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PHARMACOPOEIAL DISCUSSION GROUP

CORRECTION 1

CODE: G-20
NAME: CHROMATOGRAPHY
(Correction of the sign-off document signed on 28 September 2021)

Item to be corrected:

ADJUSTEMENTS OF CHROMATOGRAPHIC CONDITIONS (isocratic and gradient elution)

Read:

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

It is understood that sign-off covers the technical content of the draft and each party will adapt it as necessary to conform to the usual presentation of the pharmacopoeia in question; such adaptation includes stipulation of the particular pharmacopoeia's reference materials and general chapters.

Harmonised provisions:

Provision	**EP**	**JP**	**USP**
Introduction	+	+	+
Definitions	+	+	+
System suitability	+	+	+
Adjustment of chromatographic conditions (1) (2)	+	+	+
Quantitation	+	+	+
Other considerations	+	+	+

(1) Thin layer chromatography will not be stipulated by JP.

(2) JP will not stipulate the sentence 'For some parameters, the adjustments are explicitly defined in the monograph to ensure the system suitability'

Non-harmonised provisions: N/A

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Local requirements

EP	JP	USP
Introduction: reference to corresponding Ph. Eur. chapters on TLC, LC, GC ... In planar chromatography, *retardation factors* R_{Fst} and R_{Fi} used instead of t_{Rst} and t_{Ri}. *Relative retention time, RRT*: not stipulated as synonym of relative retention *System suitability*: guidance of the way to determine the S/N ratio (solution to be used)	*Introduction*: The sentence handling of the existing JP local chapter Liquid Chromatography <*2.01*> is added. *Resolution*: definition of baseline separation *System suitability*: the sentence in the sign-off text '*The following requirements are to be fulfilled, in addition to any other system suitability criteria stated in the monograph*' will be replaced by '*When the following criteria are specified in the system suitability tests, each requirement is to be fulfilled unless otherwise prescribed.*' *Adjustment of chromatographic conditions*: paragraph added excluding adjustment for certain tests for biotechnological/ biological products such as peptide mapping, glycosylation analysis of glycoprotein, and molecular heterogeneity (LC separation pattern specified as a profile); sentence added excluding adjustment for crude drugs and related drugs. *Adjustment of the flow rate after adjustment of the column dimensions* (isocratic and gradient LC): read 'the flow rate **may require** adjustment' and 'The flow rate **can be** adjusted for both the change....'	*Introduction*: last paragraph not stated. *Sections on General Procedures and Chromatographic columns are included* *System suitability*: number of injections depending on the RSD requirement. *Adjustment of chromatographic conditions* – LC mobile phase: examples of binary and ternary mixtures added; LC column dimensions (gradient elution): different wording for TPP to SPP requirements *Other considerations* – detector response: different wording included

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European Pharmacopoeia

Signature	Name	Date
DocuSigned by: Cathie VIELLE 5D202E6E19D1466...	C. Vielle	3 Nov. 2023

Japanese Pharmacopoeia

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Yoshi Saito for K. Nakai	Yoshiro Saito	Nov. 13, 2023

United States Pharmacopeia

Signature	Name	Date
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G-20 CHROMATOGRAPHY

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. 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. Principles of separation, apparatus and methods are given in the corresponding general chapters.

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 <Name> 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. Idealised chromatograms are represented as a sequence of Gaussian peaks on a baseline (Figure 1).

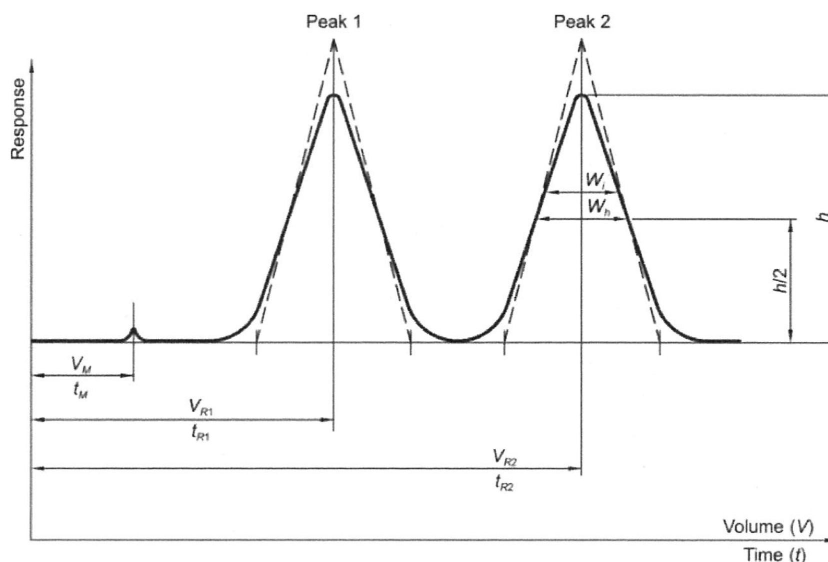

Figure 1

V_M = hold-up volume;
t_M = hold-up time;
V_{R1} = retention volume of peak 1;
t_{R1} = retention time of peak 1;

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36 V_{R2} = retention volume of peak 2;
37 t_{R2} = retention time of peak 2;
38 W_h = peak width at half-height;
39 W_i = peak width at the inflexion point;
40 h = height of the peak;
41 h/2 = half-height of the peak.
42
43 **Distribution constant (K_0)**
44 In size-exclusion chromatography, the elution characteristics of a component in a particular
45 column may be given by the distribution constant (also referred to as distribution coefficient),
46 which is calculated using the following equation:

47 $$K_0 = \frac{t_R - t_0}{t_t - t_0}$$
48
49 t_R = retention time;
50 t_0 = retention time of an unretained compound;
51 t_t = total mobile phase time.
52
53 **Dwell volume (D) (also referred to as V_D):**
54 The dwell volume (also known as gradient delay volume) is the volume between the point at
55 which the eluents meet and the inlet of the column. It can be determined using the following
56 procedure.

