type), or lead (Pb type), and is suitable for separation of disachharides and lesser sugars. Sugar retention in this method is based on the formation of a complex consisting of the sugar hydroxyl group and the metal counter ion, and involves the stability of the complex formed by the replacement of the hydrated water in the metal counter ion with the sugar. The base of the separation is the size exclusion method in which the exclusion limit molecular weight is about 1000, however, differences in retention occur even among monosaccharides with the same molecular weight depending on the type of the metal counter ion and the number and positions of the saccharide hydroxyl groups. With the Na type, since it is the same as size exclusion in principle, only glucose and fructose are separated. With the Ca type, sugar alcohols are retained selectively, while with the Pb type, sugar alcohols are even more strongly retained, and some monosaccharides are also separated.
Since only water is used as the mobile phase with this method, it can be said to be an environmentally friendly separation method. However, the separation cannot be controlled on the basis of mobile phase. In addition, separation of disaccharides is difficult, and with the Ca type and Pb type, it is necessary to set the column temperature to about 80°C to prevent peak splitting and degeneration due to anomer separation.

• Normal phase

This method is suitable for separation of monosaccharides to oligosaccharides. In particular, since the differences in oligosaccharide sugars can be distinguished one by one, separation of disaccharides is easy. In the normal phase method, an amino group bonded to a silica or polymer substrate is generally used as the packing material. For the mobile phase, an acetonitrile and water mixture is used, with the elution speed increasing as the water ratio increases. The larger the molecular weight of the saccharide, the slower it elutes.
With this method, the retention of sugars occurs due to the sugar distribution onto the water concentrated in the stationary phase. Since the amino group of the stationary phase reacts with the aldehyde group of the sugar to form a Schiff base, large peak tailing can occur especially with pentoses (arabinose, ribose, etc.). This can be controlled by the addition of salt to the mobile phase. In addition, it is important to take note that there is a limit to the separation of monosaccharides.

• Anion Exchange

The pKa of neutral sugars is about 12, so with a strongly basic mobile phase, they can be retained on anion-exchange resin. In this case, an approximately 0.1M sodium hydroxide solution can be used. Generally, elution will occur sequentially from monosaccharides to oligosaccharides. Use of the gradient method by changing the concentration of the sodium hydroxide enables separation of many constituents in one operation.

• Borate complex anion exchange

Polyoxy compounds like sugars react quickly with boric acid and borates to form a negatively charged complex. In other words, by using a boric acid buffer solution in the mobile phase, saccharides can be separated by anion exchange. This method is excellent for separation of monosaccharides to disaccharides, and in particular, use of the gradient method by changing the concentration of the boric acid buffer solution and pH enables efficient separation of many constituents.

As described here, separation of sugars is not so easy. Separation mode must be carefully chosen depending on target components and impurities.

Detection of Sugars

Here we will talk about sugar detection methods, especially focusing on neutral sugars.

• Types of Detection Methods

When selecting a detection method for a particular substance, we probably all examine the structure of that substance first. Sugars mainly constitute of OH functional groups. Sugar detection methods in HPLC can be broadly categorized as follows.

- Ultraviolet-visible detection
  - Direct detection (190 - 195 nm)
  - Derivatization (pre-column, post-column)
- Differential refractive index detection
- Fluorescence detection
  - Derivatization (pre-column, post-column)
- Electrochemical detection
  - Cu electrode, Au electrode
- Evaporative light scattering detection

Each of these will now be explained briefly.

• Ultraviolet-visible (UV) Detection

Ultraviolet absorption of sugars is only obtained in the range 190 - 195 nm. Therefore, direct UV detection is practically impossible unless only water is used as the mobile phase and the sample is extremely clean. As the derivatization method to allow UV detection, the post-column method that uses color reactions, such as the orcinol - sulfuric acid method, were formerly used. However, this method is no longer used due to its drawbacks such as the requirement for corrosion-resistant instruments and the troublesome handling procedures. As for pre-column derivatization, there it no practical method either.

• Differential Refractive Index Detection

The differential refractive index method is a common detection method that is suitable for approximate detection when there is difference between the refractive index of the sample constituents and the mobile phase. This method is widely used in sugar analysis. Originally, there was not much expectation for the detection sensitivity of this method. However, as a result of recent improvement in instrumentation performance, detection is now possible even at nmol levels with aqueous mobile phases. Nevertheless detection selectivity is poor due to the versatility of the method, and gradient elution cannot be used, making this method ineffective for oligosaccharide analysis using the normal-phase method and multi-constituent analysis using the borate complex anion exchange method. Further, in the normal-phase method, the baseline may fluctuate during high sensitivity analyses. This is caused by a small deviation in the equilibration of the mobile phase (acetonitrile/water mixture) on the surface of the stationary phase due to small changes in the temperature, which causes a change in the refractive index that is detectable. In addition, in the borate complex anion exchange method, borate ghost peaks (system peaks) that look like sugar peaks may appear.

