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⟨621⟩ CHROMATOGRAPHY

To view the Notice from the Expert Committee that posted in conjunction with this accelerated revision, please click www.uspnf.com/rb-notice-gc-621-20241122.

Change to read:

(The sections *System Sensitivity* and *Peak Symmetry* will become official on ▲June 1, 2026▲ (RB 1-Dec-2024) as indicated.)

INTRODUCTION

Chromatographic separation techniques are multistage separation procedures in which the components of a sample are distributed between two phases, one of which is stationary while the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), ion exchange, etc., or may be based on differences in the physicochemical properties of the molecules such as size, mass, volume, etc.

Portions of the present general chapter text that are national *USP–NF* text, and therefore not part of the harmonized text, are marked with symbols (♦♦) to specify this fact.

♦This chapter describes general procedures, definitions, and calculations of common parameters and generally applicable requirements for system suitability.

The types of chromatography useful in qualitative and quantitative analysis employed in USP procedures are column, gas, paper, thin-layer (including high-performance thin-layer chromatography), and pressurized liquid chromatography (commonly called high-pressure or high-performance liquid chromatography).

GENERAL PROCEDURES

This section describes the basic procedures used when a chromatographic method is described in a monograph. The following procedures are followed unless otherwise indicated in the individual monograph.

Paper Chromatography

STATIONARY PHASE

The stationary phase is a sheet of paper of suitable texture and thickness. Development may be ascending, in which the solvent is carried up the paper by capillary forces, or descending, in which the solvent flow is also assisted by gravitational force. The orientation of paper grain with respect to solvent flow is to be kept constant in a series of chromatograms. (The machine direction is usually designated by the manufacturer.)

APPARATUS

The essential equipment for paper chromatography consists of a vapor-tight chamber with inlets for addition of solvent and a rack of corrosion-resistant material about 5 cm shorter than the inside height of the chamber. The rack serves as a support for solvent troughs and for antisiphon rods that, in turn, hold up the chromatographic sheets. The bottom of the chamber is covered with the prescribed solvent system or mobile phase. Saturation of the chamber with solvent vapor is facilitated by lining the inside walls with paper wetted with the prescribed solvent system.

SPOTTING

The substance or substances analyzed are dissolved in a suitable solvent. Convenient volumes delivered from suitable micropipets of the resulting solution, normally containing 1–20 µg of the compound, are placed in 6- to 10-mm spots not less than 3 cm apart.

DESCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. A spotted chromatographic sheet is suspended in the apparatus, using the antisiphon rod to hold the upper end of the sheet in the solvent trough. [Note—Ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack, the chamber walls, or the fluid in the chamber.]
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. After equilibration of the chamber, the prepared mobile phase is introduced into the trough through the inlet.
4. The inlet is closed, and the mobile solvent phase is allowed to travel the desired distance down the paper.
5. The sheet is removed from the chamber.

6. The location of the solvent front is quickly marked, and the sheet is dried.
7. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

ASCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. The mobile phase is added to the bottom of the chamber.
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. The lower edge of the stationary phase is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.
4. When the solvent front has reached the desired height, the chamber is opened, the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.
5. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

Thin-Layer Chromatography

STATIONARY PHASE

The stationary phase is a relatively thin, uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate (typically called the plate). The stationary phase of thin-layer chromatography (TLC) plates has an average particle size of 10–15 µm, and that of high-performance TLC (HPTLC) plates has an average particle size of 5 µm. Commercial plates with a preadsorbent zone can be used if they are specified in a monograph. Sample applied to the preadsorbent region develops into sharp, narrow bands at the preadsorbent–sorbent interface. The separations achieved may be based on adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase.

APPARATUS

A chromatographic chamber made of inert, transparent material and having the following specifications is used: a flat-bottom or twin trough, a tightly fitted lid, and a size suitable for the plates. The chamber is lined on at least one wall with filter paper. Sufficient mobile phase or developing solvent is added to the chamber so that, after impregnation of the filter paper, a depth appropriate to the dimensions of the plate used is available. The chromatographic chamber is closed and allowed to equilibrate. [Note—Unless otherwise indicated, the chromatographic separations are performed in a saturated chamber.]

DETECTION/VISUALIZATION

An ultraviolet (UV) light source suitable for observations under short- (254 nm) and long- (365 nm) wavelength UV light and a variety of spray reagents to make spots visible are often used.

SPOTTING

Solutions are spotted on the surface of the stationary phase (plate) at the prescribed volume in sufficiently small portions to obtain circular spots of 2–5 mm in diameter (1–2 mm on HPTLC plates) or bands of 10–20 mm × 1–2 mm (5–10 mm × 0.5–1 mm on HPTLC plates) at an appropriate distance from the lower edge and sides of the plate. [Note—During development, the application position must be at least 5 mm (TLC) or 3 mm (HPTLC) above the level of the mobile phase.] The solutions are applied on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots, or 4 mm (2 mm on HPTLC plates) between the edges of bands, then allowed to dry.

PROCEDURE

1. Place the plate in the chamber, ensuring that the spots or bands are above the surface of the mobile phase.
2. Close the chamber.
3. Allow the mobile phase to ascend the plate until the solvent front has traveled three-quarters of the length of the plate, or the distance prescribed in the monograph.
4. Remove the plate, mark the solvent front with a pencil, and allow to dry.
5. Visualize the chromatograms as prescribed.
6. Determine the chromatographic *retardation factor* (R_F) values for the principal spots or zones.
7. Presumptive identification can be made by observation of spots or zones of identical R_F value and about equal magnitude obtained, respectively, with an unknown and a standard chromatographed on the same plate. A visual comparison of the size or intensity of the spots or zones may serve for semiquantitative estimation. Quantitative measurements are possible by means of densitometry (absorbance or fluorescence measurements).

Column Chromatography

SOLID SUPPORT

Purified siliceous earth is used for normal-phase separation. Silanized chromatographic siliceous earth is used for reverse-phase partition chromatography.

STATIONARY PHASE

The solid support is modified by the addition of a stationary phase specified in the individual monograph. If a mixture of liquids is used as the stationary phase, mix the liquids before the introduction of the solid support.

MOBILE PHASE

The mobile phase is specified in the individual monograph. If the stationary phase is an aqueous solution, equilibrate with water. If the stationary phase is a polar organic fluid, equilibrate with that fluid.

APPARATUS

Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in inside diameter and 200–300 mm in length. Attached to it is a delivery tube, without stopcock, about 4 mm in inside diameter and about 50 mm in length.

Apparatus preparation: Pack a pledget of fine glass wool in the base of the tube. Combine the specified volume of stationary phase and the specified amount of solid support to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp using gentle pressure to obtain a uniform mass. If the specified amount of solid support is more than 3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion. If the assay or test requires a multisegment column with a different stationary phase specified for each segment, tamp after the addition of each segment, and add each succeeding segment directly to the previous one. Pack a pledget of fine glass wool above the completed column packing. [Note—The mobile phase should flow through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.]

