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Fast and quantitative high-performance liquid chromatography method for the determination of 9-fluorenylmethoxycarbonyl release from solid-phase synthesis resins

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Abstract

Base catalyzed cleavage of 9-fluorenylmethoxycarbonyl (FMOC) group and subsequent analysis by UV spectrophotometry is a commonly used technique for measuring the loading of functional groups on solid supports. Recent works suggest that using 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) instead of piperidine makes it possible to use gas chromatography for quantitation, but due to the long deprotection time used, the method is not high-throughput. We observed that the dibenzofulvene released after DBU deprotection could be measured by reversed-phase (RP) HPLC. We have optimized the concentration of DBU as well as the time of the deprotection and coupled with a fast RP-HPLC separation results in a highly reproducible, high-throughput method. The measured loading correspond well with the manufacturer's data on several commercial resins. Using this method we have quantitated the amine loading on several polystyrene resins and we have found that the total amount of functional group can be more than twice the amount of the available ones. We concluded that the differences were the function of the resin loading as well as the level of crosslinking.

Keywords: Polystyrene resins; Fluorenylmethoxycarbonyl compounds; Diazabicycloundecene; Dibenzofulvene

1. Introduction

The N^{α}-9-fluorenylmethyloxycarbonyl (FMOC) group was introduced by Carpino and Han [1] as a base labile protecting group for primary and secondary amines. This protecting group has been widely utilized in organic chemistry and as a mild orthogonal alternative to N^{α}-*tert.*-butyloxycarbonyl (t-BOC) in solid-phase peptide synthesis. The FMOC group is stable toward acids and hydrolysis but readily cleaved by a variety of organic bases [2]. Piperidine is the most popular one to remove FMOC since it is inexpensive and has relatively few side effects. The base abstracts the acidic proton at the 9 position of the fluorene ring system; β elimination follows to give a reactive dibenzofulvene (DBF) intermediate which is trapped by the excess secondary amine [3] (Fig. 1). Tris(2-aminomethyl)amine [4], 4-(aminomethyl)piperidine [5] and tetrabutylammonium fluoride [6] have also been used for FMOC removal. Recently the non-nucleophilic 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) in *N*,*N*-dimethylformamide (DMF) has been utilized as an alternative to piperidine [7,8]. DBU does not form an adduct with DBF (Fig. 1) and for that reason originally was applied for continuous solid-phase peptide synthesis so the reactive fulvene can be washed quickly.

FMOC, DBF and DBF/piperidine are extremely

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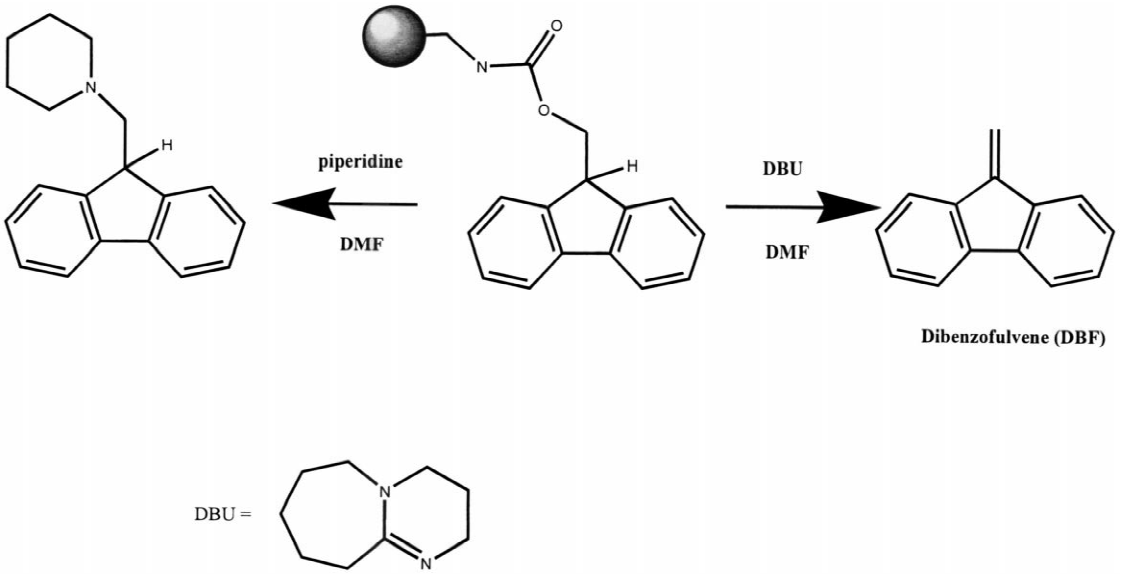

