Partnering to Advance Human Health

Case study

Analytical method development for synthetic peptide purity and impurities content by UHPLC - illustrated case study

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Introduction

Peptide-related impurities found in synthetic peptides may be process-related (deletion sequences, insertion impurities, isomers, truncated sequences, incomplete deprotection, by-products generated during synthesis or the final cleavage) or peptide-degradation products. The overall purity of a synthetic peptide (and hence the total impurity level) is related to the size of the peptide. A standard amino acid coupling cycle should be >99% efficient, meaning for a peptide containing 20 amino acids, an overall crude purity of >82% would be expected, or >74% for a peptide with 30 amino acids. The potential number of individual impurities observed in a crude peptide could theoretically be in the millions. However, most of these impurities should be removable during the purification step. The purity of synthetic peptides is usually measured by reversed-phase ultra-high-performance chromatography (RP-UHPLC) with ultraviolet (UV) detection at 210-230 nm in combination with mass spectrometry (MS).

The case study is presented to illustrate the different challenges encountered during peptide analytical method development and to outline Almac's approach to develop a suitable stability indicating UHPLC purity method. With experience of the development and validation of complex analytical methods for a wide range of peptides over the last 10 years, Almac Sciences has developed a strategy to efficiently develop purity methods. This strategy is based on a systematic screening protocol, method optimisation and scrutinisation of the method by LC-MS prior to method validation and forced degradation studies. The analytical method development work presented was performed by Almac Sciences for a biotech client looking for a synthetic peptide manufacturer with expertise in analytical method development.

Analytical target profile

Before starting the method development work, the Analytical Target Profile (ATP) was defined based on regulatory requirements and the acceptance criteria required to ensure the analytical procedure is fit for its intended purpose.

For a typical peptide purity method, the method should be able to quantify the impurities relative to the peptide within the range between limit of quantitation (LOQ) and 120% of the specification limit. The sensitivity of the method must be at least 0.10% area (Ph. Eur. General Monograph: Substances for pharmaceutical use (2034)). The precision of the method must be such that the reportable results between replicate sample preparations for individual impurities fall within acceptable limits for percent absolute difference. The method should be stability indicating if used for stability testing and the peak purity and absence of co eluting impurities should be confirmed by LC-MS.

Systematic screening preparation

Before starting the screening, a suitable diluent is determined. The predicted isoelectric point of the peptide and the acidic or basic nature of the residues in the structure are a good indication for the choice of diluent. Some peptides may also require an additional solubility study to determine a suitable diluent.

The material loading on the column, optimum wavelength detection and the mobile phase gradient are assessed in a preliminary run to ensure the quality of the screening experiment. As a guide for peptides, in general, a concentration range of approximately 0.2 mg/mL to 1.0 mg/mL, with an injection volume range of 1μL to 5μL, represents a suitable column loading and UV detector response.

For this case study, a salt form selection of the peptide was first performed based on solubility, stability studies and counterion content as presented in Table 1.

Table 1: Peptide salt form selection summary

Parameters	Salt form A	Salt form B	Salt form C
Solubility in water and buffers	+	+++	++
Stability (solid form and in solution)	More stable	Less stable	Not performed
Counterion content compared with theoretical value	Comparable	Not comparable	Not performed

Solubility was assessed by comparison: least soluble to most soluble described as +, ++ and +++.

The salt form A was selected as it was found to be more stable, both in solid form and in solution. The salt form B was more soluble. However, this was aided by the presence of residual acid, which also decreased stability.

The peptide solubility of the candidate salt form was then assessed in different diluents and found to be soluble in mildly acidic aqueous / organic mixtures. The aqueous / organic proportions and pH of the diluent were selected to be compatible with the different eluent compositions used for the column screening experiments (no sample precipitation was observed).

The test solution was prepared with a concentration of 0.5 mg/mL. The chromatographic conditions presented in Table 2 were selected for performing the initial run with acetonitrile as organic eluent.

