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Case study

Accurate determination of resin substitution in solid phase peptide synthesis - an illustrated case study for a highly moisture sensitive resin (2-chlorotrityl chloride resin)

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Introduction

Solid phase peptide synthesis (SPPS) is generally the method of choice for the chemical synthesis of peptides, allowing routine synthesis of virtually any type of peptide sequence, including complex or cyclic peptide products. In large-scale peptide manufacturing, resin substitution is a critical quality attribute which strongly impacts the financial viability of the synthesis and potentially the drug substance quality. Solid phase peptide synthesis (SPPS) is governed by the resin substitution which is of utmost importance for calculating the required input of amino acids and reagents which in turn impacts the associated yield of the synthesis. 2-chlorotrityl chloride (2-CTC) is one such resin that offers several advantages in SPPS with multiple applications. Although literature [1,2] provides several methods to determine such substitution, for highly moisture sensitive resins like 2-CTC resin, the accurate substitution determination becomes very difficult. In this case study, the determination of substitution of 2-CTC resin by the process of loading Fmoc-amino acid to the 2-CTC resin and the subsequent Ultra High Performance Liquid Chromatography (UHPLC) measurement of the liberated Fmoc-amino acid after cleaving the loaded resin is discussed. A multi-point standardisation method that involves the standard solutions of the corresponding Fmoc-amino acid to be quantified was used.

Fmoc-Leu-OH is used to load the resin and as reference standard for the quantification. Unconventional apparatus like Becton Dickinson (BD) syringe with frit, Stuart SB3 rotator and multipoint stirrer plate are used to make the process suitable to perform in analytical labs. A robust process was established for the loading and successfully validated for a UHPLC quantification method along with the resin loading step.

About 2-chlorotrityl chloride resin (2-CTC)

2-CTC resin (Figure 1) has been widely used in SPPS for the preparation of peptides with several applications [3,4,5], including cyclic peptides [6,7,8]. Although it was used initially to make protected peptides in convergent synthesis, later this resin has proved to be quite useful in synthesis of unprotected peptides [9]. The use of 2-CTC resin offers

several advantages in the synthetic process of peptides. It can be cleaved under extremely mild acid conditions by leaving Boc/tBu based protecting groups in place during the preparation of protected peptide fragments. Amino acids can be attached to 2-CTC resin with very little or no racemization. The steric bulk of 2-CTC resin also inhibits diketopiperazine (DKP) formation, a major by product in the synthesis of peptides with C-terminal proline.

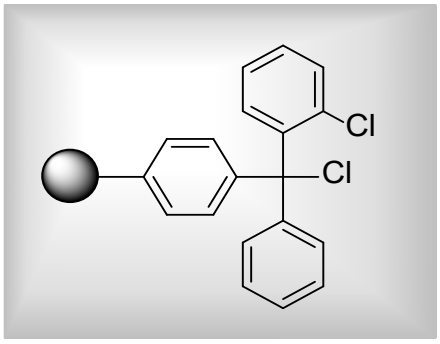


Figure 1: Chemical structure of 2-chlorotrityl chloride (2-CTC) resin

What is resin substitution?

Resin substitution or loading is the number of reactive sites per gram of resin, and it is typically expressed in units of mmoles per gram of resin.

Degradation risk with moisture

The success of SPPS is dependent on a resin and its performance. The resins used as the solid support are considered the heart of SPPS. The main requirement of a solid support is to be chemically stable throughout all conditions of the synthesis. Also, it should allow for an easy interaction of the amino acids towards the growing peptide which is anchored onto the support.

Due to its chemical structure, the 2-CTC resin is highly sensitive to moisture, and over time its load capacity tends to decrease, forming the alcohol i.e hydroxy-trityl-resin (Figure 2) [10]. Given that the alcohol is non-reactive, poor substitutions in the incorporation of the first amino acid are obtained. This causes a decrease in overall synthetic yields of peptides and affects the accurate determination of substitution values during the analysis. Hence the hydrolysis should be controlled to get the true values of substitution during analysis.