If a solution of the analyte is incorporated into the stationary phase, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of solid support and several drops of the solvent used to prepare the sample solution before adding the final portion of glass wool.

PROCEDURE

1. Transfer the mobile phase to the column space above the column packing, and allow it to flow through the column under the influence of gravity.
2. Rinse the tip of the chromatographic column with about 1 mL of mobile phase before each change in composition of mobile phase and after completion of the elution.
3. If the analyte is introduced into the column as a solution in the mobile phase, allow it to pass completely into the column packing, then add mobile phase in several small portions, allowing each to drain completely, before adding the bulk of the mobile phase.
4. Where the procedure indicates the use of multiple chromatographic columns mounted in series and the addition of mobile phase in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with mobile phase before the addition of each succeeding portion.

Gas Chromatography (GC)

LIQUID STATIONARY PHASE

This type of phase is available in packed or capillary columns.

PACKED COLUMN GC

The liquid stationary phase is deposited on a finely divided, inert solid support, such as diatomaceous earth, porous polymer, or graphitized carbon, which is packed into a column that is typically 2–4 mm in internal diameter and 1–3 m in length.

CAPILLARY COLUMN GC

In capillary columns, which contain no packed solid support, the liquid stationary phase is deposited on the inner surface of the column and may be chemically bonded to it.

SOLID STATIONARY PHASE

This type of phase is available only in packed columns. In these columns the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase. [Note—Packed and capillary columns must be conditioned before use until the baseline and other characteristics are stable. The column or packing material supplier provides instructions for the recommended conditioning procedure.]

APPARATUS

A gas chromatograph consists of a carrier gas source, injection port, column, detector, and recording device. The injection port, column, and detector are temperature controlled and may be varied as part of the analysis. The typical carrier gas is helium, nitrogen, or hydrogen, depending on the column and detector in use. The type of detector used depends on the nature of the compounds analyzed and is specified in the individual monograph. Detector output is recorded as a function of time, and the instrument response, measured as peak area or peak height, is a function of the amount present.

TEMPERATURE PROGRAM

The length and quality of a GC separation can be controlled by altering the temperature of the chromatographic column. When a temperature program is necessary, the individual monograph indicates the conditions in table format. The table indicates the initial temperature, rate of temperature change (ramp), final temperature, and hold time at the final temperature.

PROCEDURE

1. Equilibrate the column, injector, and detector with flowing carrier gas until a constant signal is received.
2. Inject a sample through the injector septum, or use an autosampler.
3. Begin the temperature program.
4. Record the chromatogram.
5. Analyze as indicated in the monograph.

Liquid Chromatography

The term "liquid chromatography" (LC), as used in the compendia, is synonymous with high-pressure liquid chromatography and high-performance liquid chromatography. LC is a separation technique based on a solid stationary phase and a liquid mobile phase.

STATIONARY PHASE

Separations are achieved by partition, adsorption, or ion-exchange processes, depending on the type of stationary phase used. The most commonly used stationary phases are modified silica or polymeric beads. The beads are modified by the addition of long-chain hydrocarbons. The specific type of packing needed to complete an analysis is indicated by the “L” designation in the individual monograph (see also the section *Chromatographic Columns*). The size of the beads is often described in the monograph as well. Changes in the packing type and size are covered in the *System Suitability* section of this chapter.

CHROMATOGRAPHIC COLUMN

The term "column" includes stainless steel, lined stainless steel, and polymeric columns, packed with a stationary phase. The length and inner diameter of the column affects the separation, and therefore typical column dimensions are included in the individual monograph. Changes to column dimensions are discussed in the *System Suitability* section of this chapter. Compendial monographs do not include the name of appropriate columns; this omission avoids the appearance of endorsement of a vendor’s product and natural changes in the marketplace. See the section *Chromatographic Columns* for more information.

In LC procedures, a guard column may be used with the following requirements, unless otherwise is indicated in the individual monograph: (a) the length of the guard column must be NMT 15% of the length of the analytical column, (b) the inner diameter must be the same or smaller than that of the analytical column, and (c) the packing material should be the same as the analytical column (e.g., silica) and contain the same bonded phase (e.g., C18). In any case, all system suitability requirements specified in the official procedure must be met with the guard column installed.

MOBILE PHASE

The mobile phase is a solvent or a mixture of solvents, as defined in the individual monograph.

APPARATUS

A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device.

GRADIENT ELUTION

The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. The gradient elution profile is presented in the individual monograph as a gradient table, which lists the time and proportional composition of the mobile phase at the stated time.

PROCEDURE

1. Equilibrate the column and detector with mobile phase at the specified flow rate until a constant signal is received.
2. Inject a sample through the injector, or use an autosampler.
3. Begin the gradient program.
4. Record the chromatogram.
5. Analyze as directed in the monograph.

CHROMATOGRAPHIC COLUMNS

A complete list of packings (L), phases (G), and supports (S) used in *USP–NF* tests and assays is located in *USP–NF*, *Reagents, Indicators, and Solutions—Chromatographic Columns*. This list is intended to be a convenient reference for the chromatographer in identifying the pertinent chromatographic column specified in the individual monograph.♦

DEFINITIONS

The system suitability and acceptance criteria in monographs have been set using parameters as defined below. With some equipment, certain parameters, such as the signal-to-noise ratio and resolution, can be calculated using software provided by the manufacturer. It is the responsibility of the user to ensure that the calculation methods used in the software are equivalent to the requirements of the *US Pharmacopeia* and to make any necessary corrections if this is not the case.

Chromatogram: A graphical or other representation of detector response, effluent concentration, or other quantity used as a measure of effluent concentration versus time or volume. Idealized chromatograms are represented as a sequence of Gaussian peaks on a baseline (*Figure*

1).

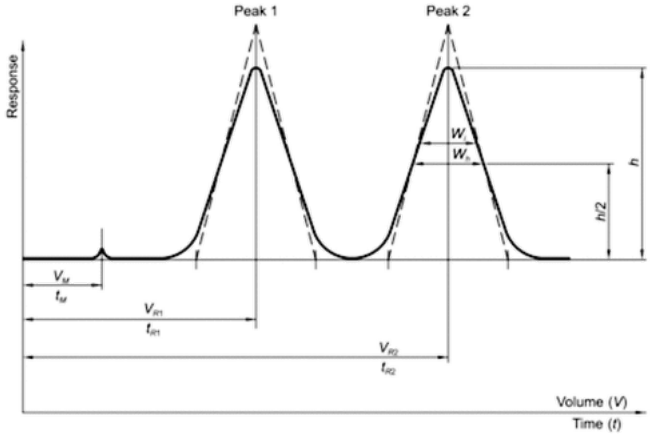

Figure 1.