Table 2: Initial screening conditions

<table>
<tr><th>Method conditions</th><th colspan="3">Details</th></tr>
<tr><td rowspan="5">Mobile Phase Gradient</td><td>Time (min)</td><td>% Aqueous</td><td>% Organic</td></tr>
<tr><td>0.00</td><td>80</td><td>20</td></tr>
<tr><td>25.00</td><td>21</td><td>79</td></tr>
<tr><td>25.01</td><td>80</td><td>20</td></tr>
<tr><td>32.00</td><td>80</td><td>20</td></tr>
<tr><td>Flow Rate</td><td colspan="3">0.22 mL/min</td></tr>
<tr><td>Column Temperature</td><td colspan="3">40°C</td></tr>
<tr><td>Detection Wavelengths</td><td colspan="3">Photodiode array (PDA)</td></tr>
</table>

The optimum wavelength was determined to be 225nm and the injection volume set to 3 µL based on peak shape and absorbance.

Systematic screening protocol

The first step in method development is the screening, performed on a UHPLC system with a column manager, using the initial screening conditions. Different parameters were assessed for this case study and are summarised in Table 3.

Aqueous eluent with varying pH value is obtained by using the AutoBlend Plus functionality of Waters H-Class system. The equipment automatically adjusts the ratio of the different eluents (formic acid, ammonium hydroxide, water, and acetonitrile) to obtain the pH value desired for the mobile phase.

A series of stationary phases were selected for screening experiments to provide the greatest breadth of selectivity. Several types of column chemistries from different manufacturers are available at Almac Sciences for screening purposes.

The effect of pH and TFA concentration of the aqueous mobile phase are assessed on two different analytical runs, with two sets of the same type of UHPLC column, as ion pairing reagents such as TFA may modify the column chemistry permanently.

The results of the screening experiments are available typically within a couple of days and are reviewed to select the most promising column and chromatographic conditions or to determine if additional screening is required. After this screening, it is possible to determine if the method development appears to be achievable with minor method optimisation or if it will be more challenging, with additional screening and additional development time required.

The results of the case study are presented in Table 3.

Table 3: Systematic screening parameters and results summary (conditions selected for further optimisation numbered 1-7)

Organic Eluent	Aqueous Eluent	Column 1	Column 2	Column 3	Column 4
Acetonitrile	pH3				
	pH5				
	pH7				
	pH9				
	pH11				
100% Acetonitrile	TFA 0.05%				
	TFA 0.1%				
	TFA 0.2%	1			2
50% Acetonitrile: 50% Methanol	TFA 0.05%				
	TFA 0.1%				3
	TFA 0.2%				
100% Methanol	TFA 0.05%		4		5
	TFA 0.1%	6			7
	TFA 0.2%				

These conditions are unsuitable with visually fewest impurities resolved and unsuitable resolution of closely eluting impurities or baseline artefacts suggest method could be unsuitable.

Based on the initial screen these conditions visually resolve the most impurities and best resolution of closely eluting impurities. Baseline profile is suitable

Based on the initial screen these conditions could be examined as a backup if required.

Initial method optimisation

Following the screening experiments, the second step is method optimisation. First, the best conditions are assessed with an unstressed and a stressed sample solution to identify degradation impurities. The sample degradation conditions are discussed with the chemistry team with the aim to stress the sample to achieve degradation at a level of between 10 to 20% area.

Only the best conditions with an adequate resolution of degradation impurities will then be optimised. Based on the chromatographic profile obtained, different method parameters are modified during method optimisation.

In this case study, eleven chromatographic conditions gave the best resolution (green). Of those, the decision was made to optimise the seven TFA Ion Paired method conditions (Conditions 1 to 7 in Table 3) due to the poor solubility of the sample in the basic eluent.

