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Jordan K. Magtaan
TU Dublin - Tallaght Campus, magtaanj@tcd.ie

Marc Devocelle
RCSI, mdevocelle@rcsi.ie

Fintan Kelleher
Technological University Dublin, fintan.kelleher@tudublin.ie

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Regeneration of Aged DMF for Use in Solid-Phase Peptide Synthesis

Jordan Kevin Magtaan,[a] Marc Devocelle[b] and Fintan Kelleher[a]*

[a] *Molecular Design and Synthesis Group, Centre of Applied Science for Health, Institute of Technology Tallaght, Dublin 24, Ireland*
[b] *Department of Chemistry, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland.*

Corresponding Author: Fintan Kelleher

Address: Department of Science,
Institute of Technology Tallaght,
Tallaght,
Dublin 24,
Ireland.

Phone Number: (+353 1) 404 2869

e-mail address: fintan.kelleher@ittdublin.ie

Abbreviations:

ACN	Acetonitrile
CYR	Cyrene
DBU	1,8-Diazabicyclo(5.4.0)undec-7-ene
DBF	Dibenzofulvene
DIPEA	*N*,*N*-Diisopropylethylamine (Hünig's base)
DMA	Dimethylamine
DMAc	*N*,*N*-Dimethylacetamide
DMF	Dimethylformamide
DNFB	Dinitrofluorobenzene (Sanger's reagent)
EHS	Environmental, health and safety
EtOH	Ethanol
EtOAc	Ethyl acetate
FA	Formic acid
Fmoc	Fluorenylmethyloxycarbonyl
GVL	γ-Valerolactone
HCTU	2-(6-Chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
NFM	*N*-Formylmorpholine
NMP	*N*-Methylpyrrolidinone
RA-MBHA	Rink Amide 4-methylbenzhydrylamine polystyrene
REACH	Registration, evaluation, authorisation and restriction of chemicals
ROUT	Robust regression and Outlier removal
SPPS	Solid-phase peptide synthesis

Abstract

DMF, which is still the most commonly used solvent for Fmoc-SPPS, has the potential for degradation over time on exposure to air (and water vapour) and storage, to give dimethylamine and formic acid impurities. In particular, dimethylamine can lead to unwanted deprotection of the Fmoc group during for example the initial loading of Fmoc amino acids in SPPS, which leads reduced calculated loading values. We have found that treatment of such aged DMF by simple sparging with an inert gas (N_2), or vacuum sonication, can regenerate the DMF in order to restore loading levels back to those found for newer, fresh, DMF samples.

Introduction

Dimethylformamide (DMF) is one of the most commonly used solvents in solid-phase peptide synthesis (SPPS). In recent years, there has been increasing concern over the environmental, health and safety (EHS) profile of DMF, with many solvent selection guides categorising DMF as hazardous.[1–3] Furthermore, DMF is also suspected to possess teratogenic properties and in recent years, has become subjected to regulations such as the EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation along with *N*,*N*-dimethylacetamide (DMAc), and *N*-methylpyrrolidinone (NMP).[4,5] In light of the aforementioned issues, there has been an increase of interest within the peptide science community in the use of greener solvents for SPPS. Albericio, North and Lopez recently reported successful synthesis of peptides using green SPPS strategies and proposed several different possible greener alternatives to DMF for solid-phase synthesis.[6–15] Despite these issues, DMF remains a popular solvent for fluorenylmethyloxycarbonyl-based SPPS (Fmoc-SPPS),[6,16] and is likely to remain a popular solvent in academic settings for the foreseeable future.

It has long been known that DMF is not stable over a long period of time and, in the presence of water, degrades to the secondary amine, dimethylamine (DMA) and formic acid (FA) (Figure 1), though storage under an inert gas has been suggested as a possible way of reducing degradation.[17,18] During synthesis, use of newly manufactured DMF with minimal DMA content to reduce unwanted reactions and storing DMF under an inert atmosphere after every use to mitigate DMA formation, are also recommended.[18] In situations where the regular purchase of newly manufactured DMF is not possible, it may be necessary to treat aged DMF instead to remove any DMA.[19]

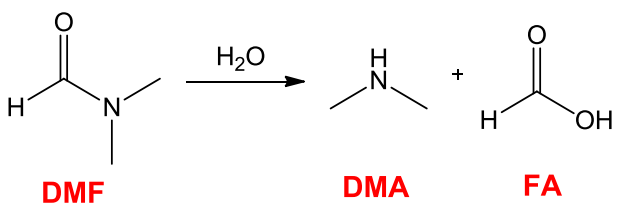

Figure 1: Scheme for the hydrolysis of DMF to DMA and FA.[18,20]

Due to unwanted Fmoc removal, the presence of significant amounts of DMA in DMF can be particularly problematic for SPPS that utilises the base-labile Fmoc protecting group for the temporary protection of the α-amino group.[19,21–24] Some protocols recommend treating DMF to reduce DMA content prior to synthesis, include sparging DMF with an inert gas such as nitrogen.[19]

A rapid colourimetric test involving the use of Sanger's reagent (dinitrofluorobenzene, DNFB) for the detection of DMA in DMF has also been described in the literature. During the DNFB test, a DMF sample suspected of having a significant amount of DMA is mixed with a solution of 1 mg/mL DNFB in 95% EtOH, in a 1:1 ratio and left to stand in the dark for 30 min. The absorbance of the mixture is measured versus a DNFB/95% EtOH solution blank. DMF samples that give absorbance values of less than 0.15 are deemed to be of satisfactory purity.[18,25,26]

The initial resin loading, which is typically reported in mmol per gram of resin ($mmol.g^{-1}$), has been identified as a critical parameter in SPPS.[27] In this technique, the initial anchored unit critically contributes to the final yield of the peptide since it serves as the limiting reagent for all subsequent couplings.[27–29] Following initial loading and during peptide assembly, constant loading or a slight progressive decrease in loading has been described as an indication of good progress of synthesis.[30] After cleavage of the peptide from the solid support, synthetic yields are typically reported based on the loading of the initial anchored unit.[27,28,31–34] Resin loading can be estimated spectrophotometrically with UV-Vis spectroscopy using methods based on DBU/DMF and dibenzofulvene (DBF) generation or piperidine/DMF and DBF-piperidine adduct (λ_{max} ≈ 267 nm, 290 nm, 301 nm) generation.[35–37]

