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# Quantitative Assessment of Preloaded 4-Alkoxybenzyl Alcohol Resins for Solid-Phase Peptide Syntheses by 1D and 2D HR-MAS NMR

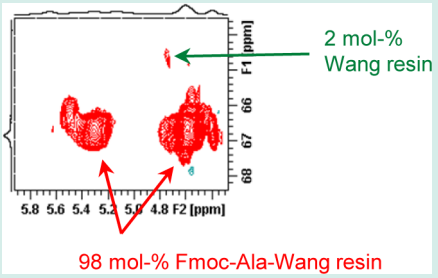
Daniel Rentsch,*[,†] Christian Stähelin,[‡] Markus Obkircher,[‡] Roland Hany,[†] Marina Simeunovic,[†] Daniel Samson,[‡] Günther Loidl,[‡] and Fritz Dick[‡]

[†]Swiss Federal Laboratories for Materials Science and Technology (Empa), Laboratory for Functional Polymers, Überlandstrasse 129, CH-8600 Dübendorf, Switzerland

[‡]Bachem AG, Hauptstrasse 144, CH-4416 Bubendorf, Switzerland

Supporting Information

**ABSTRACT:** The quality of preloaded Wang resins is very important for the success of solid-phase peptide syntheses (SPPS). A critical factor is the capping of remaining hydroxyl groups after loading with the first amino acid, since these free alcohols lead to truncated sequences during the following SPPS steps. Because the detection of hydroxyl groups by color tests is difficult and unreliable, the capping efficiency is often controlled by time-consuming peptide test syntheses. Here, we describe a two-dimensional, high resolution magic angle spinning NMR method for the quantitative determination of remaining 4-alkoxybenzyl alcohols in Fmoc-Xaa-Wang resins with a detection limit of 1 mol-%. The NMR method was validated with samples of known ratios between Fmoc-Ala-Wang and 4-alkoxybenzylalcohol resin. Application to a set of preloaded Fmoc-Ala- and Fmoc-Thr(tBu)-Wang test resins demonstrated that the full range of essential amino acids can be quantified without further spectrometer calibration. Compared to established test synthesis protocols, the NMR method represents not only advantages in terms of time and cost savings but also eliminates all inaccuracies due to further sample treatment like SPPS and cleavage from the resin.




**KEYWORDS:** *4-alkoxybenzyl alcohol resin, Bz-Wang resin, Fmoc-Ala-Wang, solid-phase, peptide synthesis, HSQC, HR-MAS NMR, SPPS, quantitative*

## INTRODUCTION

One important requirement for the preparation of high purity peptides by SPPS in good yields is the use of top grade starting materials. Compared to the state-of-the-art quality control of amino acid derivatives, solvents and reagents, the quality assessment of resins is a more challenging task, since the range of analytical tools is restricted because of the insolubility of resins such as the family of preloaded Wang resins (also known as 4-alkoxybenzyl alcohol resins).[1−4] Three main factors are believed to play an important role in the performance of preloaded Fmoc-Xaa-Wang resin during subsequent SPPS. Chain abortion after a certain number of amino acids in a growing peptide can occur due to the extent and quality of cross-linking in the polystyrene matrix or by too high loading of the starting resin leading to steric hindrance at the coupling positions. The amino acid loading can be determined with sufficient accuracy by UV detection of the Fmoc-chromophore after deprotection by piperidine/DMF,[5,6] and the cross-linking can be estimated by the swelling properties of the resin.

The third crucial parameter is the presence of free 4-alkoxybenzyl alcohol resin due to incomplete end-capping of substoichiometrically loaded Fmoc-Xaa-Wang resin. This can lead to truncated sequences in the first steps of subsequent amino acid coupling reactions. From our experience we know that the capping reaction does not always work properly and the development of an independent testing method is highly important. Contrary to literature reports,[7−10] we found that color tests are unreliable for the quantification of hydroxyl functionalities on the resins, and IR methods suffer from the relatively high lower limit of detection of 0.05 mmol/g for $CH_2$−OH groups.[11] The capping efficacy can be estimated by test syntheses, in which an amino acid sequence is attached stepwise onto the preloaded resin. The quality is deduced by the amount of truncated sequences found in HPLC analysis of the cleaved crude peptide. Because of the expenditure of time of a typical test sequence, however, the quality test of resins via chemical syntheses is expensive.

Here, we report on a magic angle spinning (MAS) NMR method for the quantitative determination of free resin alcohols without further sample preparation. MAS NMR of swollen beads at moderate spinning rates is sufficient for the suppression of anisotropic interactions and resolved resin NMR spectra can be recorded. Conventional MAS NMR probes were already successfully used to determine the loading of resins quantitatively,[12] but the spectral resolution of these NMR spectra was too low to draw further conclusions regarding the nature of the polystyrene backbone, the loading quality or the completeness of the capping.

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**Table 1. Net Resin Weights Used for the Preparation of Test Mixtures, Calculated Molar Compositions $x_w$, and Relative Amounts Determined by 1D $^{13}$C NMR**

| | | | relative amount of 4-alkoxybenzyl alcohol resin | | |
|---|---|---|---|---|---|
| sample | $m_{\text{4-alkoxybenzyl alcohol resin}}$ (g) | $m_{\text{Fmoc-Ala-Wang resin}}$ (g) | w/w-% | $x_w$ (mol-%)[a] | mol-%[b] |
| **1a** | 10.00 | 5.21 | 65.7 | 82.9 | 82.7 |
| **1b** | 10.02 | 10.03 | 50.0 | 71.7 | 73.0 |
| **1c** | 7.50 | 10.00 | 42.9 | 65.5 | 67.7 |
| **1d** | 5.00 | 10.01 | 33.3 | 55.8 | 55.9 |
| **1e** | 4.00 | 10.00 | 28.6 | 50.3 | 54.4 |
| **1f** | 2.00 | 10.00 | 16.7 | 33.6 | 34.7 |
| **1g** | 1.00 | 10.01 | 9.1 | 20.2 | 16.9 |
| **1h** | 0.500 | 10.04 | 4.7 | 11.2 | |
| **1i** | 0.201 | 10.00 | 2.0 | 4.8 | |
| **1j** | 0.103 | 10.00 | 1.0 | 2.5 | |
| **1k** | 0.050 | 10.00 | 0.5 | 1.3 | |

[a]Calculated using eq 2, with loadings of 1.265 mmol/g for 4-alkoxybenzyl alcohol and of 0.500 mmol/g for Fmoc-Ala-Wang resin. [b]Obtained by line shape simulation of 1D $^{13}$C NMR spectra (spectra shown in Figures S-8 and S-9, Supporting Information).

