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Revision 1

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**PHARMACOPOEIAL DISCUSSION GROUP
SIGN-OFF DOCUMENT**

CODE: B-05

NAME: PEPTIDE MAPPING

REVISION 1

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	+	+	+
Development of a peptide mapping identity test procedure	+	+	+
Points to consider prior to validation	+	+	+
Validation	+	+	+
Summary	+	+	+

Legend

+ will adopt and implement; – will not stipulate

Non-harmonized provisions

None.

Local requirements

None.

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December 2022

European Pharmacopoeia

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1 B05 BIOTECHNOLOGY-DERIVED ARTICLES—PEPTIDE MAPPING (sign-off)

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INTRODUCTION

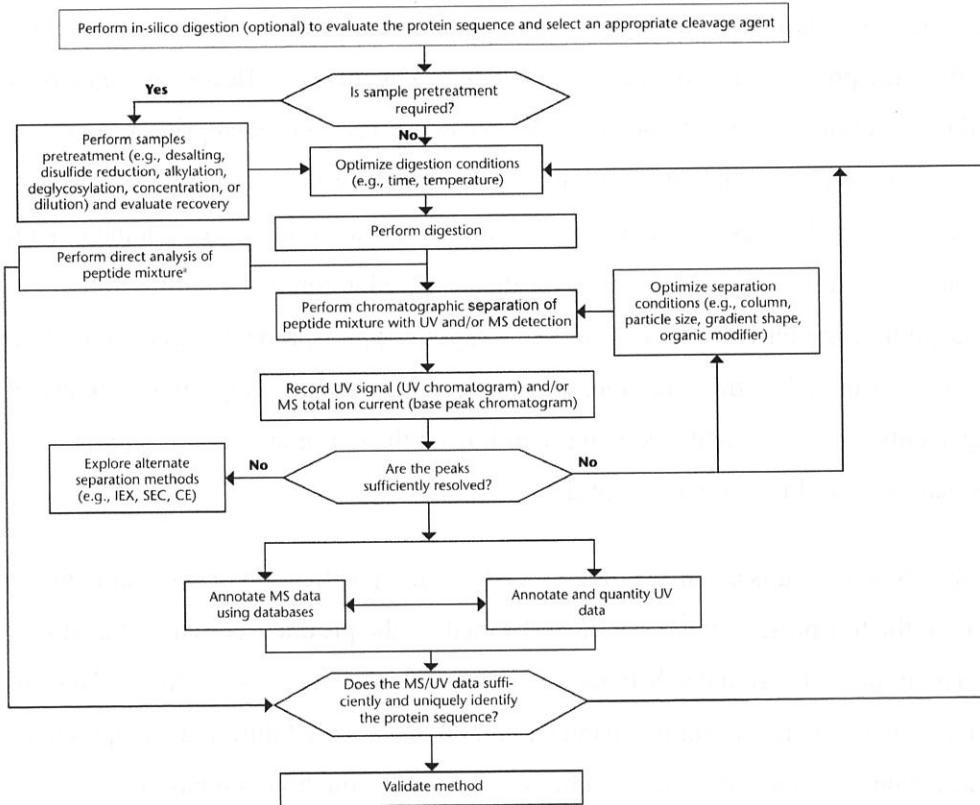
4 Proteins can exist as large complex structures, with some molecules in the population
5 displaying heterogeneity in their amino acid sequence due to improper assembly, degradation or
6 post-translational modification. The high molecular mass of proteins combined with their
7 complexity makes it particularly challenging to chemically identify an intact protein product
8 using a single analytical method. It is possible to cleave the test protein into smaller fragments
9 which can be identified with sufficient mass resolution to determine the amino acid sequence of
10 the protein. This process is the basis of the protein identification technique commonly known as
11 peptide mapping. The peptide mapping technique involves a digestion step in which the protein
12 is selectively cleaved at amide bonds between specific amino acid residues to yield a predictable
13 set of peptides. Analytical chromatographic separation, detection, and identification of the
14 peptide mixture reveal information on the amino acid sequence of the protein which can be used
15 to identify the protein. Peptide mapping is a comparative procedure; the results from the test
16 protein are contrasted with the results of the reference standard or material similarly treated to
17 determine the identity of the test protein. This comparative identification confirms that the
18 primary structure of the test protein matches that of the reference protein.