Overloading of resins is known to potentially cause problems during synthesis.[27] Lower loading levels are more commonly employed in SPPS since high loadings have been associated with synthetic failures.[28] In general, high loadings introduce greater steric crowding and as a result, decreased reactive site accessibility within the resin. Overloading has also been attributed to increased likelihood of intermolecular interactions between the growing peptide chains and as a consequence, aggregation and failure of synthesis.[27,28,38]

DMA has been identified to be able to potentially remove Fmoc groups from Fmoc-protected amines.[17,19,39] If aged DMF is used during Fmoc-SPPS, its presence can lead to unwanted Fmoc removal particularly during the initial loading and peptide assembly stages of SPPS.[18] Another consequence of unwanted Fmoc removal during synthesis is a possible decrease in purity of the final peptide product.[19,39]

Materials and Methods

The initial loading was carried out using Fmoc-Gly-OH (≥ 99.9%) from Iris Biotech GmbH in disposable 2 mL MultiSynTech GmbH peptide reactors (Part# V020PE061) and Luer stoppers (Part# V000LS100). HCTU (≥ 99.0%) and Rink Amide-MBHA-PS (RA-MBHA resins, 0.580 $mmol.g^{-1}$ capacity, crosslinking 1% DVB) resins were donated by IPSEN Manufacturing Ireland Ltd. Piperidine (≥ 99.5% AcroSeal®, Lot# A0362067) and DBU (≥ 99.0%, Lot# BCBL8308V) were purchased from Acros Organics and Sigma Aldrich Ireland, respectively. DIPEA (≥ 99%, Lot# STBF0608V) was also purchased from Sigma Aldrich Ireland. UV-Vis spectroscopy studies were carried out using 1 mL volume and 1 cm path length Hellma Analytics Quartz SUPRASIL® cuvettes on a Hitachi U-2900 spectrophotometer and the software used was the UV Solutions software. Vacuum sonication was carried out with a Decon F5100b sonicator and a BUCHI Labortechnik AG V-700 pump. The silica gel (40-63 µm, pH 6.7, Lot# 17D074111) and the basic alumina (50-200 µM, pH 9-10, Lot# A0386697) were purchased from VWR and Acros Organics, respectively. The DNFB (≥ 98%, Lot# A0392098) for the colourimetric DNFB tests was purchased from Acros Organics. Formic acid (>98%, Lot# BCBP4740V) was purchased from Fisher Scientific. Measurements of refractive indices (n^{20}_{D}) were carried out using an Index Instruments Automatic Refractometer model PTR2a at 20 °C, using a sodium yellow light source (589 nm) and set at continuous mode. For statistical analyses and data processing, PRISM 6.0 was used to analyse the results using ANOVA with Tukey-Kramer's test ($\alpha = 0.05$) and to detect possible data outliers ($Q = 1\%$ or $\alpha = 0.05$). [40,41] Values of *p* that are less than 0.05 mean that there is a statistically significant difference, which is also marked by asterisks. The solvents were used before the stated date of expiration and stored in dark safety storage cabinets for flammable organic solvents away from direct sunlight.

Table 1: *Range of solvents used in these studies (other than DMF)*

	Grade and Percentage Purity
Dichloromethane (DCM)	Analytical Grade, ≥ 99.99%
Methanol (MeOH)	≥ 99.5%
Ethanol (EtOH)	≥ 99.8%
γ-Valerolactone (GVL)	Food Chemicals Codex, Food Grade, ≥ 99.0%
***N*-Formylmorpholine (NFM)**	≥ 99%
Cyrene (CYR)	≥ 99%

The age and therefore the date of manufacture of the different DMF samples was central to the study and are mentioned below (Table 2). Manufacture dates were obtained by direct contact with the solvent manufacturer, certificates of analyses or information on the container of the DMF sample. For comparison, different grades of newer DMF samples were also used.

Table 2: *Summary of the DMF used during the studies.*

	Grade and Percentage Purity	**Manufactured On:**	**Opened On:**
Aged DMF	Grade not specified, ≥ 99.5%	Feb 2016	July 2016[‖]
Newer DMF	Reagent grade, ≥ 99.0%	Feb 2017	April 2018[‡]
	HPLC grade, ≥ 99.9%	Feb 2017	June 2018[‡]
	Peptide synthesis grade, ≥ 99.8%	Dec 2017	June 2018[‡]

	Extra dry grade, ≥ 99.8%	May 2018	June 2018‡

‖ = Exposed to air many times after opening the sealed bottle. ‡ = Used immediately after opening the sealed bottle and stored under N_2 after use.

Table 3: *Notation used to describe different DMF samples*

Notation	Description of Treatments of Aged DMF
aN^{I}-N^{II}	Treated aged DMF where N^{I} denotes type of treatment and N^{II} denotes duration of treatment in minutes, where applicable
N^{1}	
1	Passed through basic alumina
2	Passed through silica gel
3	Sparged with inert gas (N_2)
4	Sonicated under vacuum
5	Passed through basic alumina and then silica gel
6	Sparged with inert gas and then sonicated under vacuum
7	Passed through silica gel, sparged with inert gas and then sonicated under vacuum
8	Passed through basic alumina, sparged with inert gas and and then sonicated under vacuum

	Description of the Grades of New DMF
b	New, reagent grade DMF
c	New, HPLC grade DMF
d	New, Peptide synthesis grade DMF
e	New, Anhydrous grade DMF

Example: a3-15 = Aged DMF treated by sparging with nitrogen gas for 15 minutes.

General methods for treating aged DMF (see Table 3)**:**

Treatments 1 & 2: Basic Alumina or Silica method

Basic alumina or silica gel (1.0 – 1.5 g) was placed in a chromatography column. Aged DMF (50 mL) was passed through the basic alumina or silica gel and was collected by gravity in an amber bottle that has been flushed four times with N_2 for 5 sec each time. The aged DMF was passed through basic alumina or silica gel one

more time, using a different column with fresh basic alumina or silica gel, collected once more in an amber bottle that has been flushed with N_2 as described above. **a1** and **a2** were stored under an inert atmosphere and were immediately used for the initial loading experiments.