The development of high resolution (HR)-MAS NMR methods significantly improved resolution and enabled quantitative results,[13−15] but they cannot be applied whenever resonance overlap occurs or peaks of very low relative signal intensities have to be quantified next to strong signals. The development of gradient equipped HR-MAS probes allows the recording of the whole range of 2D NMR techniques used in typical solution state high resolution applications.[13,16] We focused on HR-MAS HSQC (heteronuclear single quantum coherence) NMR and considered in a first step that for quantitative applications, modified HSQC sequences (Q- or QQ-HSQC; "quantitative" or "quick and quantitative" HSQC) and more recently improved versions applying shaped pulses on the $^{13}$C channel and constant-time CPMG-INEPT transfer (Carr-Purcell-Meiboom Gill- type polarization transfer) periods could be applicable.[17−19] However, all mentioned NMR methods failed because of their sensitivity to considerable variations of nuclear relaxation rates since our aim was quantification of low abundant polymer-bound species with chemical groups both in the direct vicinity and further apart from the solid support. In addition, the necessary phase cycle to obtain quantitative NMR spectra with a desired signal-to-noise ratio results in excessively long measurement times.

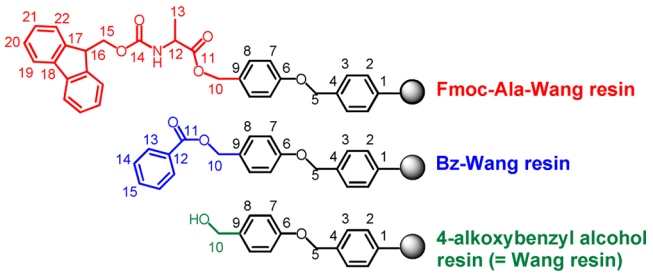
Therefore, we decided to apply nonquantitative versions of HSQC pulse sequences on a set of samples with precisely known compositions for calibration of cross peak volumes. To improve the resolution and reduce the NMR acquisition time, spectral aliasing methodology was further applied in the $^{13}$C dimension.[20] Preliminary results have shown that HSQC NMR cross signals of a 2 wt-% mixture of 4-alkoxybenzyl alcohol resin with Fmoc-Ala-Wang resin could be detected easily.[21] For our investigations, we set a target of 1 mol-% for the limit of detection (LOD) to meet the necessary quality standards. Using samples of known compositions, the NMR method was first calibrated. To demonstrate the generality, the method was then applied to several commercially available resins that were also analyzed via a specific peptide test synthesis followed by analysis of the cleaved peptides by Ultra Performance Liquid Chromatography (UPLC).

## RESULTS AND DISCUSSION

Fmoc-Ala-Wang resin was synthesized according to known procedures,[22,23] and mixtures with different amounts of the starting material 4-alkoxybenzyl alcohol resin were prepared through weighing followed by thorough mixing (Table 1). The precise sample composition is not fully specified by the net weights, since the loadings of the two starting materials are a priori not known and net weights are defined by the unity weight-%, whereas NMR signal integration results in relative amounts given in mol-%. Furthermore, Fmoc-Ala-Wang resin was only loaded with a substoichiometric amount of Fmoc-Ala-OH and remaining free hydroxyl groups were subsequently capped by benzoylation. The loading of the Fmoc-Ala-Wang resin used for calibration (0.500 mmol/g) was determined with UV spectroscopy.

The structures of the chemical compounds present simultaneously in the mixtures of Fmoc-Ala-Wang, Bz-Wang, and 4-alkoxybenzyl alcohol resin are shown in Scheme 1, including

**Scheme 1. Chemical Structures of Resin Bound Species**




the numbering of positions used for $^{1}$H and $^{13}$C NMR chemical shift assignments (Table S-1, Supporting Information).

$^{1}$H MAS NMR signals were generally broad and unresolved. Resonances H-19 of Fmoc-Ala-Wang resin at 7.92 ppm and H-13 of Bz-Wang resin at 8.26 ppm, however, were virtually free of overlaps from other signals (Figure 1A). Integration of these $^{1}$H NMR signals yielded relative amounts of Bz-Wang and Fmoc-Ala-Wang resin (Table S-2, Supporting Information). Integrations were carried out by line shape simulation of the high frequency spectral region (Figures 1B and S-3, Supporting Information) or by using DMFIT.[24] A fraction of 50.6 ± 0.6 mol-% Bz-Wang relative to Fmoc-Ala-Wang resin was obtained from eleven reference samples **1a**−**1k** (Table S-2, Supporting Information).