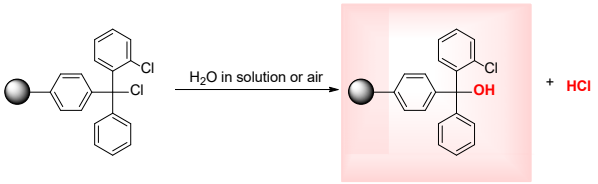


Figure 2: Formation of alcohol when exposed to moisture

Pre-activation of 2-chlorotrityl chloride resin

Hydrolysis during storage as well as the relative high price of the 2-CTC resin are two major problems for its use on large scale synthesis. In this context, resin pre-activation guarantees an adequate degree of substitutions. Pre-activation of hydrolysed resin is possible with chlorinating agents like acetyl chloride, thionyl chloride or oxalyl chloride The hydrolysed hydroxy-trityl-resins are treated with chlorinating agents under mild reaction conditions to reform the actual chloro-trityl-resins (Figure 3). The method is useful for the recycling of the resin during solid phase peptide and organic synthesis [11].

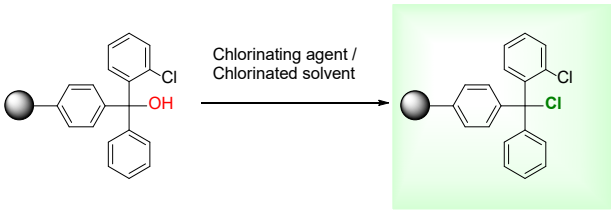


Figure 3: Pre-activation of 2-chlorotrityl chloride resin

Why resin substitution is important?

Due to the increased demand of peptides for both research and industrial purposes, peptide synthesis is a process that is constantly under review, seeking new procedures and developments to make it more efficient. One of the most important advantages of solid phase peptide synthesis

is the high yields it can generate in the coupling steps, which is the cornerstone of its broad application. Choosing the right solid support and considering its accurate substitution value in coupling steps produces higher yields in peptide synthesis. In SPSS, the nominal batch size is calculated using the starting resin substitution. The resin substitution is a critical quality attribute as it constitutes the basis for the calculation of a whole set of important process parameters, such as the quantity of amino acids and reagents required for SPSS. Due to relative high price for resin and amino acids, the accurate resin substitution values will help to add only required quantity of amino acids and reagents during synthesis which benefit the financial viability of the synthesis. High substitution allows for less resin and amino acids/reagents to be used with the active sites more packed and result in higher efficiency. Lower substitution can cause incomplete reactions for more difficult couplings and impacts potentially the peptide quality causing more impurities. Due to these factors, the accurate determination of resin substitution is important, and it helps to achieve financial benefits such as good peptide quality and synthetic yields.

What is needed for resin substitution?

To overcome the financial, quality and yield challenges posed, a reliable method for substitution determination is of utmost importance as the substitution of a resin defines the SPPS batch size and batch size-dependent process parameters (e.g. the quantity of amino acid to be charged, calculated synthesis yields, etc.).

Analytical methods for the determination of resin substitution

Historical methods for the determination of resin substitution can be found using potentiometric titrators and UV spectrophotometers [1,2]. The titration methods estimate the active chloride where the resin was hydrolyzed with sodium/potassium hydroxide and then titrated with silver nitrate. However, this method not only estimates the chloride bound on the resin and has the potential to include chloride from the Hydrochloric acid, which makes this method less accurate. Few UV methods were reported for Fmoc-substituted resins where substitution determination is often performed by suspending the Fmoc-protected starting resin in 20% (v/v) piperidine in DMF to generate the dibenzofulvene-piperidine adduct that is quantified by ultraviolet-visible spectroscopy. The spectrometric measurement is performed at the maximum absorption wavelength of the dibenzofulvene-piperidine adduct. The substitution is determined based on Fmoc deprotection. However, these are more suitable for the Fmoc-substituted resins.

In the present case study, a robust procedure is established to determine the accurate & precise substitution of 2-CTC resin using UHPLC, which involves a 3 step process:

- The first step is loading step: Loading Fmoc-Leu-OH to the 2-CTC resin using DIPEA as base.
- The second step is cleaving step: Cleaving the loaded resin using 2% TFA solution.
- The third step is a quantification step: The liberated Fmoc-Leu-OH is quantified against an external standard of Fmoc-Leu-OH using UHPLC.

Challenges

- Sensitive 2-CTC resin often faces moisture-driven stability challenges that can accelerate the formation of inactive 2-chlorotrityl alcohol resin. This will reduce the load capacity of resin; hence this should be controlled during the analysis to obtain true results for the substitution of 2-CTC resin.
- Accurate and precise determination of resin substitution; a critical quality

attribute in the process validation of peptides.