19 Peptide mapping's ability to detect gross alterations in the primary structure has resulted in
20 many applications for the determination of protein quality which are outside the scope of this
21 chapter. The purity of the test protein with regard to amino acid misincorporation or other
22 misassembly such as disulfide bond scrambling, post-translational modifications, and
23 degradation can be determined using a quantitative peptide map. Peptide mapping comparison
24 during scale up or manufacturing changes can support studies of process consistency.
25 Additionally, peptide mapping can be used to determine the degree and specific amino acid
26 location of modifications such as glycosylation and conjugation (e.g., degree of pegylation). The
27 focus of this chapter will be on the use of peptide mapping for the chemical identification of a
28 protein product where specificity is the primary attribute of the analytical method.

29

30 DEVELOPMENT OF A PEPTIDE MAPPING IDENTITY TEST PROCEDURE—POINTS TO
31 CONSIDER

32 Prior to development of an identity test method procedure it is important to understand the
33 application and level of specificity required to differentiate the identity of the test protein from
34 other products processed in the same facility. In some instances orthogonal methods may be
35 required to differentiate samples of structurally related proteins. Each protein presents unique
36 characteristics that must be well understood so that the scientific approach used during
37 development of the peptide map procedure will result in an analytical method that can be
38 validated with sufficient specificity. The amino acid sequence of the test protein should be
39 evaluated in order to select pretreatment and cleavage conditions resulting in optimal peptide
40 length for analysis. Depending on application, complete or nearly complete sequence coverage is
41 important, because there may be no prior knowledge of the alterations to the protein during
42 development. The following points should be considered during development of a peptide
43 mapping analytical technique. These elements are also presented graphically in Figure 1.



*Peptide mass fingerprint

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Figure 1: Identify Peptide Map Method and Target Performance Parameters

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PRETREATMENT

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Isolation and purification may be necessary for analysis of bulk drugs, dosage forms, or reference standards or materials containing interfering excipients or carrier proteins. Residual interfering substances may impact enzymatic cleavage efficiency and appearance of the peptide map. The impact of residual substances or the sample purification process on the final test peptide map should be assessed during the development process.

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53 The tertiary structure of proteins may hinder full access of the cleavage enzyme to all cleavage
54 sites resulting in unacceptable sequence coverage. The treatment of proteins with chaotropic
55 agents (e.g., guanidinium chloride, urea) and surfactants (e.g., sodium dodecyl sulfate) can be
56 used to unfold the protein prior to digestion. Denaturing agents can affect enzyme activity and
57 additional purification (e.g. diafiltration) or dilution steps may be needed prior to digestion. It
58 may be necessary to reduce and alkylate the disulfide bonds prior to digestion in order to allow
59 the enzyme to have full access to cleavage sites; however, the cysteine-to-cysteine linkage
60 information is then lost. Common reagents for disulfide reduction include dithiothreitol and
61 trialkylphosphine compounds such as tris (2-carboxyethyl) phosphine. Reagents for alkylating
62 reduced cysteines include iodoacetamide, iodoacetic acid, and 4-vinylpyridine. The use of
63 alkylating agents may create adducts which will impact the chromatographic separation and alter
64 the molecular weight of the affected peptide.

65 Since peptide mapping is a comparative procedure, any purification or pretreatment steps
66 performed on the test protein must also be performed on the product reference standard or
67 material. The impact of residual substances, purification procedures, or pretreatment of the
68 protein on method specificity and precision should be investigated during development and
69 considered for inclusion in robustness studies conducted for method validation.