With the relative amount of 50.6 mol-% Bz-Wang and the loading of 0.500 mmol/g for Fmoc-Ala-Wang resin, a loading of 0.512 mmol/g was calculated for Bz-Wang resin. The loading of the starting material could then be evaluated using eq 1, where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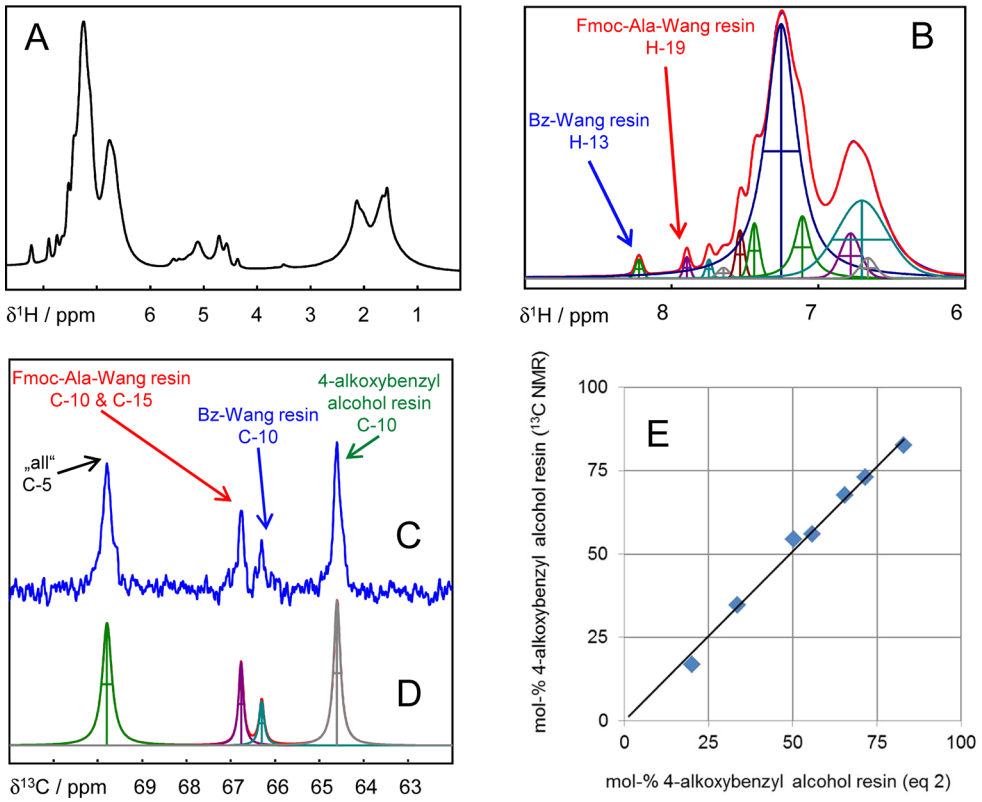


**Figure 1.** (A) Full $^{1}$H HR-MAS NMR spectral region of **1a**, (B) $^{1}$H NMR line shape simulation of the expanded high frequency region, (C) experimental and (D) simulated 1D $^{13}$C NMR spectra of **1a**, and (E) relative amounts of 4-alkoxybenzyl alcohol resin evaluated via eq 2 and correlated with 1D $^{13}$C NMR data. All line shape simulations were performed using DMFIT.

$l_{Wang}$ stands for the loading of 4-alkoxybenzyl alcohol resin in mmol/g, and $l_i$ represents the loadings of Fmoc-Ala-Wang and Bz-Wang resin. $\Delta MG_i$ are the increases of molecular weights of Fmoc-Ala-Wang and Bz-Wang resin with respect to the precursor 4-alkoxybenzyl alcohol resin (293.32 and 104.11 g/mol, respectively).

$$l_{Wang} = \sum [l_i/(1 - \sum (l_i \times \Delta MG_i/1000)] \quad (1)$$

Applying eq 1, we obtained a loading of 1.265 mmol/g for 4-alkoxybenzyl alcohol resin. Relative molar amounts of 4-alkoxybenzyl alcohol resin ($x_w$) in the test mixtures were calculated using eq 2, with the masses $m$ and loadings l of the two resins (Table 1).

$$x_w = 100 \times ((m_{Wang} \times l_{Wang})/(m_{Wang} \times l_{Wang} + m_{Fmoc} \times l_{Fmoc})) \quad (2)$$

We mention that residual solvent dioxane was detected in the $^{1}$H NMR spectrum of 4-alkoxybenzyl alcohol resin, while diisopropyl ether was found in Fmoc-Ala-Wang resin used for the preparation of test mixtures **1a**−**1k**. The absolute amounts of residual solvents were determined by integration of the respective $^{1}$H NMR resonances relative to signals assigned to 4-alkoxybenzyl alcohol resin and to Fmoc-Ala-Wang resin, respectively. Since both solvents were found in similar quantities (1.2 w/w-% for both resins, calculated according to equation S-1, Figure S-4, Supporting Information), the preparation of mixed samples by weighing resins is supposed to contain no systematic error because of solvent impurities in the starting material.

Because of the low cavity volume of the HR-MAS spinner, only small amounts of resin could be measured and signal-to-noise ratios (S/N) of 1D $^{13}$C NMR spectra were limited (Figure 1C). Nevertheless, relative amounts of 4-alkoxybenzyl alcohol to Fmoc-Ala-Wang resin obtained by line shape simulations (Figure 1D) showed an excellent correlation with the calculated data using eq 2. A slope of 1.017 ($R^2$ = 0.991) was obtained for samples **1a**−**1g** with 4-alkoxybenzyl alcohol resin contents between ∼65 and 9 w/w-%, when the linear fit was forced through the origin (Figure 1E). This confirms the overall procedure to evaluate the initial loading of 4-alkoxybenzyl alcohol resin. For sample **1g** a measurement time of 14 h was necessary to obtain a quantitative $^{13}$C NMR spectrum where the resonance at 64.7 ppm assigned to 4-alkoxybenzyl alcohol resin could still be integrated (Figure S-9, Supporting Information). A measurement time of a few hours was enough to obtain a sufficient S/N for e.g. samples **1a**−**1d**. For smaller fractions of 4-alkoxybenzyl alcohol resins in samples **1h**−**1k**, 1D $^{13}$C NMR could not be applied.