Fig. 1. Piperidine vs. DBU cleavage of the FMOC group.

good chromophores and the latter one has been used extensively to quantitate FMOC groups by UV spectroscopy [3]. Recently Newcomb et al. [9] used gas chromatography (GC) to quantitate FMOC by measuring the released DBF after DBU deprotection. The GC method has been shown to be less labor intensive than the standard UV method but the deprotection step has been taking very long (1 h) which significantly limits the throughput when parallel chemistry capabilities are not available.

Aminomethyl derivatives of crosslinked polystyrene resins have been important since the early 1970s for the preparation of supports for solid-phase peptide synthesis [3]. Recently these supports have been widely utilized to prepare combinatorial libraries of small molecules, as well as polymer-supported reagents and scavengers [10,11]. For all of these applications it is very important to know the exact amount of the reactive amino functionality on the polymer surface, as this affects purity and yield.

The objective of this study was to offer a fast and quantitative alternative to this GC method using reversed-phase high-performance liquid chromatography (RP-HPLC) to measure the released DBF. The effect of the DBU concentration and the length of the deprotection time has been investigated and optimized. We demonstrate that only a few minutes of deprotection is sufficient as opposed to an hour [9] and FMOC-amino acids can be utilized in place of FMOC-ethanolamine for constructing calibration curves. Several amino polystyrene resins were analyzed with this method by reacting them with FMOC-amino acids and it will be shown that the total amount of nitrogen (determined by elemental analysis) and the available nitrogens can differ significantly as a function of the base matrix and loading density.

2. Materials and methods

2.1. Materials

FMOC-containing polystyrene resins (Rink Amide, Sieber, NovaSyn Sieber-TG, FMOC-ileucine-Wang), FMOC-alanine and *O*-benzotriazol-1-yl-*N*,*N*,*N′*,*N′*-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem (San Diego, CA, USA). ArgoPore-NH, ArgoPore-NH-HL and ArgoGel-Trisamine were obtained from Argonaut Technologies (San Carlos, CA, USA). DMF, DBU, *N*-methylmorpholine (NMM), trifluoroacetic acid (TFA) and nitrobenzoic acid were purchased from Aldrich (Milwaukee, WI, USA). HPLC-