- Range of equipment like BD syringe with frit, Stuart SB3 rotator and multipoint stirrer plate are required to perform the studies in analytical labs.
- The proposed procedure is not a traditional UHPLC method. It includes a two-step treatment prior to quantification by UHPLC; loading of 2-CTC Resin and cleaving the loaded resin.
- The first step is very critical, due to the need of a synthetic process for complete loading of resin. This process needs to be developed in an analytical lab with high accuracy and transferrable for release purposes.
- Subsequently, the esterified resin has to be completely cleaved to determine its precise loading value.

The ultimate goal

The goal was to establish a robust synthetic process for loading and cleaving steps in analytical labs, developing a robust analytical method for quantification, perform validation and transfer to for its intended release purpose.

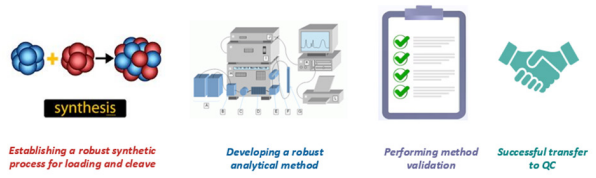


Figure 3: Steps for the ultimate goal

Materials and methods:

Chemicals and reagents:
All reagents and solvents of LC or analytical grade, including acetonitrile (ACN), Dichloromethane (DCM), Dimethylformamide (DMF), Hexane, Toluene, Trifluoroacetic acid (TFA) and N,N-Diisopropylethylamine (DIPEA) were used in study. The high pure Milli-Q water used was obtained through a Milli-Q integral water purification system.

Equipment and instruments:
20 mL BD syringe with PE frit (PP– Reactor 20ml with PE frit V200PE100), B Braun Sterican Needles 0.80 x 120mm, Product code: 4665643, Stuart SB3 rotator, Memmert vacuum oven and GC head space vials were used in the esterification step. Ikamag R015P Multipoint stirrer plate, volumetric flasks and magnetic stir bars were used in the cleave step. Each Chemical and test samples were accurately weighed using Mettler Toledo analytical balance. The quantification of Fmoc-Leu-OH was performed on Waters Acquity UHPLC system with a UV detector and standard flow cell. The data was collected and processed using empower software.

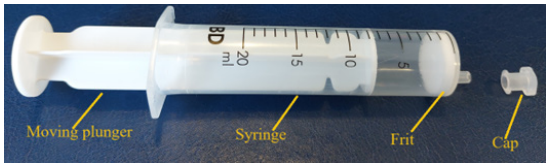


Figure 4: BD syringe

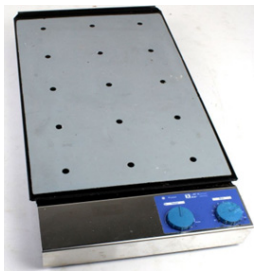


Figure 5: Stuart SB3 rotator



Figure 6: Multi point stirrer

Procedure used in the study:

First step - Loading step:
Loading Fmoc-Leu-OH to the 2-CTC resin using DIPEA as base.

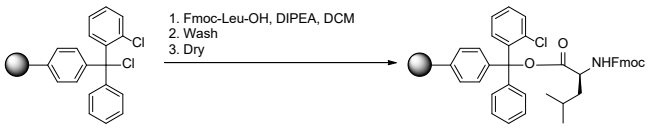


Figure 7: Loading 2-CTC Resin with Fmoc-Leu-OH

Step 1: Add Fmoc-Leu-OH (2.0-2.3 eq) to a solution of DIPEA (2.3-2.6 eq) in DCM (Amino acid concentration 0.6 mol/L)
Step 2: Transfer the solution from step 1 into a BD syringe containing pre-weighed 2-CTC resin (1 eq weighed under nitrogen blanket) using a syringe needle.
Step 3: Close the BD syringe with its cap and attach to the rotator for spinning (40 rpm, 4 hrs)
Step 4: Drain the mixture in the syringe and wash the resin with 6 x 6mL DMF, 4 x 6mL toluene, and 4 x 5 mL hexane, for 2 mins per each wash by spinning on the rotator.
Step 5: Dry the loaded resin in a vacuum oven at ambient temperature until constant weight is observed.
Step 6: Store the loaded dried resin in a GC head space vial under nitrogen in a freezer.

