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Calculating Resin Functionalization in Solid-Phase Peptide Synthesis Using a Standardized Method based on Fmoc Determination

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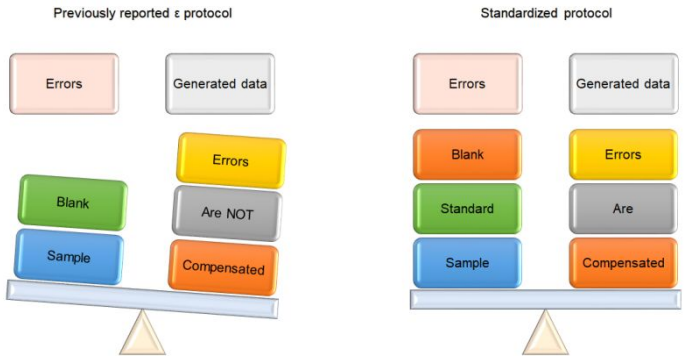

Abstract

Solid-phase synthesis is the method of choice for peptide preparation in both research and industrial settings. The whole synthetic process is governed by the initial functionalization of the resin. Although the literature provides several methods to determine such functionalization, the addition of an Fmoc-amino acid and the posterior spectrophotometric measurement of the dibenzofulvene adduct formed after Fmoc removal is the most widely used for this purpose. However, a range of molar extinction coefficient (ε) values and even wavelengths are currently used in the field, with no standardization of the method. Here, we propose a single-point standardization method that involves a standard solution of the corresponding amino acid to be checked that is prepared freshly at the time of the analysis.

Peptides are key chemical entities with broad applications in drug discovery and related scientific areas. The “peptide boom” in these fields of research has been possible thanks to the development of the solid-phase peptide synthesis (SPPS) approach, which is now the strategy of choice for the preparation of these molecules [3]. This simple method is based on the use of a solid polymeric protecting group (resin), usually for the *C*-carboxylic function. This resin facilitates the physical manipulations-all reactions in a simple reaction vessel-and, more importantly, the chemical reactions. Thus, as the reagents and soluble side-products can be removed by simple filtration and washings, excess of reagents can be used to optimize the course of reactions. At the end of the process, the peptide is released from the resin by cleaving the bond between the first amino acid and the resin [4].

The so-called 9-fluorenymethyloxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) strategy [4, 5] is the most widely used for SPPS in both research purposes [6] and large-scale industrial applications [7]. In this approach, the α-amino function is protected by Fmoc, which is removed with piperidine or another secondary amine, and the side chains are protected by *t*Bu and related groups for, which are removed with trifluoroacetic acid (TFA).

The success of the overall synthetic process is dependent on each individual step [4-7]. However, accurate knowledge of the functionalization (number of reactive sites per gram) of the resin is crucial for controlling the synthesis and for assuring optimum performance, namely the use of the correct amounts of reagents, and for determination of the overall yield. In another words, the functionalization of the resin in the SPPS approach is comparable with the concept of the molecular weight of reactants in solution chemistry. Furthermore, the determination of the incorporation of the first amino acid onto the resin, mostly for *C*-terminal carboxylic resins [Wang and 2-chlorotrityl chloride (CTC) resins], which cannot be monitored by a colorimetric test [8], is key for assuring efficient synthesis [9].

The functionalization/loading of the resin can be determined directly on the resin or after the incorporation of an amino acid, which can be the first amino acid of the peptide sequence or simply any amino acid used for the purpose of this measurement. The literature provides several methods to calculate the functionalization/loading, namely: (i) elemental analysis [10, 11]; (ii) quantitative carbon nuclear magnetic resonance spectroscopy (^{13}C NMR) [12]; (iii) picrate adduct determination [13]; (iv) perchloric acid in acetic acid titration [11]; (v) chloride titration [14]; (vi) Schiff bases formation [15]; (vii) quantitative ninhydrin test [16]; (viii) amino acid analysis [3]; (ix) monitoring the change in the resin's weight after the whole process; (x) dibenzofulvene adduct spectrophotometric [17-19] or (xi) gas chromatography (GC) determination [19]; and (xii) Edman degradation and mass spectrometry [20]. Furthermore, it has been reported that the concomitant application of more than one of these methods usually leads to divergent results [13, 14].