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DIGESTION

72 The choice of a cleavage technique is protein dependent. Some of the more common cleavage
73 agents, both enzymatic and chemical, and their specificity are shown in Table 1. There may be
74 specific reasons for using other cleavage agents or combinations of methods.

75 Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin, EC 3.4.21.4	C-terminal side of Arg and Lys
	Chymotrypsin, EC 3.4.21.1	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)

	Pepsin A (Pepsin), EC 3.4.23.1	Low-specificity digest
	Lysyl endopeptidase (Lys-C endopeptidase), EC 3.4.21.50	C-terminal side of Lys
	Glutamyl endopeptidase (Glu-C endoproteinase; V8 protease); (from <i>S. aureus</i> strain V8), EC 3.4.21.19	C-terminal side of Glu and Asp
	Peptidyl-Asp metalloendopeptidase (Asp-N endoproteinase), EC 3.4.24.33	N-terminal side of Asp
	Clostripain (Arg-C endopeptidase), EC 3.4.22.8	C-terminal side of Arg
Chemical	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thiocyanobenzoic acid	N-terminal side of Cys
	O-Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	3-Bromo-3-methyl-2-(2-nitrophenylthio-3H-indole (BNPS-skatole)	Trp

76 Factors that impact the effectiveness and reproducibility of protein digestion include pH,
77 digestion buffer, temperature, time, and ratio of digest enzyme/reagent to protein. The optimal
78 digestion mixture pH is generally determined by the enzyme or reagent. Chemical stability of
79 the peptides including amino acid side chains and protein modifications at the selected pH must
80 be considered. For example, a highly acidic environment (e.g., pH 2, formic acid) is necessary
81 when using cyanogen bromide as a cleavage agent; however, a slightly alkaline environment (pH
82 8) is optimal when using trypsin as a cleavage agent.

83 The optimal temperature is dependent on the cleavage reagent; for example, most enzymes
84 have optimum activity in a range of 25°–37°. The temperature can define the specificity of the
85 enzyme to some extent. In these cases the adjustment of the temperature can be used to optimize
86 the digestion conditions for certain proteins. Ideally, the digestion temperature will minimize
87 sample-related chemical side reactions, such as deamidation, and protein aggregation while
88 maximizing the susceptibility of the test protein to digestion while maintaining the activity of the
89 cleavage agent.

90 It is necessary to ensure the digestion time is sufficient for intended use to avoid variable
91 digests. A simple time-course study should be performed to ensure sufficient digestion with
92 minimal peptide fragments resulting from partial digestion. Time of digestion varies from
93 minutes to days and aliquots of a single reaction may be appropriately stabilized for analysis to
94 determine the time required for complete digestion of the protein.

95 A sufficient cleavage agent should be used to attain the desired level of digestion within a
96 practical time period (i.e., 2–20 h), while the amount of the cleavage agent is minimized to avoid
97 its contribution to the peptide map. For an enzymatic digest, the protein-to-protease mass ratio
98 between 20:1 and 200:1 is generally used. In cases where the cleavage agent is unstable, the
99 cleavage efficiency may be improved by making multiple additions of the cleavage agent.
100 Enzymes may be bound to a solid support to allow the use of higher relative amounts of protease
101 while avoiding enzyme autolysis contamination and contribution of enzyme fragments to the
102 peptide map. Chemical cleavage reagents are usually used in significant molar excess, and may
103 need to be removed at the end of the digestion.

104 The optimal concentration of the test protein in the digestion should be empirically
105 determined. The concentration should be low enough to minimize the potential aggregation of
106 intact and partially digested proteins but must be sufficient to result in acceptable limit of
107 detection of peptides following chromatographic separation with the selected detection method.
108 Sample dilution or sample concentration by techniques such as centrifugal filtration may be
109 required. Any dilution or concentration steps performed on the test protein must also be
110 performed on the product reference standard or material. Protein recovery should be evaluated
111 for any concentration step and the impact of dilution or concentration on method specificity and
112 precision should be investigated during development and considered for inclusion in robustness
113 studies conducted for method validation.