V_M = hold-up volume

t_M = hold-up time

V_{R1} = retention volume of peak 1

t_{R1} = retention time of peak 1

V_{R2} = retention volume of peak 2

t_{R2} = retention time of peak 2

W_h = peak width at half-height

W_i = peak width at the inflection point

h = height of the peak

$h/2$ = half-height of peak

Distribution constant (K_0): In size-exclusion chromatography, the elution characteristics of a component in a particular column may be given by the distribution constant (also referred to as distribution coefficient), which is calculated using the following equation:

$$K_0 = \frac{t_R - t_0}{t_t - t_0}$$

t_R = retention time

t_0 = retention time of an unretained compound

t_t = total mobile phase time

Dwell volume (D) (also referred to as V_D): The dwell volume (also known as gradient delay volume) is the volume between the point at which the eluents meet and the inlet of the column. It can be determined using the following procedure.

COLUMN: Replace the chromatographic column by an appropriate capillary tubing (e.g., 1 m × 0.12 mm).

MOBILE PHASE: See *Table 1*.

MOBILE PHASE A: Water

MOBILE PHASE B: 0.1% v/v solution of acetone in water

Table 1

Time (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0–20	100→0	0→100
20–30	0	100

FLOW RATE: Set to obtain sufficient back-pressure (e.g., 2 mL/min).

DETECTION: Spectrophotometer at 265 nm

Determine the time ($t_{0.5}$), in minutes, when the absorbance has increased by 50% (*Figure 2*).

$$D = t_D \times F$$

t_D = $t0.5 - 0.5tG$ (min)

t_G = pre-defined gradient time, 20 min

F = flow rate (mL/min)

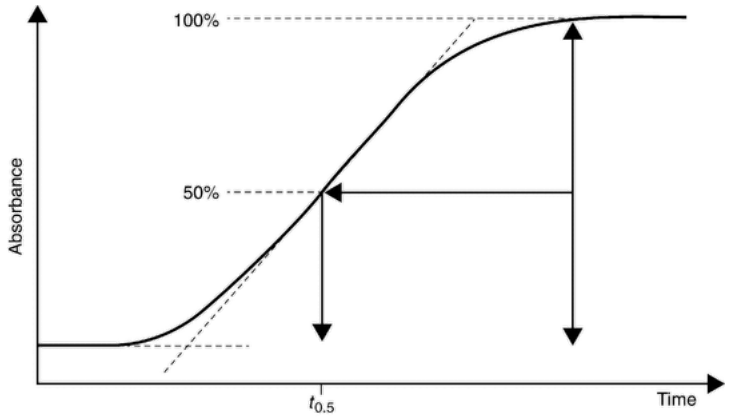

Figure 2.

[NOTE—where applicable, this measurement is performed with the autosampler in the "inject" position so as to include the injection loop volume in the dwell volume.]

Hold-up time (t_M): Time required for elution of an unretained component (see *Figure 1*, baseline scale being in minutes or seconds).

In size-exclusion chromatography, the term retention time of an unretained compound (t_0) is used.

Hold-up volume (V_M): Volume of the mobile phase required for elution of an unretained component. It may be calculated from the hold-up time and the flow rate (F) in milliliters per minute using the following equation:

$$V_M = t_M \times F$$

In size-exclusion chromatography, the term "retention volume" of an unretained compound V_0 is used.

Peak: Portion of a chromatogram recording the detector response when a single component (or two or more unresolved components) is eluted from the column.

The peak response may be represented by the peak area or the peak height (h).

Peak-to-Valley Ratio (p/v): The peak-to-valley ratio may be employed as a system suitability criterion when baseline separation between two peaks is not achieved (see *Figure 3*).

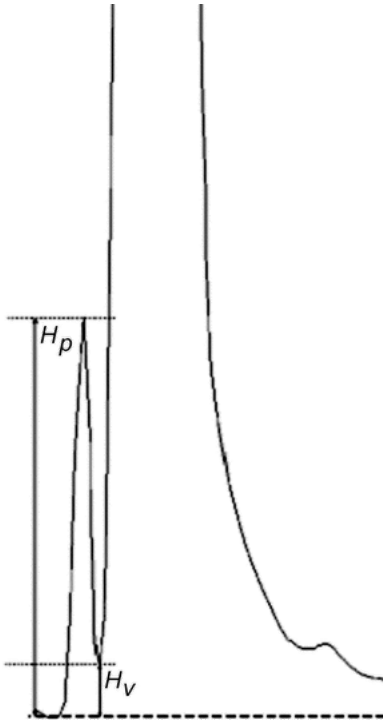

Figure 3.

$$p/v = \frac{H_p}{H_v}$$

H_p = height above the extrapolated baseline of the minor peak

H_v = height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks

Plate height (*H*) (height equivalent to one theoretical plate [HETP]): Ratio of the column length (*L*), in micrometers, to the plate number (*N*):

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

Plate number (*N*) (number of theoretical plates): A number indicative of column performance (column efficiency) can only be calculated from data obtained under either isothermal, isocratic, or isodense conditions, depending on the technique, as the plate number, using the following equation, the values of t_R and W_h being expressed in the same units:

$$N = 5.54 \left(\frac{t_R}{W_h}\right)^2$$

t_R = retention time of the peak corresponding to the component

W_h = peak width at half-height (h/2)

The plate number varies with the component as well as with the column, the column temperature, the mobile phase, and the retention time.

Reduced plate height (*h*): Ratio of the plate height (*H*), in micrometers, to the particle diameter (d_p) in micrometers:

$$h = \frac{H}{d_p}$$

Relative retardation (R_{rel}): The relative retardation, used in thin-layer chromatography, is calculated as the ratio of the distances travelled by the spot of the compound of interest and a reference compound (*Figure 4*).

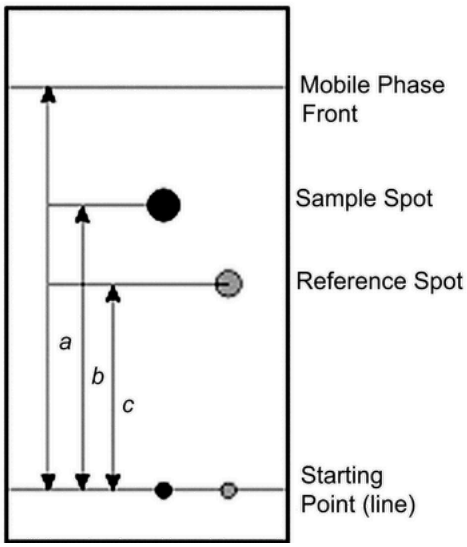

Figure 4.

$$R_{rel} = b/c$$

a = migration distance of the mobile phase

b = migration distance of the compound of interest

c = migration distance of the reference compound

Relative retention (r): Relative retention is calculated as an estimate using the following equation:

$$r = \frac{t_{Ri} - t_M}{t_{Rst} - t_M}$$

t_{Ri} = retention time of the peak of interest

t_{Rst} = retention time of the reference peak (usually the peak corresponding to the substance to be examined)

t_M = hold-up time

Relative retention, unadjusted (r_G)♦or (RRT)♦

The unadjusted relative retention is calculated using the following equation:

$$r_G = \frac{t_{Ri}}{t_{Rst}}$$

Unless otherwise indicated, values for relative retention stated in monographs correspond to unadjusted relative retention.