• Fluorescence Detection

The fluorescence detection method is excellent with respect to sensitivity and detection selectivity. However, the sample constituents must be fluorescent. Since sugars are not fluorescent, various fluorescence derivatization methods are being investigated. In the pre-column method, pyridylamino derivatization using the 2-aminopyradine reagent is widely used for glycoprotein sugar chain structural analysis, in which detection is possible at pmol levels. However, the derivatization process of the pre-column method typically involves some degree of time and trouble. In this respect, the derivatization reaction in the post-column method is conducted automatically, making it a preferable method for routine analyses. In the 1980s, many methods using 2-cyanoacetamide, ethanolamine, etc. were devised as post-column methods. In the same period, we found a strongly fluorescent derivative that was formed in the heating reaction between the basic amino acid arginine and a saccharide in the presence of boric acid, and we constructed an analytical system that uses this reaction. This system is still being used by many of our customers.

• Electrochemical detection

Electrochemical detection is also a high-sensitivity detection method, in which Cu and Au electrodes are used in the detection of sugars. In particular, by combining the Au electrode with the pulse mode, detection of sugar is possible at pmol levels. However, since it is necessary to maintain the reaction liquid in a strongly basic state, depending on the mobile phase conditions, high-concentration sodium hydroxide must be added to the column eluent using a separate pump. In addition, the detection selectivity is not very high and separation from impurities may be difficult in some cases.

• Evaporative Light Scattering Detection

The evaporative light scattering method nebulizes the column eluent to remove the mobile phase through evaporation, directs light at the remaining solute, and detects the scattered light. This method is applicable to the detection of any non-volatile sample constituents. Detectors using this method have been on the market for as long as 20 years, but because of the disadvantages such as low sensitivity and difficulty in use, they have not been so popular. However, with the recent appearance of instruments with improved performance on the market, they are gradually attracting attention as general-purpose detectors. Since these detectors allow gradient elution, they are especially effective in oligosaccharide analysis by the normal-phase method. However, it is important to note that the detection principle does not allow the use of non-volatile mobile phases (e.g., phosphate buffer solutions).

As described above, the detection method, as well as the separation method, must be carefully selected when analyzing sugars.

Detection of Sugars - Continued

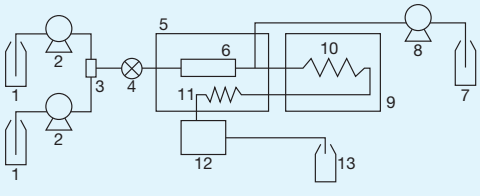
We received many letters requesting detailed information about post-column fluorescence detection for sugars. Thus we feature post-column fluorescence detection using arginine reagent in this issue.

• When is Post-column Fluorescence Detection Necessary?

Several methods are available for sugar detection. Roughly speaking, sugar samples that taste sweet can generally be detected by the differential refractive index detector. Actually, in the food industry, the differential refractive index detector is widely used. However, what about fermented products such as soy sauce? Glucose is the major sugar constituent in soy sauce, and it can be detected by the differential refractive index detector. However, in many cases, the analysis targets are sugars that exist in trace amounts, such as ribose, mannose, arabinose, galactose, and xylose. These sugars cannot be detected by the differential refractive index detector. The electrochemical detector is the next possible candidate from the viewpoint of sensitivity. However, the many amino acids present in soy sauce interfere with the electrochemical detection of the sugars of interest. The post-column fluorescence method is effective in this situation.

• Post-column Derivatization Using Arginine Reagent

We found a strongly fluorescent derivative (excitation wavelength 340 nm, fluorescence wavelength 430 nm) that was formed in a heated reaction (150°C) between the basic amino acid arginine and a reducing sugar in the presence of boric acid [1], and developed a post-column fluorescence detection HPLC system using this reaction. Fig. 1 shows the flow line diagram of that system.