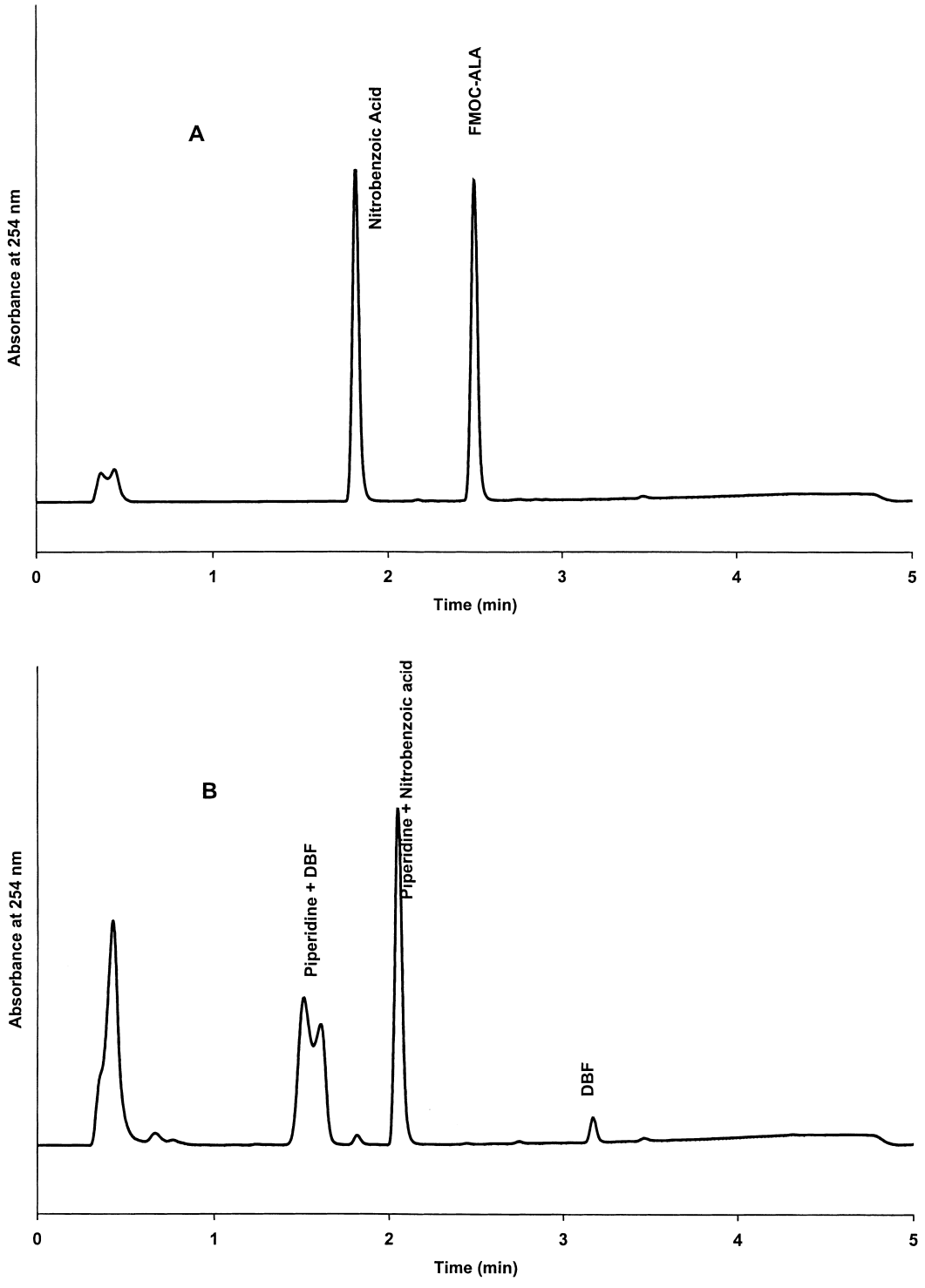

Fig. 2. Reversed-phase chromatograms of (A) nitrobenzoic acid and FMOC-alanine; (B) piperidine deprotection of FMOC-alanine; (C) DBU deprotection of FMOC-alanine. For experimental details see Materials and Methods.

grade water and acetonitrile were obtained from EM Science (Gibbstown, NJ, USA).

2.2. Reversed-phase HPLC analysis

The HPLC measurements were carried out on an HP Model 1100 system equipped with high-pressure gradient pump, an autoinjector set at 5 μl, a variable-wavelength UV–Vis detector set at 254 nm and a 3D Chemstation Data System (Hewlett-Packard, Palo Alto, CA, USA).

The column used for the separations was a Zorbax Rapid Resolution SB-C8, 50×4.6 mm I.D., 3.5 μm particle size, obtained from Hewlett-Packard. All separations were carried out at room temperature.

Mobile phase A was 0.1% TFA in water while mobile phase B was 0.1% TFA in acetonitrile. The flow-rate was kept at 2 ml/min throughout the separation. The samples were eluted with a gradient from 15% B to 95% B in 3.5 min.

2.3. Coupling of FMOC-alanine to amino resins

Samples of dried resins were measured (~10 μmol amine equivalent) into 15-ml centrifuge tubes and washed 3×12 ml DMF. We added to each vessel 2 ml DMF, 1 ml FMOC-alanine solution (0.1 *M* in DMF) followed by 1 ml HBTU solution (0.1 *M* HBTU, 0.4 *M* NMM in DMF). The reaction vessels were shaken overnight on a wrist action shaker (Model 75, Burrel, Philadelphia, PA, USA). At the end of the reaction, the gels were washed with 6×12 ml DMF and left in the centrifuge tube for DBU deprotection as is described in the following section.