Treatment 3: Sparging method

Aged DMF (50 mL) was placed in an amber bottle that has been flushed four times with N_2 for 5 sec each. A modified silicone tube fitted with a 3 mL syringe and a Luer needle was connected to a N_2 line and the syringe component of the modified tube was submerged under the aged DMF. The N_2 gas was switched on and the aged DMF was bubbled for 15 or 30 min. **a3-15** and **a3-30** were stored under an inert atmosphere and immediately used for the initial loading experiments.

Treatment 4: Vacuum sonication method

Aged DMF (50 mL) was placed in an amber bottle that has been flushed with N_2 for 5 sec each. The bottle was attached to a vacuum pump via an adaptor and the bottle was placed in a sonicator. The vacuum pump and the sonicator were switched on simultaneously and the solvent was vacuum sonicated for 15 or 30 min. **a4-15** and **a4-30** were stored under an inert atmosphere and were immediately used for the initial loading experiment.

Treatments 5-8: Combination of treatments

Combinations of the treatments were also carried out to regenerate aged DMF as described above.

General method for the Initial Loading of Fmoc-Gly-OH onto RA-MBHA Resins:

In a 2 mL disposable MultiSynTech manual peptide synthesis reactor, Rink Amide-MBHA-PS resin (50 mg, 0.580 $mmol.g^{-1}$ capacity) was added. The resin was swollen in DCM (1 mL) and agitated for 15 min. The resin-bound Fmoc groups were removed using 96:2:2 DMF:DBU:Piperidine (0.5 mL) with agitation for 15 min (new reagent-grade DMF (≥ 99.0%) was used).[42] The resin was washed with the solvent† (1 mL) and then washed 8 more times (two alternating washes of 2 x 1 mL solvent† and 2 x 1 mL MeOH). Fmoc removal was monitored visually using the qualitative Kaiser test in test tubes.[43] The resins were washed with DCM (2 x 0.5 mL) and then

suspended in DCM (1 mL) with agitation for 15 min. Fmoc-Gly-OH (3 equiv.) and HCTU (3 equiv.) dissolved in the solvent† (0.4 mL) was added to the reactor followed by agitation for 5 min. In the case of greener solvents, sonication for up to 20-25 minutes was required to form a solution. DIPEA (6 equiv.) dissolved in the solvent† (0.1 mL) was added to the reactor followed by agitation for 40 min. The coupling reaction was carried out at room temperature. The resin was rewashed with the solvent† (1 mL) and then washed 8 more times (two alternating washes of 2 x 1 mL solvent† and 2 x 1 mL MeOH). The resin was washed 2 more times with MeOH (1 mL) and the excess MeOH was removed by attaching the reactor to a 5 mL syringe via a luer-lok connector and pulling the plunger of the second syringe to the 5 mL mark. The resin was dried overnight *in vacuo* at room temperature in preparation for the spectrophotometric estimation of initial resin loading.

† = Aged DMF, treated DMF, new DMF, GVL, NFM and CYR.

General method for the spectrophotometric estimation of resin loading: [37,44,45]

Dry resin was placed carefully into a preweighed 2 mL microtube and the weight of the dry resin was determined by difference (6-15 mg). New reagent grade (≥ 99.0%) DMF was used during the spectrophotometric estimation of loading. DMF (0.8 mL) was added to the microtube to swell the resin. The microtube was vortexed for 5 sec and then placed on a microtube rack which was placed on a gyratory rocker. The microtube rack was gently agitated at 70 rpm for 15 min on the gyratory rocker at room temperature. The microtube was vortexed once more for 5 sec and then piperidine (0.2 mL) was added to generate the DBF-piperidine adduct. The microtube was vortexed twice for 10 sec to ensure good mixing, placed back on a microtube rack which was placed on a gyratory rocker again and gently agitated as previously. The resin was vortexed again for 10 sec, allowed to settle and three 100-fold dilutions of the supernatant were prepared in 2 mL microtubes, using DMF as the diluent. $A_{290\ nm}$ was obtained (triplicate, n = 3) for each of the three supernatant dilutions versus a DMF blank in 1 mL quartz cuvettes (1 cm path length). For one of the 1:100 dilutions, loading (L, in $mmol.g^{-1}$) was calculated for each replicate measurement of $A_{290\ nm}$, using the formula in Eqn. 1 and the mean L was determined. The calculations for L and mean L were repeated for the two remaining 1:100 dilutions to obtain a total of 3 mean L values. Spectrophotometric estimation of

resin loading was performed two more times, as described above, to obtain a total of nine (nonuplicate, n = 9) mean loading values. The grand mean was calculated and taken as the initial resin loading in mmol.g^{-1}.

$$Loading\ (mmol.g^{-1}) = \frac{A_{290\ nm} \times D \times V}{\varepsilon_{290\ nm} \times m \times \Lambda} \quad \text{Eqn 1}$$

Where $A_{290\ nm}$ = absorbance at 290 nm, D = dilution factor (100), V = volume (1 mL), $\varepsilon_{290\ nm}$ = molar absorptivity at 290 nm expressed in mL.mmol^{-1}.cm^{-1} (6089 mL.mmol^{-1}.cm^{-1}), m = weight of resins (g) and Λ = path length (1 cm)

Results and Discussion

Comparison of initial resin loading values

To the best of our knowledge, there have not been any comparative studies in the literature on which method of treating aged and partially degraded DMF is the most efficient at DMA removal and consequently, improved resin loading. As outlined earlier, initial capacity has been identified as a critical parameter in SPPS since the loading level of the first anchored amino acid unit dictates the maximum possible yield of the peptide.[27–29] In the following study, we used the loading of the simplest Fmoc-amino acid, Fmoc-Gly-OH onto a solid support to probe the effect of using aged DMF treated by different methods for the initial anchoring. We calculated the initial resin loading of Fmoc-Gly-OH on Rink Amide-MBHA-PS resins (0.580 mmol.g^{-1} capacity, 1% crosslinking) by spectrophotometric methods. Fmoc-Gly-OH was anchored onto the solid-support using aged DMF during the washing steps and the highly important coupling step and compared with the loading using treated DMF (**a1** to **a8**) and new DMF samples of different grades (**b** to **e**).