♦**Relative retention time (RRT):** See *Relative retention, unadjusted*♦

Resolution (R_s): The resolution between peaks of two components (*Figure 1*) may be calculated using the following equation:

$$R_S = \frac{1.18(t_{R2} - t_{R1})}{W_{h1} + W_{h2}}$$

$$t_{R2} > t_{R1}$$

t_{R1}, t_{R2} = retention times of the peaks

W_{h1}, W_{h2} = peak widths at half-height

In quantitative thin-layer chromatography, using densitometry, the migration distances are used instead of retention times and the resolution between peaks of two components may be calculated using the following equation:

$$R_S = \frac{1.18a(R_{F2} - R_{F1})}{W_{h1} + W_{h2}}$$

$$R_{F2} > R_{F1}$$

R_{F1}, R_{F2} = retardation factors of the peaks

W_{h1}, W_{h2} = peak widths at half-height

a = migration distance of the solvent front

Retardation factor (R_F): The retardation factor, used in thin-layer chromatography, is the ratio of the distance from the point of application to the center of the spot and the distance simultaneously travelled by the solvent front from the point of application (*Figure 4*).

$$R_F = \frac{b}{a}$$

b = migration distance of the component

a = migration distance of the solvent front

Retention factor (k): The retention factor (also known as mass distribution ratio (D_m) or capacity factor (k')) is defined as:

$$k = \frac{\text{amount of component in stationary phase}}{\text{amount of component in mobile phase}} = K_c \frac{V_S}{V_M}$$

K_C = distribution constant (also known as equilibrium distribution coefficient)

V_S = volume of the stationary phase

V_M = volume of the mobile phase

The retention factor of a component may be determined from the chromatogram using the following equation:

$$k = \frac{t_R - t_M}{t_M}$$

t_R = retention time

t_M = hold-up time

Retention time (t_R): Time elapsed between the injection of the sample and the appearance of the maximum peak response of the eluted sample zone (*Figure 1*, baseline scale being in minutes or seconds).

Retention volume (V_R):

Volume of the mobile phase required for elution of a component. It may be calculated from the retention time and the flow rate (F), in milliliters per minute, using the following equation:

$$V_R = t_R \times F$$

Retention time of an unretained compound (t_0): In size-exclusion chromatography, retention time of a component whose molecules are larger than the largest gel pores (*Figure 5*).

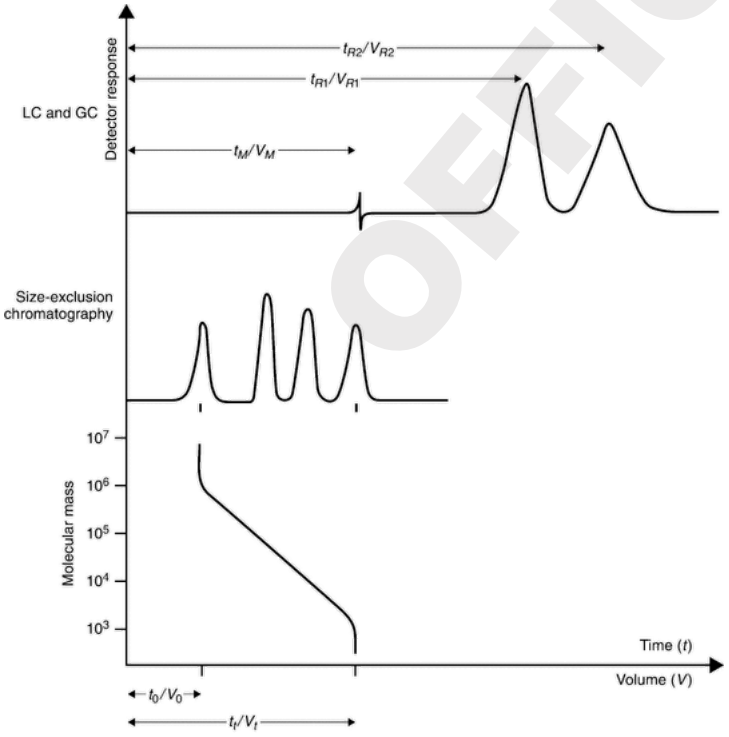

Figure 5.

Retention volume of an unretained compound (V_0): In size-exclusion chromatography, retention volume of a component whose molecules are larger than the largest gel pores. It may be calculated from the retention time of an unretained compound and the flow rate (F), in

milliliters per minute, using the following equation:

$$V_0 = t_0 \times F$$

Separation factor (α): Relative retention calculated for two adjacent peaks (by convention, the value of the separation factor is always >1):

$$\alpha = k_2 / k_1$$

k_1 = retention factor of the first peak

k_2 = retention factor of the second peak

Signal-to-noise ratio (*S/N*): The short-term noise influences the precision and accuracy of quantitation. The signal-to-noise ratio is calculated using the following equation:

$$S/N = \frac{2H}{h}$$

H = height of the peak (*Figure 6*) corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance of at least 5 times the width at half-height

h = range of the noise in a chromatogram obtained after injection of a blank (*Figure 7*), observed over a distance of at least 5 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found