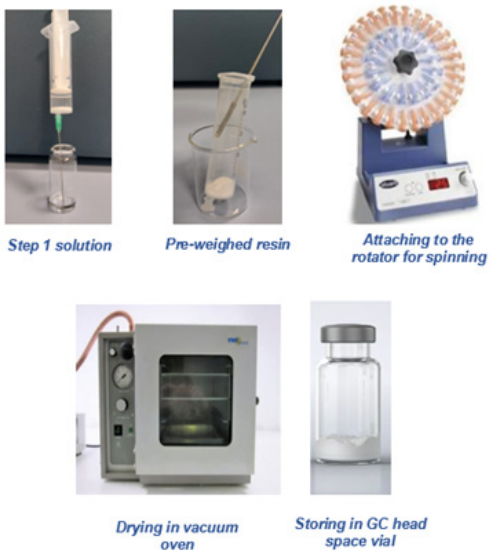


Figure 8: Experimental set up for loading step

Second step - Cleave step:
Cleaving the loaded resin using 2% TFA solution.

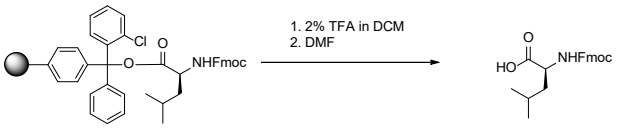


Figure 9: Cleaving the loaded resin with TFA

Step 1: Weigh 50.0mg loaded resin into 50mL volumetric flask, add 4mL 2%TFA in DCM and stir for 30 minutes at 200 rpm, use multi point stirrer plate to stir multiple solutions.
Step 2: Make up to the volume with DMF, allow the contents to settle for 5 min and use the supernatant solution as test solution for the quantification using UHPLC.

Third step - Quantification step:
In the final step, the liberated Fmoc-Leu-OH is quantified against an external standard of Fmoc-Leu-OH using UHPLC. The UHPLC method is optimised to separate DMF, other solvents and impurities from the Fmoc-Leu-OH peak. The final method uses waters UHPLC with standard flow cell.

Standard solutions:
A series of solutions for Fmoc-Leu-OH reference standard ranging from 50% to 150% of the nominal standard concentration (0.40 mg/mL) are prepared to plot a multi-point calibration curve for qualification.

Sample solutions:
The supernatant solutions from step 2 of cleave step are used as test sample for quantification.

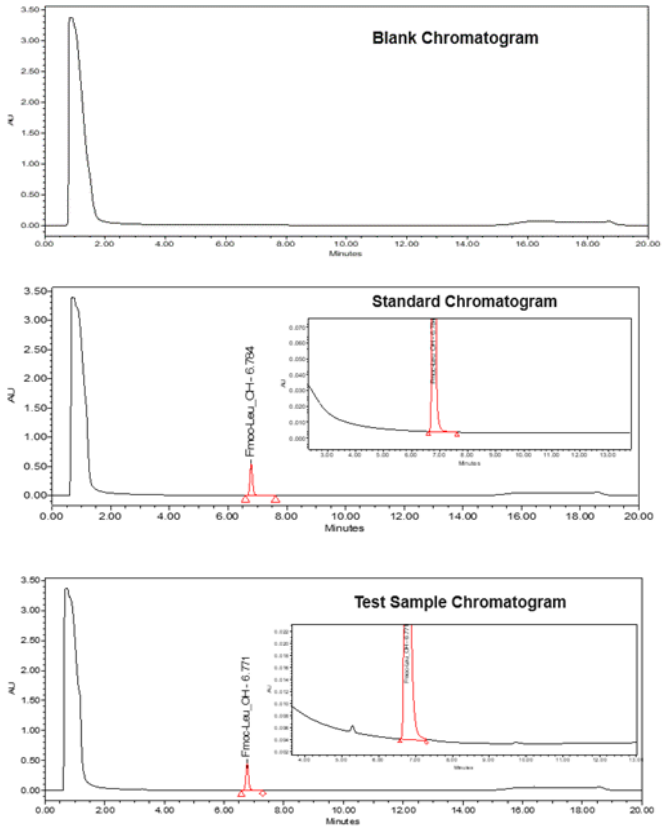


Figure 10: Example chromatograms

Calculations:

The resin substitution is calculated using the following equation:

$$\textit{Substitution}\ (\text{mmol/g}) = \frac{A_{sample} \times W2 \times \text{mmol standard} \times V_{sample}}{A_{standard} \times W1 \times W3 \times V_{standard}}$$

Where

A_{sample}	: Peak area of test sample
$A_{standard}$	: Peak area of standard
mmol standard	: Weight/MW OF fMOC-L-LEU-OH
W1	: Weight of starting resin (g)
W2	: Weight of esterified resin (g)
W3	: Weight of sample (g)
V_{sample}	: Final volume of sample
$V_{standard}$	: Final volume of standard

Overcoming the Challenges

- *Mitigating the degradation risk*

Moisture is a critical factor that must be controlled as it affects resin activation, causing a decrease in substitution value. To mitigate the risk of degradation from moisture, the 2-CTC resin was stored under nitrogen atmosphere in a tightly sealed container. Container was placed in a dry place and at ambient temperature. Nitrogen blanket was used while handling the 2-CTC resin for weighing operations. Importantly, all glassware and apparatus used in the procedure are thoroughly dried before use. With this, the quality of 2-CTC resin will be maintained, and thus accurate results can be obtained.