57 *Column*: replace the chromatographic column by an appropriate capillary tubing (e.g.
58 1 m × 0.12 mm).

59 *Mobile phase*:
60 – *mobile phase A*: *water*;
61 – *mobile phase B*: 0.1 per cent *V/V* solution of *acetone* in *water*;

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 – 20	100 → 0	0 → 100
20 - 30	0	100

62 *Flow rate*: set to obtain sufficient back-pressure (e.g. 2 mL/min).

63 *Detection*: spectrophotometer at 265 nm.

64 Determine the time ($t_{0.5}$) in minutes when the absorbance has increased by 50 per cent
65 (Figure 2).

66 $$D = t_D \times F$$

67 $t_D = t_{0.5} - 0.5t_G$, in minutes;
68 t_G = pre-defined gradient time (= 20 min);
69 F = flow rate, in millilitres per minute.

70

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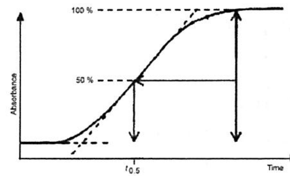

Figure 2

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 (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 millilitres 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 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 3).

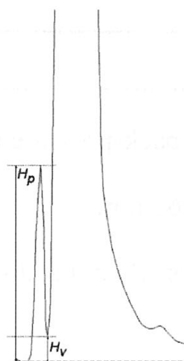

Figure 3

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

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101 H_p = height above the extrapolated baseline of the minor peak;
102 H_v = height above the extrapolated baseline at the lowest point of the curve separating the minor
103 and major peaks.
104
105 **Plate height (*H*) (synonym: Height equivalent to one theoretical plate (HETP))**
106 Ratio of the column length (*L*), in micrometers, to the plate number (*N*):
107

108 $$H = \frac{L}{N}$$

109
110 **Plate number (*N*) (synonym: Number of theoretical plates)**
111 A number indicative of column performance (column efficiency). It can only be calculated
112 from data obtained under either isothermal, isocratic or isodense conditions, depending on the
113 technique, as the plate number, using the following equation, the values of t_R and w_h being
114 expressed in the same units:

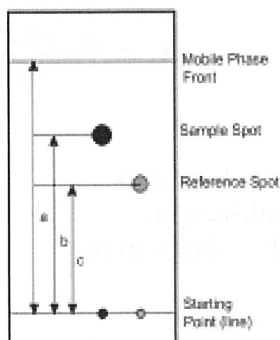
115 $$N = 5.54\left(\frac{t_R}{w_h}\right)^2$$

116 t_R = retention time of the peak corresponding to the component;
117 w_h = peak width at half-height ($h/2$).
118
119 The plate number varies with the component as well as with the column, the column
120 temperature, the mobile phase and the retention time.
121
122 **Reduced plate height (*h*)**
123 Ratio of the plate height (*H*), in micrometers, to the particle diameter (d_p) in micrometers:
124

125 $$h = \frac{H}{d_p}$$

126
127 **Relative retardation (*R rel*)**
128 The relative retardation, used in thin-layer chromatography, is calculated as the ratio of the
129 distances travelled by the spot of the compound of interest and a reference compound
130 (Figure 4).

131 $$R\,rel = b/c$$



132 Figure 4

133 a = migration distance of the mobile phase;
134 b = migration distance of the compound of interest;

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135 c = migration distance of the reference compound.
136
137 **Relative retention (r)**
138 Relative retention is calculated as an estimate using the following equation:

$$r = \frac{t_{Ri} - t_M}{t_{Rst} - t_M}$$
139

140 t_{Ri} = retention time of the peak of interest;
141 t_{Rst} = retention time of the reference peak (usually the peak corresponding to the substance to
142 be examined);
143 t_M = hold-up time.
144
145 **Relative retention, unadjusted (r_G) or RRT**
146 Unadjusted relative retention is calculated using the following equation:

147
$$r_G = \frac{t_{Ri}}{t_{Rst}}$$

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

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

151 **Resolution (R_s)**
152 The resolution between peaks of 2 components (Figure 1) may be calculated using the
153 following equation:

$$R_s = \frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$
154

155 $t_{R2} > t_{R1}$
156 t_{R1}, t_{R2} = retention times of the peaks;
157 w_{h1}, w_{h2} = peak widths at half-height.

158 In quantitative thin-layer chromatography, using densitometry, the migration distances are
159 used instead of retention times and the resolution between peaks of 2 components may be
160 calculated using the following equation:

$$R_s = \frac{1.18a(R_{F2} - R_{F1})}{w_{h1} + w_{h2}}$$
161

162 $R_{F2} > R_{F1}$
163 R_{F1}, R_{F2} = retardation factors of the peaks;
164 w_{h1}, w_{h2} = peak widths at half-height;
165 a = migration distance of the solvent front.
166
167

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

172
$$R_F = \frac{b}{a}$$

173 b = migration distance of the compound of interest;
174 a = migration distance of the solvent front.
175

176 **Retention factor (k)**
177 The retention factor (also known as mass distribution ratio (D_m) or capacity factor (k')) is
178 defined as:
179

180
$$k = \frac{\text{amount of component in stationary phase}}{\text{amount of component in mobile phase}} = K_C \frac{V_S}{V_M}$$
181

182 K_C = distribution constant (also known as equilibrium distribution coefficient);
183 V_S = volume of the stationary phase;
184 V_M = volume of the mobile phase.
185

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

188
$$k = \frac{t_R - t_M}{t_M}$$
189 t_R = retention time;
190 t_M = hold-up time.
191