2.4. DBU cleavage of FMOC-bearing resins

Samples of vacuum dried resins (approximately 10 μmol FMOC based on loading) were placed in a 15-ml centrifuge tube followed by 10 ml of DBU/DMF solution (2–10% DBU content) and 0.2 ml nitrobenzoic acid solution (10 mg/ml in DMF) addition. The suspensions were shaken for 5–60 min, centrifuged at 4500 rpm for 2 min using a Model GS-15 centrifuge (Beckman, Palo Alto, CA, USA) and the supernatants were analyzed by RP-HPLC by the above-described method.

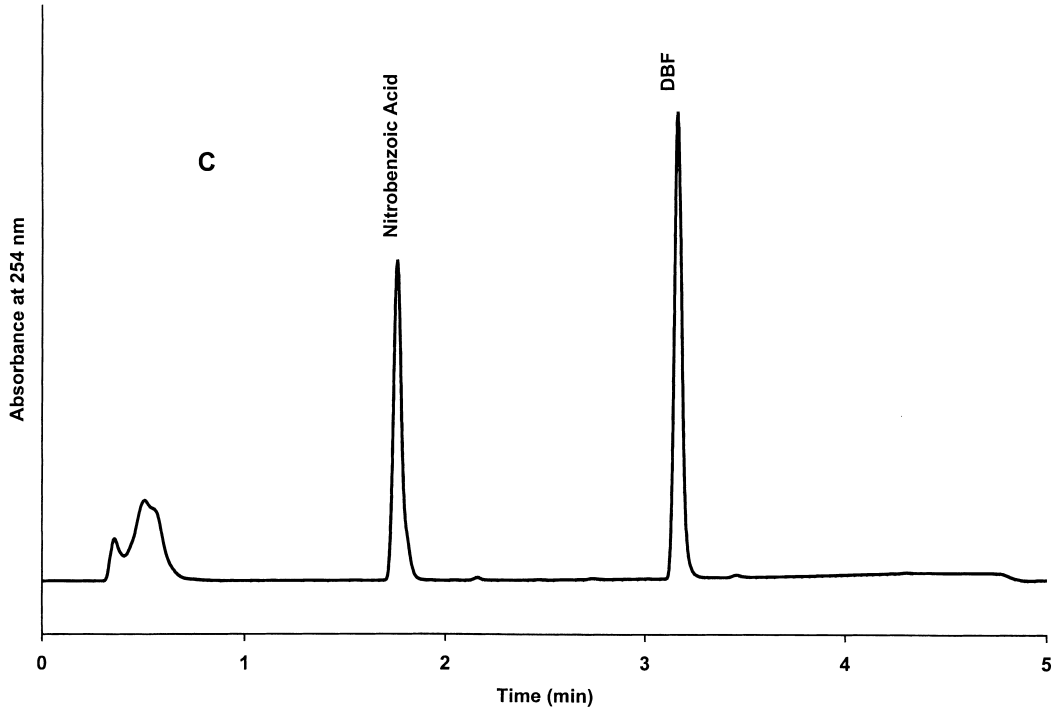

Fig. 2 (*continued*).

2.5. Calibration curve for HPLC analysis of FMOC deprotection

FMOC-alanine (3.7 mg; 11.9 μmol) was dissolved in 10 ml of 2% DBU and the solution was shaken at room temperature for 5 min. This stock then was diluted serially and nitrobenzoic acid solution (10 mg/ml in DMF, 0.1 ml to every 5 ml standard