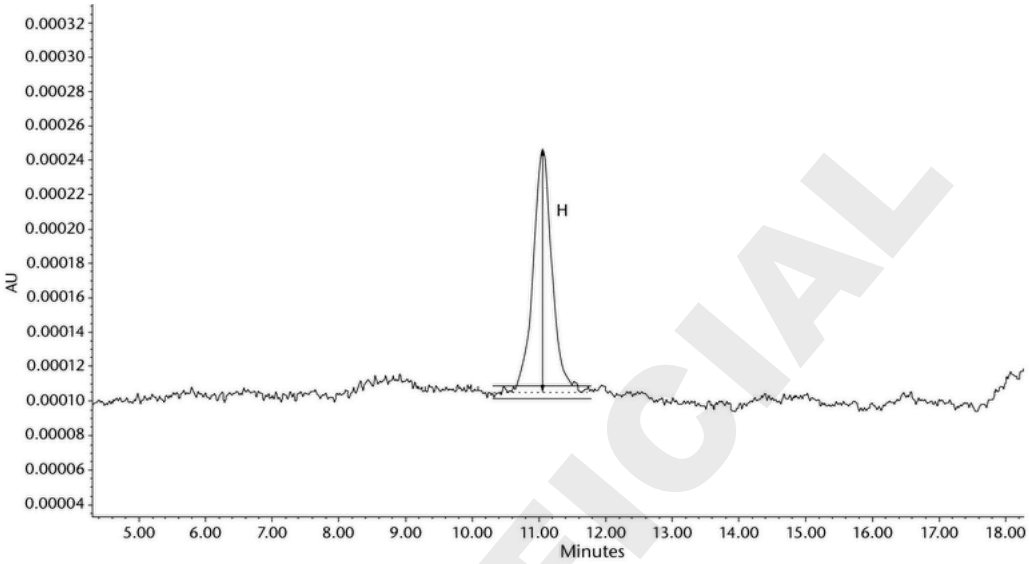

Figure 6. Chromatogram of the reference solution

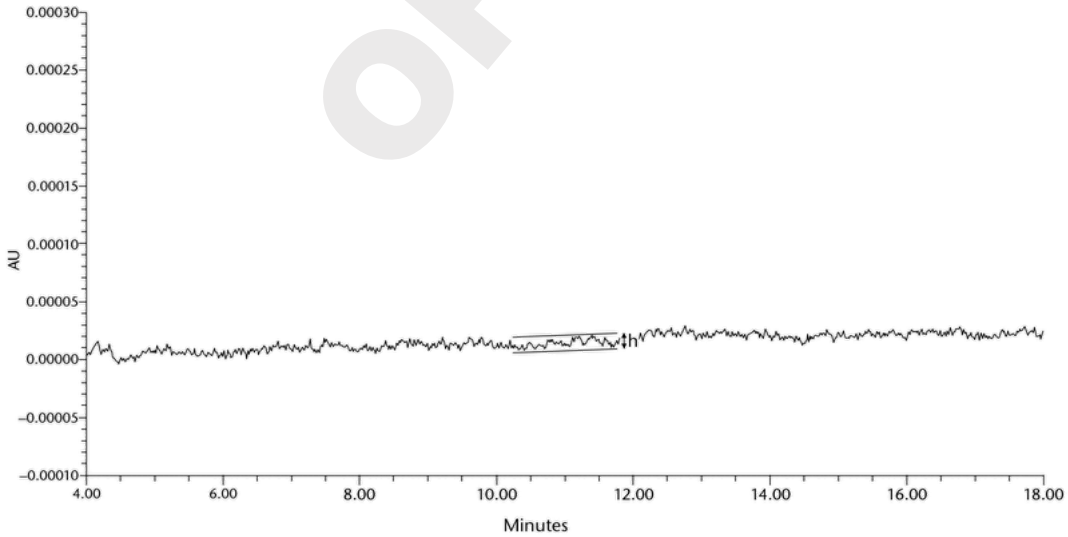

Figure 7.

Symmetry factor (A_s): The symmetry factor of a peak (also known as the asymmetry factor or tailing factor) (*Figure 8*) is calculated using the following equation:

$$A_s = \frac{W_{0.05}}{2d}$$

$W_{0.05}$ = width of the peak at one-twentieth of the peak height

d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height

An A_s value of 1.0 signifies symmetry. When A_s > 1.0, the peak is tailing. When A_s < 1.0, the peak is fronting.

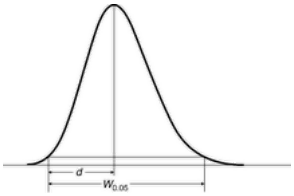

Figure 8.

System repeatability: The repeatability of response is expressed as an estimated percentage relative standard deviation (%RSD) of a consecutive series of measurements for not fewer than three injections or applications of a reference solution, and is calculated using the following equation.

$$\%RSD = \frac{100}{\bar{y}}\sqrt{\frac{\Sigma(y_i - \bar{y})^2}{n-1}}$$

y_i = individual values expressed as peak area, peak height, or ratio of areas by the internal standardization method

$\bar{y}$ = mean of individual values

n = number of individual values

Total mobile phase time (t_t): In size-exclusion chromatography, retention time of a component whose molecules are smaller than the smallest gel pores (*Figure 5*).

Total mobile phase volume (V_t): In size-exclusion chromatography, retention volume of a component whose molecules are smaller than the smallest gel pores. It may be calculated from the total mobile phase time and the flow rate (F), in milliliters/minute, using the following equation:

$$V_t = t_t \times F$$

Change to read:

SYSTEM SUITABILITY

[NOTE—This section only covers liquid chromatography and gas chromatography]

The various components of the equipment employed must be qualified and be capable of achieving the performance required to conduct the test or assay. The system suitability tests represent an integral part of the analytical procedure and are used to ensure adequate performance of the chromatographic system. Column plate number, retention factor (mass distribution ratio), system repeatability, signal-to-noise, symmetry factor, and resolution/peak-to-valley ratio are the parameters that may be employed in assessing the performance of the chromatographic system. When it is indicated in the individual monograph, in case of complex chromatographic profiles (e.g., for biotechnological and biological products) visual comparison of the profiles can be used as a system suitability test. Factors that may affect the chromatographic behavior include:

- Composition and temperature of the mobile phase
- Ionic strength and pH of the aqueous component, of the mobile phase
- Flow rate, column dimensions, column temperature, and pressure
- Stationary phase characteristics including, type of chromatographic support (particle-based or monolithic), particle or pore size, and porosity, and specific surface area
- Reverse phase and other surface modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading, etc.)

Retention times and relative retentions may be provided in monographs for information purposes only, unless otherwise stated in the monograph. There are no acceptance criteria applied to relative retentions.

Compliance with the system suitability criteria is required throughout the chromatographic procedure. No sample analysis is acceptable unless the suitability of the system has been demonstrated.

The following requirements are to be fulfilled, in addition to any other system suitability criteria stated in the monograph. When specific requirements are stated in the monograph, they supersede the requirements mentioned in this chapter.

System Repeatability

♦When a relative standard deviation requirement is specified in an individual monograph, if the requirement is 2.0 or less the calculation is based on data from five replicate injections of the analyte, if the requirement is more than 2.0% data from six replicate injections are used.♦

In an assay of an active substance or an excipient, where the target value is 100% for a pure substance, and a system repeatability requirement is not specified, the maximum permitted relative standard deviation ($\%RSD_{max}$) for the defined limits is calculated for a series (n = 3 to 6) of injections of the reference solution. The maximum permitted relative standard deviation of the peak response does not exceed the appropriate value given in *Table 2*.

$$\%RSD_{\max} = \frac{KB\sqrt{n}}{t_{90\%,n-1}}$$

K = constant (0.349), obtained from the expression $K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\%,n-1}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required percentage relative standard deviation determined on 6 injections for B = 1.0

B = upper limit given in the definition of the individual monograph minus 100%

n = number of replicate injections of the reference solution ($3 \le n \le 6$)

$t_{90\%,n-1}$ = Student's t at the 90% probability level (double sided) with $n-1$ degrees of freedom

Table 2. Maximum Permitted Relative Standard Deviation of Peak Response (Assay)

	Number of Individual Injections (n)			
	3	4	5	6
B (%)	Maximum Permitted Relative Standard Deviation (%)			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

B = upper limit of content given in the individual monograph minus 100%

▲System Sensitivity

The signal-to-noise ratio is used to define the system sensitivity. The limit of quantitation (corresponding to a signal-to-noise ratio of 10) is equal to or less than the reporting threshold.