The sample stressing conditions used for method optimisation were mild acid and heat / humidity. Based on the resolution of the impurities on the tail of the main peak, only two of the seven evaluated sets of chromatographic conditions were deemed suitable for further optimisation:

- *Condition 2: Column 4, 100% acetonitrile organic eluent, 0.20% TFA aqueous eluent*
- *Condition 3: Column 4, 50% acetonitrile and 50% methanol, 0.10% TFA*

The following method attributes were assessed:

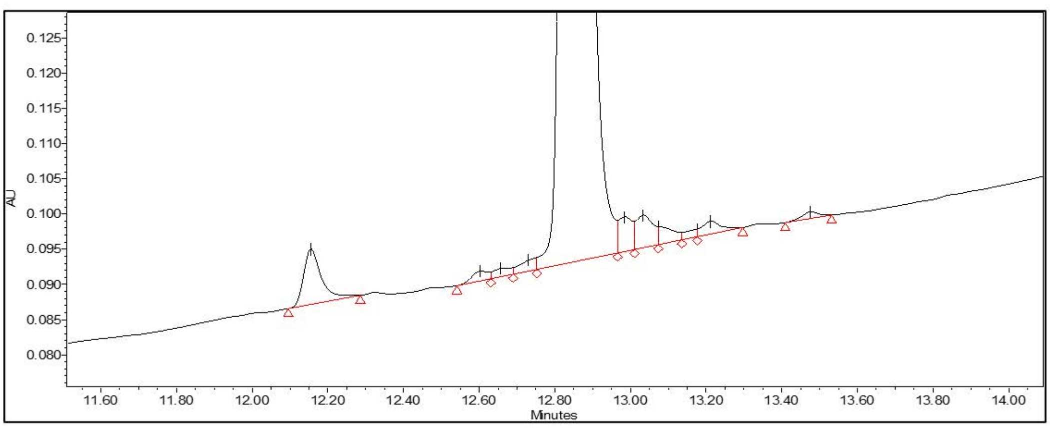
- *gradient range*
- *flow rate*
- *column temperature*

After this final round of optimisation, the final method conditions selected for evaluation of peak purity by LC-MS (based on condition 2) are presented in Table 4 and the chromatography is presented in Figure 1

Table 4: Method parameters selected after initial optimisation

<table>
<tr><th>Method conditions</th><th colspan="3">Details</th></tr>
<tr><td rowspan="7">Mobile phase Gradient</td><td>Time</td><td>% A</td><td>% B</td></tr>
<tr><td>0.00</td><td>80</td><td>20</td></tr>
<tr><td>20.00</td><td>40</td><td>60</td></tr>
<tr><td>25.01</td><td>80</td><td>20</td></tr>
<tr><td>27.00</td><td>80</td><td>20</td></tr>
<tr><td colspan="3">A: 0.2% TFA in water</td></tr>
<tr><td colspan="3">B: 0.2% TFA in 100% acetonitrile</td></tr>
<tr><td>Flow Rate</td><td colspan="3">0.30 mL/min</td></tr>
<tr><td>Column Temperature</td><td colspan="3">50°C</td></tr>
<tr><td>Detection Wavelength</td><td colspan="3">225nm</td></tr>
</table>

Figure 1: Example chromatogram using optimised, initial method parameters described in Table 4



Peak purity is evaluated by MassHunter Molecular Feature Extraction algorithm. Any compounds detected under the main peak above 0.3% volume are assessed to determine if they are impurities from the sample, or adducts, in-source fragments or clusters formed during analysis.

During the case study, the peak purity of the peptide in both freshly prepared and heat / humidity stressed samples was evaluated by mass spectrometry. Although compounds were detected under the tail of the peptide peak, these compounds were well resolved in the UV chromatogram. Therefore, the main peak was pure in both freshly prepared and heat / humidity degraded samples. All the observed peaks under the peptide main peak resulted from adducts, in-source fragments or clusters formed during analysis on the mass spectrometry equipment.

