2.00 Chromatography

This test is harmonized with the European Pharmacopoeia and the U. S. Pharmacopeia.

The corresponding part of the attributes/provisions which are agreed as non-harmonized within the scope of the harmonization is marked with symbols (◆ ◆) and the corresponding parts which are agreed as the JP local requirement other than the scope of the harmonization are marked with symbols (◇ ◇).

Information on the harmonization with the European Pharmacopoeia and the U. S. Pharmacopeia is published on the website of the Pharmaceuticals and Medical Devices Agency.

1. Introduction

Chromatographic separation techniques are multi-stage separation procedures in which the components of a sample are distributed between 2 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 physico-chemical properties of the molecules such as size, mass, volume, etc. This chapter contains definitions and calculations of common parameters and generally applicable requirements for system suitability. ◇The prescription described in Liquid Chromatography <2.01> other than the prescription of this test can be applied to the system suitability of liquid chromatography.◇ Principles of separation, apparatus and methods are given in the corresponding general tests.

2. 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 Japanese Pharmacopoeia 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 2.00-1).

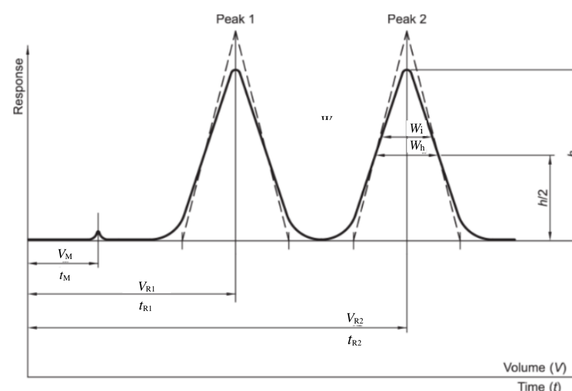


Figure 2.00-1

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 inflexion 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.

Mobile phase A: water.

Mobile phase B: 0.1 vol% solution of acetone in water.

Time (min)	Mobile phase A (vol%)	Mobile phase B (vol%)
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 ($t_{0.5}$) (minutes) when the absorbance has increased by 50% (Figure 2.00-2).

$$D = t_D \times F$$

t_D: $t_{0.5} - 0.5\ t_G$ (minutes)

t_G: Pre-defined gradient time (= 20 minutes)

F: Flow rate (mL/minute)

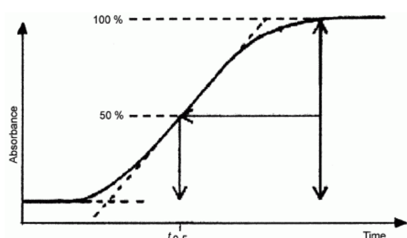


Figure 2.00-2

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 (Figure 2.00-1, 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 mL/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 2 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 (Figure 2.00-3).

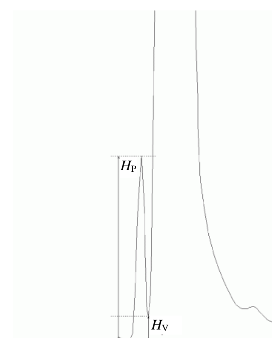


Figure 2.00-3

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

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) (synonym: Height equivalent to one theoretical plate (HETP))

Ratio of the column length (L) (μm) to the plate number (N):

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

Plate number (N)

A number indicative of column performance (column efficiency). It 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)

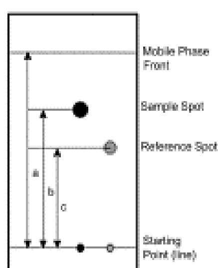
Ratio of the plate height (H)(μm) to the particle diameter (d_p) (μm):

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

156 **Relative retardation (R_{rel})**
157 The relative retardation, used in thin-layer chromatog-
158 raphy, is calculated as the ratio of the distances travelled by
159 the spot of the compound of interest and a reference com-
160 pound (Figure 2.00-4).