Expanded HSQC NMR regions of **1a** recorded with full and reduced spectral widths in the $^{13}$C dimension are shown in Figure 2A and 2B. Resonances 10 of Fmoc-Ala-Wang and Bz-Wang resin (red and blue, respectively, assignments of chemical shifts in Tables S-1 and S-3, Supporting Information) are better resolved in the aliased spectrum (Figure 2B), with the additional benefit of a 4-fold reduction in NMR acquisition time to 3h. The reduced spectral width of 6.2 ppm was optimized to separate correlation signals as far as possible without excessively extending the t1 increment applied for data acquisition.[20] Changes in the positions of cross signals are observed since the measurement of a HSQC NMR spectrum with reduced spectral width in the $^{13}$C dimension generates folding of resonances. Additionally, cross signals assigned to positions 12 and 16 of


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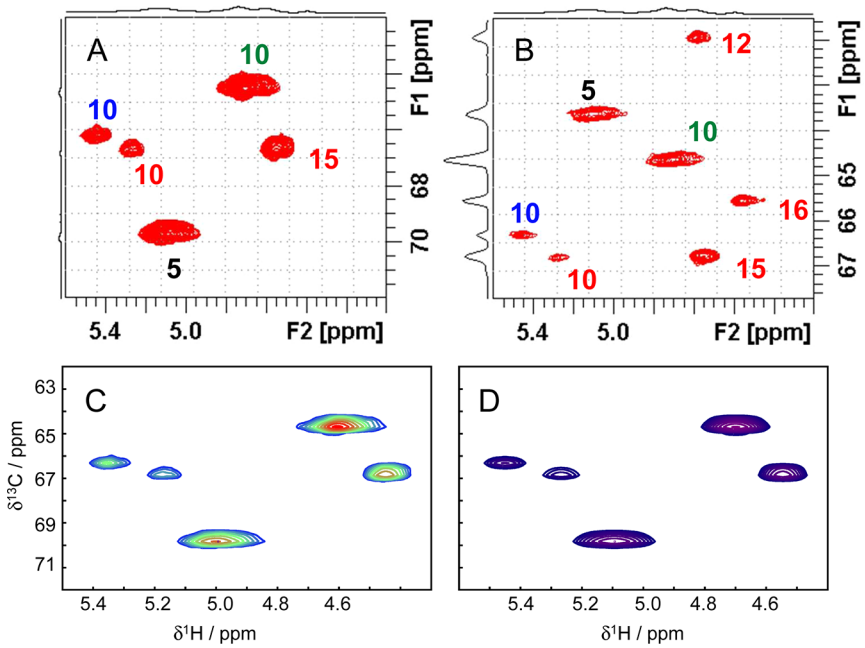


**Figure 2.** $^{1}H-^{13}C$ HSQC NMR spectra of **1a**. (A) recorded with full and (B) reduced widths in F1 with signal assignments (for chemical structures see Scheme 1), (C) experimental, and (D) simulated line shapes using DMFIT.

**Table 2. Experimental ($x_w^{exp}$) Volumes of HSQC Cross Peaks of 4-Alkoxybenzyl Alcohol with Respect to Fmoc-Ala-Wang Resin (Pos 10 or 15), Relative Polarization Transfer Rates *r* and Calculated ($x_w^{calcd}$) Amounts of 4-Alkoxybenzyl Alcohol. For Clarity, Column 2 is Repeated from Table 1**

| sample | $x_w$, eq 2 [mol-%] | $x_w^{exp}(10)$ [vol-%] | $r(10)^a$ eq 3 | $x_w^{exp}(15)$ [vol-%] | $r(15)^a$ eq 3 | $x_w^{calcd}(10)$ eq 4 [mol-%] | $x_w^{calcd}(15)$ eq 4, [mol-%] |
|---|---|---|---|---|---|---|---|
| **1a** | 82.9 | 88.3 | 0.646 ± 0.043 | 69.3 | 2.150 ± 0.121 | 82.3 | 82.0 |
| **1b** | 71.7 | 82.3 | 0.542 ± 0.020 | 57.0 | 1.903 ± 0.029 | 74.2 | 72.8 |
| **1c** | 65.5 | 76.2 | 0.592 ± 0.018 | 48.8 | 1.994 ± 0.052 | 66.5 | 65.8 |
| **1d** | 55.8 | 65.9 | 0.655 ± 0.043 | 38.1 | 2.056 ± 0.034 | 54.5 | 55.4 |
| **1e** | 50.3 | 61.0 | 0.649 ± 0.024 | 31.8 | 2.176 ± 0.074 | 49.2 | 48.5 |
| **1f** | 33.6 | 45.1 | 0.617 ± 0.013 | 19.4 | 2.104 ± 0.105 | 33.7 | 32.7 |
| **1g** | 20.2 | 28.3 | 0.640 ± 0.011 | 10.0 | 2.267 ± 0.076 | 19.7 | 18.3 |
| **1h** | 11.2 | 15.3 | 0.703 ± 0.073 | 5.2 | 2.317 ± 0.195 | 10.1 | 10.0 |
| **1i** | 4.8 | 5.8 | 0.912 ± 0.318 | 1.8 | 3.105 ± 1.155 | 3.7 | 3.6 |
| **1j** | 2.5 | 3.4 | 0.748 ± 0.111 | 1.0 | 2.585 ± 0.370 | 2.2 | 2.0 |
| **1k** | 1.3 | 1.0 | 1.368 ± 0.308 | 0.3 | 4.460 ± 0.937 | 0.6 | 0.6 |

$^a$The weighted average is $r(10) = 0.62 \pm 0.01$ and $r(15) = 2.02 \pm 0.02$.