114 The digestion step can introduce ambiguities in the peptide map as a result of side reactions,
115 such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine
116 residues, carbamylation of lysine residues, or formation of pyroglutamic groups created from the
117 deamidation of glutamine at the N-terminus of a peptide. Autolysis may introduce extraneous
118 peaks produced by the proteolytic enzyme digesting itself. The intensities of autolysis peptide

119 peaks are dependent on the enzyme to substrate ratio and the modifications and quality of the
120 enzyme used. To avoid autolysis, reagent solutions of proteolytic enzymes should be prepared at
121 a pH which inhibits enzyme activity or the reagent solutions should be prepared immediately
122 before use. Modified enzymes, where changes are made to the protease to prevent autolysis, may
123 be used. Commercial preparations of trypsin (often called “proteomics grade”) are available in
124 which the lysine residues of the enzyme have been methylated or acetylated to reduce the
125 number of autolytic cleavage sites. To identify digestion artifacts, a blank determination is
126 performed using a digestion control with all the reagents except the test protein.

127

128

SEPARATION

129 Chromatographic separation of the peptide mixture resulting from the digestion step is meant
130 to resolve its complexity so that a valid interpretation of the data is meaningful and reproducible.
131 The complexity of the peptide map will ultimately dictate the optimal set of chromatography
132 conditions, column, and mobile phases. Method optimization experiments will be required to
133 obtain the highest quality reproducible chromatogram. The molecular weight of the test protein
134 will also influence the complexity of the map and the optimal separation.

135 Many techniques (e.g., ion-exchange high performance liquid chromatography [HPLC],
136 hydrophobic interaction HPLC, and capillary electrophoresis) have been used to separate
137 peptides for peptide map analysis. However, reversed phase HPLC (RP-HPLC) is the most
138 common method for the peptide mapping separation step and will be the focus of this chapter.

139 The selection of a chromatographic column is empirically determined for each protein.
140 Columns with different pore sizes (80–1000 Å) or nonporous based on silica, polymeric, or
141 hybrid supports have been shown to give adequate separation. Columns with particle sizes <2
142 µm are available and are typically more efficient than those with 3–5 µm particle sizes.
143 Generally, octyl or octadecylsilyl bonded phases are ideal for peptides. Octadecylsilane (C18)
144 with 300 Å or smaller pores is the most commonly employed bonded phase for the peptide
145 mapping separation step.

146 The most common mobile phase for the RP-HPLC separation of peptides is water with
147 acetonitrile as the organic modifier; however other organic modifiers such as methanol,
148 isopropyl alcohol, or n-propyl alcohol can be employed. Solvents such as the propyl alcohols in
149 the mobile phase may be useful for separating samples that contain many highly hydrophobic
150 peptides; however, it should be noted that hydrophilic or small peptides may possibly elute in a
151 column void volume. Mobile phase additives such as acids, bases, buffer salts, and ion-pairing
152 reagents are generally needed to produce high quality chromatographic separations of peptides.
153 The most common mobile phase additive has been trifluoroacetic acid (TFA) with typical
154 concentrations of 0.05%–0.2% being employed. The use of phosphate as an additive is less
155 common but can be useful in cases where UV detection is used. Volatile acids and salts can be
156 used in the mobile phase to improve compatibility with mass spectrometer detection. While TFA
157 has a significant positive impact on the quality of peptide separation, sensitivity with mass
158 spectrometer detection can suffer with TFA due to ion suppression. Formic acid, acetic acid, or
159 combinations of these with TFA increase mass spectrometer sensitivity by reducing ion
160 suppression. Temperature control of the chromatographic column is necessary to achieve good
161 reproducibility. The column temperature may be used to optimize peptide separation or improve
162 the retention or elution of certain peptides since the resolution typically increases with
163 temperature for a reversed-phase column.