Simple methods of regenerating aged DMF were chosen for rapidity and convenience and to reduce costs. For this reason, distillation of aged DMF was not performed.[46] Aged DMF was treated using simple methods such as passing through silica gel to scavenge DMA (**a2**). As mentioned in the introductory section, sparging aged DMF with an inert gas has been recommended in the literature for treating partially degraded DMF. We also chose sparging with N_2 (**a3**) as a method of treatment. To investigate whether the presence of FA in aged DMF, which may lead to unwanted formylation reactions or premature resin cleavage, could affect the

loading measurements, aged DMF was also treated by passing through basic alumina to scavenge FA (**a1**) but not DMA. Sonication under reduced pressure is a common technique for degassing solvents for use in HPLC.[47] Aside from sparging, we also attempted sonication under reduced pressure as a possible way of removing dissolved DMA gas in aged DMF (**a4**). Combinations of treatments (**a5** to **a8**) were also performed. Like the new DMF samples used in this experiment, aged DMF was noted to be clear and colourless but had a potent fishy smell compared to the new DMF samples.

DCM, which is a known high-swelling solvent for PS-based resins, was used for the swelling steps.[48] For the removal of the resin-bound Fmoc group of RA-MBHA resins before the attachment of Fmoc-Gly-OH, 96:2:2 DMF:DBU:Piperidine was the chosen solution for deprotection.[42] With this method of deprotection, we found an average of 89% Fmoc removal, associated with qualitative Kaiser test results (a deep purple-ultramarine colour) (n = 9). The initial loading strategy chosen involved the stand-alone coupling reagent 2-(6-Chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and Hünig's base (*N*,*N*-diisopropylethylamine, DIPEA).[49]

Photometry-based estimations of resin loading involving the generation of the DBF-piperidine adduct from the resin-bound Fmoc group is well-documented in the literature; it is a standard and one of the most common methods of evaluating resin loading.[50,51] The DBF-piperidine adduct has absorption maxima at around 267 nm, 290 nm, and 301 nm, with spectrophotometric determinations of resin loading generally obtained at around 290 nm and 301 nm. In the study presented, determinations were performed at 290 nm, because substitution determination at this wavelength has been recently described by Eissler *et al* as more reliable.[37,44] The results of the comparative loading experiments are shown in Table 4 and Figure 2. Most loading measurements were found to be within the common loading levels of 0.3–0.5 mmol.g^{-1} and loading percentages of 50-70%, which are deemed as acceptable loading levels.[28,52]

From a data analysis perspective, we had to address the issue of large spread observed during some experiments. For the sets of data, we found that the spread, in terms of the coefficient of variation (CV (%)), ranged between 1.8% and 18.5%.

Possible data outlier detection was performed using the robust regression and outlier removal (ROUT) method (Q = 1%) and Grubbs' test (α = 0.05).[53] In all cases, no significant outliers were detected using both methods, despite the relatively large spread observed in some cases.[54]

Table 4: *Mean (n = 9) initial resin loading (mmol.g^{-1}) values for the loading of Fmoc-Gly-OH onto Rink Amide-MBHA-PS resins (0.580 mmol.g^{-1} capacity) when aged DMF was used during amino acid attachment versus DMF^{b} to DMF^{e} and DMF^{a1} to DMF^{a8}. ± denote 95% confidence intervals (CI) and sample standard deviations (s) are in brackets. Percent coefficient of variation = CV (%). Descriptive statistical (DS) analysis was performed using the PRISM 6.0 software.*

	Fmoc-Gly-OH Resin Loading (mmol.g^{-1})	**CV (%)**
Aged DMF	0.238 ± 0.034 (0.044)	18.5
a1	0.267 ± 0.015 (0.020)	7.4
a2	0.325 ± 0.010 (0.014)	4.2
a3-15	0.297 ± 0.015 (0.019)	6.5
a3-30	0.352 ± 0.009 (0.011)	3.1
a4-15	0.309 ± 0.021 (0.027)	8.7
a4-30	0.362 ± 0.013 (0.016)	4.5
a5	0.321 ± 0.013 (0.017)	5.2
a6-30	0.363 ± 0.009 (0.012)	3.2
a7-30	0.376 ± 0.011 (0.015)	4.0
a8-30	0.366 ± 0.009 (0.012)	3.3
b	0.369 ± 0.010 (0.013)	3.5
c	0.361 ± 0.018 (0.023)	6.3
d	0.355 ± 0.010 (0.013)	3.8
e	0.356 ± 0.005 (0.007)	1.8