1. Mobile Phases
2. Pumps for Mobile Phases
3. Gradient Mixer
4. Injector
5. Column Oven
6. Column
7. Reaction Reagent
8. Pump for Reaction Reagent
9. Reaction Bath
10. Reaction Coil
11. Cooling Coil
12. Fluorescence Detector
13. Waste

Fig.1 Flow Line Diagram

This method can be applied to each of the representative sugar separation methods described in issue No. 49. However, combining it with the borate complex anion exchange method is the most effective. Although the borate complex anion exchange method is excellent for the separation of monosaccharides and disaccharides, gradient elution is necessary to improve the separation efficiency and the differential refractive index detection cannot be used. The post-column fluorescence detection combined with the borate complex anion exchange method using the arginine reagent is widely used.

• Merits of Arginine Reagent

There are several variations for post-column fluorescence detection of reducing sugars. Among these variations, the arginine reagent method is superior to the other options in that the reagent is easily obtained, moderately priced and safe to use. The arginine reagent method also detects the non-reducing disaccharide, sucrose. Although the sensitivity is about 1/10 the sensitivity of the reducing disaccharide maltose, the capability of detecting sucrose is a great advantage.

• More About the Arginine Reagent

We investigated the method of adding the arginine reagent to the mobile phase. This method eliminates the necessity of using the mixer and reagent delivery pump, which often deteriorate efficiency in the post-column method. The entire system can also be simplified. The results showed that in the borate complex anion exchange method the arginine in the mobile phase did not influence in any way the sugar separation, and the reaction in the reaction coil heated to 150°C was carried out with good efficiency.

• Soy Sauce Analysis

We analyzed sugars in soy sauce using the system described above. Fig. 2 shows the results of analysis of commercial soy sauce. It is evident that trace sugars other than glucose were also detected.

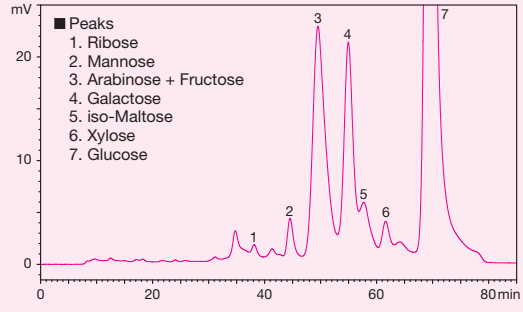


Fig. 2 Soy Sauce Analysis

1) H.Mikami and Y.Ishida:Bunseki Kagaku, 32, E207 (1983)
2) H.Mikami and Y. Ishida :10th International Symposium on Column Liquid Chromatography (1986)

Evaporative Light Scattering Detector

After the evaporative light scattering detector (ELSD) was introduced, we received many letters requesting more detailed information about ELSD. This article features ELSD.

• What is ELSD?

The ELSD (Evaporative Light Scattering Detector) is an instrument which nebulizes the column eluent to remove the mobile phase solvent through evaporation, then directs light at the remaining non-volatile constituents and detects the scattered light. Since any substances that do not evaporate with the mobile phase are detected, this instrument is sometimes referred to as a "universal detector". Actually, ELSD has been on the market for more than 20 years, but because of its relatively poor sensitivity and stability, it has not been very popular. However, especially in response to increased requirements in the pharmaceutical field, many improvements have been made in recent years, greatly enhancing the ELSD performance and thereby increasing its popularity.

• Principles of ELSD

Fig. 1 illustrates the detection principle of the Shimadzu ELSD-LT. The ELSD detection process is divided into 3 steps: (1) nebulizing the column eluent, (2) evaporating the mobile phase, and (3) detecting the scattered light from non-volatile sample constituents. The column eluent is nebulized by the flow of nitrogen or air from the nebulizer. As it proceeds through the temperature-controlled drift tube, the mobile phase is removed through evaporation, and only the non-volatile components are sent to the detector. In the detector, light is irradiated onto these constituent droplets, and the resultant scattered light is detected by the photomultiplier. Fig. 2 illustrates the structure of the detection unit.

• ELSD vs. RID

RIDs (Refractive Index Detectors) are also "universal detectors" that detect any substance even without UV absorption. The RID detects the difference in the refractive index between the sample constituents and mobile phase. Since there is normally some difference between these refractive indices, fundamentally, anything can be detected by RID. Let us compare the ELSD and RID, which are both universal but based on completely different detection principles.