192 **Retention time (t_R)**
193 Time elapsed between the injection of the sample and the appearance of the maximum peak
194 response of the eluted sample zone (Figure 1, baseline scale being in minutes or seconds).
195

196 **Retention volume (V_R)**
197 Volume of the mobile phase required for elution of a compound. It may be calculated from
198 the retention time (t_R) and the flow rate (F) in millilitres per minute using the following
199 equation:

200
$$V_R = t_R \times F$$
201

202 **Retention time of an unretained compound (t_0)**
203 In size-exclusion chromatography, retention time of a component whose molecules are larger
204 than the largest gel pores (Figure 5).
205

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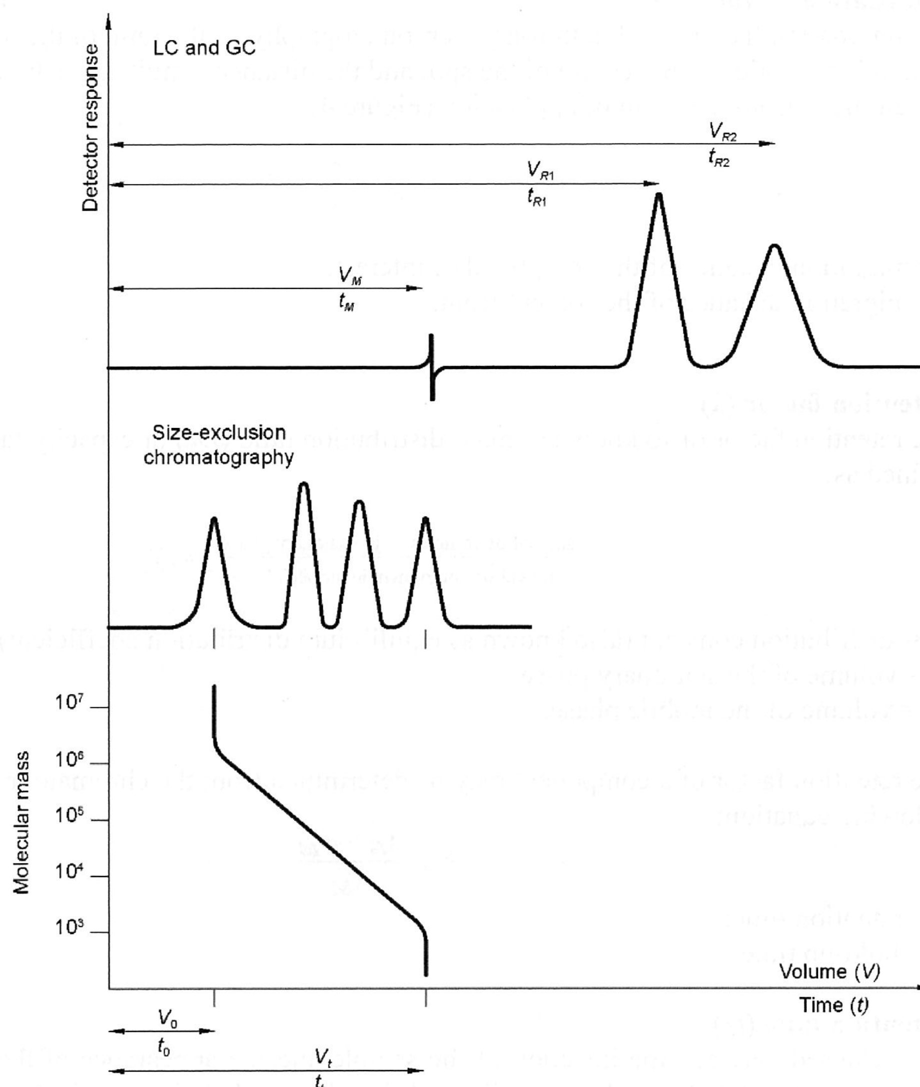

207
208
209 Figure 5

210 **Retention volume of an unretained compound (V_0)**

211 In size-exclusion chromatography, retention volume of a component whose molecules are
212 larger than the largest gel pores. It may be calculated from the retention time of an unretained
213 compound (t_0) and the flow rate (F) in millilitres per minute using the following equation:

214 $$V_0 = t_0 \times F$$

215 **Separation factor (α)**

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

218 $$\alpha = k_2/k_1$$

219 k_1 = retention factor of the first peak;
220 k_2 = retention factor of the second peak.
221

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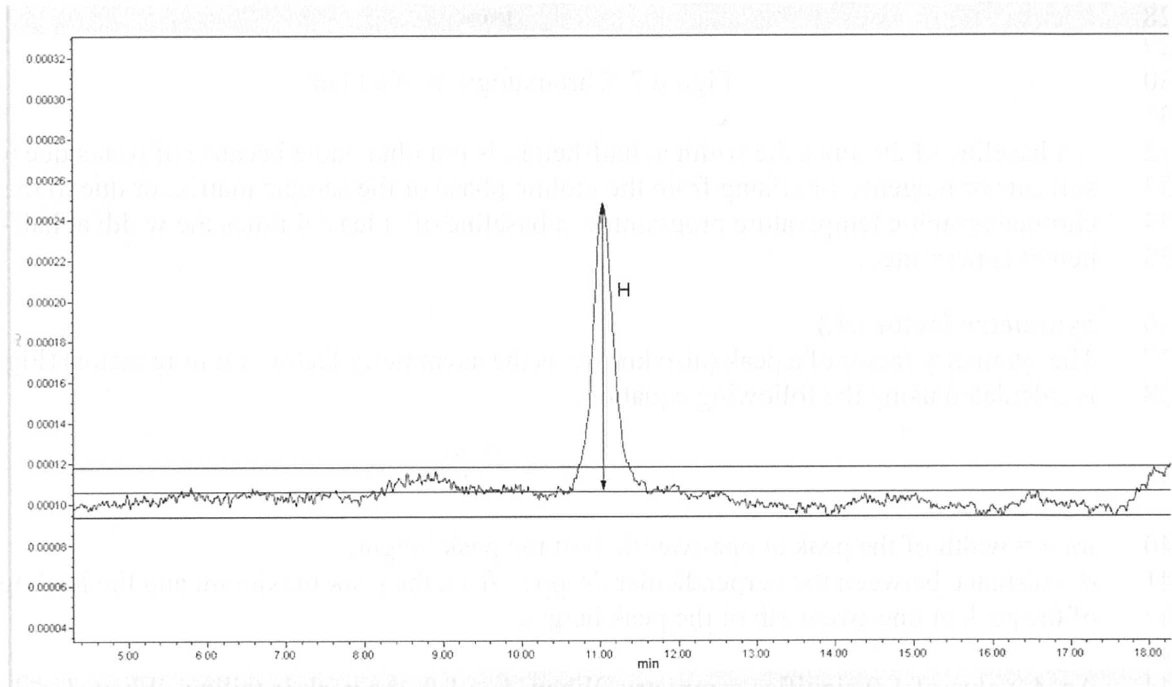
222 **Signal-to-noise ratio (*S/N*)**