In fact, most of the aforementioned approaches are laborious and have important drawbacks. In addition, some require sophisticated instruments. In this regard, elemental analysis (i) is an indirect method that can introduce interferences. For example, the calculation of the functionalization of a CTC resin on the basis of Cl content will determine two Cl, one bound to the phenyl ring and the other to the benzylic ring. Calculations of the functionalization of resins on the basis of N content will also determine the presence of N attributable to the precursor where the amino function has not been properly converted. In the quantitative ^{13}C NMR method (ii), apart from the advanced instrumentation required, there are some additional disadvantages. For example, the results can be affected by high broadening of the carbon resonance. Moreover, this method involves a relatively long analysis time per sample (2-3 h) and it is useful only for Wang and CTC resins [12] .

Methods (iii-vii) are based on the titration of the amine group. The most widely used approach is probably the one developed by Gisin (iii), where picrate is formed, and then is subsequently displaced with *N,N*-diisopropylethylamine (DIEA), and is measured spectrophotometrically [13]. However, although this method was automatized, the explosivity associated with picric acid precludes its use. Titration with perchloric acid (iv) involves the use of picric acid in combination with HOAc as a solvent. However, the latter is not the most appropriate solvent for swelling the polystyrene resin—a capacity that is key for accessibility and therefore for accurate measure of functionalization [15]. The Schiff base formation method (vi) is considered a time-consuming procedure, requiring about 12 h of treatment prior to determination [15]. Finally, in the ninhydrin test (vii) the colorimetric response is dependent on the used amino acid.

For high accuracy, amino acid analysis (viii), which is a tedious method involving several chemical manipulations, requires the incorporation of several amino acids and an automatic analyzer, which is not commonly found in laboratories nowadays [3, 11]. Furthermore, both this method and the weight change method (ix) are performed after the synthesis has been completed and also require the use of large amounts of resin to obtain accurate measurements of functionalization.

With the advent of the Fmoc/*t*Bu strategy and taking advantage that the Fmoc moiety contains an aromatic system that absorbs in the UV region, the use of Fmoc for measuring functionalization has been broadly adopted, using mostly a UV spectrophotometer [17, 18] (x). As shown in Figure 1, the Fmoc group is removed through a β-elimination reaction by treatment with a secondary amine such as piperidine, with concomitant formation of highly reactive dibenzofulvene (DBF). The latter reacts with the excess of piperidine to give the adduct *N*-fluorenyl-piperidine (DBF adduct), which shows a characteristic UV absorbance at a maxima of 301 nm [17, 18].