Following method optimisation, the main peak was spectrally pure, and the method resolved heat degradation impurities from the main peak. However, as more manufacturing experience was gained, inconsistencies in the impurity profile obtained between the batches were observed using the optimised method.

The peak purity assessment was performed by LC-MS and three new impurities were detected under the main peak across the four batches, as presented in Table 5 below. Further method optimisation was required to resolve the new impurities from the main peak.

Table 5: Summary of co-eluting impurities

Batch	*1*	*2*	*3*	*4*
Impurities detected under main peak	*one capped truncated impurity (<0.3%)*	*one capped truncated impurity (<0.3%)*	*one deletion impurity, two capped truncated impurities*	*one deletion impurity, two capped truncated impurities*

Deletion: deletion of one (or more) amino acid in the peptide sequence

Capped truncated impurities: if the amino acid coupling does not go to completion during the synthesis, the remaining uncoupled peptide is acetylated with acetic anhydride to give the corresponding capped truncate.

Further method optimisation and finalisation

Further method optimisation is often required, commonly when peptide manufacturing process development is ongoing in parallel. Various method parameters are evaluated to identify the critical method parameters that control the resolution of the peptide related impurities.

In this case study, when analysing additional batches of material, new impurities co-eluting with the front and the tail of the main peak were observed, making resolution challenging. The following parameters were modified and their impact on the resolution of the impurities assessed:

- *Shallower gradient*
- *TFA concentration of aqueous eluent*
- *Column temperature*
- *Composition of organic eluent (methanol and acetonitrile mix)*

A final method based on this assessment showed improved separation of the three co-eluting impurities compared to the previous method conditions. Peak purity determination by LC-MS was performed using the new method conditions, and the main peak was found to be spectrally pure.

The final challenge was the inadequate sensitivity of the method, with a signal to noise ratio below 10:1 for a solution prepared at 0.10% of nominal sample concentration. The recovery of peak area for the sensitivity solution was low (60%) when compared to the nominal sample solution. A blocking agent was added to the sensitivity solution to prevent non-specific binding of the peptide on the glassware, system, or column. As a result, peak area recovery and sensitivity were found to be suitable (recovery increased to 86%).

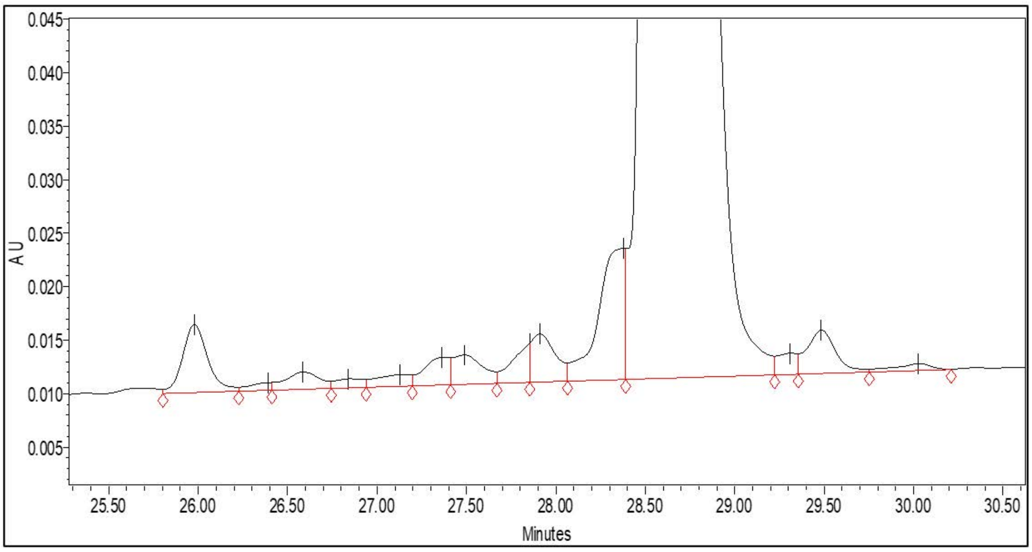
Final method, forced degradation study and method validation

The final method parameters are presented in Table 6 and a chromatogram of the peptide is presented in Figure 2.