161 $$R_{rel} = b/c$$

162 a: Migration distance of the mobile phase
163 b: Migration distance of the compound of interest
164 c: Migration distance of the reference compound
165



166
167 Figure 2.00-4
168

169 **Relative retention (r)**
170 Relative retention is calculated as an estimate using the fol-
171 lowing equation:

172 $$r = \frac{t_{Ri} - t_M}{t_{Rst} - t_M}$$

173 t_{Ri}: Retention time of the peak of interest
174 t_{Rst}: Retention time of the reference peak (usually the peak
175 corresponding to the substance to be examined)
176 t_M: Hold-up time

177 **Relative retention, unadjusted (r_G) or (RRT)**
178 Unadjusted relative retention is calculated using the fol-
179 lowing equation:

180 $$r_G = \frac{t_{Ri}}{t_{Rst}}$$

181 Unless otherwise indicated, values for relative retention
182 stated in monographs correspond to unadjusted relative re-
183 tention.

184 **Relative retention time (RRT):**
185 see Relative retention, unadjusted.

186 **Resolution (R_S)**
187 The resolution between peaks of 2 components (Figure
188 2.00-1) may be calculated using the following equation:

189 $$R_S = \frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

190 t_{R1}, t_{R2}: Retention times of the peaks, $t_{R2} > t_{R1}$
191 w_{h1}, w_{h2}: Peak widths at half-height

192 ◇Complete separation means the resolution of not less than
193 1.5, and is also referred to as baseline separation.◇
194 In quantitative thin-layer chromatography, using densi-
195 tometry, the migration distances are used instead of retention
196 times and the resolution between peaks of 2 components may
197 be calculated using the following equation:

198 $$R_S = \frac{1.18a(R_{F2} - R_{F1})}{w_{h1} + w_{h2}}$$

199 $R_{F2} > R_{F1}$
200 R_{F1}, R_{F2}: Retardation factors of the peaks
201 w_{h1}, w_{h2}: Peak widths at half-height
202 a: Migration distance of the solvent front

203 **Retardation factor (R_F)**
204 The retardation factor, used in thin-layer chromatography,
205 is the ratio of the distance from the point of application to the
206 center of the spot and the distance simultaneously travelled
207 by the solvent front from the point of application (Figure
208 2.00-4).

209 $$R_F = \frac{b}{a}$$

210 b: Migration distance of the compound of interest
211 a: Migration distance of the solvent front

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

215 $$k = \frac{\text{amount of component in stationary phase}}{\text{amount of component in mobile phase}} = K_C \frac{V_S}{V_M}$$

216 K_C: Distribution constant (also known as equilibrium dis-
217 tribution coefficient);
218 V_S: Volume of the stationary phase
219 V_M: Volume of the mobile phase

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

222 $$k = \frac{t_R - t_M}{t_M}$$

223 t_R: Retention time
224 t_M: Hold-up time

225 **Retention time (t_R)**
226 Time elapsed between the injection of the sample and the
227 appearance of the maximum peak response of the eluted sam-
228 ple zone (Figure 2.00-1, baseline scale being in minutes or
229 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: mL/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 2.00-5).

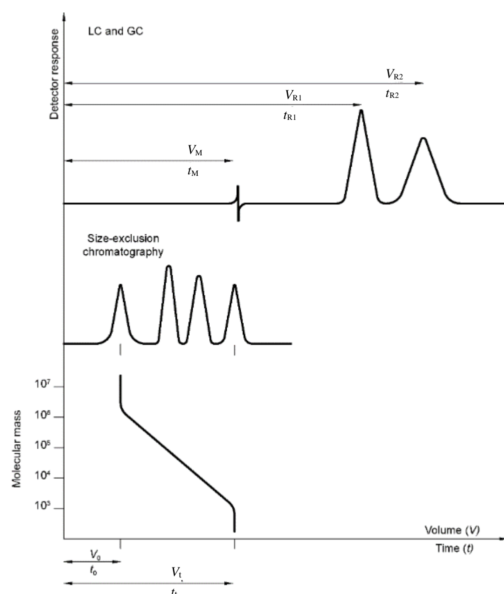


Figure 2.00-5

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: mL/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 2.00-6) 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 equal to 20 times the width at half-height

h: Range of the noise in a chromatogram obtained after injection of a blank (Figure 2.00-7), observed over a distance equal to 20 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.