Peak Symmetry

Unless otherwise stated, in a test or assay, the symmetry factor (tailing factor) of the peak used for quantification is 0.8–1.8.▲ (Official 1-Jun-2026)

Change to read:

ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions described have been validated during the elaboration of the monograph.

The extent to which the various parameters of a chromatographic test may be adjusted without fundamentally modifying the pharmacopeial analytical procedures are listed below. Changes other than those indicated require revalidation of the procedure.

Multiple adjustments can have a cumulative effect on the performance of the system and are to be properly evaluated by the users. This is particularly important in cases where the separation pattern is described as a profile. In those cases, a risk assessment has to be carried out.

Any adjustments must be made on the basis of the pharmacopeial procedure.

If adjustments are made to a pharmacopeial procedure, additional verification tests may be required. To verify the suitability of the adjusted pharmacopeial procedure, assess the relevant analytical performance characteristics potentially affected by the change.

When a pharmacopeial procedure has been adjusted according to the requirements stated below, no further adjustments are allowed without appropriate revalidation.

Compliance with the system suitability criteria is required to verify that conditions for satisfactory performance of the test or assay are achieved.

Adjustment of conditions with gradient elution (HPLC) or temperature programming (GC) is more critical than with isocratic (HPLC) or isothermal (GC) elution, since it may shift some peaks to a different step of the gradient or to different elution temperatures, potentially causing partial or complete coelution of adjacent peaks or peak inversion, and, thus leading to the incorrect assignment of peaks and to the masking of peaks or a shift such that elution occurs beyond the prescribed elution time.

For some parameters the adjustments are explicitly defined in the monograph to ensure the system suitability.

Thin-Layer Chromatography

Composition of the mobile phase: the amount of the minor solvent components may be adjusted by ±30% relative or ±2% absolute, whichever is the larger; no other component is altered by more than 10% absolute. A minor component comprises less than or equal to (100/*n*)%, *n* being the total number of components of the mobile phase. For a minor component at 10% of the mobile phase, a 30% relative adjustment allows a range of 7%–13% whereas a 2% absolute adjustment allows a range of 8%–12%, the relative value therefore being the larger; for a minor component at 5% of the mobile phase, a 30% relative adjustment allows a range of 3.5%–6.5% whereas a 2% absolute adjustment allows a range of 3%–7%, the absolute value being the larger in this case.

pH of the aqueous component of the mobile phase: ±0.2 pH units, unless otherwise prescribed

Concentration of salts in the buffer component of a mobile phase: ± 10%

Application volume: 10%–20% of the prescribed volume if using fine particle size plates (2–10 µm)

Liquid Chromatography: Isocratic Elution

COLUMN PARAMETERS AND FLOW RATE

Stationary phase: No change of the identity of the substituent (e.g., no replacement of C18 by C8); the other physicochemical characteristics of the stationary phase, i.e., chromatographic support, surface modification and extent of chemical modification must be similar; a change from totally porous particle (TPP) columns to superficially porous particle (SPP) columns is allowed provided the above-mentioned requirements are met.

Column dimensions (particle size, length): The particle size and/or length of the column may be modified, provided that the ratio of the column length (*L*) to the particle size (*dp*) remains constant or in the range between −25% to +50% of the prescribed *L/dp* ratio.

Adjustments from totally porous to superficially porous particles: For the application of particle-size adjustment from totally porous to superficially porous particles, other combinations of *L* and *dp* can be used, provided that the plate number (*N*) is within −25% to +50%, relative to the prescribed column.

These changes are acceptable, provided that system suitability criteria are fulfilled, and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.

Internal diameter: ▲The internal diameter of the column may be adjusted even in the absence of a change in particle size and/or length .▲ (RB 1-Dec-2024)

Caution is necessary when the adjustment results in smaller peak volumes due to a smaller particle size or smaller internal column diameter, a situation that may require adjustments to minimize extra-column band broadening by factors such as instrument connections, detector cell volume and sampling rate, and injection volume.

When the particle size is changed, the flow rate requires adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). The flow rate is adjusted for both the change in column diameter and particle size using the following equation:

$$F_2 = F_1 \times \left[\left(dc_2^2 \times dp_1\right) / \left(dc_1^2 \times dp_2\right)\right]$$

F_1 = flow rate indicated in the monograph (mL/min)

F_2 = adjusted flow rate (mL/min)

dc_1 = internal diameter of the column indicated in the monograph

dc_2 = internal diameter of the column used (mm)

dp_1 = particle size indicated in the monograph (µm)

dp_2 = particle size of the column used (µm)

When a change is made from ≥3-µm to <3-µm particles in isocratic separations, an additional increase in linear velocity (by adjusting the flow rate) may be justified, provided that the column performance does not drop by more than 20%. Similarly, when a change is made from <3-µm to ≥3-µm particles, an additional reduction of linear velocity (flow rate) may be justified to avoid reduction in column performance by more than 20%.

After an adjustment due to a change in column dimensions, an additional change in flow rate of ±50% is permitted.

Column temperature: ± 10°C, where the operating temperature is specified, unless otherwise prescribed

Further adjustments in analytical procedure conditions (mobile phase, temperature, pH, etc.) may be required, within the permitted ranges described under *System Suitability* and *Adjustment of Chromatographic Conditions* in this chapter.

MOBILE PHASE

Composition: The amount of the minor components of the mobile phase can be adjusted by ±30% relative. However, the change in any component cannot exceed ±10% absolute. A minor component comprises less or equal than (100/n) %, *n* being the total number of components of the mobile phase:

♦Examples of adjustments for binary and ternary mixtures are given below.

BINARY MIXTURES

Specified ratio of 50:50: 30% of 50 is 15% absolute, but this exceeds the maximum permitted change of ±10% absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60–60:40.

Specified ratio of 2:98: 30% of 2 is 0.6% absolute. Therefore, the maximum allowed adjustment is within the range of 1.4: 98.6–2.6: 97.4.

TERNARY MIXTURES

Specified ratio of 70:25:5: For the second component, 30% of 25 is 7.5% absolute. Therefore, the second component may be adjusted within the range of 32.5%–17.5% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100%. Therefore, mixture ranges of 62.5: 32.5: 5 to 77.5: 17.5: 5 or 68.5: 25: 6.5 to 71.5: 25: 3.5 would meet the requirement.♦

- *pH of the aqueous component of the mobile phase*: ±0.2 pH units, unless otherwise prescribed
- *Concentration of salts in the buffer component of a mobile phase*: ±10%
- *Flow rate*: In absence of a change in column dimensions, an adjustment of the flow rate by ±50% is permitted

DETECTOR WAVELENGTH

No adjustment permitted.