Fmoc-Ala-Wang resin were folded into Figure 2B whereas these resonances lie outside the spectral region in Figure 2A.

Relative peak volumes between the cross signal H-10/C-10 of 4-alkoxybenzyl alcohol and H-10/C-10, such as H-15/C-15 of Fmoc-Ala-Wang resin were determined for both the full spectral width and the aliased HSQC NMR spectra either by using the standard Bruker integration software (TopSpin 2.1, pl6, integration regions shown in Figure S-5, Supporting Information) or by complete line shape simulation using DMFIT. Figure 1C and 1D illustrate the almost perfect simulation for **1a**. Applying both integration methods to regular and aliased HSQC NMR data resulted in a set of two times four values of relative peak volumes for each mixture **1a**−**1k** (Tables S-4 and S-5, Supporting Information). Volume integrals showed some variations, particularly for low relative amounts of 4-alkoxybenzyl alcohol resin in samples **1i**−**1k**, but no systematic differences between integration methods or HSQC spectra with different F1 spectral widths were observed.

Mean values of these 4-alkoxybenzyl alcohol resin relative peak volumes ($x_w^{exp}$) are summarized in Table 2, and the graphical comparison between peak volumes and the fractions calculated with eq 2 showed nonlinear trends (Figure 3A). This can be explained by different $^{1}H$ and $^{13}C$ magnetization polarization transfer rates for each of the correlated nuclei couple. The polarization transfer depends on a number of factors such as relaxation rates, coupling constant and multiplicity.[19] The data trend suggests that the transfer rates follow the decreasing order of H-15/C-15 of Fmoc-Ala-Wang, H-10/C-10 of 4-alkoxybenzyl alcohol followed by H-10/C-10 of Fmoc-Ala-Wang resin.

For samples **1a**−**1k** with known amounts $x_w$ and HSQC peak volumes $x_w^{exp}$ of 4-alkoxybenzyl alcohol (Table 2), relative polarization transfer rates r of H-10/C10 of 4-alkoxybenzyl alcohol to H-10/C-10 and to H-15/C-15 of Fmoc-Ala-Wang resin were calculated with eq 3 (Table 2, derivation of equation in Supporting Information).

$$r = (100/x_w^{exp} - 1)/(100/x_w - 1) \quad (3)$$

Weighted averages $r(10) = (0.62 \pm 0.01)$ and $r(15) = (2.02 \pm 0.02)$ were obtained and their ratio $r(10)/r(15)$ resulted in


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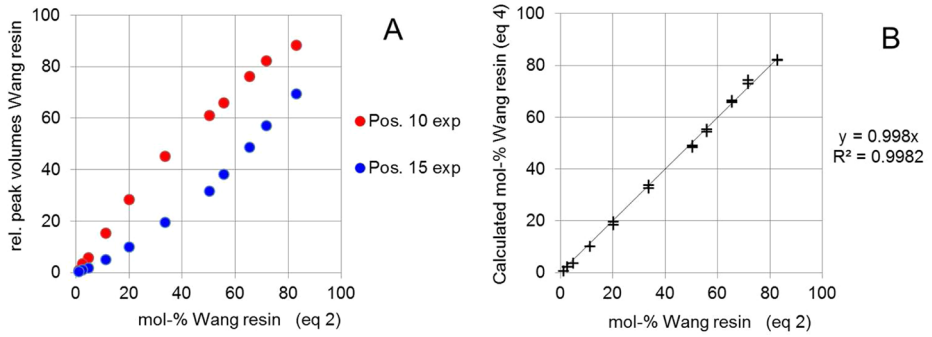


**Figure 3.** 4-Alkoxybenzyl alcohol resin (Wang) contents correlated to (A) experimental and (B) linearized HSQC peak volumes.

theoretical relative peak volumes of 23.5% and 76.5% for cross peaks 10 and 15 of Fmoc-Ala-Wang resin, respectively. Indeed, average volumes of 23.0% and 77.0% were obtained for the eleven reference samples **1a**−**1k**, confirming our approach for data treatment. From the barely detectable HSQC cross signals of **1k** after 24 h NMR measuring time (Figures S-10 and S-11, Supporting Information), LOD of the developed NMR method is suggested to be approximately 1 mol-%. This LOD is nearly a factor of 5 lower as reported in earlier experiments, in which the H-10/C-10 cross signal of 4-alkoxybenzyl alcohol resin was observed in a concentration of 4.8 mol-%.[21] Longer measuring times or stronger NMR fields will decrease the LOD further.

With known transfer rates *r*, experimental HSQC NMR peak volumes can be transformed by eq 4, thus eliminating transfer rate differences between various cross signals. The resulting linearized data $x_w^{calcd}$ can directly be compared with $x_w$. Equation 4 was also used to calculate $x_w^{calcd}$ for samples **1a**−**1k** (Table 2). Figure 3B shows the excellent agreement between the fractions of 4-alkoxybenzyl alcohol known in these samples ($x_w$) and obtained with NMR ($x_w^{calcd}$).

$$x_w^{calcd} = 100/(1 + (100/x_w^{exp} - 1)/r) \quad (4)$$

To verify the described method, two series of Fmoc-Ala-Wang (**1**−**5**) and Fmoc-Thr(tBu)-Wang resins (**6**−**10**) were analyzed with NMR and standard peptide test synthesis. These use tests were performed with an automated synthesizer, the 16-mer peptides were cleaved from the resin and the crude products were analyzed by UPLC (Figures S28−S37, Supporting Information). To deduce the quality of the capping, the sum of the UPLC peak areas of the first five truncated sequences Fmoc-[1−*n*]-OH (*n* = 15, 13, 12, 11, 10) was compared to the peak area of the desired product (Table S9, Supporting Information). Further deletion or truncated sequences were not taken into account. The deletion sequence Fmoc-[1−14]-OH was not detectable in the crude product since the first coupling in the test synthesis was performed with an Fmoc-dipeptide to avoid subsequent diketopiperazine formation.