164

165

DETECTION

166 While RP-HPLC is the most common separation method employed with peptide mapping for
167 identity testing, the most common detection method is ultraviolet (UV) light absorption at 214
168 nm. The peptides resulting from protein digestion may not contain amino acids with aromatic
169 side chains that absorb light at higher wavelengths (e.g., 280 nm) so detection at 214 nm (i.e.,
170 wavelength where peptide bonds absorb light) is essential to ensure sequence coverage of the
171 protein while taking care to minimize background due to the mobile phase. Other detection
172 methods may also be suitable.

173 The limitation of UV detection is that it provides no peptide structural information. Mass
174 spectrometry is a useful detection method which provides mass information to aid in

175 identification of peptides, as well as selectivity in cases when peptides co-elute. In most
176 applications, the RP-HPLC effluent can be directly introduced into the mass spectrometer,
177 provided that the mobile phase is compatible. Specific mobile phase considerations are
178 dependent on the ionization method selected. Electrospray ionization (ESI) is the most common
179 method for the introduction of proteins and peptides into the mass analyzer, and volatile, water-
180 solvent mixtures provide the greatest ionization efficiency. Peptide mapping by ESI-MS is most
181 often performed in positive ion mode. Formic acid or acetic acid are commonly added to the
182 mobile phase to reduce pH and thereby enhance protonation of the peptides. Buffers and salts
183 should be minimized since they can reduce signal, and nonvolatile salts can deposit in the source.
184 As mentioned previously, TFA should be avoided because it can result in ion suppression, a type
185 of matrix interference, which may reduce the signal of some peptides, particularly when ESI is
186 used. Ion suppression may also reduce the ionization efficiency of glycosylated peptides,
187 resulting in reduced sensitivity. It is thus important to optimize conditions in order to achieve
188 optimal results for both UV and MS detection.

189

190

DATA ANALYSIS

191 Peptide mapping is a comparative procedure. To determine if the test protein is the desired
192 protein of interest, the test protein's peptide map must be compared to the peptide map of the
193 reference standard or material generated using identical pre-treatment, separation and detection
194 procedures. Visual comparison of the retention times, the peak responses (the peak area or the
195 peak height), the number of peaks, and the overall elution pattern is the first step of the
196 procedure. It is a best practice to conduct a further non-subjective analysis of the peak response
197 ratios of the critical peaks and the peak retention times. If all critical peaks in the test protein
198 digest and in the reference standard or material digest have the same retention times and peak
199 response ratios, then the identity of the test protein is confirmed. For example, peptide mapping
200 tests for monoclonal antibody samples often include a common Fc peptide that is used as a
201 reference peak. The reference peptide can be spiked into the sample digest and then peak
202 response ratios of the critical peaks and retention times can be examined in comparison with the
203 predefined acceptance criteria. The method of comparison selected should depend on the
204 complexity of the resulting peptide map and the specificity required for the particular identity
205 test application (e.g. differentiation between different protein products manufactured at the same
206 facility or differentiation of variants of the same protein product).

207 When high specificity is required, a mass spectrometer can be used for routine analyses to
208 provide insight into peptide modifications, truncations, missed cleavages, impurities, and
209 unresolved co-eluting peak(s) under a single peak.

210

211

POINTS TO CONSIDER PRIOR TO VALIDATION

212 During the development of the peptide mapping procedure, knowledge and experience are
213 gained that lead to selection of system suitability criteria and analytical method validation
214 acceptance criteria. A final review of the procedure prior to validation can ensure that the
215 procedure is ready for validation, reducing risk of failure to meet criteria. As a general
216 procedure, peptide mapping may encompass a significant range of experimental designs,
217 applications, and requirements for performance. As a consequence, in a general text, it is not
218 possible to set out specific system suitability or validation criteria. The following elements are
219 suggested for evaluation prior to starting the validation.