- *Accurate and precise determination of resin substitution*

A range of equipment like BD syringe with frit, Stuart SB3 rotator and multipoint stirrer plate were used in the study to make the process more suitable and robust to perform in the analytical labs. The quantities of Fmoc-Leu-OH, DIPEA, DCM, reaction time and rpm were optimised for complete loading of resin. Subsequently, the quantity of TFA required for cleave step was also optimised along with the time & rpm required for complete cleaving of loaded resin. A simple, rapid and reliable reversed phase UHPLC method was developed for the quantification. The developed 3-step process was shown to be a suitable technique to determine the accurate and precise resin substitution values in solid phase peptide synthesis.

Method validation:

After establishing the process, it was subjected to method validation as per ICH guidelines. The method was validated for specificity, precision (repeatability), intermediate precision (reproducibility), linearity, accuracy, solution stability and range parameters.

Table 1: Method validation

Validation parameter	Assessment	Results
Specificity	Interference in the blank and resolution of other impurities from Fmoc-Leu-OH peak	• No interference in the blank chromatogram • USP resolution of other impurities from the Fmoc-Leu-OH > 1.5
Precision for loading and cleave steps	%RSD for six individual measurements at nominal sample concentration for both loading and cleave steps	• %RSD for n=6 < 2.0% • Individual measurements range in 1.76 mmol/g to 1.79 mmol/g
Intermediate precision for loading and cleave steps	%RSD for six individual measurements at nominal sample concentration for both loading and cleave steps	• %RSD for n=6 < 2.0% • Individual measurements 1.76 mmol/g for all
Linearity of standard and loaded resin	Across a range of 50 to 150% of nominal concentrations for both Fmoc-Leu-OH standard and loaded resin. Assessment of correlation coefficient (r), Y-intercept values at 100% nominal concentrations, p- value, 95% confidence intervals, linear demonstration of plots and random distribution of residuals.	• Correlation coefficients (r) ≥0.99 • % Y-intercept at 100% nominal concentration ≤5% • p-value >0.05 • 95% confidence intervals include zero. • Plots demonstrated linear response. • Residuals randomly distributed.
Accuracy	Across a range of 50 to 150% of nominal sample concentration. Assessment of mean recovery at each level and %RSD for %recoveries at each level.	• Mean recovery at each level between 95.0 – 105.0% (n=3, each) • %RSD for % recoveries at each level ≤5.0% (n=3, each).
Stability of loaded resin and solutions	Assessment of solid loaded resin, standard stock solution, working standard solution and test sample solution	• Loaded resin stable for 1 month. • Standard stock solution stable for 24 hrs • Working standard solution stable for 24 hrs • Test sample solution stable for 24 hrs
Overall validated range	Assessment over which the process is liner, accurate and precise	• Overall validated range for resin substitution 0.864-2.632 mmol/g

Conclusion

A new approach for the determination of resin substitution that combines loading, cleaving of resin with subsequent UHPLC quantification is presented in this case study. This approach offers the advantage over the reported methods through potentiometric titration/UV showing true results for substitution. The main advantage of this method is that only active sites will react and therefore only those will be measured, hence gives the accurate results. The degradation risk was mitigated by storing/operating the material under nitrogen atmosphere. The developed 3-step process has proven to be an accurate method for the substitution determination of 2-CTC resin that governs the solid phase peptide synthesis. With these substitution values, the quantity of amino acids and reagents to be charged for a solid phase peptide synthesis can be calculated accurately. Very fast UHPLC separation combined with robust loading and cleave steps described in this work allows the application of the method for routine analysis as needed for release purposes. Method validation results have proved the entire process to be specific, precise, linear, accurate and robust as required by ICH standards. This process was shown to be a suitable technique to quantify resin substitution in solid phase peptide synthesis and can be successfully applied for routine analysis.

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