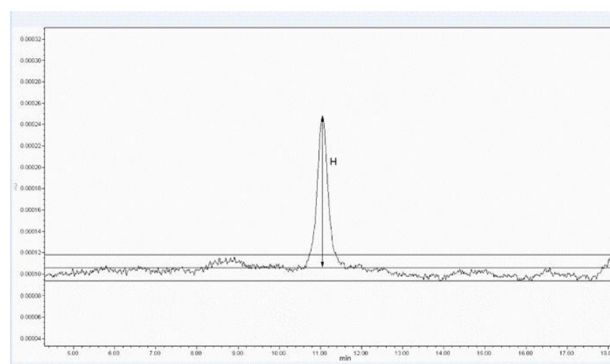


Figure 2.00-6. Chromatogram of the reference solution

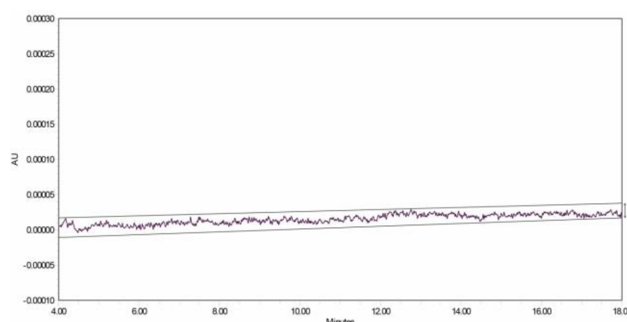


Figure 2.00-7. Chromatogram of a blank

If a baseline of 20 times the width at half-height is not obtainable because of peaks due to the solvents or reagents, or arising from the mobile phase or the sample matrix, or due to the gas chromatographic temperature program, a baseline of at least 5 times the width at half-height is permitted.

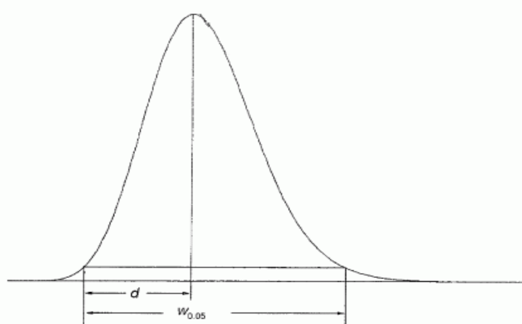
Symmetry factor (A_S)

The symmetry factor of a peak (also known as the asymmetry factor or tailing factor) (Figure 2.00-8) is calculated using the following equation:

290 $$A_S = \frac{w_{0.05}}{2d}$$

291 $w_{0.05}$: Width of the peak at one-twentieth of the peak height
292 d: Distance between the perpendicular dropped from the
293 peak maximum and the leading edge of the peak at one-
294 twentieth of the peak height

295 An A_S value of 1.0 signifies symmetry. When $A_S > 1.0$, the
296 peak is tailing. When $A_S < 1.0$, the peak is fronting.
297



298

299 Figure 2.00-8
300

301 **System repeatability**
302 The repeatability of response is expressed as an estimated
303 percentage relative standard deviation (%RSD) of a consec-
304 utive series of measurements for not fewer than 3 injections
305 or applications of a reference solution, and is calculated using
306 the following equation.

307 $$\%RSD = \frac{100}{\overline{y}}\sqrt{\frac{\Sigma(y_i - \overline{y})^2}{n-1}}$$

308 y_i: Individual values expressed as peak area, peak height,
309 or ratio of areas by the internal standardization method;
310 $\overline{y}$: Mean of individual values
311 n: Number of individual values
312
313 Total mobile phase time (t_t)
314 In size-exclusion chromatography, retention time of a
315 component whose molecules are smaller than the smallest gel
316 pores (Figure 2.00-5).
317
318 Total mobile phase volume (V_t)
319 In size-exclusion chromatography, retention volume of a
320 component whose molecules are smaller than the smallest gel
321 pores. It may be calculated from the total mobile phase time
322 and the flow rate (F) (mL/minute) using the following equa-
323 tion.