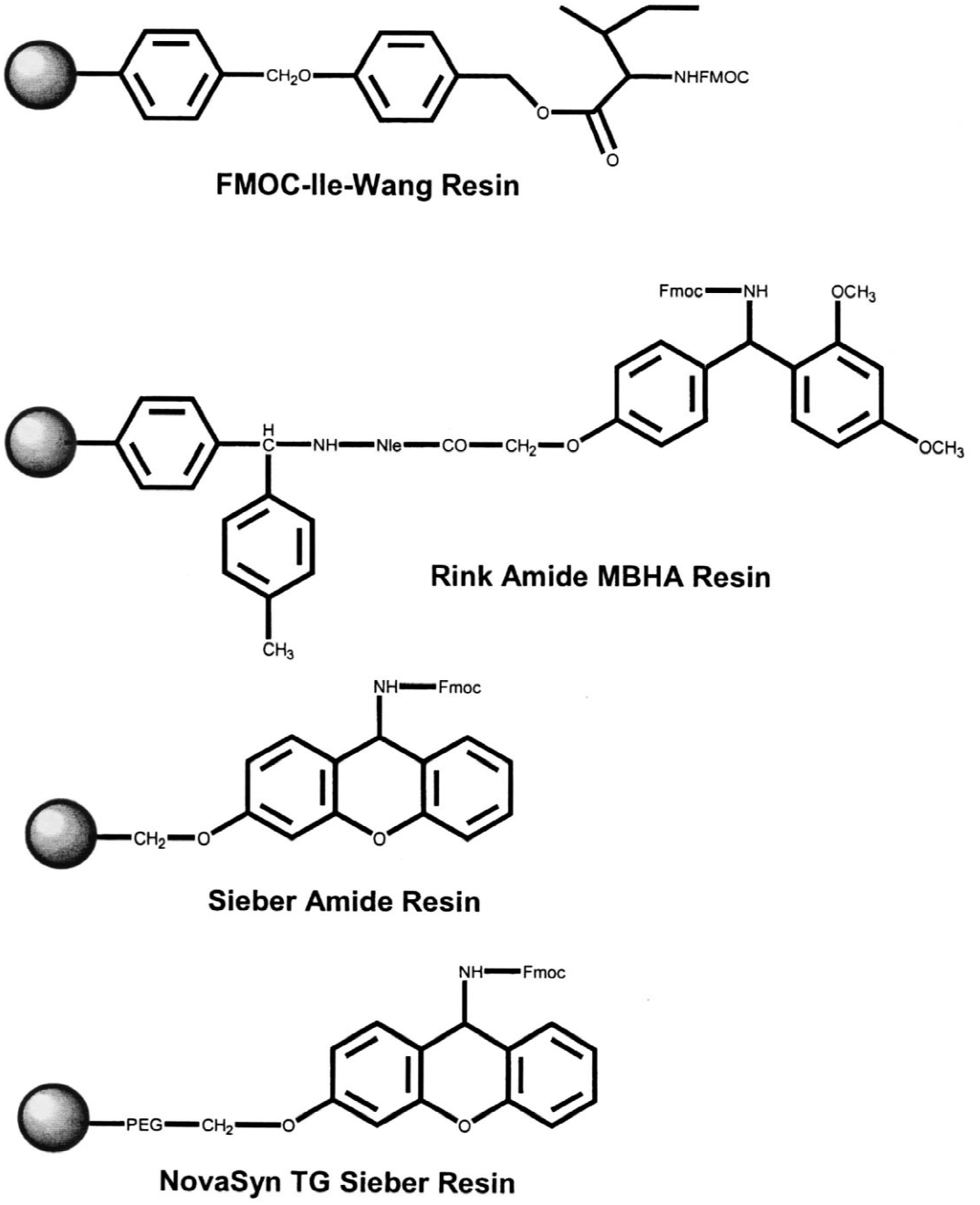

Fig. 3. The structures of the FMOC containing handles on different commercial solid-phase synthesis resins used in this study.

solution) was added and the samples were analyzed by HPLC. A standard curve was constructed plotting the ratio of the peak areas of dibenzofulvene and nitobenzoic acid vs. the concentration of FMOC-alanine in DMF. The slope and intercept of the resulting calibration curve were calculated by the method of the least squares.

3. Results and discussion

Our goal was to adapt the recently developed GC method for FMOC loading on solid-phase synthesis resins for RP-HPLC which is our main analytical method. We set out to further investigate the cleavage conditions since the long cleavage time used by Newcomb et al. [9] can seriously limit the throughput of the analysis. Their investigation showed that the reattachment of the dibenzofulvene to the free amine on the surface might not be a serious problem contrary to Wade et al. [7].