Merits of ELSD

The primary advantage of the ELSD is its sensitivity. The S/N in sugar analysis using the ELSD is 5 - 10 times higher than that of the RID. Secondly, the RID is associated with baseline drift. With the RID, an extremely small difference in refractive index between the sample constituents and mobile phase is detected, and therefore it is necessary to maintain the mobile phase refractive index stable at all times. However, as the refractive index is affected by slight fluctuations in temperature and flow rate, it is difficult to obtain a straight baseline in high sensitivity analysis even with the newest technology. Naturally gradient elution is impossible when using the RID. The ELSD, on the other hand, is free from this problem. Especially, the capability of gradient elution provides a powerful merit for analyzing multiple substances without UV absorption. Furthermore, with the RID, if the sample solvent is different from the mobile phase, the peak of the solvent may interfere with the analyte peak. The ELSD is also free from this problem.

Cautions with ELSD

With the RID, there is no restriction with the mobile phase. With the ELSD, however, the mobile phase must be volatile. This means phosphate buffers cannot be used. Also, the ELSD detects not only target substances but also dirt and dust. Care is required for impurities in the mobile phase and soluble column packing materials, because they cause noise in detection. Another thing to point out is linearity. The RID provides linearity over a wide range. However, with the ELSD, substance quantity and the intensity of scattering light are exponentially related, and the calibration curve needs to be linearized using the double logarithm.

• Application Fields of ELSD

The ELSD has been used to analyze sugars and lipids, which cannot be detected by UV absorption. In addition to this, the ELSD is currently attracting increasing attention as an instrument for impurity verification in the pharmaceutical field. (Refer to the issue No. 51.) It is also being used in analysis of herbal medicines and other natural substances. It is expected that the ELSD will be more popular as the range of its

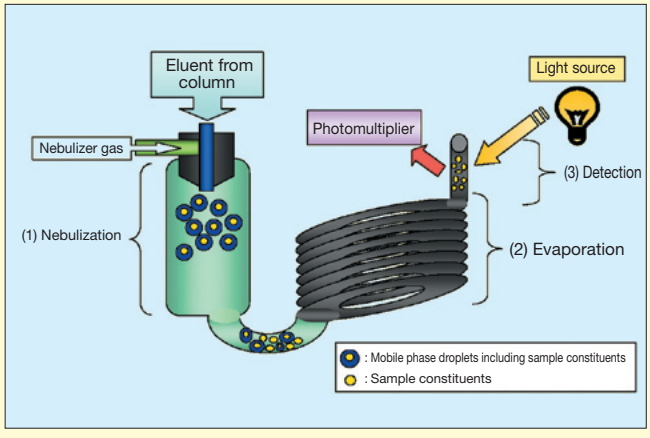


Fig. 1 Detector Principle (ELSD-LT)

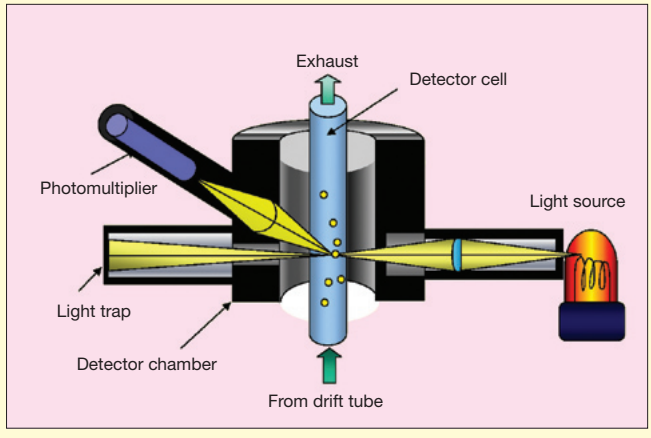


Fig. 2 Detector Structure (ELST-LT)

Methods of Amino Acid Analysis

Amino acids are attracting increasing attention with the heightening of health consciousness. Amino acids are most commonly analyzed by HPLC. Here we discuss the methods of amino acid analysis, focusing primarily on the methods of detection.

• Types of Detection Methods

The only means available to detect amino acids with UV absorption is to utilize the absorption of the carboxyl group (-COOH) at 200 - 210 nm. Although amino acids with benzene rings can be detected at 250 - 280 nm, it is generally difficult to directly analyze amino acids with high selectivity and sensitivity.
Accordingly, the derivatization method has been used. Most amino acids have amino groups (-NH2, -NHR) in their structure, and a derivatization reagent is used to selectively react with these groups.

• Pre-column Derivatization

With this method, amino acids are derivatized before injection, and the resultant substances are separated and detected using HPLC. Fig. 1 illustrates the outline of this process.