324 $$V_t = t_t \times F$$

325 **3. System suitability**

326 This section only covers liquid chromatography and gas
327 chromatography.
328 The various components of the equipment employed must
329 be qualified and be capable of achieving the performance re-
330 quired to conduct the test or assay.
331 The system suitability tests represent an integral part of the
332 analytical procedure and are used to ensure adequate perfor-
333 mance of the chromatographic system. Column plate number,
334 retention factor (mass distribution ratio), system repeatability,
335 signal-to-noise, symmetry factor and resolution/peak-to-val-
336 ley ratio are the parameters that may be employed in as-
337 sessing the performance of the chromatographic system.
338 When stated in the individual monograph, in cases of com-
339 plex chromatographic profiles (e.g., for biotechnological/bi-
340 ological products), visual comparison of the profiles can be
341 used as a system suitability test.
342 Factors that may affect the chromatographic behavior in-
343 clude:
344 ・Composition and temperature of the mobile phase;
345 ・Ionic strength and pH of the aqueous component of the mo-
346 bile phase;
347 ・Flow rate, column dimensions, column temperature and
348 pressure;
349 ・Stationary phase characteristics including type of chroma-
350 tographic support (particle-based or monolithic), particle or
351 pore size, porosity, specific surface area;
352 ・Reversed phase and other surface-modification of the sta-
353 tionary phases, the extent of chemical modification (as ex-
354 pressed by end-capping, carbon loading etc.).

355 Retention times and relative retentions may be provided in
356 monographs for information purposes only, unless otherwise
357 stated in the monograph. There are no acceptance criteria ap-
358 plied to relative retentions.
359 Compliance with the system suitability criteria is required
360 throughout the chromatographic procedure. No sample anal-
361 ysis is acceptable unless the suitability of the system has been
362 demonstrated.
363 ◇When the following criteria are specified in the system
364 suitability tests, each requirement is to be fulfilled unless oth-
365 erwise prescribed.◇

366 **System repeatability – assay of an active substance or an**
367 **excipient**
368 In an assay of an active substance or an excipient, where
369 the target value is 100% for a pure substance, and a system
370 repeatability requirement is not specified, the maximum per-
371 mitted relative standard deviation ($\%RSD_{max}$) for the defined
372 limits is calculated for a series (n = 3 to 6) of injections of the
373 reference solution.
374 The maximum permitted relative standard deviation of the
375 peak response does not exceed the appropriate value given in
376 Table 2.00-1.

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

K: Constant (0.349), obtained from the expression,

$K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\%,5}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required relative standard deviation (percentage) determined on 6 injections for B=1.0

B: (Upper limit given in the definition of the individual monograph - 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 2.00-1 – Maximum permitted relative standard deviation (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 - 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 quantitation is 0.8 to 1.8.

4. 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 pharmacopoeial 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 pharmacopoeial procedure.

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

When a pharmacopoeial analytical 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.

◇In the tests of biotechnological/biological products such as peptide mapping, glycosylation analysis and tests related to molecular heterogeneity, the separation pattern obtained by liquid chromatography may be set for acceptance criteria as a profile. In such a test method, the method shown in this section may not be applicable.◇

◇Crude drugs and related drugs are outside the scope of this section.◇

4.1. 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 (d_p) remains constant or in the range between –25% to +50% of the prescribed L/d_p ratio.
- Adjustment 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 d_p can be used provided that the plate number (N) is within –25% to +50%, relative to the prescribed column. These 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.

- Internal diameter: In absence of a change in particle size and/or length, the internal diameter of the column may be adjusted.

Caution is necessary when the adjustment results in smaller peak volumes, due to a smaller particle size or a smaller internal 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 ◇may require◇ adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). The flow rate ◇can be adjusted◇ for both the change in column diameter and particle size using the following equation:

$$F_2 = F_1 \times [(d_{c2}^2 \times d_{p1})/(d_{c1}^2 \times d_{p2})]$$

F_1: Flow rate (mL/minute) indicated in the monograph
F_2: Adjusted flow rate (mL/minute)
d_{c1}: Internal diameter (mm) of the column indicated in the monograph
d_{c2}: Internal diameter (mm) of the column used
d_{p1}: Particle size (μm) indicated in the monograph
d_{p2}: Particle size (μm) of the column used

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 test method.

Mobile phase:

- Composition: The amount of the minor solvent components may be adjusted by ±30% relative. 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. No 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.
- 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.

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

V_{inj1}: Injection volume (μL) indicated in the monograph
V_{inj2}: Adjusted injection volume (μL)
L_1: Column length (cm) indicated in the monograph
L_2: New column length (cm)
d_{c1}: Column internal diameter (mm) indicated in the monograph
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.