First we reexamined FMOC-amino acids as DBF source for calibration curves. Newcomb et al. [9] had problems using FMOC-glycine in 20% piperidine/DMF due to precipitate formation. We found, however, that using FMOC-alanine as standard did not have this problem in DBU/DMF or piperidine/DMF deprotection. Adding nitrobenzoic acid as internal standard did not effect the deprotection step. Fig. 2 depicts the separations of the resulting mixtures after DBU as well as piperidine deprotection. It can be seen that the DBU deprotection results in only two peaks (nitrobenzoic acid and dibenzofulvene) while no FMOC-alanine was left. The piperidine, however complexes not only with the dibenzofulvene but with the nitrobenzoic acid, too. The DBF/piperidine adduct is a wide, hard to analyze peak. This result is a further proof that the piperidine deprotection method is not well suited for chromatographic analysis.

Using FMOC-alanine and nitrobenzoic acid we have constructed a standard curve. Plotting the ratio of the peak areas of the DBF and nitrobenzoic acid as a function of the molar concentration of the

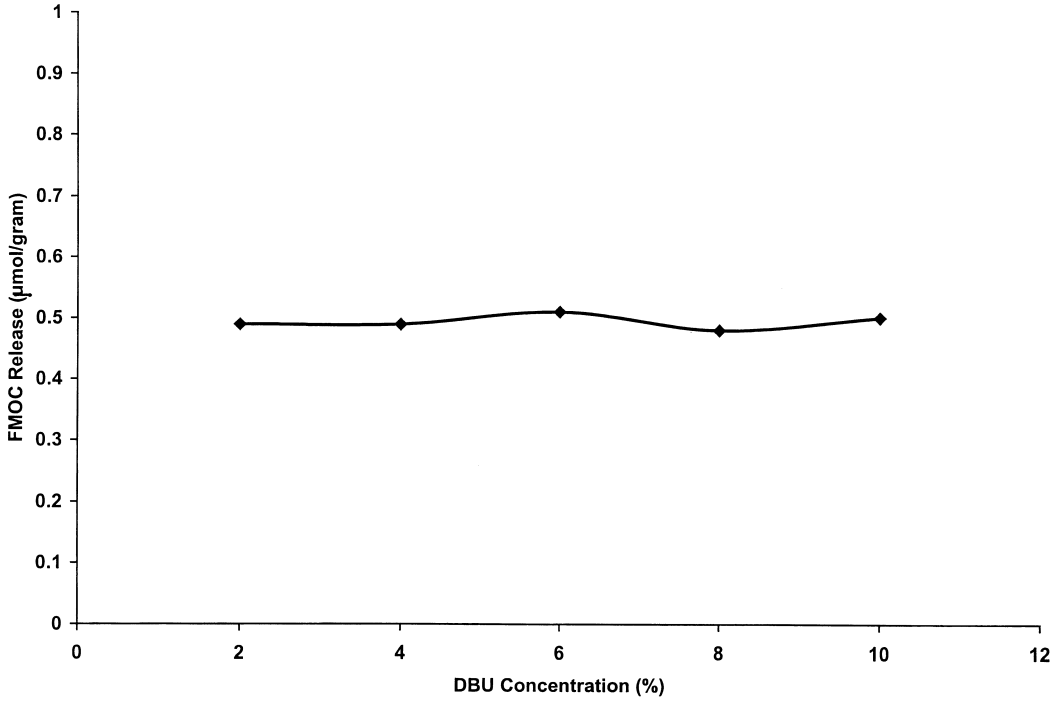

Fig. 4. Effect of the DBU concentration in DMF on the release of DBF from preloaded FMOC-isoleucine-Wang resin. The total loading based on the manufacturer's data is 0.46 mmol FMOC/g dry resin. The deprotection time was kept constant at 20 min.

FMOC-Ala resulted in a straight line which can be described with the following equation: $y=2.5x+0.05$ where y is the ratio of the peak areas of the DBF to nitrobenzoic acid and x is the concentration of FMOC-Ala in μmol/ml. The regression coefficient was 0.998. The linearity and near zero intercept are further proof that the deprotection is full, while no other compounds than dibenzofulvene are formed and nitrobenzoic acid did not interfere with the deprotection, respectively.