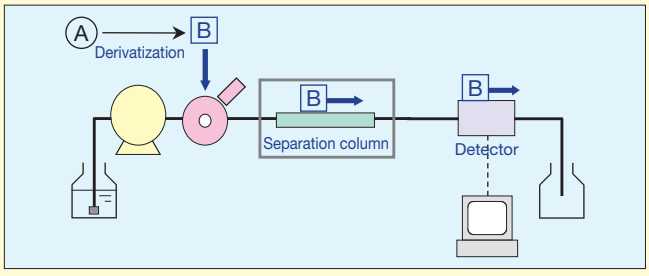


Fig. 1 Pre-column Derivatization

The merits of this method are as follows:

- Reagent consumption can be minimized by setting up a small reaction system.
- As a result of the merit above, expensive reagents can be used, making possible high sensitivity analysis with low background.
- Even if a portion of the derivatization reagent remains unreacted, it is separated in the column and therefore does not affect the analysis results.

This method also has a disadvantage. Since the sample is mixed directly with the derivatization reagent, the reaction efficiency is largely influenced by the sample matrix (co-existing constituents and solvents, etc.).
Considering the advantages and disadvantages, it is said that pre-column derivatization is suitable for high sensitivity analysis of some specific samples.
Representative pre-column derivatization reagents for amino acid analysis include o-phthalaldehyde, phenyl isothiocyanate, fluorescamine, and dansyl chloride. The procedures to cause reaction differ for each reagent. Some reagents cause reaction simply by being mixed with the sample at room temperature, and others require heating. There are also reagents that require cleanup after the reaction.
Reversed-phase chromatography (RPC) is often used for the separation of the reaction product. Since RPC is capable of quick analysis with high resolution, high-throughput analysis is possible if the conditions are set optimally.

• Post-column Derivatization

With the post-column derivatization method, the amino acids are first separated in the column, then the derivatization reagent is introduced into the eluent to cause reaction, and finally, the resultant compounds are guided to the detector. Fig. 2 illustrates a typical flow line diagram of this process.
The merits of this method are as follows:

- The reaction can be automated, improving quantitation performance and reproducibility.
- Sample constituents are separated before derivatization, minimizing the influence of the matrix. As a result the method is applicable to a wide range of samples.

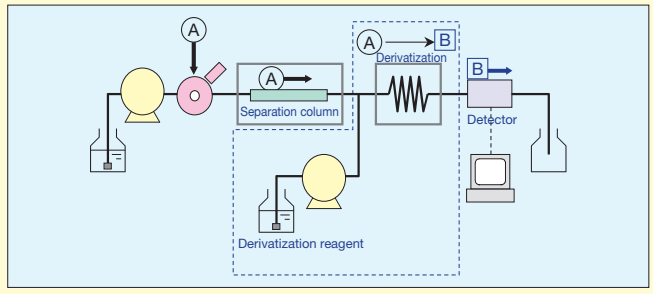


Fig. 2 Post-column Derivatization

The disadvantages are the difficulty in enhancing sensitivity, and the large consumption of reagent (reagents need to be continuously delivered).
Considering the advantages and disadvantages, post-column derivatization is an excellent technique for quantitation in routine analyses that can be applied to a wide range of samples once the reaction system is optimized.
Since the reaction reagent continuously flows into the detector, only specific reagents can be used in post-column derivatization, to prevent unreacted reagent from being detected. Currently only two reagents are available for analysis of amino acids: ninhydrin for UV-VIS detection and o-phthalaldehyde for fluorescence detection.
The most common separation method is cation-exchange chromatography. Since amino acids are ampholite ions that have both amino and carboxyl groups in their structure, in cation exchange, constituents with higher acidity (constituents that easily become anions) elute faster, and constituents with higher basicity (constituents that easily become cations) elute more slowly.
By the way, why is the cation-exchange method is used even though the mainstream of HPLC is reversed-phase chromatography? This is because the separation of amino acids and other substances including amino groups (amines), which is difficult with the reversed-phase chromatography, can be conducted easily and with good efficiency using cation-exchange.
Since the derivatization reagents used in amino acid analysis possess reactivity for amino groups, they also react with amines, and those may be detected as peaks. Amines generally do not have an anion type functional group like a carboxyl group, showing a stronger basicity than amino acids. Therefore they elute more slowly than amino acids in cation-exchange. This is how the cation-exchange method prevents amines from interfering with the quantitation of amino acids.
As described above, cation-exchange chromatography, which can separate amino acids and amines, and post-column derivatization, which realizes selective reaction with amino acids, provide a perfect combination.
Fig. 3 shows an example of a chromatogram obtained by analyzing an amino acid beverage by the post-column derivatization method using o-phthalaldehyde as the reagent.