4.2. 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 (d_p) remains constant or in the range between –25% to

+50% of the prescribed L/d_p 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 d_p can be used provided that the ratio $(t_R/w_h)^2$ is within –25% to +50%, relative to the prescribed column, for each peak used to check the system suitability, as stated in this chapter and the individual monograph.

These 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.

- Internal diameter: In absence of a change in particle size and/or length, the internal diameter of the column may be adjusted.

Caution is necessary when the adjustment results in smaller peak volumes, due to a smaller particle size or a smaller internal 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 ◇may require◇ adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). The flow rate ◇can be adjusted◇ for both the change in column diameter and particle size using the following equation:

$$F_2 = F_1 \times [(d_{c2}^2 \times d_{p1})/(d_{c1}^2 \times d_{p2})]$$

F_1: Flow rate (mL/minute) indicated in the monograph

F_2: Adjusted flow rate (mL/minute)

d_{c1}: Internal diameter (mm) o the column indicated in the monograph

d_{c2}: Internal diameter (mm) of the column used

d_{p1}: Particle size (μm) indicated in the monograph

d_{p2}: Particle size (μm) of the column used

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 $L\times d_c^2$). 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.

$$t_{G2} = t_{G1} \times (F_1 / F_2)\ [(L_2 \times d_{c2}^2) / (L_1 \times d_{c1}^2)]$$

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

(1) adjust the column length and particle size according to L/d_p,

(2) adjust the flow rate for changes in particle size and column diameter, and

(3) adjust the gradient time of each segment for changes in column length, diameter and flow rate. The example below illustrates this process.

Variable	Original Conditions	Adjusted Conditions	Comment
Column length (L) (mm)	150	100	User's choice
Column diameter (d_c) (mm)	4.6	2.1	User's choice
Particle size (d_p) (μm)	5	3	User's choice
L/d_p	30.0	33.3	(1)
Flow rate (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) 11% increase within allowed L/d_p change of –25% to +50%

(2) calculated using $F_2 = F_1\ [(d_{c2}^2 \times d_{p1}) / (d_{c1}^2 \times d_{p2})]$

(3) calculated using $t_{G2} = t_{G1} \times (F_1 / F_2)\ [(L_2 \times d_{c2}^2) / (L_1 \times d_{c1}^2)]$

- Column temperature: ±5°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/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

648 time(s) obtained with the original conditions. This require-
649 ment does not apply when the column dimensions are
650 changed.
651 ・The composition of the mobile phase and the gradient are
652 such that the first peaks are sufficiently retained and the last
653 peaks are eluted.
654 ・pH of the aqueous component of the mobile phase: ±0.2 pH
655 units, unless otherwise prescribed.
656 ・Concentration of salts in the buffer component of a mobile
657 phase: ±10%

658 Where compliance with the system suitability criteria can-
659 not be achieved, it is preferable to consider the dwell volume
660 or to change the column.

661 **Dwell volume** The configuration of the equipment em-
662 ployed may significantly alter the resolution, retention time
663 and relative retentions described. Should this occur, it may
664 be due to a change in dwell volume. Monographs preferably
665 include an isocratic step before the start of the gradient pro-
666 gram so that an adaptation can be made to the gradient time
667 points to take account of differences in dwell volume be-
668 tween the system used for analytical procedure development
669 and that actually used. It is the user's responsibility to adapt
670 the length of the isocratic step to the analytical equipment
671 used. If the dwell volume used during the elaboration of the
672 monograph is given in the monograph, the time points (t min)
673 stated in the gradient table may be replaced by adapted time
674 points (t_c min), calculated using the following equation.

675 $$t_c = t - (D - D_0) / F$$

676 D: Dwell volume (mL)
677 D_0: Dwell volume (mL) used for development of the ana-
678 lytical procedure
679 F: Flow rate (mL/min)

680 The isocratic step introduced for this purpose may be omit-
681 ted if validation data for application of the analytical proce-
682 dure without this step is available.

683 **Detector wavelength**: No adjustment permitted.

684 **Injection volume**: When the column dimensions are
685 changed, the following equation may be used for adjusting
686 the injection volume.