INJECTION VOLUME

When the column dimensions are changed, the following equation may be used for adjusting the injection volume:

$$V_{inj2} = V_{inj1} \left(L_2 \; {d_{c2}}^2\right) / \left(L_1 \; {d_{c1}}^2\right)$$

V_{inj1} = injection volume indicated in the monograph (µL)

V_{inj2} = adjusted injection volume (µL)

L_1 = column length indicated in the monograph (mm)

L_2 = new column length (mm)

dc_1 = column internal diameter indicated in the monograph (mm)

dc_2 = new column internal diameter (mm)

This equation may not be applicable to changes from TPP columns to SPP columns.

Even in the absence of any column dimension change, the injection volume may be varied provided System Suitability criteria remain within their established acceptability limits. When the injection volume is decreased, special attention is given to (limit of) detection and repeatability of the peak response(s) to be determined. An increase is permitted provided, in particular, linearity and resolution of the peak(s) to be determined remain satisfactory.

Liquid Chromatography: Gradient Elution

Adjustment of chromatographic conditions for gradient systems requires greater caution than for isocratic systems.

COLUMN PARAMETERS AND FLOW RATE

Stationary phase: No change of the identity of the substituent (e.g., no replacement of C18 by C8); the other physicochemical characteristics of the stationary phase i.e., chromatographic support, surface modification, and extent of chemical modification must be similar; a change from totally porous particle (TPP) columns to superficially porous particle (SPP) columns is allowed provided the above-mentioned requirements are met.

Column dimensions (particle size, length): The particle size and/or length of the column may be modified provided that the ratio of the column length (*L*) to the particle size (*dp*) remains constant or in the range between −25% to +50% of the prescribed *L/dp* ratio.

Adjustments from totally porous to superficially porous particles: For the application of particle-size adjustment from totally porous to superficially porous particles, other combinations of *L* and *dp* can be used provided that the ratio $(t_R/W_h)^2$ is within −25% to +50%, relative to the prescribed column ♦for all the peaks used to determine the system suitability parameters♦.

These changes are acceptable, provided that system suitability criteria are fulfilled, and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.

Internal diameter: ▲The internal diameter of the column may be adjusted even in the absence of a change in particle size and/or length .▲ (RB 1-Dec-2024)

Caution is necessary when the adjustment results in smaller peak volume resulting from smaller particle size or smaller internal column diameter, a situation which may require adjustments to minimize extra-column band broadening by factors such as instrument connections, detector cell volume and sampling rate, and injection volume.

When the particle size is changed, the flow rate requires adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). Flow rate is adjusted for both the change in column diameter and particle size using the following equation:

$$F_2 = F_1 \times \left[\left(dc_2^{\,2} \times dp_1\right) / \left(dc_1^{\,2} \times dp_2\right)\right]$$

F_1 = flow rate indicated in the monograph (mL/min)

F_2 = adjusted flow rate (mL/min)

dc_1 = internal diameter of the column indicated in the monograph (mm)

dc_2 = internal diameter of the column used (mm)

dp_1 = particle size indicated in the monograph (µm)

dp_2 = particle size of the column used (µm)

A change in column dimensions, and thus in column volume, impacts the gradient volume which controls selectivity. Gradients are adjusted to the column volume by changing the gradient volume in proportion to the column volume. This applies to every gradient segment volume. Since the gradient volume is the gradient time, t_G, multiplied by the flow rate, F, the gradient time for each gradient segment needs to be adjusted to maintain a constant ratio of the gradient volume to the column volume (expressed as $L \times dc^2$). Thus, the new gradient time, t_{G2} can be calculated from the original gradient time, t_{G1}, the flow rate(s), and the column dimensions as follows:

$$t_{G2} = t_{G1} \times \left(F_1 / F_2\right) \left[\left(L_2 \times dc_2^{\,2}\right) / \left(L_1 \times dc_1^{\,2}\right)\right]$$

Thus, the change in conditions for gradient elution requires three steps:

1. Adjust the column length and particle size according to *L/dp*.
2. Adjust the flow rate for changes in particle size and column diameter.
3. Adjust the gradient time of each segment for changes in column length, diameter, and flow rate. The example below illustrates this process.

Table 3

Variable	Original Conditions	Adjusted Conditions	Comment
Column length (*L*), in mm	150	100	User's choice
Column diameter (*dc*), in mm	4.6	2.1	User's choice
Particle size (*dp*), in µm	5	3	User's choice
L/dp	30.0	33.3	(1)
Flow rate, in mL/min	2.0	0.7	(2)
Gradient adjustment factor (t_{G2}/t_{G1})	—	0.4	(3)
Gradient conditions	—	—	—
B (%)	Time (min)	Time (min)	
30	0	0	—
30	3	(3 × 0.4) = 1.2	—
70	13	[1.2 + (10 × 0.4)] = 5.2	—
30	16	[5.2 + (3 × 0.4)] = 6.4	—

1. An 11% increase within allowed *L/dp* change of −25% to +50%
2. Calculated using $F_2 = F_1 \left[(dc_2^{\,2} \times dp_1)/(dc_1^{\,2} \times dp_2)\right]$
3. Calculated using $t_{G2} = t_{G1} \times (F_1/F_2) \left[(L_2 \times dc_2^{\,2})/(L_1 \times dc_1^{\,2})\right]$

Column temperature: ±5° C, where the operating temperature is specified, unless otherwise prescribed

Further adjustments in procedure conditions (mobile phase, temperature, pH, etc.) may be required, within the permitted ranges described under *System Suitability* and *Adjustment of Chromatographic Conditions* in this chapter.

MOBILE PHASE

Composition/gradient: Adjustments of the composition of the mobile phase and the gradient are acceptable provided that:

- The system suitability criteria are fulfilled.
- The principal peak(s) elute(s) within ±15% of the retention time(s) obtained with the original conditions; this requirement does not apply when the column dimensions are changed.
- The composition of the mobile phase and the gradient are such that the first peaks are sufficiently retained and the last peaks are eluted.

pH of the aqueous component of the mobile phase: ±0.2 pH units, unless otherwise prescribed

Concentration of salts in the buffer component of a mobile phase: ±10%

Where compliance with the system suitability criteria cannot be achieved, it is often preferable to consider the dwell volume or to change the column.

DWELL VOLUME

The configuration of the equipment employed may significantly alter the resolution, retention time, and relative retentions described. Should this occur, it may be due to a change in dwell volume. Monographs preferably include an isocratic step before the start of the gradient program so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the system used for analytical procedure development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used. If the dwell volume used during the elaboration of the monograph is given in the monograph, the time points (t min) stated in the gradient table may be replaced by adapted time points (t_c min), calculated using the following equation:

$$t_c = t - \frac{(D - D_0)}{F}$$

D = dwell volume (mL)

D_0 = dwell volume used for development of the method (mL)

F = flow rate (mL/min)

The isocratic step introduced for this purpose may be omitted if validation data for application of the analytical procedure without this step is available.