220 It should be noted that the scope of this document does not include routine application of mass
221 spectrometry (MS)–based peptide mapping applications; however, the application of mass
222 spectrometry for structural identification of peptides during the development of peptide mapping
223 methods is a best practice. Mass spectrometric detection can be utilized to evaluate the following
224 performance parameters.

225

Coverage

226 Coverage refers to the percentage of the amino acid sequence identified in the peptide map to
227 the target protein sequence. Although no specific figure can be identified for all applications, in
228 many cases, coverage approaching 95% has been found to be an acceptable performance target
229 for a peptide mapping procedure.

230

Specific Bond Cleavages

231 The specific bonds cleaved by the chosen enzyme or chemical digestion procedure should be
232 identified and listed.

233 Major Peaks

234 The major peptides recovered from the specific bond cleavages should be identified and listed.

235 Partial Cleavages

236 Peptide bonds susceptible to partial or incomplete cleavage and their associated
237 chromatographic peaks or signals should be identified.

238 Minor/Non-specific Cleavages

239 The extent of cleavage at non-specific bonds should be identified and limited or controlled.

240 Protease-derived Peaks

241 If a protease is used for the test protein digestion then any peaks above background derived
242 from the protease should be identified and, where appropriate, limited.

243 Undigested “Core” Protein

244 Undigested or partially digested protein (often called “core”) should be identified and limited.

245 Mean Peptide Length

246 It describes the peptide set produced by the combination of the chosen protease and/or
247 chemical cleavage reagent and the test protein. This is a trade-off between smaller peptides,
248 which show a higher level of structural selectivity with peptide mapping but produce a more
249 complex map with more peaks, and longer peptides which produce simpler maps but with less
250 resolving capacity for structural variants. No specific peptide length is suitable for all
251 applications, but a mean peptide length of 10–20 residues is often considered appropriate.

252 Resolution Capacity

253 Resolution capacity refers to the capacity of the separation system to resolve the peptide set
254 generated by the protease or chemical cleavage reagent. For example, a digest may produce 30

255 peptides but only 20 peaks due to co-elutions or nonrecoveries. Problematic separations should
256 be identified and resolved by appropriate chromatographic procedures and, if necessary,
257 controlled by the use of peptide reference standard or material or system performance criteria.

258 System Suitability Criteria Selection

259 System suitability criteria should be developed to ensure that the elements of the procedure for
260 protein digestion, separation, and detection have successfully provided a structural identification
261 of the test protein at the level of unambiguity required for the application. System suitability
262 criteria evaluated during routine analysis for identity tests will typically include an assessment of
263 the reference protein digest chromatogram and may include such performance characteristics as:

- 264 • Qualitative similarity to reference chromatogram
- 265 • Extent of digestion
- 266 • Partial cleavages
- 267 • Non-specific cleavages
- 268 • Peak heights/signal-to-noise ratio
- 269 • Peak shape
- 270 • Peak retention time
- 271 • Resolution of specific peaks

272 For test method procedures that require sample isolation, purification, or concentration, a
273 sample recovery criteria should be determined and included as part of the system suitability
274 assessment. In cases where digestion artifacts may be present, assessment of a blank digestion
275 control may be needed to demonstrate a lack of interference.

276

277 VALIDATION

278 Before validating a peptide mapping procedure, the procedure should have been developed to
279 its final form and documented with system suitability criteria. Each time the procedure is
280 performed the results are evaluated against the system suitability criteria to determine if the
281 procedure has successfully provided reproducible results consistent with previous testing

311 To facilitate the determination of the precision (repeatability and intermediate precision) of the
312 peptide mapping procedure, an empirical method of quantifying peak responses (peak areas or
313 peak heights) and peak retention factor should be part of the procedure. One approach is to make
314 peak response and peak retention time comparisons that are expressed relative to a highly
315 reproducible reference peak within the same chromatogram. The precision results obtained
316 during the analytical procedure validation are reported and should meet the acceptance criteria of
317 the validation. Failure of the precision results to meet the acceptance criteria can lead the analyst
318 to reassess the digestion and/or separation steps in the procedure.