223 The short-term noise influences the precision and accuracy of quantitation. The signal-to-noise
224 ratio is calculated using the following equation:

225
$$S/N = \frac{2H}{h}$$

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 equal to 20 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 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.

226


227 Figure 6. Chromatogram of the reference solution

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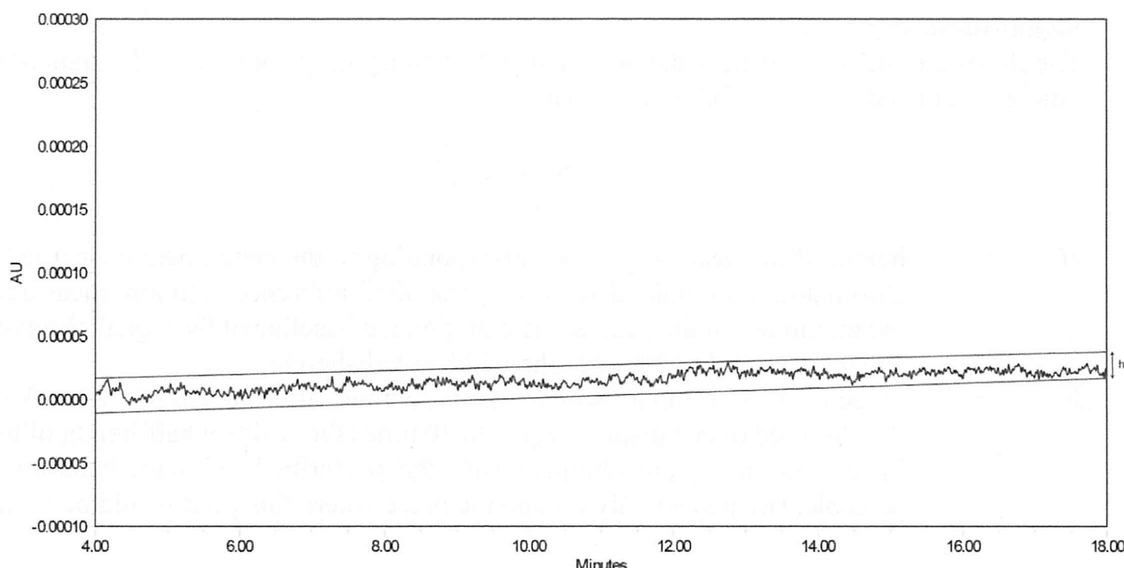

228
229
230 Figure 7. Chromatogram of a blank
231
232 If a baseline of 20 times the width at half-height is not obtainable because of peaks due to the
233 solvents or reagents, or arising from the mobile phase or the sample matrix, or due to the gas
234 chromatographic temperature programme, a baseline of at least 5 times the width at half-
235 height is permitted.

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

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

239

240 $w_{0.05}$ = width of the peak at one-twentieth of the peak height;
241 d = distance between the perpendicular dropped from the peak maximum and the leading edge
242 of the peak at one-twentieth of the peak height.
243
244 An A_s value of 1.0 signifies symmetry. When $A_s > 1.0$, the peak is tailing. When $A_s < 1.0$, the
245 peak is fronting.

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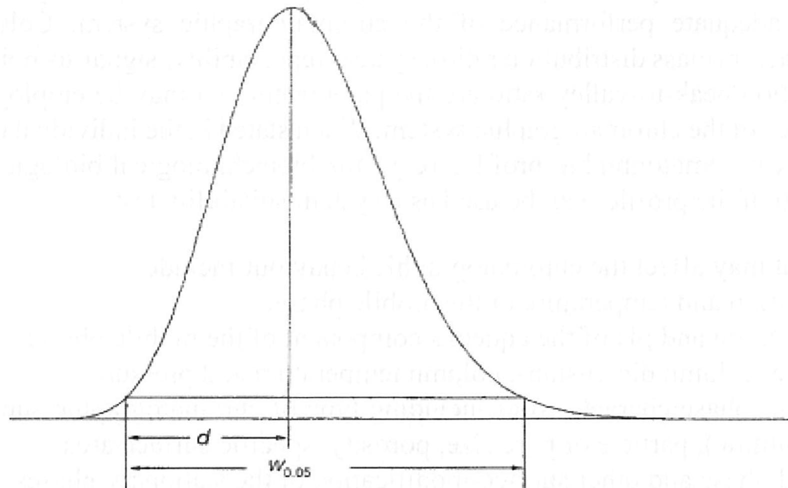