Detector wavelength: No adjustment permitted

Injection volume: When the column dimensions are changed, the following equation may be used for adjusting the injection volume:

$$V_{inj2} = V_{inj1} \left(L_2 d c_2^2\right) / \left(L_1 d c_1^2\right)$$

V_{inj1} = injection volume indicated in the monograph (µL)

V_{inj2} = adjusted injection volume (µL)

L_1 = column length indicated in the monograph (cm)

L_2 = new column length (cm)

d_{c1} = column internal diameter indicated in the monograph (mm)

d_{c2} = new column internal diameter (mm)

This equation may not be applicable to changes from TPP columns to SPP columns.

Even in the absence of any column dimension change, the injection volume may be varied provided system suitability criteria remain within their established acceptability limits. When the injection volume is decreased, special attention is given to (limit of) detection and repeatability of the peak response(s) to be determined. An increase is permitted provided, in particular, linearity and resolution of the peak(s) to be determined remain satisfactory.

Gas Chromatography

COLUMN PARAMETERS

Stationary phase

Particle size: Maximum reduction of 50%; no increase permitted (packed columns)

Film thickness: −50% to +100% (capillary columns)

Column dimensions

Length: −70% to +100%

Internal diameter: ±50%

Column temperature: ±10%

Temperature program: Adjustment of temperatures is permitted as stated above; adjustment of ramp rates and hold times of up to ±20% is permitted.

Flow rate: ±50%

The above changes are acceptable provided system suitability criteria are fulfilled and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.

Injection volume and split ratio: May be varied provided system suitability criteria remain within their established acceptability limits. When the injection volume is decreased, or the split ratio is increased special attention is given to (limit of) detection and repeatability of the peak response(s) to be determined. An increase in injection volume or decrease in split ratio is permitted provided, in particular, linearity and resolution of the peak(s) to be determined remain satisfactory.

Injection port temperature and transfer-line temperature in static headspace conditions: ±10° C, provided no decomposition or condensation occurs

QUANTITATION

The following quantitation approaches may be used in general texts or monographs.

External Standard Method

Using a calibration function: Standard solutions with several graded amounts of a reference standard of the compound to be analyzed are prepared in a range that has been demonstrated to give a linear response, and a fixed volume of these standard solutions is injected. With the chromatograms obtained, a calibration function is prepared by plotting the peak areas or peak heights on the ordinate against the amount of reference standard on the abscissa. The calibration function is generally obtained by linear regression. Then, a sample solution is prepared according to the procedure specified in the individual monograph. The chromatography is performed under the same operating conditions as for the preparation of the calibration function, the peak area or peak height of the compound to be analyzed is measured, and the amount of the compound is read out or calculated from the calibration function.

Using one-point calibration: In an individual monograph, generally one of the standard solutions with a concentration within the linear range of the calibration function and a sample solution with a concentration close to that of the standard solution are prepared, and the chromatography is performed under fixed conditions to obtain the amount of the component by comparing the responses obtained. In this method, all procedures, such as the injection procedure, must be carried out under constant conditions.

Internal Standard Method

Using a calibration function: In the internal standard method, a stable compound is chosen as an internal standard which shows a retention time close to that of the compound to be analyzed, and whose peak is well separated from all other peaks in the chromatogram. Several standard solutions containing a fixed amount of the internal standard and graded amounts of a reference standard of the compound to be analyzed are prepared. Based on the chromatograms obtained by injection of a fixed volume of individual standard solutions, the ratio of peak area or peak height of the reference standard to that of the internal standard is calculated. A calibration function by plotting these ratios on the ordinate against the amount of the reference standard or the ratio of the amount of reference standard to that of the internal standard on the abscissa is prepared. The calibration function is generally obtained by linear regression. Then, a sample solution containing the internal standard in the same amount as in the standard solutions used for the preparation of the calibration function is prepared according to the procedure specified in the individual monograph. The chromatography is performed under the same operating conditions as for the preparation of the calibration function. The ratio of the peak area or peak height of the compound to be analyzed to that of the internal standard is calculated, and the amount of the compound is read out or calculated from the calibration function.

Using one-point calibration: In an individual monograph, generally one of the standard solutions with a concentration within the linear range of the calibration function and a sample solution with a concentration close to that of the standard solution, both containing a fixed amount of the internal standard, are prepared, and the chromatography is performed under fixed conditions to determine the amount of the compound to be analyzed by comparing the ratios obtained.

Normalization procedure: Provided linearity of the peaks has been demonstrated, individual monographs may prescribe that the percentage content of a component of the substance to be examined is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix, and those at or below the disregard limit or reporting threshold.

OTHER CONSIDERATIONS

Detector response: ♦The detector sensitivity is the signal output per unit concentration or unit mass of a substance (also known as response factor) in the mobile phase entering the detector. The relative detector response factor, commonly referred to as relative response factor, expresses the sensitivity of a detector for a given substance relative to a standard substance.

In tests for related substances, any correction factors indicated in the monograph are applied.♦

Interfering peaks: Peaks due to solvents and reagents or arising from the mobile phase or the sample matrix are disregarded.

Measurement of peaks: Integration of the peak area of any impurity that is not completely separated from the principal peak is preferably performed by tangential skim (*Figure 9*).

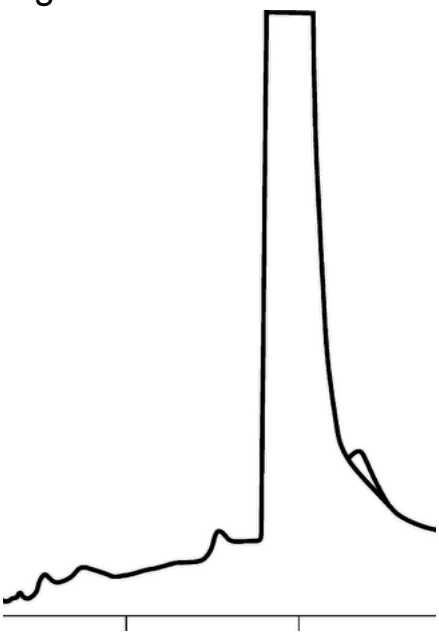

Figure 9.

REPORTING THRESHOLD

When the related substances test prescribes a limit for the total of impurities or a quantitative determination of an impurity, it is important to choose an appropriate reporting threshold and appropriate conditions for the integration of the peak areas.

In such tests the reporting threshold, i.e., the limit above which a peak is reported, is defined generally 0.05%.

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
<621> CHROMATOGRAPHY	Edmond Biba Senior Scientific Liaison	GCCA2020 General Chapters - Chemical Analysis 2020

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