In Table 3 relative HSQC peak volumes (Tables S-6 and S-7, Supporting Information) and linearized data (using eq 4) are shown together with results from the peptide test syntheses. We were not able to detect H-10/C-10 HSQC NMR cross peaks assigned to 4-alkoxybenzyl alcohol resin in **1** and **2** even for extended measuring times. Both samples consisted of commercially available batches used as starting material for a wide spectrum of reactions and were classified with the predicate "excellent capping" according to SPPS since only minor amounts of truncated sequences were found. We suppose that such small quantities of deletion sequences tend to be overrated because of integration effects in UPLC, since any UV active impurities present in the background and baseline noise account to the integrals.

**Table 3. Relative Volumes of HSQC Cross Peaks of 4-Alkoxybenzyl Alcohol Resin in Fmoc-Ala-Wang Resins 1−5 and Fmoc-Thr(tBu)-Wang Resins 6−10 (Pos 10 and 15), Linearized Data and Results from UPLC after Peptide Test Syntheses**

| compound | sample | $x_w^{exp}(10)$ [vol-%] | $x_w^{exp}(15)$ [vol-%] | $x_w^{calcd}$(10 and 15)[a] [mol-%] | deletion sequences[b] [%] |
|---|---|---|---|---|---|
| Fmoc-Ala-Wang resin | **1** | *c* | *c* | | 0.6 |
| | **2** | *c* | *c* | | 1.2 |
| | **3** | 4.2 | 1.0 | 2.3 | 2.3 |
| | **4** | 8.6 | 4.5 | 7.0 | 4.5 |
| | **5** | 69.7 | 38.8 | 57.2[d] | 36.7 |
| Fmoc-Thr(tBu)-Wang resin | **6** | 1.2 | 0.4 | 0.8 | 0.8 |
| | **7** | 4.5 | 1.4 | 2.8 | 1.6 |
| | **8** | 12.0 | 4.0 | 7.8 | 3.4 |
| | **9** | 14.3 | 4.8 | 9.3 | 4.7 |
| | **10** | 76.6 | 41.8 | 63.1[d] | 44.4 |

[a]Peak volumes after linearization via eq 4, using polarization transfer rates $r(10) = 0.62$ and $r(15) = 2.02$. [b]Determined by UPLC for a 16 AA sequence after cleavage from the resin. The sum of truncated sequences for the first five AAs from the C-terminus of the peptide is indicated. [c]No correlation signal H-10/C-10 of 4-alkoxybenzyl alcohol resin was observed in HSQC NMR spectra. [d]Simulation of quantitative 1D HR-MAS $^{13}C$ NMR spectra resulted in relative amounts of 55.6 mol-% (**5**) and 63.6 mol-% (**10**) 4-alkoxybenzyl alcohol resin.

HSQC NMR spectra of **3** (Figure S-16, Supporting Information) and **4** were recorded within 6 and 2 h. Thereby, **3** was prepared on purpose with insufficient capping for the peptide test synthesis and **4** was purchased by another supplier. Amounts of 2.3 mol-% and 7.0 mol-% of free 4-alkoxylbenzyl alcohol resin were determined by NMR, respectively. The amount of 4-alkoxybenzyl alcohol resin found in sample **4** was slightly higher than the sum of deletion sequences from the peptide test synthesis (4.5%, Table 3). For **5**, an even larger difference between the two methods was detected. The same trend could be observed within a series of Fmoc-Thr(tBu)-Wang resins (**6**−**10**). The reason for that observation is that even with increasing amounts of free Wang resin all remaining 4-alkoxybenzyl alcohols can be detected by NMR, whereas with the peptide test sequence only those free hydroxyl groups lead to truncated sequences that are sterically accessible for coupling reactions. Resins **6**−**9** originated from a test series to determine completeness of capping as function of solvent and reaction time (see Experimental Procedures).


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Resins **5** and **10** were synthesized without a capping step after loading. The relative amounts of 57 and 63 mol-% 4-alkoxybenzyl alcohol resin determined by 2D NMR agreed with data obtained from quantitative 1D $^{13}C$ NMR (56 mol-% and 64 mol-%, Table 3). From a chemical point of view, it is obvious that these values are much higher than those found with SPPS (37% and 44%), since not all coupling sites are accessible due to sterical hindrance. In addition, for practical reasons just the first five truncated sequences are considered for the use test, therefore it is plausible that other truncated sequences may sum up for higher amounts of residual alkoxybenzyl alcohol groups.

Of all essential amino acids, only threonine, proline, and valine, as well as some commonly used protecting groups for arginine and asparagine, show cross signals in the HSQC NMR spectral region of interest ($^{1}H$, 4.2−5.6 ppm; $^{13}C$, 60−70 ppm).[25] We have tested the applicability of the HSQC NMR method to some of these potentially disturbing candidates and further examples to cover all classes of amino acids. As depicted in Figures S-39 and S-40 (Supporting Information) no resonances were observed in all HSQC spectra that would prevent the quantification of the $CH_2$−OH groups from present 4-alkoxybenzyl alcohol resin. A further advantage of the NMR method is that important structural information such as the occurrence of byproducts (Figure S-38, Supporting Information) can be obtained directly without peptide cleavage or sample preparation. Moreover, the NMR method as such can also be applied on a "normal" high resolution NMR probe with z gradient using chloroform swollen beads; of course with loss of resolution and especially of sensitivity (HSQC NMR spectra of **1e** are shown in Figure S-41, Supporting Information).