246

247 Figure 8

248 **System repeatability**
249 The repeatability of response is expressed as an estimated percentage relative standard deviation
250 (%RSD) of a consecutive series of measurements for not fewer than 3 injections or applications
251 of a reference solution, and is calculated using the following equation:

252
$$\%RSD = \frac{100}{\bar{y}} \sqrt{\frac{\sum(y_i - \bar{y})^2}{n-1}}$$

253

254 y_i = individual values expressed as peak area, peak height, or ratio of areas by the internal
255 standardisation method;
256 $\bar{y}$ = mean of individual values;
257 n = number of individual values.
258
259 **Total mobile phase time (t_t)**
260 In size-exclusion chromatography, retention time of a component whose molecules are
261 smaller than the smallest gel pores (Figure 5).
262
263 **Total mobile phase volume (V_t)**
264 In size-exclusion chromatography, retention volume of a component whose molecules are
265 smaller than the smallest gel pores. It may be calculated from the total mobile phase time and
266 the flow rate (F) in millilitres per minute using the following equation:

267
$$V_t = t_t \times F$$
268

269 ***SYSTEM SUITABILITY***

270 *This section only covers liquid chromatography and gas chromatography.*

271 The various components of the equipment employed must be qualified and be capable of
272 achieving the performance required to conduct the test or assay.

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273 The system suitability tests represent an integral part of the analytical procedure and are used
274 to ensure adequate performance of the chromatographic system. Column plate number,
275 retention factor (mass distribution ratio), system repeatability, signal-to-noise, symmetry factor
276 and resolution/peak-to-valley ratio are the parameters that may be employed in assessing the
277 performance of the chromatographic system. When stated in the individual monograph, in cases
278 of complex chromatographic profiles (e.g., for biotechnological/biological products), visual
279 comparison of the profiles can be used as a system suitability test.

280 Factors that may affect the chromatographic behaviour include:
281 – composition and temperature of the mobile phase;
282 – ionic strength and pH of the aqueous component of the mobile phase;
283 – flow rate, column dimensions, column temperature and pressure;
284 – stationary phase characteristics including type of chromatographic support (particle-based
285 or monolithic), particle or pore size, porosity, specific surface area;
286 – reversed phase and other surface-modification of the stationary phases, the extent of
287 chemical modification (as expressed by end-capping, carbon loading etc.).

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

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

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

297 • ***System repeatability – assay of an active substance or an excipient***
298
299 In an assay of an active substance or an excipient, where the target value is 100 per cent for a
300 pure substance, and a system repeatability requirement is not specified, the maximum
301 permitted relative standard deviation ($\%RSD_{max}$) for the defined limits is calculated for a
302 series (n = 3 to 6) of injections of the reference solution. The maximum permitted relative
303 standard deviation of the peak response does not exceed the appropriate value given in
304 Table 1.

305 $$\%RSD_{max} = \frac{KB\sqrt{n}}{t_{90\%,n-1}}$$

306 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
307 required percentage relative standard deviation determined on 6 injections for B = 1.0;

308 B = upper limit given in the definition of the individual monograph minus 100 per cent;

309 n = number of replicate injections of the reference solution ($3 \leq n \leq 6$);

310 $t_{90\%,n-1}$ = Student's t at the 90 per cent probability level (double sided) with $n-1$ degrees of
311 freedom.

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312 *Table 1 – Maximum permitted relative standard deviation (assay)*

	Number of individual injections *n*			
	3	4	5	6
B (per cent)	*Maximum permitted relative standard deviation (per cent)*			
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

313 B = upper limit of content given in the individual monograph minus 100 per cent.
314
315 • ***System sensitivity***
316 The signal-to-noise ratio is used to define the system sensitivity. The limit of quantitation
317 (corresponding to a signal-to-noise ratio of 10) is equal to or less than the reporting threshold.
318
319 • ***Peak symmetry***
320 Unless otherwise stated, in a test or assay, the symmetry factor (tailing factor) of the peak
321 used for quantitation is 0.8 to 1.8.
322
323 **ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS**

324 The chromatographic conditions described have been validated during the elaboration of the
325 monograph.
326 The extent to which the various parameters of a chromatographic test may be adjusted without
327 fundamentally modifying the pharmacopoeial analytical procedures are listed below. Changes
328 other than those indicated require revalidation of the procedure.
329
330 Multiple adjustments can have a cumulative effect on the performance of the system and are to
331 be properly evaluated by the users. This is particularly important in cases where the separation
332 pattern is described as a profile. In those cases, a risk assessment has to be carried out.
333
334 Any adjustments must be made on the basis of the pharmacopoeial procedure.
335
336 If adjustments are made to a pharmacopoeial procedure, additional verification tests may be
337 required. To verify the suitability of the adjusted pharmacopoeial procedure, assess the
338 relevant analytical performance characteristics potentially affected by the change.
339
340 When a pharmacopoeial analytical procedure has been adjusted according to the requirements
341 stated below, no further adjustments are allowed without appropriate revalidation.

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

344 Adjustment of conditions with gradient elution (HPLC) or temperature programming (GC) is
345 more critical than with isocratic (HPLC) or isothermal (GC) elution, since it may shift some
346 peaks to a different step of the gradient or to different elution temperatures, potentially causing
347 partial or complete coelution of adjacent peaks or peak inversion, and thus leading to the

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348 incorrect assignment of peaks, and to the masking of peaks or a shift such that elution occurs
349 beyond the prescribed elution time.

350 For some parameters, the adjustments are explicitly defined in the monograph to ensure the
351 system suitability.
352
353 **Thin-layer chromatography**

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

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

365 *Concentration of salts in the buffer component of a mobile phase*: ± 10 per cent.