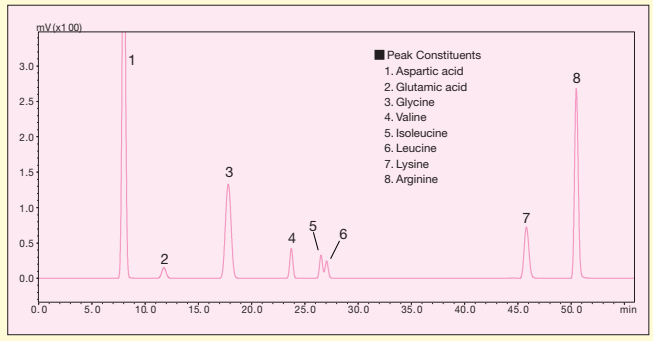


Fig. 3 Commercial Amino Acid Beverage Analysis

Analysis of Organic Acids

Organic acids, along with amino acids and sugars, are often analyzed for "deliciousness" in food products. Here we will discuss the analysis of organic acids using HPLC.

• Separation

There are three major modes for the separation of organic acids: anion-exchange, ion exclusion and reversed-phase. We briefly describe each mode below.

• Anion-exchange mode (Fig. 1)

In this mode, separation is realized by the negative ions and organic acid ions (negative ions) in the mobile phase competing for the positive ions on the column packing. The retention behavior on the column surface differs depending on the organic acid radius and ion valence. Separation of many organic acids requires gradient elution, and thereby a long analysis time.

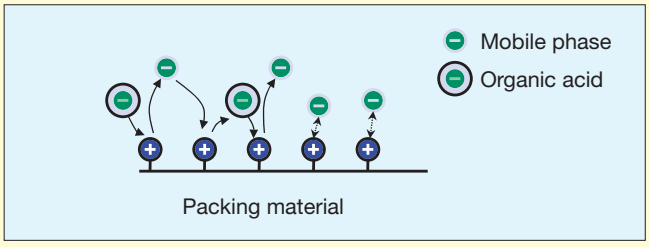


Fig. 1 Anion-exchange Mode

• Ion Exclusion Mode (Fig. 2)

This is the most common mode for the analysis of organic acids. H-type ion exchange resin is used as the packing material, and the organic acids are separated according to the degree of Donnan exclusion between the stationary phase (H-type ion exchange group) and the mobile phase. In this mode, strong acids are subjected to a large electrostatic exclusion by the negative electrical charge of the stationary phase, and cannot enter the pores of the packing material. With weak acids like organic acids, however, the degree of infiltration into the pores is determined by the size of the electrical charge (pKa), causing differences in elution time, namely separation. According to this principle, organic acids elute in the sequence of their pKa values (from small to large), with all of them having eluted before the elution time of the neutral substances (the time when the pores are completely infiltrated). In actual analysis, however, highly hydrophobic organic acids may elute more slowly due to hydrophobic interaction with the packing material matrix. This mode is widely used because of its operational simplicity.

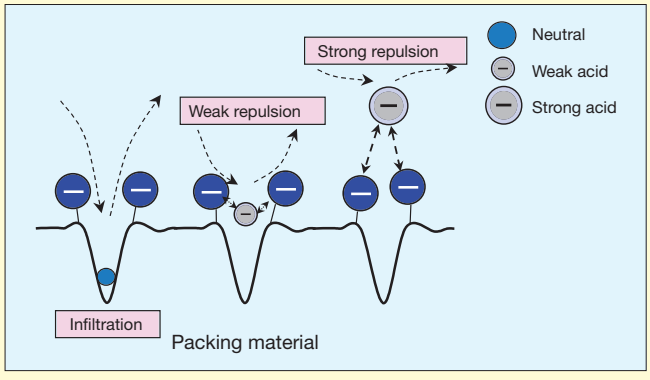


Fig. 2 Ion Exclusion Mode

• Reversed-phase Mode

Although this is the most versatile HPLC mode, it is not widely used for the analysis of organic acids. This is because sufficient retention or selectivity cannot be achieved due to the hydrophilic properties of organic acids. Recently, reversed-phase columns that also retain highly hydrophilic substances are appearing on the market.