Table 6: Final method parameters

<table>
<tr><th>Method conditions</th><th colspan="3">Details</th></tr>
<tr><td rowspan="7">Mobile phase Gradient</td><td>Time</td><td>% A</td><td>% B</td></tr>
<tr><td>0.0</td><td>70</td><td>30</td></tr>
<tr><td>35.0</td><td>49</td><td>51</td></tr>
<tr><td>35.1</td><td>70</td><td>30</td></tr>
<tr><td>40.0</td><td>70</td><td>30</td></tr>
<tr><td colspan="3">A: 0.1% TFA in water (v/V)</td></tr>
<tr><td colspan="3">B: 0.1% TFA in 82% acetonitrile: 18% methanol (v/v)</td></tr>
<tr><td>Flow Rate</td><td colspan="3">0.30 mL/min</td></tr>
<tr><td>Column Temperature</td><td colspan="3">40°C</td></tr>
<tr><td>Detection Wavelength</td><td colspan="3">225nm</td></tr>
</table>

Figure 2: Example chromatogram using finalised method described in Table 6



The final steps are a forced degradation study and method validation. The aim of the forced degradation study is to check that the method is stability indicating. The target degradation rate for the stressed sample is a decrease of purity between 10-20% area. The extent of the method validation is based on the clinical phase of the peptide project.

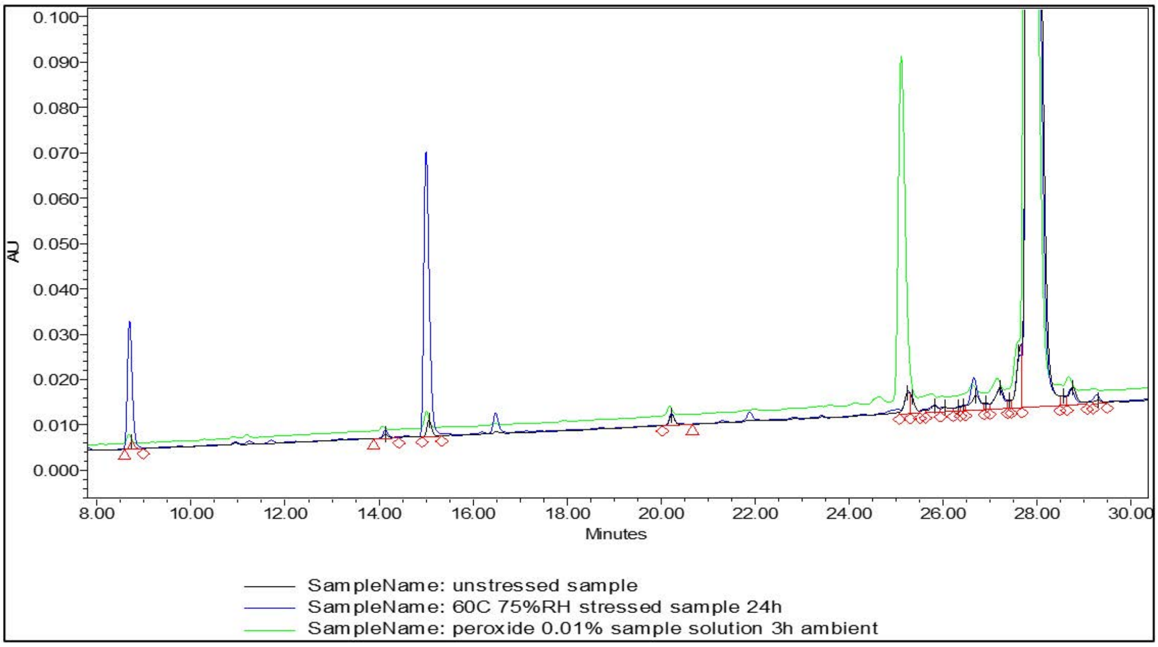
In this case study, during forced degradation studies, the target degradation was reached for both conditions (60C/75%RH and oxidative conditions) as presented in Table 7. The corresponding chromatograms are presented in Figure 3.