319 Robustness

320 Robustness may be evaluated during the development of analytical procedures. It is not
321 necessarily repeated, but it may be included as a part of method validation. Factors such as
322 composition of the mobile phase, protease quality or chemical reagent purity, column variation
323 and age, digestion temperature, and digest stability are likely to affect the overall performance of
324 the test and its reproducibility. Tolerances for each of the key parameters are evaluated and
325 baseline limits established in case the test is used for routine lot release purposes.

326 Variations in purification, pretreatment, dilution, or concentration procedures of the protein
327 sample can have an impact on recovery, test system, and the chromatogram. The variations and
328 their impacts should be identified during the development process and controlled. Impact of
329 residual substances remaining after sample preparation on method specificity and precision
330 should be considered. Critical parameters identified during development should be included in
331 robustness studies conducted for method validation.

332 Many protein fragmentation strategies employ the use of proteolytic enzymes. As a result, the
333 digestion portion of the peptide mapping procedure is inherently more sensitive to minor
334 variation of test parameters. These parameters may include all or a subset of the following:
335 digestion pH, buffer, buffer concentration, ionic strength, digestion temperature, digestion
336 kinetics, test protein concentration, protease quantity, protease quality, and the stability of the
337 digest. Using a design-of-experiments approach, the identified critical parameters are
338 systematically studied to understand their impact on method variability. Those digestion

339 parameters where small variations have been shown to impact the precision of the peptide
340 mapping procedure should be carefully controlled within the test procedure using operating
341 ranges established and validated by these studies.

342 To evaluate the protease quality or chemical reagent purity, a sample of the reference standard
343 or material is prepared and digested with different lots of cleavage agent. The chromatograms for
344 each digest are compared in terms of peak areas, peak shape, and number. The same procedure
345 can be applied to other critical chemicals or pretreatment procedures used during sample
346 preparation, such as reducing and S-carboxymethylation reagents.

347 The length of time a digest can be held before proceeding to the separation step of the
348 procedure, as well as the conditions under which the digest is stored before separation, are
349 assessed. Several aliquots from a single digest are stored under different storage conditions and
350 resolved by the chromatographic method. These maps are then evaluated for significant
351 differences.

352 During the separation step, column-to-column variability, even within a single column lot, can
353 affect the performance of the peptide mapping procedure. To evaluate column lot differences, the
354 reference standard or material of the protein of interest is digested and the digest is subjected to
355 separation using different column lots from a single manufacturer. The resulting peptide maps
356 are then evaluated in terms of the overall elution profile, retention times, and resolution
357 according to predetermined acceptance criteria.

358 To evaluate the lifetime of a column in terms of robustness, a single digest of the reference
359 standard or material can be analyzed using the peptide mapping procedure with columns that
360 vary by the injection number history (e.g., 10 injections per column to 250 injections per
361 column). The resulting peptide maps can then be compared for significant differences in peak
362 broadening and overall resolution. As a column ages, an increase in back pressure might be
363 observed that can affect the peptide map. System suitability or assay validity criteria can be
364 designed to be diagnostic of column aging or other events that may affect the peptide mapping
365 results.

366

367

SUMMARY

368 The peptide mapping procedure consists of multiple steps possibly including protein isolation,
369 denaturation, chemical modification (e.g., blocking sulfhydryl groups) if necessary, protein
370 digestion, peptide separation and detection, and data analysis. Each step should be optimized
371 during development to result in a well-qualified analytical procedure for the peptide mapping
372 identity test. In combination with the use of a suitable reference standard or material, system
373 suitability criteria should be chosen that evaluate if all the steps in the procedure worked together
374 properly to produce a successful peptide map of that reference standard or material that is
375 consistent with the validation of the analytical procedure. When properly developed, validated,
376 and performed, the analytical peptide mapping procedure can be used to verify the identity of the
377 test protein which is a critical quality attribute of the product.