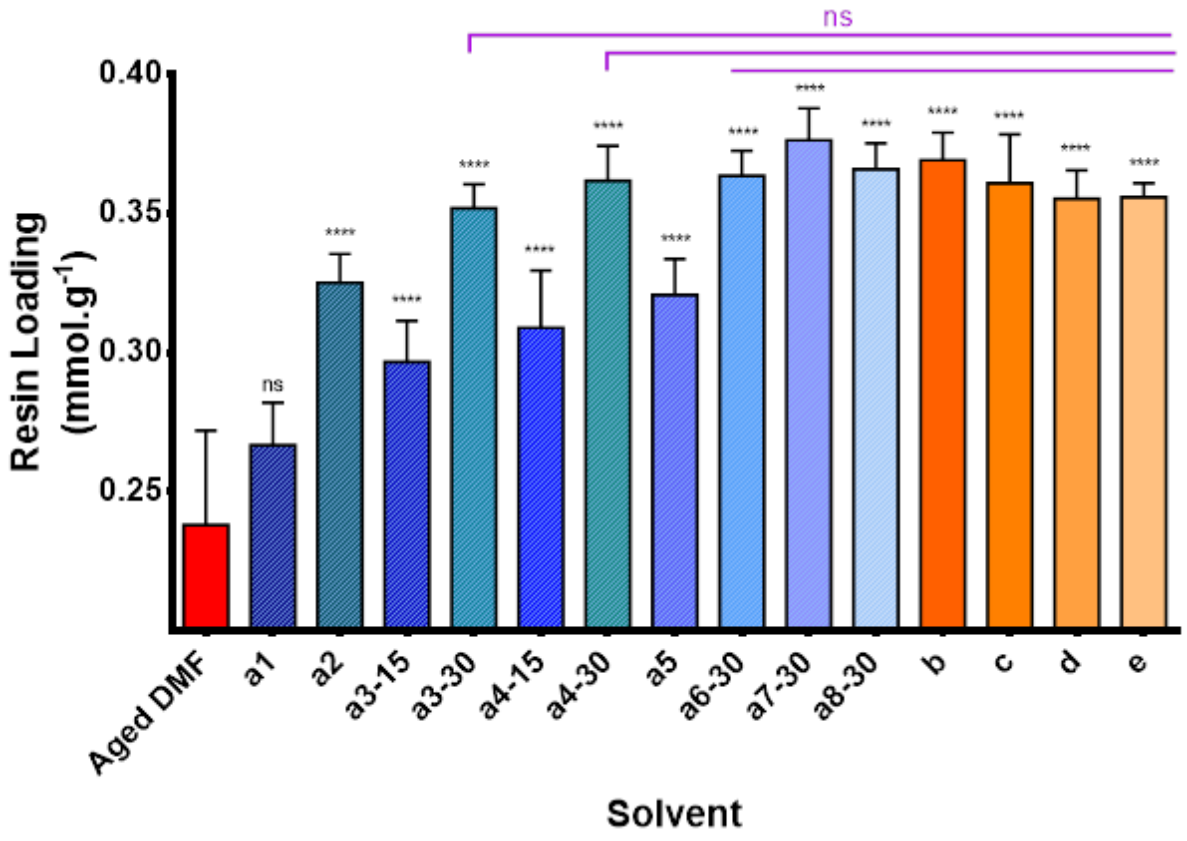

Figure 2: *Comparison of the loading in mmol.g^{-1} (n = 9) of Fmoc-Gly-OH onto Rink Amide-MBHA-PS resins (0.580 mmol.g^{-1} capacity) in aged DMF, treated aged DMF and new DMF samples. Loading estimations were based on DBF-piperidine adduct formation during the removal of resin-bound Fmoc groups. Error bars denote 95% CI. ns denotes p > 0.05 (not significant) and **** denotes p < 0.0001 (extremely significant) relative to aged DMF. p-values were calculated by univariate analysis (UVA) with a one-way analysis of variance (ANOVA) with Tukey-Kramer's test (post hoc) using the PRISM 6.0 software, where α = 0.05. The purple horizontal lines indicate no statistically significant difference between a3-30, a4-30, a6-30, a7-30, a8-30, b, c, d and e.*

As outlined earlier, it has been well-documented in the literature that DMF degrades to DMA and FA via hydrolysis and the presence of traces of DMA can result in Fmoc-amino deprotections.[17–19] As shown above, there is strong statistical evidence that there is a significant difference in mean initial resin loading values due to the solvent used during the attachment of Fmoc-Gly-OH onto the resin. The measured resin loading when aged DMF was used during the attachment of Fmoc-Gly-OH was significantly lower ($p \leq 0.0001$) than when new DMF samples (**b** to **e**) were used (Table 4 and Figure 2). Interestingly, we also noted that the spectrophotometrically measured initial resin loading values, when newer DMF samples of different grades were used during the attachment to the resin, were not

significantly different to each other, despite some being older than the others or opened on slightly different dates (ns, Tukey-Kramer test). We also found that vortexing Fmoc-Gly-OH in an aged DMF sample even for just 5 min resulted in a small and significant (unpaired t-test, $p < 0.05$) decrease in Fmoc-Gly-OH (%), by UPLC and based on peak areas, compared to vortexing in **d,** which averaged at 2.1% (n = 5).

When using treated DMF, the measured values improved, with the exception of basic alumina-treated aged DMF (**a1**). The mean loading when **a1** was used was higher than when aged DMF was used. However, our results indicate that the scavenging of FA did not contribute to a statistically significant improvement in loading value. Using aged DMF treated for just 15 minutes resulted in a significant ($p \leq 0.0001$) improvement in the measured resin loading. The initial loading of Fmoc-Gly-Rink-MBHA-PS resins using DMF with longer treatment times was also calculated. When treatment times were extended from 15 minutes to 30 minutes, we observed an even greater improvement in loading levels, which is similar to that of newer DMF samples, as reflected by the mean loading values (ns, Tukey-Kramer test). We found that the loading values when using sparged aged DMF (**a3-30**) and sonicated under vacuum (**a4-30**), each for 30 min, did not significantly differ from each other and to newer DMF (**b** to **e**). However, it is important to mention that DMF is also known to be subjected to thermal degradation to DMA and carbon monoxide and since sonication can heat samples, care must be taken when choosing this method of DMF treatment.[20]

We also noted that the resin loading values obtained when aged DMF samples, that underwent a combination of treatments (**a5** to **a8**), were used during the attachment of Fmoc-Gly-OH, did not significantly differ to aged DMF that had been sparged with N_2 (**a3-30**) or vacuum sonicated (**a4-30**) for 30 min. As such, we deem it unnecessary to carry out combinations of treatments and that sparging with an inert gas or vacuum sonication would suffice. However, it is important to note that the duration of the treatment time would likely vary and be highly dependent on the age, quality and quantity of the DMF sample.[18]

As described in the introduction, there has been a surge in interest in implementing green chemistry principles on SPPS in recent years, now termed greener solid-

phase peptide synthesis (GSPPS).[9] In line with the 5th principle of green chemistry and for comparison, we also attached Fmoc-Gly-OH onto RA-MBHA resins using greener solvents. Greener solvents chosen in this part of the study include *N*-formylmorpholine (NFM), cyrene (CYR) and γ-valerolactone (GVL). Both NFM and GVL have been proposed as alternatives to DMF for GSPPS by Albericio *et al*.[6] CYR is a bioderived solvent that has been described as a green dipolar aprotic solvent by Clark *et al*.[55] Since alternative solvents to DMF are used, unwanted Fmoc removal by DMA is avoided. However, the ring-opening of GVL by amines is of concern. Studies on ring opening of GVL in a SPPS context under certain stress conditions has been recently reported by Albericio *et al*.[14] Despite this, successful GSPPS of "difficult" peptides using GVL has also been reported, also by Albericio *et al*.[6]