687 $$V_{inj2} = V_{inj1} (L_2 d_{c2}^2) / (L_1 d_{c1}^2)$$

688 V_{inj1}: Injection volume (μL) indicated in the monograph
689 V_{inj2}: Adjusted injection volume (μL)
690 L_1: Column length (cm) indicated in the monograph
691 L_2: New column length (cm)
692 d_{c1}: Column internal diameter (mm) indicated in the mon-
693 ograph
694 d_{c2}: New column internal diameter (mm)

695 This equation may not be applicable to changes from TPP
696 columns to SPP columns.
697 Even in the absence of any column dimension change, the
698 injection volume may be varied provided system suitability
699 criteria remain within their established acceptability limits.
700 When the injection volume is decreased, special attention is
701 given to (limit of) detection and repeatability of the peak re-
702 sponse(s) to be determined. An increase is permitted pro-
703 vided, in particular, linearity and resolution of the peak(s) to
704 be determined remain satisfactory

705 **4.3. Gas chromatography**

706 **Column parameters**

707 Stationary phase:
708 Particle size: Maximum reduction of 50%. No increase
709 permitted (packed columns).
710 Film thickness: −50% to +100% (capillary columns)
711 Column dimensions:
712 Length: −70% to +100%;
713 Internal diameter: ±50%;
714 Column temperature: ±10%;
715 Temperature program: Adjustment of temperatures is per-
716 mitted as stated above. Adjustment of ramp rates and hold
717 times of up to ±20% is permitted.

718 **Flow rate**: ±50%.
719 The above changes are acceptable provided system suita-
720 bility criteria are fulfilled, and selectivity and elution order of
721 the specified impurities to be controlled are demonstrated to
722 be equivalent.

723 **Injection volume and split ratio**: may be varied provided
724 system suitability criteria remain within their established ac-
725 ceptability limits. When the injection volume is decreased, or
726 the split ratio is increased, special attention is given to (limit
727 of) detection and repeatability of the peak response(s) to be
728 determined. An increase in injection volume or a decrease in
729 split ratio is permitted provided, in particular, linearity and
730 resolution of the peak(s) to be determined remain satisfactory.

731 **Injection port temperature and transfer-line temperature**
732 **in static head-space conditions**: ±10°C, provided no decom-
733 position or condensation occurs.

734 **5. Quantitation**

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

737 **5.1. External standard method**

738 Using a calibration function
739 Standard solutions with several graded amounts of a refer-
740 ence standard of the compound to be analyzed are prepared
741 in a range that has been demonstrated to give a linear re-
742 sponse, and a fixed volume of these standard solutions is in-
743 jected. With the chromatograms obtained, a calibration func-
744 tion 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, must be carried out under constant conditions.

5.2. 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.

5.3 Normalisation 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.

6. Other considerations

6.1. Detector response

The detector sensitivity is the signal output per unit concentration or unit mass of a substance in the mobile phase entering the detector. The relative detector response factor, commonly referred to as response factor, expresses the sensitivity of a detector for a given substance relative to a standard substance. The correction factor is the reciprocal of the response factor. In tests for related substances any correction factors indicated in the monograph are applied (i.e. when the response factor is outside the range 0.8-1.2).

6.2. Interfering peaks

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

6.3. 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 2.00-9).

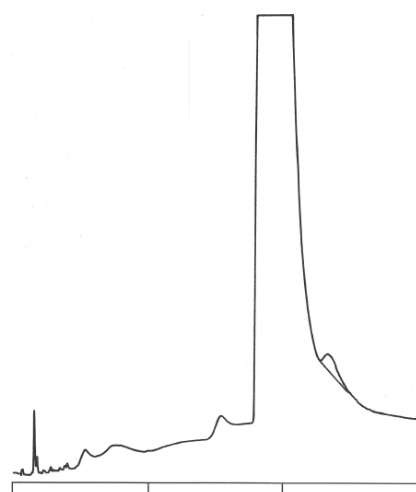


Figure 2.00-9

6.4. Reporting threshold

When the related substances test prescribes a limit for the

833 total of impurities or a quantitative determination of an im-
834 purity, it is important to choose an appropriate reporting
835 threshold and appropriate conditions for the integration of the
836 peak areas. In such tests the reporting threshold, i.e. the limit
837 above which a peak is reported, is generally 0.05%.