The main peak was pure in both unstressed and stressed samples when assessed by LC-MS.

Table 7: Forced degradation study conditions and results

Sample Conditions	Storage time	Purity	Degradation
Unstressed	N/A	92.0 %area	N/A
Heat/humidity 60C/75%RH	24h	78.4%area	13.6%area
Oxidative conditions	3h	79.9%area	12.1%area

Figure 3: Chromatograms from forced degradation study



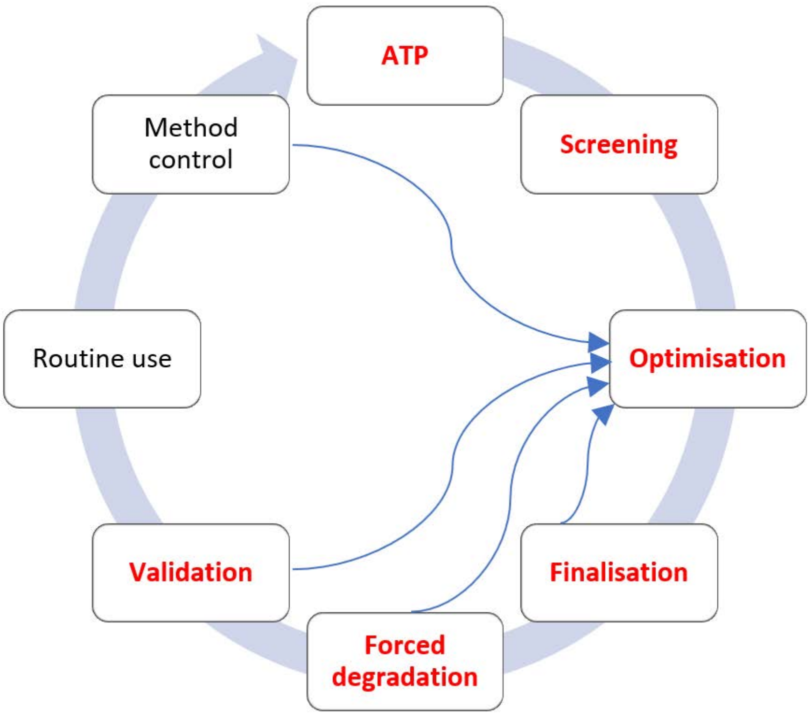
For this case study, the method was successfully validated for specificity, sensitivity (limit of detection and limit of quantitation), linearity, repeatability and stability of solution.

Conclusion

A clear strategy is key for efficient analytical method development as illustrated in this challenging case study. First, a clear objective was defined with the Analytical Target Profile (ATP). A systematic screening was then performed to determine suitable chromatographic parameters. These parameters were optimised, and the method obtained scrutinised by LC-MS to confirm peak purity of the main peak. Further optimisation was needed to finalise the method following the detection of deletion and capped truncated impurities beneath the main peak in new batches of peptide. Validation of the method and a forced degradation study were successfully performed.

The flow chart in Figure 4 presents the different steps in the lifecycle of an analytical method and key activities highlighted in red, all of which are discussed in this case study. The blue arrows illustrate how the intermediate steps help in optimising a method. It also shows the importance of an efficient method development strategy to avoid several additional method optimisations.

Figure 4: Summary flow chart of peptide UHPLC method lifecycle



Finally, the systematic screening and method optimisation approach is currently undergoing further development using software for Analytical Quality by Design. The existing UHPLC equipment with column manager, UV, PDA and QDA detector enables the tracking of impurities by mass during method development, further enhancing the efficiency of method development for synthetic peptide purity and impurities content by UHPLC.

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