• Detection

The easiest detection method is the direct UV direct detection, in which the carboxyl group absorption at 200 - 210 nm is detected. However, since many organic substances show absorption in this wavelength region, there is great interference due to impurities, making accurate quantitation difficult unless the sample is extremely clean. Although differential refractive index detectors can be used, they do not provide sufficient selectivity and sensitivity. In direct detection using the electrical conductivity detector, it is necessary to lower the electrical conductivity of the mobile phase background.

Post-column methods that selectively detect organic acids with high sensitivity include the visible absorption detection using pH indicators and electrical conductivity detection using pH buffers. The pH-indicator method, which utilizes the color change of pH indicators in response to organic acids, is slightly poor in linearity and procedural simplicity. The pH buffer method is excellent for its sensitivity, selectivity and accuracy.

• pH-buffer Method

In the ion exclusion mode widely applied in analysis of organic acids, acidic mobile phase is normally used and therefore, the background electrical conductivity is kept high. The pH-buffer method continuously adds a pH buffer reagent to the column eluent to bring it near a neutral pH and dissociate the organic acids. Then the dissociated organic acids are detected by an electrical conductivity detector with high sensitivity. This is a very effective method that lowers the mobile phase background, and at the same time enhances sensitivity by dissociating the organic acids.

The pH-buffer method enables highly sensitive and selective detection of organic acids, as well as analysis of samples over a wide range of concentrations, thanks to its wide range of calibration curve linearity. This method is adopted in Shimadzu's organic acid analysis system. Fig. 3 shows the results of soy sauce analysis. Using this system, organic acid analysis even in samples like soy sauce can be conducted with good accuracy, without interference from impurities.

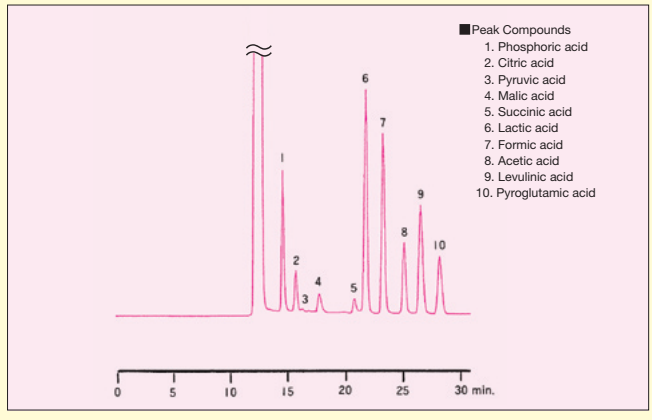


Fig. 3 Soy Sauce Chromatogram

Size Exclusion Chromatography

"Size exclusion chromatography" and "GPC" are terms we often hear. Let us return to the basics to understand just what kind of information can be obtained with these methods.

• Size Exclusion Chromatography (SEC)

This is a separation mode that utilizes the phenomenon in which solute molecules either infiltrate or are excluded from the pores of porous column packing according to the pore diameter (size). In general, a technique that uses hydrophobic packing material and non-aqueous (organic solvent) mobile phase to analyze the molecular weight distribution of synthetic polymers is called "Gel Permeation Chromatography (GPC)". Another technique that uses hydrophilic packing material and aqueous mobile phase for separation and molecular weight distribution analysis of polysaccharides, proteins and other aqueous macromolecules is called "Gel Filtration Chromatography (GFC).

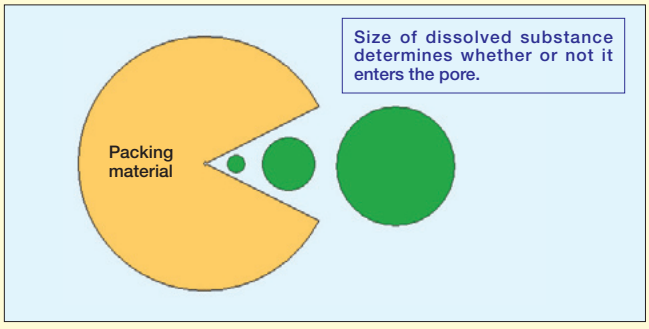


Fig. 1 Principle of Size Exclusion Chromatography

• Information Obtained in Molecular Weight Distribution Analysis

Most polymers, unlike low molecular weight substances, are a set of homologues with different molecular weights. Conventionally, the molecular weights of polymers have been analyzed with the light scattering, osmotic pressure, viscosity and other methods. However, these methods merely output the mean value, assuming that the analyzed substance consists of a single molecule. As a result, substances with the same result (mean value) may actually have significantly different molecular weight distributions. Size exclusion chromatography is an excellent method for ascertaining the differences in the physical properties of polymers.