Table 5: *Mean (n = 9) initial resin loading (mmol.g^{-1}) values for the loading of Fmoc-Gly-OH onto Rink Amide-MBHA-PS resins (0.580 mmol.g^{-1} capacity) when aged DMF was used during amino acid attachment versus greener solvent systems. ± denote 95% confidence intervals (CI) and sample standard deviations (s) are in brackets. Percent coefficient of variation = CV (%). Descriptive statistical (DS) analysis was performed using the PRISM 6.0 software. Data for* ***d*** *from Table 4 is also shown for comparison.*

	Fmoc-Gly-OH Resin Loading (mmol.g^{-1})	**CV (%)**
Aged DMF	0.238 ± 0.034 (0.044)	18.5
GVL	0.327 ± 0.017 (0.022)	6.7
NFM	0.325 ± 0.005 (0.006)	1.8
CYR	0.234 ± 0.031 (0.040)	17.0
d	0.355 ± 0.010 (0.013)	3.8

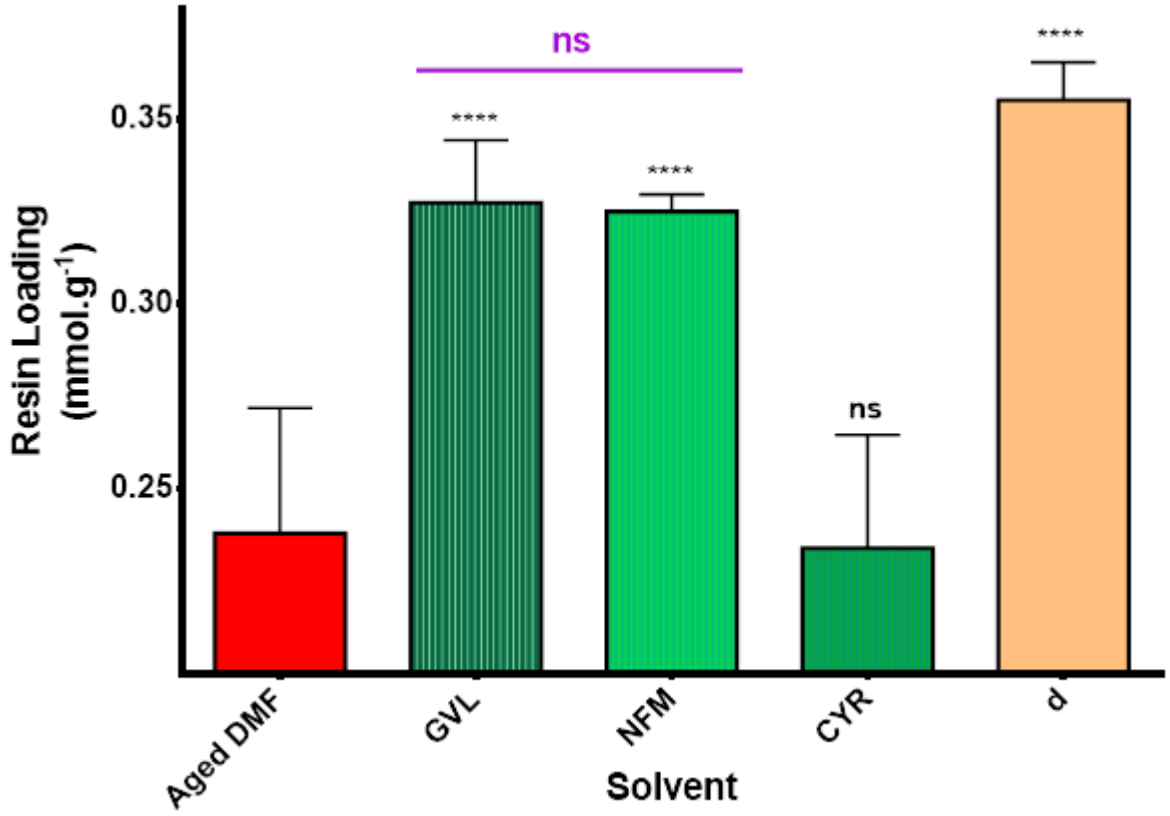

Figure 3: *Comparison of the loading in mmol.g^{-1} (n = 9) of Fmoc-Gly-OH onto Rink Amide-MBHA-PS resins (0.580 mmol.g^{-1} capacity) in aged DMF, and greener solvents. Loading estimations were based on DBF-piperidine adduct formation during the removal of resin-bound Fmoc groups. Error bars denote 95% CI. ns denotes p > 0.05 (not significant), ** denotes p = 0.001 to 0.01 (very significant) and **** denotes p < 0.0001 (extremely significant) relative to aged DMF. p-values were calculated by UVA with a one-way ANOVA with Tukey-Kramer's test (post hoc) using the PRISM 6.0 software, where α = 0.05. Data for* ***d*** *from Figure 3 is also shown for comparison.*

As shown in Table 5 and Figure 3, we observed that compared to aged DMF , the initial loading of Fmoc-Gly-OH onto the RA-MBHA resin is significantly higher (p < 0.05) when greener solvent systems were used, with the exception of CYR. However, in comparison to the greener solvents, mean initial loading values were higher when newer DMF samples were used. These greener solvent systems also required an additional sonication step to dissolve HCTU.

Testing the Quality of Aged DMF and Treated Aged DMF

Two simple methods were selected for evaluating the quality of the DMF samples, namely the DNFB test and refractometry. The DNFB test has been described in the literature as a rapid means of assessing the quality of DMF and detecting the presence of dissolved DMA gas.[18,26,56] DMA content of aged and treated DMF was assessed using the colourimetric DNFB test accordingly.[18] The absorption maximum of the chromophore was determined to be 452 nm in our laboratory. $A_{452\ nm}$ was obtained (quintuplicate, n = 5) for the aged and treated DMF samples and the mean of the absorbance values was used to assess whether these DMF samples pass the DNFB test or not. In all cases, all of the treated aged DMF samples passed the DNFB test, signifying that the DMA content is low. Surprisingly, even the aged DMF sample also passed the DNFB test. It indicated that the DMA content of the latter samples were at an acceptable concentration. Although aged DMF also passed the DNFB test, it was noted that the mean absorbance value was highest in aged DMF, indicating greater quantities of DMA compared to treated DMF ($A_{\text{Aged DMF}} > A_{\text{Treated DMF}}$). This is consistent with the lower values of the loading achieved when aged DMF was used during the attachment of Fmoc-Gly-OH.