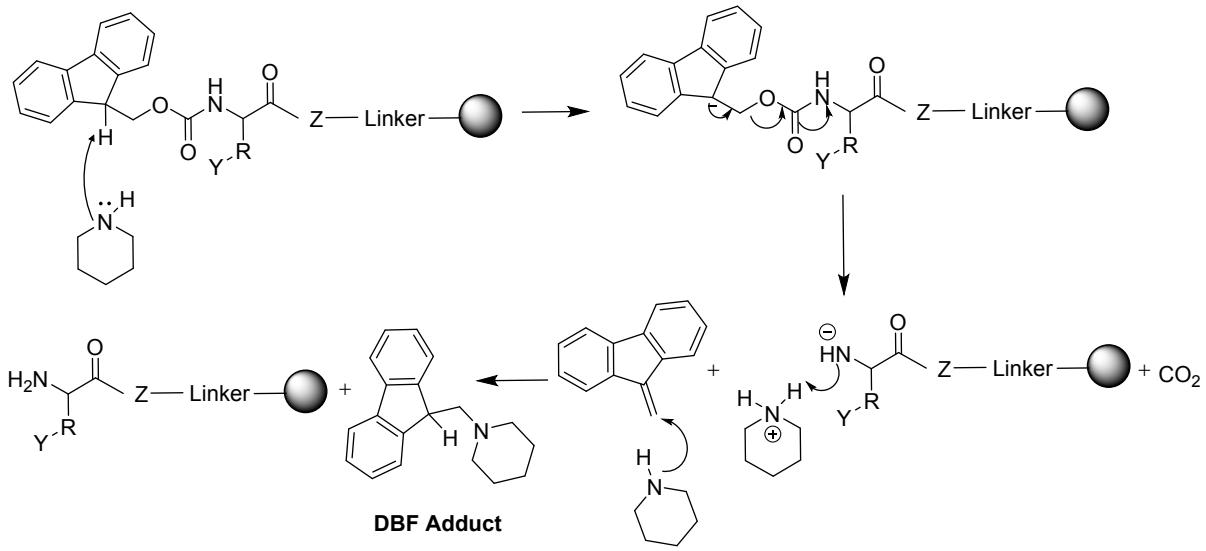

Figure 1. Mechanism for Fmoc group removal. R = amino acid side chain; Y = side chain protecting group (if required); Z = O, NH. Fmoc-amino acid can be directly loaded on amino methyl resin and then the linker is avoided.

A similar method was developed for the determination of the carboxylic groups present in a resin. This approach involves the incorporation of fluorenylmethanol with *N,N*-diisopropylcarbodiimide (DIC) and 4-dimethylaminopyridine (DMAP) and posterior spectrophotometric determination of the DBF adduct after treatment with piperidine [21].

In this method, the loading is calculated using the Beer-Lambert law. Refer to equation (1):

$$Abs = \varepsilon \times c \times l \qquad (1)$$

where Abs is the absorbance, ε is the molar extinction coefficient, c is the concentration, and l is the light path length.

However, the literature reveals that this approach has resulted in large discrepancies. In most cases, loading determination is calculated with reference to a previously determined ε value at a corresponding wavelength for the DBF adduct. Table 1 shows the ε value at different wavelengths reported by various industrial and academic groups. Of note, while group # 1 used the ε at 304 nm, groups # 3-10 used the ε at 301 nm. Interestingly, for the four groups using 301 nm, the reported ε varies from 5.304 to 8.100 L $mmol^{-1}cm^{-1}$, which indicates more than 50% of variability. Thus, there is

no consensus about the ε value or the wavelength to be used, and a small variation in the calibration of the UV spectrometer could bring about an error in the wavelength and therefore in the ε value. Furthermore, the aging of the lamp in the UV spectrophotometer will result in an alteration in the value for ε and therefore an increase in the error. At research scale, these errors would probably have limited impact, but work at a multi-kilogram scale calls for an accurate determination of the loading.

Some groups (#1 and 2) claim that 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) shows more sensitivity for Fmoc cleavage than piperidine [19, 22]. However, the values (at different wavelengths) of these two reagents are totally different. Nevertheless, whatever base is used for Fmoc removal, a reference standard of the corresponding amino acid is required at the time of analysis.

Table 1. Molar extinction coefficient (ε) reported by various research groups

Entry	ε (L $mmol^{-1}cm^{-1}$)	Wavelength (nm)	Reference
1	8.794	294	[22]
	7.624	304	
	6.234 (DBU)	299	
2	9.254 (DBU)	307	[19]
3	8.021	301	[18]
	6.089	289.8	
4	6.700	302	[23]
5	7.100	301	[24]
6	8.100	301	[25]
7	7.200	301	[26]
8	5.304	301	[27]
9	7.205	301	(a)
10	7.800	301	(b) [23]

The above values were estimated using 20% piperidine-DMF as the Fmoc cleavage base unless otherwise stated; (a) previously determined in the author's lab; (b) the most used value (however, the research group that determined this value is unknown).

To assure the quality of the data generated in an analytical study, a standardized test is required. According to the Harmonized Tripartite Guideline of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the analytical method must have the capacity to accommodate deliberate changes in its standard parameters (wavelength, pH, temperature, among others). Such a test is called a "robustness test" [28]. If the method fails to accommodate such changes, it is considered invalid and, in turn, it will give false results. Of note, these changes might arise because of a systematic error on the part of the analyst or low efficiency of the instrument itself (old apparatus). Accordingly, it is recommended that a standard solution be tested along with the sample solution, thus exposing both solutions to the same alterations or systematic errors (if any) in the same manner and to the same extent. Hence, the undesirable effect on the final data is omitted, because other influences are already compensated for.

Two main standardized methods can be used to quantify resin loading values on the basis of the UV absorbance of the DBF adduct released:

(i) Multi-point standardization method (calibration curve).