• What is Mean Molecular Weight?

When actually measuring molecular weight and molecular weight distribution, the polymer sample must be dissociated in a solvent to disperse the molecules. If the polymer shows a single state in the solvent, the molecular weight can be ideally measured. Since a polymer is a set of homologues with different molecular weights, the pattern of molecular weight distribution, as well as the mean value of the molecular weights and parameter for determining the width of molecular weight distribution are required. The mean molecular weight is expressed as the number average molecular weight ($\overline{M}n$), weight average molecular weight ($\overline{M}w$), Z average molecular weight ($\overline{M}z$), and viscosity average molecular weight ($\overline{M}v$). The molecular weight ascribed to the peak apex in size exclusion chromatography is the change between $\overline{M}n$ and $\overline{M}w$.

The parameter for determining the width of molecular weight distribution is the degree of distribution ($\overline{M}w$ / $\overline{M}n$). If this value is near 1.0, the distribution is narrow, and as the value increases, the distribution widens.

• Calibration Curve

The following equation generally applies to the relationship between the elution position (Ve) and molecular weight (M) of a polymer

Log M = b-cVe (where b and c are constants)

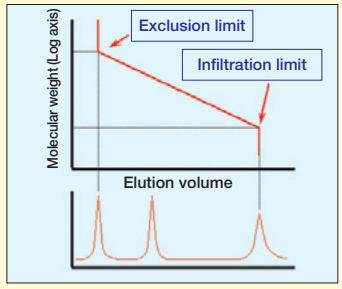


Fig. 2 Calibration Curve and Chromatogram

A calibration curve is a graphical expression of this relationship. The vertical axis is molecular weight, and the horizontal axis is elution position. By using a molecular weight marker with known molecular weight, the elution volume (retention time) is obtained to generate a calibration curve. Solutes that are larger than a given size are completely excluded from entering the stationary phase mesh. This size is the "exclusion limit". Conversely, solutes that are smaller than a given size completely infiltrates the stationary phase mesh, and they elute at about the same position. This size is the "infiltration limit". The calibration curve is generated for the region between the exclusion limit and the infiltration limit.

Since it is difficult for one packing material (column) to cover a wide range of molecular weights, size exclusion columns are available with several packings with the same properties but different pore sizes. It is necessary to select the appropriate column depending on the target molecular weight range.

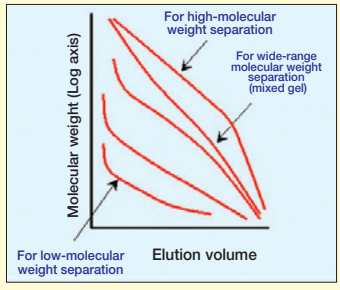


Fig. 3 Column and Calibration Curve

When the sample molecular weight distribution extends over a wide range, the columns necessary for covering the molecular weight distribution range are selected, and are then connected in series. Or, a column with packing having different pore diameters (mixed gel) can also be used.

• Cautionary Points

It should be noted that in size exclusion chromatography, the molecular weight and actual molecule size do not necessarily correspond. Even if two substances have the same molecular weight, the actual sizes are different if one of them is coarse and the other is dense. Moreover, depending on the type of solvent used for dissolution, the structure may become coarse or dense.

A marker with a known molecular weight is used to calculate the molecular weight distribution. This molecular weight marker and the actual sample must be of the same substance to obtain the actual molecular weight distribution.

Furthermore, if there is interaction (adsorption, ion exchange, etc.) between the sample compounds and the packing material, the correct molecular weight distribution cannot be obtained. The principles of size exclusion chromatography are relatively easy to understand, however, a deeper understanding is required to conduct actual analysis and interpret the analysis results.

JQA-0376

Founded in 1875, Shimadzu Corporation, a leader in the development of advanced technologies, has a distinguished history of innovation built on the foundation of contributing to society through science and technology. We maintain a global network of sales, service, technical support and applications centers on six continents, and have established long-term relationships with a host of highly trained distributors located in over 100 countries. For information about Shimadzu, and to contact your local office, please visit our Web site at **www.shimadzu.com**

SHIMADZU

SHIMADZU CORPORATION. International Marketing Division
3. Kanda-Nishikicho 1-chome, Chiyoda-ku, Tokyo 101-8448, Japan
Phone: 81(3)3219-5641 Fax. 81(3)3219-5710
URL http://www.shimadzu.com

Printed in Japan 3295-03613-30ATD