366 *Application volume*: 10-20 per cent of the prescribed volume if using fine particle size plates
367 (2-10 µm).

368 **Liquid chromatography: isocratic elution**

369 ***Column parameters and flow rate***
370 ➢ *Stationary phase*: no change of the identity of the substituent (e.g. no replacement
371 of C18 by C8); the other physico-chemical characteristics of the stationary phase,
372 i.e. chromatographic support, surface modification and extent of chemical
373 modification must be similar; a change from Totally Porous Particle (TPP)
374 columns to Superficially Porous Particle (SPP) columns is allowed provided the
375 above-mentioned requirements are met.
376
377 ➢ *Column dimensions (particle size, length)*: the particle size and/or length of the
378 column may be modified provided that the ratio of the column length (*L*) to the
379 particle size (*dp*) remains constant or in the range between − 25 per cent to + 50
380 per cent of the prescribed *L*/*dp* ratio.
381 *Adjustments from totally porous to superficially porous particles*: for the
382 application of particle-size adjustment from totally porous to superficially porous
383 particles, other combinations of *L* and *dp* can be used provided that the plate
384 number (*N*) is within − 25 per cent to + 50 per cent, relative to the prescribed
385 column.
386 These changes are acceptable provided the system suitability criteria are fulfilled,
387 and selectivity and elution order of the specified impurities to be controlled are
388 demonstrated to be equivalent.
389
390 ➢ *Internal diameter*: the internal diameter of the column may be adjusted even in the
391 absence of a change in particle size and/or length.

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392 Caution is necessary when the adjustment results in smaller peak volumes, due to a smaller
393 particle size or a smaller internal diameter, a situation which may require adjustments to
394 minimize extra-column band broadening by factors such as instrument connections, detector
395 cell volume and sampling rate, and injection volume.
396
397 When the particle size is changed, the flow rate requires adjustment, because smaller-
398 particle columns will require higher linear velocities for the same performance (as measured
399 by reduced plate height). The flow rate is adjusted for both the change in column diameter
400 and particle size using the following equation:
401
402
$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

F_1 = flow rate indicated in the monograph, in millilitres per minute;
F_2 = adjusted flow rate, in millilitres per minute;
403 dc_1 = internal diameter of the column indicated in the monograph, in millimetres;
404 dc_2 = internal diameter of the column used, in millimetres;
405 dp_1 = particle size indicated in the monograph, in micrometres;
406 dp_2 = particle size of the column used, in micrometres.
407
408 When a change is made from ≥ 3-µm to < 3-µm particles in isocratic separations, an
409 additional increase in linear velocity (by adjusting the flow rate) may be justified, provided
410 that the column performance does not drop by more than 20 per cent. Similarly, when a
411 change is made from < 3-µm to ≥ 3-µm particles, an additional reduction of linear velocity
412 (flow rate) may be justified to avoid reduction in column performance by more than 20 per
413 cent.
414
415 After an adjustment due to a change in column dimensions, an additional change in flow rate
416 of ± 50 per cent is permitted.

417 ➢ *Column temperature*: ± 10 °C, where the operating temperature is specified, unless
418 otherwise prescribed.

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

422 ***Mobile phase***

423 ➢ *Composition*: the amount of the minor solvent components may be adjusted by
424 ± 30 per cent relative (see examples under Thin-layer chromatography); no
425 component is altered by more than 10 per cent absolute. A minor component
426 comprises less than or equal to (100/*n*) per cent, *n* being the total number of
427 components of the mobile phase;
428 ➢ *pH of the aqueous component of the mobile phase*: ± 0.2 pH units, unless
429 otherwise prescribed;
430 ➢ *Concentration of salts in the buffer component of a mobile phase*: ± 10 per cent;
431 ➢ *Flow rate*: in absence of a change in column dimensions, an adjustment of the flow
432 rate by ± 50 per cent is permitted.

433 ***Detector wavelength***: no adjustment permitted.

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434 ***Injection volume***: when the column dimensions are changed, the following equation may
435 be used for adjusting the injection volume:
436 $$V_{inj2} = V_{inj1} (L_2 d_{c2}^2) / (L_1 d_{c1}^2)$$
437
438 V_{inj1} = injection volume indicated in the monograph, in microlitres;
439 V_{inj2} = adjusted injection volume, in microlitres;
440 L_1 = column length indicated in the monograph, in millimetres;
441 L_2 = new column length, in millimetres;
442 d_{c1} = column internal diameter indicated in the monograph, in millimetres;
443 d_{c2} = new column internal diameter, in millimetres.

444 This equation may not be applicable to changes from TPP columns to SPP columns.
445
446 Even in the absence of any column dimension change, the injection volume may be varied
447 provided System Suitability criteria remain within their established acceptability limits. When
448 the injection volume is decreased, special attention is given to (limit of) detection and
449 repeatability of the peak response(s) to be determined. An increase is permitted provided, in
450 particular, linearity and resolution of the peak(s) to be determined remain satisfactory.

451 **Liquid chromatography: gradient elution**

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

454 ***Column parameters and flow rate***
455 ➢ *Stationary phase*: no change of the identity of the substituent (e.g. no replacement of
456 C18 by C8); the other physico-chemical characteristics of the stationary phase, i.e.
457 chromatographic support, surface modification and extent of chemical modification
458 must be similar; a change from Totally Porous Particle (TPP) columns to
459 Superficially Porous Particle (SPP) columns is allowed provided the above-
460 mentioned requirements are met.
461
462 ➢ *Column dimensions (particle size, length)*: the particle size and/or length of the
463 column may be modified provided that the ratio of the column length (L) to the
464 particle size (dp) remains constant or in the range between − 25 per cent to +50 per
465 cent of the prescribed L/dp ratio.
466 *Adjustments from totally porous to superficially porous particles*: for the application
467 of particle-size adjustment from totally porous to superficially porous particles,
468 other combinations of L and dp can be used provided that the ratio $(t_R/w_h)^2$ is within
469 – 25 per cent to + 50 per cent, relative to the prescribed column, for each peak used
470 to check the system suitability, as stated in this chapter and the individual
471 monograph.
472 These changes are acceptable provided system suitability criteria are fulfilled, and
473 selectivity and elution order of the specified impurities to be controlled are
474 demonstrated to be equivalent.