In this method, a ε of DBF is established, and then the sample is measured on the basis of its UV absorbance. ε is a measure of the ability of any substance to absorb UV light at a certain wavelength. Its value is obtained by rearrangement of Beer-Lambert's law. Refer to equation (2):

$$\varepsilon = \frac{Abs}{C \times l} \qquad (2)$$

Where ε is specific for each solution at a defined wavelength. Sensor aging, as well as the stability of the UV source, directly influence the absorbance of samples. Furthermore, the working temperature also has a pronounced effect on the ε value [29]. Therefore, to assure the quality and exactness of the final results, these uncertainty factors have to be taken into consideration [30].

A multi-point standardization method provides high accuracy. However, it is a tedious approach in which several calibration points must be prepared and verified on the same day as the analysis. Otherwise, the use of a previously established ε value (see above) is likely to favour misleading or inexact results.

(ii) Single-point standardization method.

In this method, a single standard solution is considered rather than a set of standard solutions with several concentrations. The UV absorbance of the sample is then measured and compared with this standard solution. Refer to equations 3-5:

$$K = \frac{standard\ Abs}{standard\ concentration} \qquad (3)$$

$$Sample\ concentration = \frac{sample\ Abs}{K} \qquad (4)$$

Then, by substituting K (sensitivity) from equation (3) in equation (4):

$$Sample\ concentration = \frac{sample\ Abs \times standard\ concentration}{standard\ Abs} \qquad (5)$$

A single-point standardization method is fast and reliable for determining the concentration of unknown analytes in many applications. However, it has some drawbacks in comparison with the multi-point method. In this regard, as only one standard solution is prepared, any error in the preparation will be carried over into all the following steps. Moreover, another obstacle for the single-point method can be the wide concentration range of analytes, which can sometimes be far from that of the standard solution prepared. Nevertheless, preparing two or more standard solutions (of the same concentration) can support the validity of the standard prepared, hence circumventing the first drawback. Moreover, during loading determination in SPPS, the amount of linker loaded (functional groups) is usually known and provided by the manufacturer. Therefore, efforts can be made to bring the final concentrations of the sample being examined and the standard solution as close as possible. Accordingly, the second drawback of this method can also be avoided. Thus, the single-point method is considered a procedure of choice, showing versatility and offering a rapid alternative to the tedious multi-point approach. Importantly, the ε value must be calculated and used on the same day as the analysis (fresh preparation), otherwise uncertain data may be generated.

Here we review the method of calculating resin functionalization on the basis of the spectrophotometric determination of the DBF adduct and using a single-point standardization method, which does not rely on the ε value taken from a previously established calibration curve.

Table 2 shows the loading values for all the Fmoc-amino acids incorporated separately onto Wang resin. The loading values were calculated twice, once based on a published ε value of 7.800 L mmol^{-1}cm^{-1} (# 10, Table 1) [23], and once based on the absorbance of a freshly prepared standard solution of each amino acid. Two standard solutions of each corresponding amino acid were prepared, and their absorbances were proved to be matching. Table 2 shows the low functionalization obtained when considering a previously estimated ε (application of other wavelengths and/or ε would show larger discrepancies). On the contrary, the method that considered sample and standard solutions provided satisfactory data that reflect the true loading values and are closer to those reported by the supplier (1.00-1.10 mmol/g).

Table 2. Loading values using distinct methods of calculation

Entry	Fmoc-Amino acid	1st Protocol: (Previously established ε 7.800 L mmol^{-1}cm^{-1})	2nd Protocol: (versus Fmoc-AA freshly prepared solution)
1	Gly	0.44	0.74
2	Ala	0.54	1.10
3	Ser	0.36	0.72
4	Thr	0.55	1.09
5	Cys	0.47	0.71
6	Phe	0.45	0.92
7	Val	0.43	0.81
8	Trp	0.30	0.71
9	His	0.33	0.57
10	Leu	0.44	0.84
11	Lys	0.41	0.71
12	Ile	0.30	0.82
13	Tyr	0.22	0.45
14	Pro	0.29	0.74
15	Met	0.30	0.81
16	Asn	0.29	0.68
17	Asp	0.31	0.70
18	Glu	0.27	0.69
19	Gln	0.27	0.57
20	Arg	0.26	0.45

1st protocol: calculated using a previously established ε value (7.800 L mmol^{-1}cm^{-1}) (equation 6) [23], 2nd protocol: calculated versus a freshly prepared standard solution of the corresponding Fmoc-amino acid (equation 7).