To further test the quality of the aged DMF and treated DMF samples, we measured their refractive indices (RI, n^{20}_D) (septuplicate, n = 7) and compared the n^{20}_D values with the values obtained for newer DMF samples. Refractometry is an established technique for the rapid assessment of the quality and purity of liquid organic samples, since impurities can lead to RI values different to that of a purer sample. Compared to other methods such as GC, refractometry is a rapid and generally inexpensive test for gaining an insight on the purity of a liquid sample.[57] Nevertheless, its disadvantage as an analytical technique is that it cannot distinguish between the different impurities that may be present in a sample. As previously mentioned, FA has been described in the literature to form alongside DMA during the degradation of DMF. Solutions of FA in reagent grade (≥ 99.0%) DMF (**b**) over a broad concentration range were prepared to assess the impact of concentration of FA on the RI of DMF. For reference, the RI of reagent grade DMF (**b**) was also obtained. Since DMA is a gas, we deemed it impractical to attempt to make solutions of DMA in DMF.

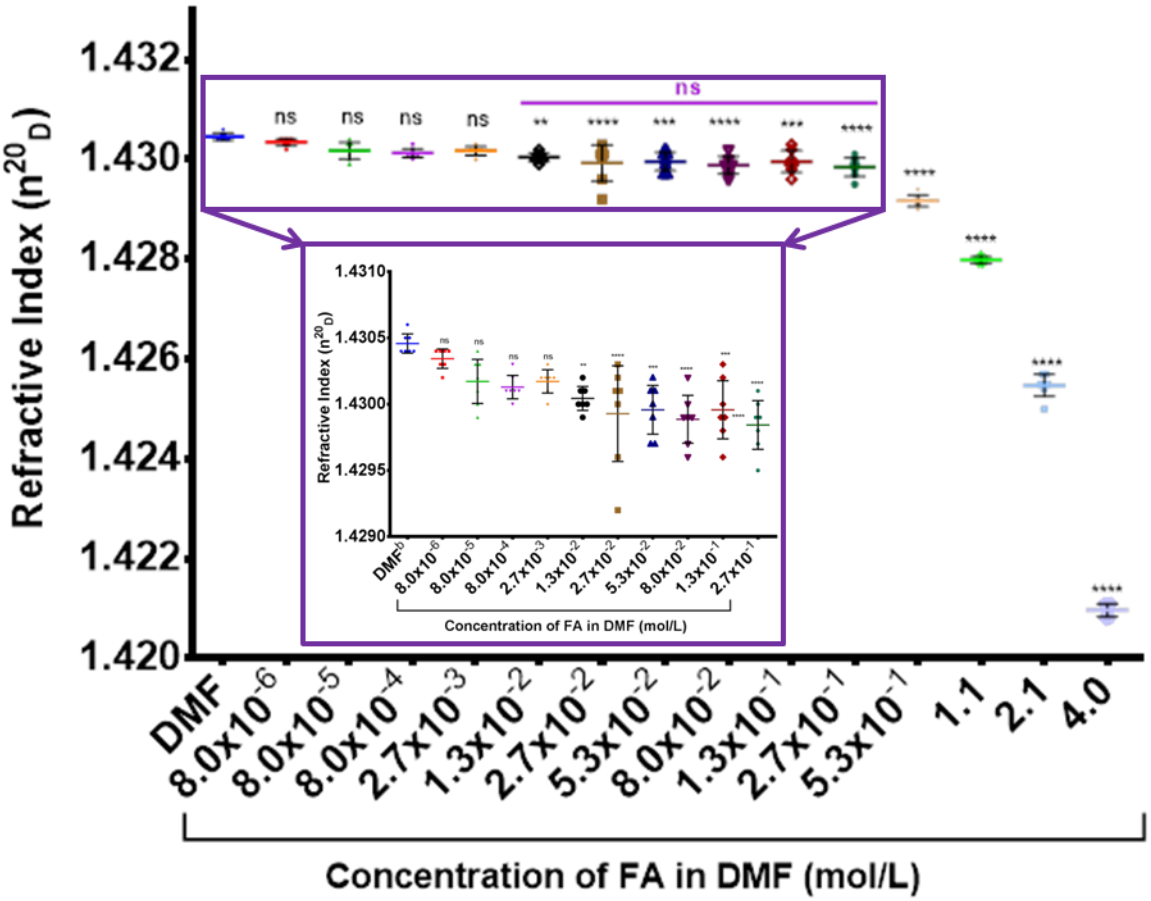

Figure 4: *Interval plot of the effect of increasing the concentration FA in reagent grade (≥ 99.0%) DMF (**b**) on the refractive index. n = 7. Error bars denote 95% CI. ns denotes p > 0.05 (not significant), ** denotes p = 0.001 to 0.01 (very significant), *** denotes p = 0.0001 to 0.001 (extremely significant) and **** denotes p < 0.0001 (extremely significant) relative to reagent grade DMF (**b**). p-values were calculated by UVA with a one-way ANOVA with Tukey-Kramer's test (post hoc) using the PRISM 6.0 software, where α = 0.05. The RI values for DMF^b and the FA/DMF solutions up to 2.7x10^{-1} M have been magnified.*

At very low concentrations of FA in DMF, we found that the mean RI values were slightly lower than pure **b**, but we found that the difference was not statistically significant (ns, Tukey-Kramer test, Figure 4). However, from 1.3x10^{-2} mol/L, we began to observe a statistically significant decrease in RI values as the concentration of FA increased. Between 1.3x10^{-2} M and 2.7x10^{-1} M, the RI values were not significantly different to each other, but were statistically significantly different to the RI of newer reagent grade DMF. After establishing that FA impurities can lead to a statistically significant decrease in the RI of pure DMF, and thus may be useful in evaluating the purity of DMF samples, we measured the RI of all our DMF samples.