475 ➢ *Internal diameter*: the internal diameter of the column may be adjusted even in
476 absence of a change in particle size and/or length.

477 Caution is necessary when the adjustment results in smaller peak volumes, due to a smaller
478 particle size or a smaller internal diameter, a situation which may require adjustments to

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479 minimize extra-column band broadening by factors such as instrument connections, detector
480 cell volume and sampling rate, and injection volume.
481
482 When the particle size is changed, the flow rate requires adjustment, because smaller-
483 particle columns will require higher linear velocities for the same performance (as measured
484 by reduced plate height). The flow rate is adjusted for both the change in column diameter
485 and particle size using the following equation:
486

487 $$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

488
489 F_1 = flow rate indicated in the monograph, in millilitres per minute;
490 F_2 = adjusted flow rate, in millilitres per minute;
491 dc_1 = internal diameter of the column indicated in the monograph, in millimetres;
492 dc_2 = internal diameter of the column used, in millimetres;
493 dp_1 = particle size indicated in the monograph, in micrometres;
494 dp_2. = particle size of the column used, in micrometres.
495
496 A change in column dimensions, and thus in column volume, impacts the gradient volume
497 which controls selectivity. Gradients are adjusted to the column volume by changing the
498 gradient volume in proportion to the column volume. This applies to every gradient segment
499 volume. Since the gradient volume is the gradient time, t_G, multiplied by the flow rate, F, the
500 gradient time for each gradient segment needs to be adjusted to maintain a constant ratio of the
501 gradient volume to the column volume (expressed as L x dc^2). Thus, the new gradient time, t_{G2}
502 can be calculated from the original gradient time, t_{G1}, the flow rate(s), and the column
503 dimensions as follows:
504

505 $$t_{G2} = t_{G1} \times (F_1 / F_2) [(L_2 \times dc_2^2) / (L_1 \times dc_1^2)]$$

506
507 Thus, the change in conditions for gradient elution requires three steps:
508 (1) adjust the column length and particle size according to L/dp,
509 (2) adjust the flow rate for changes in particle size and column diameter, and
510 (3) adjust the gradient time of each segment for changes in column length, diameter and flow
511 rate. The example below illustrates this process.
512

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513 Table 2

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 (per cent)	Time (min)	Time (min)	
30	0	0	
30	3	(3x0.4)=1.2	
70	13	[1.2+(10x0.4)]=5.2	
30	16	[5.2+(3x0.4)]=6.4	

514
515 (1) 11 per cent increase within allowed L/dp change of – 25 per cent to + 50 per cent
516 (2) calculated using $F_2 = F_1 \, [(dc_2^2 \times dp_1) / (dc_1^2 \times dp_2)]$
517 (3) calculated using $t_{G2} = t_{G1} \times (F_1 / F_2) \, [(L_2 \times dc_2^2) / (L_1 \times dc_1^2)]$
518
519 ➢ *Column temperature*: ± 5 °C, where the operating temperature is specified, unless
520 otherwise prescribed.

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

524 ***Mobile phase***

525 ➢ *Composition/gradient*: adjustments of the composition of the mobile phase and the
526 gradient are acceptable provided that:
527 – the system suitability criteria are fulfilled;
528 – the principal peak(s) elute(s) within ± 15 per cent of the retention time(s) obtained
529 with the original conditions; this requirement does not apply when the column
530 dimensions are changed;
531 – the composition of the mobile phase and the gradient are such that the first peaks
532 are sufficiently retained and the last peaks are eluted.

533 ➢ *pH of the aqueous component of the mobile phase*: ± 0.2 pH units, unless otherwise
534 prescribed.
535 ➢ *Concentration of salts in the buffer component of a mobile phase*: ± 10 per cent.

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

538 ***Dwell volume.*** The configuration of the equipment employed may significantly alter the
539 resolution, retention time and relative retentions described. Should this occur, it may be due to
540 a change in dwell volume. Monographs preferably include an isocratic step before the start of

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541 the gradient programme so that an adaptation can be made to the gradient time points to take
542 account of differences in dwell volume between the system used for analytical procedure
543 development and that actually used. It is the user's responsibility to adapt the length of the
544 isocratic step to the analytical equipment used. If the dwell volume used during the
545 elaboration of the monograph is given in the monograph, the time points (t min) stated in the
546 gradient table may be replaced by adapted time points (t_c min), calculated using the following
547 equation:

548 $$t_c = t - \frac{(D - D_0)}{F}$$
549

550 D = dwell volume, in millilitres;
551 D_0 = dwell volume used for development of the analytical procedure, in millilitres;
552 F = flow rate, in millilitres per minute.

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

555 ***Detector wavelength***: no adjustment permitted.

556 ***Injection volume***: when the column dimensions are changed, the following equation may
557 be used for adjusting the injection volume:

558 $$V_{inj2} = V_{inj1} (L_2 d_{c2}^2) / (L_1 d_{c1}^2)$$
559

560 V_{inj1} = injection volume indicated in the monograph, in microlitres;
561 V_{inj2} = adjusted injection volume, in microliters;
562 L_1 = column length indicated in the monograph, in millimetres;
563 L_2 = new column length, in millimetres;
564 d_{c1} = column internal diameter indicated in the monograph, in millimetres;
565 d_{c2} = new column internal diameter, in millimetres.