In addition, we studied the stability of the analytical solution (20% piperidine-DMF) in relation to the results (loading values/functionalization) obtained. As shown in Table S1, the standard solution must be freshly prepared, otherwise loading values will be underestimated. This observation is attributed to the increase in the absorption values recorded for the standard solutions one day (at rt) after the preparation date.

Resin capacity

Resin capacity represents the initial amount (mmol) of functional groups present on the polymeric support. For example, for CTC resin, the capacity is represented as mmol of Cl per g of resin; for Wang resin as mmol of OH per g of resin; and for aminomethyl resin as mmol of NH_2 per g of resin. On the other hand, the amount that is determined after the anchoring of the first amino acid represents the

mmol of Fmoc-amino acid per g of the Fmoc-amino acid-resin. Thus, in order to determine the amount of the functional groups (Cl, OH, NH_2) originally loaded on the resin (as supplied from the manufacturer), additional calculations must be made. Refer to equation (8).

Here we have discussed the methods used to calculate the amount of Fmoc-amino acid loaded onto a resin. Regarding the spectrophotometric method, it is widely accepted as an easy, accurate and fast approach for determining the loading value. Freshly prepared sample solution must be tested versus a freshly prepared standard solution of the corresponding amino acid. This can be done through a single or multi-point standardization methodology. We have shown that the use of an ε value derived from a previously established calibration curve cannot guarantee the accuracy the data generated because of numerous uncertainty factors, as discussed in this work. Our findings were corroborated by testing the 20 proteinogenic Fmoc-amino acids and calculating the loading values versus a previously reported ε value, and versus a freshly prepared standard (single-point method). Clear differences in the loading values between both approaches were observed. Provided that, the loading values derived from a previously established calibration curve were far from those reported by the supplier. We therefore recommend the single-point method.

EXPERIMENTAL PROCEDURES

Loading test

Sample solution preparation

About 10 mg of the dry loaded resin was weighed in a polypropylene syringe preloaded with a filter. Next, 200 µL of the deprotection solution (20% piperidine/DMF) was added, and the sample was allowed to shake for 10 min. The filtrate was then collected in a 25-mL volumetric flask (another 200 µL was added to repeat this step). Finally, the volume was made up to 25 mL with ethanol.

Blank solution preparation

400 µL of the deprotection solution (20% piperidine-DMF) was transferred to a 25-mL volumetric flask, and the volume was made up to 25 mL with EtOH.

Standard solution preparation

About 3 mg of the corresponding/same Fmoc-amino acid being analysed was transferred to a 25-mL volumetric flask. 400 µL of the deprotection solution (20% piperidine-DMF) was added and the volume was made up to 25 mL with ethanol.

Loading calculation

(i) Sample was read versus a blank solution at 301 nm. Loading was calculated using equation (6):

$$L = \frac{\text{sample Abs} \times \text{V}}{\varepsilon \times \text{l} \times \text{m}} \qquad (6)$$

where L is the loading, V is the final volume, m is the loaded resin weight.

(ii) Sample and standard solutions were read versus a blank solution at 301 nm. Loading was calculated using equation (7), derived from equation (5), and then incorporating the corresponding molecular weight term of the Fmoc-amino acid:

$$L = \frac{\text{sample Abs} \times \text{standard mass} \times 1000}{\text{standard Abs} \times \text{loaded resin mass} \times \text{Fmoc} - \text{amino acid m.wt}} \qquad (7)$$

where 1000 is to convert from mol to mmol, and m.wt is the molecular weight.

Calculation of resin capacity

To calculate the amount of linker (native functionality), the weight gain/loss after the incorporation reaction is corrected. Thus, the L value in equation (7) should be corrected, considering the molecular weight of the incorporated amino acid and that of the molecule released after the functionalization. Refer to equation (8):

$$C = \frac{\text{L}}{1 - [\text{L} \times \Delta\text{m.wt}/1000]} \qquad (8)$$

where C is the resin capacity, 1 is 1 g of the resin, Δm. wt is the difference between the molecular weight of the amino acid and the molecule released (e.g. for CTC resin, the molecule released is HCl, and for Wang or aminomethyl resins it is H_2O).