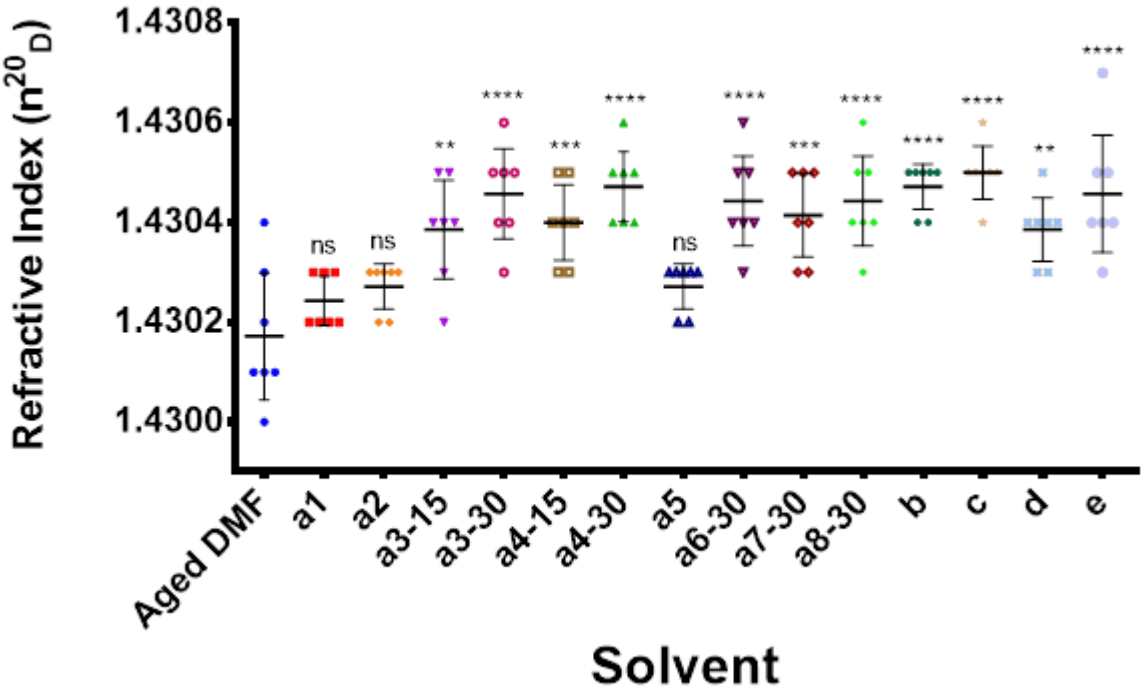

Figure 5: *Interval plot of the RI values for the different DMF samples. n = 7. Error bars denote 95% CI. ns denotes p > 0.05 (not significant), ** denotes p = 0.001 to 0.01 (very significant), *** denotes p = 0.0001 to 0.001 (extremely significant) and **** denotes p < 0.0001 (extremely significant) relative to reagent grade DMF (**b**). p-values were calculated by UVA, with a one-way ANOVA with Tukey-Kramer's test (post hoc) using the PRISM 6.0 software, where α = 0.05.*

We observed that the RI value of our aged DMF was slightly, but significantly ($p < 0.05$), different to the RI values of the newer DMF samples (Figure 5). The lowered RI value of the aged DMF compared to newer DMF samples is likely due to degradation impurities. The low magnitude of the difference in RI signifies that the quantity of impurities in aged DMF is low, which is consistent with what we found using the DNFB test. In most cases, the difference in RI values between aged and new DMF samples were not statistically significant, though there were some exceptions. Interestingly, even though we observed a significant improvement in initial resin loading when **a2** and **a5** were used during the attachment of Fmoc-Gly-OH onto the resin, the RI values of the **a2** and **a5** treated samples were not significantly different to the RI value of aged DMF (ns, Tukey-Kramer test). The RI value for **a1** was also not significantly different to that of aged DMF.

In summary, the analytical studies (DNFB test, statistical analysis and RI measurements) indicate that the quantity of degradation impurities in our aged DMF

sample is low, but has contributed to a significant decrease in the measured initial resin loading, when using the spectrophotometric method based on DBF-piperidine adduct generation for estimating loading. We found sparging with N_2 and sonication under vacuum to be effective in improving the measured loading values.

Conclusions

We emphasised the importance of the initial loading step in SPPS and the impact of DMF degradation to DMA on Fmoc-SPPS. The spectrophotometrical method of estimating resin loading based on the quantification of the DBF-piperidine adduct is one of the most common and standard methods of assessing loading efficiencies. Our results show that the use of aged DMF for the initial loading step of Fmoc-SPPS can lead to lower calculated initial resin loading values. Loading significantly improved when aged DMF that has been regenerated, by simple means of treatment such as sparging with an inert gas and sonication under vacuum, was used for attaching Fmoc-Gly-OH onto the resin. It is also highly likely that treatment times would vary depending on the volume, age and quality of the DMF sample being used.

Treating aged DMF may be sufficient to reduce the quantity of degradation impurities and hence improve the resin loading values. Where possible, the use of newer DMF samples is also strongly recommended when carrying out Fmoc-SPPS. Despite passing the DNFB test, the mean loading value when aged DMF was used was comparably lower versus newer DMF samples. During our investigation, analytical studies indicated that the quantity of impurities in our aged DMF sample was low, but sufficient enough to lead to less accurate and a significantly lower initial resin loading value.

Alternatively, one may use one of the alternative greener solvents in the published literature, such as GVL and NFM, proposed instead of DMF to totally avoid the issues associated with DMA. However, along with the lower loading levels obtained, the use of greener solvents also required an additional sonication step to dissolve the coupling reagent HCTU.

Although the study of the initial loading step is critically important, the use of DMF for the further Fmoc amino acid coupling steps is of no less importance. As such studies

are underway on examining the effects of aged, treated and newer DMF samples when used in a full SPPS protocol. The results of these studies will be reported in due course.

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Conflict of interest:

The authors declare no conflict of interest.

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