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

567 Even in the absence of any column dimension change, the injection volume may be varied
568 provided system suitability criteria remain within their established acceptability limits. When
569 the injection volume is decreased, special attention is given to (limit of) detection and
570 repeatability of the peak response(s) to be determined. An increase is permitted provided, in
571 particular, linearity and resolution of the peak(s) to be determined remain satisfactory
572

573 **Gas chromatography**

574 ***Column parameters***
575 ➢ *Stationary phase*:
576 – *particle size*: maximum reduction of 50 per cent; no increase permitted (packed
577 columns);
578 – *film thickness*: – 50 per cent to + 100 per cent (capillary columns);
579

580 ➢ *Column dimensions*:
581 – *length*: – 70 per cent to + 100 per cent;
582 – *internal diameter*: ± 50 per cent;

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583
584 ➢ *Column temperature*: ± 10 per cent;
585 ➢ *Temperature programme*: adjustment of temperatures is permitted as stated above;
586 adjustment of ramp rates and hold times of up to ± 20 per cent is permitted.

587 ***Flow rate***: ± 50 per cent.

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

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

597 ***Injection port temperature and transfer-line temperature in static head-space conditions***:
598 ± 10 °C, provided no decomposition or condensation occurs.
599

600
601 **QUANTITATION**
602
603 The following quantitation approaches may be used in general texts or monographs:
604
605 — *External standard method.*
606 • using a calibration function
607 Standard solutions with several graded amounts of a reference standard of the compound to be
608 analysed are prepared in a range that has been demonstrated to give a linear response, and a
609 fixed volume of these standard solutions is injected. With the chromatograms obtained, a
610 calibration function is prepared by plotting the peak areas or peak heights on the ordinate
611 against the amount of reference standard on the abscissa. The calibration function is generally
612 obtained by linear regression. Then, a sample solution is prepared according to the procedure
613 specified in the individual monograph. The chromatography is performed under the same
614 operating conditions as for the preparation of the calibration function, the peak area or peak
615 height of the compound to be analysed is measured, and the amount of the compound is read
616 out or calculated from the calibration function.

617 • using one-point calibration
618 In an individual monograph, generally one of the standard solutions with a concentration
619 within the linear range of the calibration function and a sample solution with a concentration
620 close to that of the standard solution are prepared, and the chromatography is performed under
621 fixed conditions to obtain the amount of the compound by comparing the responses obtained.
622 In this method, all procedures, such as the injection, must be carried out under constant
623 conditions.

624 — *Internal standard method.*
625 • using a calibration function
626 In the internal standard method, a stable compound is chosen as an internal standard which
627 shows a retention time close to that of the compound to be analysed, and whose peak is well

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628 separated from all other peaks in the chromatogram. Several standard solutions containing a
629 fixed amount of the internal standard and graded amounts of a reference standard of the
630 compound to be analysed are prepared. Based on the chromatograms obtained by injection of
631 a fixed volume of individual standard solutions, the ratio of peak area or peak height of the
632 reference standard to that of the internal standard is calculated. A calibration function by
633 plotting these ratios on the ordinate against the amount of the reference standard or the ratio
634 of the amount of reference standard to that of the internal standard on the abscissa is prepared.
635 The calibration function is generally obtained by linear regression. Then, a sample solution
636 containing the internal standard in the same amount as in the standard solutions used for the
637 preparation of the calibration function is prepared according to the procedure specified in the
638 individual monograph. The chromatography is performed under the same operating conditions
639 as for the preparation of the calibration function. The ratio of the peak area or peak height of
640 the compound to be analysed to that of the internal standard is calculated, and the amount of
641 the compound is read out or calculated from the calibration function.
642
643 • using one point calibration
644 In an individual monograph, generally one of the standard solutions with a concentration
645 within the linear range of the calibration function and a sample solution with a concentration
646 close to that of the standard solution, both containing a fixed amount of the internal standard,
647 are prepared, and the chromatography is performed under fixed conditions to determine the
648 amount of the compound to be analysed by comparing the ratios obtained.

649 — *Normalisation procedure*. Provided linearity of the peaks has been demonstrated,
650 individual monographs may prescribe that the percentage content of a component of the
651 substance to be examined is calculated by determining the area of the corresponding peak as a
652 percentage of the total area of all the peaks, excluding those due to solvents or reagents or
653 arising from the mobile phase or the sample matrix, and those at or below the disregard limit
654 or reporting threshold.
655
656 OTHER CONSIDERATIONS

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

664 **Interfering peaks**
665 Peaks due to solvents and reagents or arising from the mobile phase or the sample matrix are
666 disregarded.
667
668 **Measurement of peaks**
669 Integration of the peak area of any impurity that is not completely separated from the
670 principal peak is preferably performed by tangential skim (Figure 9).

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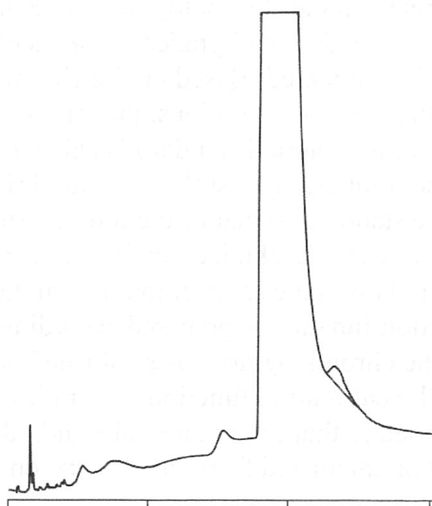

671
672 Figure 9
673
674
675 **Reporting threshold.**

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

679 In such tests the reporting threshold, i.e. the limit above which a peak is reported, is
680 generally 0.05 per cent.