ASSOCIATED CONTENT

Supporting Information

Stability of analytical solution. Experimental details.

AUTHOR INFORMATION

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Author Contributions

The strategy was designed by the all authors, the experiments were carried out mainly by OAM, and all authors discussed the results and prepared the manuscript.

AB is in the employment of Purolite, a supplier of polystyrene-based resins.

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References

1. O. Al Musaimi, D. Al Shaer, B. G. de la Torre, F. Albericio, 2017 FDA Peptide Harvest. *Pharmaceuticals (Basel).* **2018**, *11*, 10.3390/ph11020042.
2. D. Al Shaer, O. Al Musaimi, F. Albericio, B. G. d. l. Torre, 2018 FDA Tides Harvest. *Pharmaceuticals (Basel).* **2019**, *12*, 1-6, 10.3390/ph12020052.
3. R. B. Merrifield, Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide'. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154, 10.1021/ja00897a025.
4. D. M. M. Jaradat, Thirteen decades of peptide synthesis: key developments in solid phase peptide synthesis and amide bond formation utilized in peptide ligation. *Amino Acids.* **2018**, *50*, 39-68, 10.1007/s00726-017-2516-0.
5. R. Behrendt, P. White, J. Offer, Advances in Fmoc solid-phase peptide synthesis. *J. Pept. Sci.* **2016**, *22*, 4-27, 10.1002/psc.2836.

6. A. J. Mijalis, D. A. Thomas, 3rd, M. D. Simon, A. Adamo, R. Beaumont, K. F. Jensen, B. L. Pentelute, A fully automated flow-based approach for accelerated peptide synthesis. *Nat. Chem. Biol.* **2017**, *13*, 464-466, 10.1038/nchembio.2318.
7. A. A. Zompra, A. S. Galanis, O. Werbitzky, F. Albericio, Manufacturing peptides as active pharmaceutical ingredients. *Future Med. Chem.* **2009**, *1*, 361-377, 10.4155/fmc.09.23.
8. J. Vázquez, G. Qushair, F. Albericio, Qualitative colorimetric tests for solid phase synthesis. *Methods Enzymol.* **2003**, *369*, 21-35, 10.1016/S0076-6879(03)69002-6.
9. O. Al Musaimi, Y. E. Jad, A. Kumar, A. El-Faham, J. M. Collins, A. Basso, B. G. d. l. Torre, F. Albericio, Greening the Solid-Phase Peptide Synthesis Process. 2-MeTHF for the Incorporation of the First Amino Acid and Precipitation of Peptides after Global Deprotection. *Org. Process Res. Dev.* **2018**, *22,* 1809-1816, 10.1021/acs.oprd.8b00335.
10. C. L. Krumdieck, C. M. Baugh, The solid-phase synthesis of polyglutamates of folic acid. *Biochemistry.* **1969**, *8*, 1568-72
11. E. Giralt, F. Albericio, Free amine content of benzhydrylamine resins used as solid supports for peptide synthesis. *Anales de Quimica, Serie C: Quimica Organica y Bioquimica.* **1981**, *77*, 134-137
12. R. Hany, D. Rentsch, B. Dhanapal, D. Obrecht, Quantitative Determination of Resin Loading in Solid-Phase Organic Synthesis Using 13C MAS NMR. *J. Comb. Chem.* **2001**, *3*, 85-89, 10.1021/cc000066q.
13. B. F. Gisin, The monitoring of reactions in solid-phase peptide synthesis with picric acid. *Anal. Chim. Acta.* **1972**, *58*, 248-249, 10.1016/S0003-2670(00)86882-8.
14. L. C. Dorman, A non-destructive method for the determination of completeness of coupling reactions in solid phase peptide synthesis. *Tetrahedron Lett.* **1969**, *10*, 2319-2321, 10.1016/S0040-4039(01)88152-1.
15. K. Esko, S. Karlsson, J. Porath, A Method for Determining Free Amino Groups in Polymers with Particular Reference to the Merrifield Synthesis. *Acta. Chem. Scand.* **1968**, *22*, 3342-3344, 10.3891/acta.chem.scand.22-3342.
16. V. K.Sarin, S. B.H.Kent, J. P.Tam, R.B.Merrifield, Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* **1981**, *117*, 147-157, 10.1016/0003-2697(81)90704-1.
17. O. F. Luna, J. Gomez, C. Cardenas, F. Albericio, S. H. Marshall, F. Guzman, Deprotection Reagents in Fmoc Solid Phase Peptide Synthesis: Moving Away from Piperidine? *Molecules.* **2016**, *21*, 10.3390/molecules21111542.
18. S. Eissler, M. Kley, D. Bächle, G. Loidl, T. Meiera, D. Samson, Substitution determination of Fmoc-substituted resins at different wavelengths. *J. Pept. Sci.* **2017**, *23*, 757-762, 10.1002/psc.3021.
19. W. S. Newcomb, T. L. Deegan, W. Miller, J. A. P. Jr, Analysis of 9-Fluorenylmethoxycarbonyl (FMOC) Loading of Solid-Phase Synthesis Resins by Gas Chromatography. *Analytical.* **1998**, *61*, 55-60, 10.1002/(SICI)1097-0290(199824)61:1<55::AID-BIT10>3.0.CO;2-8.
20. F. Weygand, R. Obermeier, Messung des Umsatzes bei der FestkörperPeptidsynthese mit H ilfe einer massenspektrometrischen Verdünnungsanalyse. *Z. Naturforsch. B.* **1968**, *23b*, 1390
21. M. Bassedas, F. Albericio, F. López-Calahorra, Quantitative monitoring of carboxyl groups in polymers. *Anal. Chim. Acta.* **1989**, *219*, 161-163, 10.1016/S0003-2670(00)80345-1.
22. M. Gude, J. Ryf, P. D. White, An accurate method for the quantitation of Fmoc-derivatized solid phase supports. *Lett. Pept. Sci.* **2002**, *9*, 203-206, 10.1007/BF02538384.
23. C. F. V. Galeano, Z. J. R. Monroy, J. E. R. Pérez, J. E. G. Castañeda, Efficient Synthesis of Peptides with 4-Methylpiperidine as Fmoc Removal Reagent by Solid Phase Synthesis. *J. Mex. Chem. Soc.* **2014**, *58,* 386-392
24. A. Romieu, T. Bruckdorfer, G. Clave, V. Grandclaude, C. Massif, P. Y. Renard, N-Fmoc-alpha-sulfo-beta-alanine: a versatile building block for the water solubilisation of chromophores