## EXPERIMENTAL PROCEDURES

**HR-MAS NMR Studies.** To remove excess solvents that are often present in the polystyrene matrix, resins were placed in a G3 glass frit and were washed five times with approximately the double volume of $CDCl_3$ compared to the volume of swollen beads. The washed and slightly dried resins (approximately 20 mg) were then filled into 4 mm zirconia rotors (active volume of 50 $\mu$L) and swollen in $CDCl_3$. NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer equipped with a 4 mm HR-MAS probe at 400.13 ($^{1}H$) and 100.61 ($^{13}C$) MHz at ambient temperature. Sample rotation of 4000 Hz was applied, and $^{1}H$ and $^{13}C$ chemical shifts were referenced against residual solvent signals at 7.26 and 77.0 ppm, respectively. Quantitative 1D HR-MAS $^{13}C$ NMR spectra were recorded with relaxation delays of 10 s, since the longest $T_1$ relaxation time was 0.9 s for C-10 of the 4-alkoxybenzyl alcohol resin, and all other relevant resonances showed substantially lower $T_1$ values (Table S-1, Supporting Information). Relaxation data was obtained via inversion recovery experiments with fitted relaxation curves shown in Figures S-1 and S-2 (Supporting Information). Gradient selected sensitivity enhanced phase sensitive HR-MAS $^{1}H$−$^{13}C$ HSQC NMR spectra with or without multiplicity editing were acquired by gradient-selection techniques using Bruker standard pulse sequences ("hsqcedetgpsisp2.2" and "hsqcetgpsisp2.2") applying smoothed chirp shaped pulses of 0.5 and 2.0 ms for all 180° inversion and refocusing pulses on the $^{13}C$ channel, respectively. For the HSQC acquisition data matrices 1024 × 256 points (10 × 140 ppm, full spectral widths) were applied in $t_2$ and $t_1$, FIDs were multiplied by 90° shifted sine squared window functions and zero-filled to 1024 × 512 matrices prior to Fourier transformation. The spectral aliased HSQC version was run using a data matrix of 1024 × 40 points (10 × 6.2 ppm spectral widths) with applying zero-filling to 1024 × 128 points prior to Fourier transformation.

**Line Shape Simulations of 1D and 2D NMR Spectra.** Selected 1D and 2D NMR resonances were simulated using the line shape simulation software DMFIT.[24] For the simulations of $^{1}H$ NMR high frequency regions, a total of 11 resonances were fitted with the freely adjustable parameters relative signal intensity, chemical shift, line width and shape (percentage of Gaussian/Lorentzian shape), and zero order baseline correction. It was found that the two resonances of interest at 8.26 and 7.92 ppm assigned to Bz-Wang and Fmoc-Ala-Wang resin, respectively, were converging to 100% of Lorentzian shapes, whereas the broader resonances of the polymer backbone converged to shapes with significant Gaussian amounts. In the 1D $^{13}C$ NMR spectral region 62−72 ppm of the −$CH_2$−O− groups, 4 resonances with freely adjustable parameters (see above) were fitted. For the simulation of $^{1}H$−$^{13}C$ HSQC NMR spectra five resonances were defined in both spectral dimensions and selected in the cross peak matrix. The parameters relative signal intensity, chemical shift, line width, and zero order baseline were adjusted in the fitting process in both dimensions and best simulations were obtained when 100% Gaussian shapes were applied.

**Preparation of Fmoc-Ala-Wang Resin (1−5) and Fmoc-Thr(tBu)-Wang Resin (6−10).** Fmoc-Ala-Wang and Fmoc-Thr(tBu)-Wang resins were prepared by typical loading procedures via standard esterification method (DCCI/DMAP in DMF/THF at 0 °C) applying a substoichiometric amount of Fmoc-Ala-OH and Fmoc-Thr(tBu)-OH, respectively. End-capping of the remaining hydroxyl groups of the Wang resin was achieved by treatment with benzoyl chloride/pyridine.[23] Origins of resins: Fmoc-Ala Wang resin **1** (Bachem No. D-2280, Charge 1027694 MD/PIN 4193/32a) is the starting product used for the preparation of test resins **1a**−**1k** (see below). Resin **2** originates from an almost depleted Fmoc-Ala Wang batch, which has been used very successfully in syntheses at Bachem. Resin **3** was intentionally capped only in deficiency and used for test syntheses to examine the influence of residual hydroxymethyl groups. Resin **4** was bought for comparison purposes from another manufacturer and resins **5** and **10** were synthesized without applying any capping reaction. The Fmoc-Thr(tBu)-Wang resins **6**−**9** were from a test series where the completeness of capping was investigated as function of solvent and reaction time (**6**, benzoylation in DMF for 5 min; **7**−**9**, benzoylation in THF for 45, 15, and 5 min).

**Preparation of Mixtures (1a−1k) of Fmoc-Ala-Wang Resin with 4-Alkoxybenzyl Alcohol Resin for NMR Data Calibration.** Fmoc-Ala-Wang resin **1** was used for the preparation of all calibration mixtures. The mixed samples of Fmoc-Ala-Wang resins with 4-alkoxybenzyl alcohol resin used for NMR data calibration were prepared by weighing known amounts (see Table 1) into 3-L round-bottom flasks (total weight at least 10 g for each mixture). After addition of 1000 mL of DMF, the resins were mixed for 1 h on a rotary evaporator, subsequently filtered off, washed five times with 200 mL of $CHCl_3$ and dried under high vacuum at room temperature overnight. All test mixtures were prepared with the same 4-alkoxybenzyl alcohol resin that was used for the synthesis of the Fmoc-Ala-Wang resin **1**.