and fluorophores by solid-phase strategy. *Org. Biomol. Chem.* **2011**, *9,* 5337-5342, 10.1039/c1ob05730h.

25. J. Eichler, M. Bienert, A. Stierandova, M. Lebl, Evaluation of cotton as a carrier for solid-phase peptide synthesis. *Pept. Res.* **1991**, *4,* 296-307
26. J. M. Perez, I. Sucholeiki, *Fmoc Test*, in *High-Throughput Synthesis: Principles and Practices*, I. Sucholeiki, Editor. 2001, Marcel Dekker: New York, Basel. p. 31-33.
27. R. Trzcinska, P. Suder, A. Bodzon-Kulakowska, M. Skalska, A. Marcinkowski, J. Kubacki, R. Pedrys, J. Silberring, A. Dworak, B. Trzebicka, Synthesis and characterisation of PEG-peptide surfaces for proteolytic enzyme detection. *Anal. Bioanal. Chem.* **2013**, *405*, 9049-59, 10.1007/s00216-013-7082-z.
28. ICH Harmonised Tripartite Guideline. **2005**
29. B. K. Mukerji, A. K. Bhattacharji, N. R. Dhar, The Variation of the Extinction-Coefficient with Temperature. *J. Phys. Chem.* **1928**, *32*, 1834-1840, 10.1021/j150294a005.
30. F. P. Wieringa, R. A. Bezemer. *Standardizing biological UV-radiometry, an introduction to EN14255-1*. in *IUVA, DafP & DVGW European Conference on UV Radiation*. 2004. Wieringa Abstract 196th PTB Workshop NL 22 09 2004.pdf