**Evaluation of the Capping Efficiency of Fmoc-Ala-Wang Resin and Fmoc-Thr(tBu)-Wang Resin.** The quality of resin batches was determined directly by a 16 amino acid test sequence prepared on a Symphony synthesizer from PTI (Tucson, Arizona) on a 150 $\mu$mol scale. The amino acids were


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coupled with *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate and *N*,*N*-diisopropylethylamine (TBTU/DIPEA) in dimethylformamide (DMF) and Fmoc was cleaved with 20% piperidine in DMF. The test sequences were cleaved from the resin with a mixture of trifluoroacetic acid, water and 1,2-ethanethiol. The crude product was isolated by precipitation in cold diisopropyl ether and analyzed by UPLC. The individual peaks were identified with UPLC by coelution with separately synthesized and purified references of the truncated peptide sequences. Quantitative results were obtained by integration of the relevant peaks normalized to the main peak. The total amount of the first five truncated sequences Fmoc-[1−*n*]-OH (*n* = 15, 13, 12, 11, 10) was used to estimate the resin quality. UPLC conditions for Fmoc-[1-Ala[16]]-OH: Waters BEH phenyl-column (2.1 × 100 mm), 45 °C, flow 0.5 mL/min, TFA buffer system (A, 0.1% TFA in $H_2O$/acetonitrile 99:1; B, 0.1% TFA in acetonitrile), gradient: 10%-2′-10%-3′-25%-5′-25%-5′-30%-6′-45% (B), detector wavelength 220 nm, injection volume 2 μL (2 mg/mL 50% AcOH). UPLC conditions for Fmoc-[1-Thr[16]]-OH: Waters BEH PST-Column (2.1 × 150 mm), 45 °C, flow 0.5 mL/min, TFA buffer system (A, 0.1% TFA in $H_2O$/acetonitrile 99:1; B, 0.1% TFA in acetonitrile), gradient 10%-2′-10%-3′-25%-5′-25%-5′-30%-6′-45% (B), detector wavelength 220 nm, injection volume 2 μL (2 mg/mL 50%AcOH).

## CONCLUSIONS

The HR-MAS HSQC NMR method for investigating quality aspects of preloaded Wang resins is a valuable alternative to commonly used peptide test syntheses. For a limit of detection of 2 mol-% 4-alkoxybenzyl alcohol resin an NMR measurement time of ∼6 h was sufficient, longer acquisition times (∼24 h) decreased the LOD below 1 mol-% (**6**, Table 3). These times have to be compared with 20 effective working hours required to synthesize the peptide test sequence followed by UPLC analysis for one single batch of preloaded Fmoc-Xaa-Wang resin. In addition, one has to take into account the costs of all reagents and solvents, the use of a peptide synthesizer for 36 h and the constraint that for almost all amino acids different test synthesis protocols have to be developed.

The quantitative analysis can easily be implemented on NMR spectrometers in other laboratories. Therefore, a unique set of reference sample mixtures with known compositions is required. Key NMR resonances are the $CH_2$-O groups of 4-alkoxybenzyl alcohol and of Fmoc-Ala-Wang resin. Peak volumes are obtained via standard integration routines or full line shape simulation. From the relative HSQC volume fractions of 4-alkoxybenzyl alcohol resin polarization transfer rates are then extracted using eq 3. If *r* is known, the amount of 4-alkoxybenzyl alcohol resin in actual test samples with unknown compositions can be obtained with eq 4. For samples where the quantities of 4-alkoxybenzyl alcohol and Fmoc-Ala-Wang resin can be evaluated by 1D $^{13}C$ NMR (e.g., **1a**−**1f**), polarization transfer rates can directly be obtained from the corresponding HSQC NMR spectra, and can then be applied for samples with lower resin contents. This would correspond to a rapid one-point determination of *r* in cases, where a lower precision is acceptable; this procedure requires a priori no exact knowledge of reference sample composition.

The quantitative determination of residual 4-alkoxybenzyl alcohol resin in Fmoc-Ala-Wang resin was demonstrated in detail and the applicability of the method to other amino acids was confirmed with Fmoc-Thr(tBu)-Wang resin. We demonstrated that no resonances of alternative amino acids fall into the region of interest of HSQC NMR spectra and, therefore, our method can be adapted to the full range of essential amino acids attached to 4-alkoxybenzyl resin. The quantitative determination of lower amounts of remaining hydroxyl groups of the Wang linker can be of interest for the chemical community since the quality and performance of commercially available resins can be improved. Although the Trityl linker gradually displaces the Wang linker in SPPS, there exist a number of filed processes certified by the FDA or other authorities to synthesize peptides for medical applications where the Wang linker must still be used. Further applications of the 2D NMR method in the field of SPPS are imaginable for all cases where no fast chemical or spectroscopic quantitative methods are available.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text including NMR chemical shift assignments with NMR spectra; $T_1$ relaxation times and curves; quantification of Bz-Wang resin with respect to Fmoc-Ala-Wang resin by 1D $^1H$ and $^{13}C$ NMR; quantitative determination of solvents in resins by $^1H$ NMR; chemical shift assignment in aliased $^{13}C$ HSQC NMR spectra; relative peak volumes of 4-alkoxybenzyl alcohol resin relative to Fmoc-Ala-Wang resin in HSQC NMR spectra; full and expanded regions of HR-MAS $^1H$, $^{13}C$ and HSQC NMR spectra of **1a**−**1k** and of **1**−**10**; UPLC UV traces and quantitative data evaluation after use tests of **1**−**10**; HSQC NMR spectra of further Fmoc-Xaa-Wang resins; derivation of eq 3 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +41 58 765 42 38. Fax: +41 58 765 40 12. E-mail: daniel.rentsch@empa.ch.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

| | |
|---|---|
| CPMG-INEPT | Carr Purcel Meiboom Gill-type polarization transfer |
| DCCI | *N*,*N*′-dicyclohexylmethanediimine |
| DMAP | 4-dimethylaminopyridine |
| HSQC | heteronuclear single quantum coherence |
| LOD | limit of detection |
| Q-HSQC | quantitative HSQC |
| QQ-HSQC | quick and quantitative HSQC |
| UPLC | ultra performance liquid chromatography |

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