The next step was to optimize the deprotection conditions on the solid-phase. For this purpose we have used commercially available preloaded polystyrene resins. Fig. 3 shows the structures on the surfaces of the different resins. First we have treated FMOC-isoleucine on Wang-polystyrene resin (1% crosslinked 0.46 mmol/g loading density measured by the supplier) for 20 min with different concentration of DBU solutions following by the analysis of the supernatant. Fig. 4 depicts the FMOC release, calculated from the calibration curve, as a function of DBU concentration. It is clearly seen that the maximum FMOC is released using 2% DBU solution and that higher concentrations do not release more FMOC. The next step was to learn what is the minimum deprotection time needed to release all the FMOC groups. We have treated the same resin with 2% DBU solution for 5 to 60 min and the results are depicted in Fig. 5. It can be clearly seen that after 5 min the deprotection is full and any longer time is totally unnecessary and it may even increase the chance for side reactions like DBF reattaching to the resin or DBF polymerization.

In order to validate the optimized deprotection method (2% DBU, 5 min) we have measured the loading on the selected commercial polystyrene resins (Fig. 3). Table 1 summarizes the results. It can be clearly seen that the measurement is very reproducible and corresponds well with the manufacturers data for a wide range of loading density. The loading given by the manufacturers were measured by the standard UV method [3] quantifying the released DBF/piperidine adduct. This method has been used by peptide chemist for over a decade and

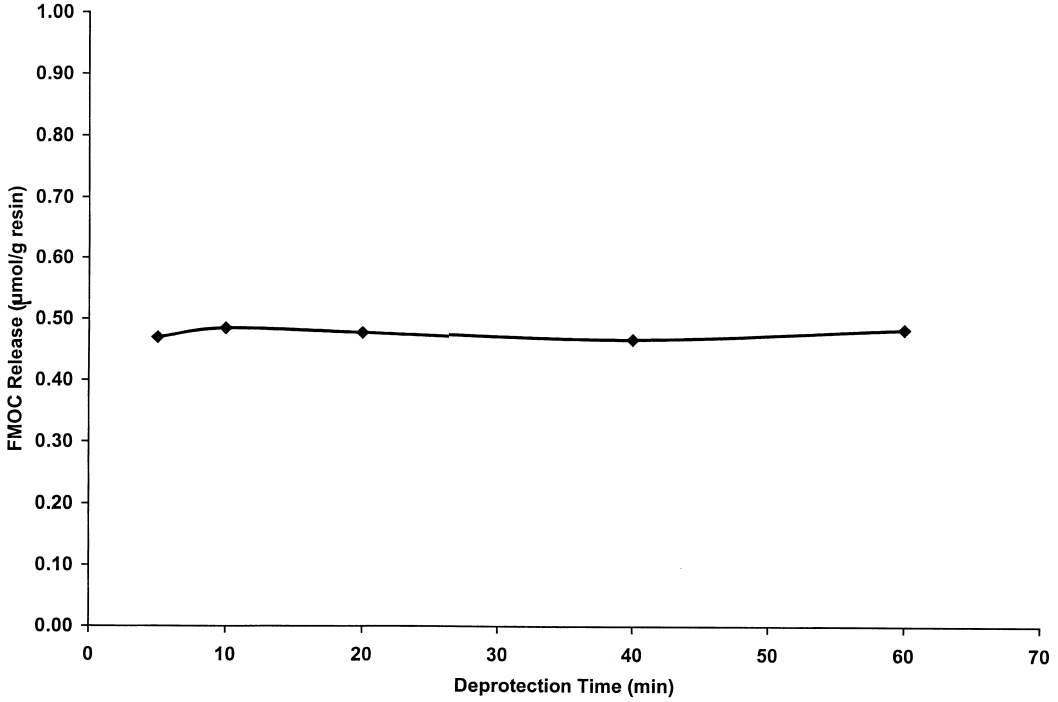

Fig. 5. Effect of the duration of the deprotection on the release DBF from preloaded FMOC-isoleucine-Wang resin. The DBU concentration was kept at 2%.

Table 1
FMOC loading on different activated polystyrene resins measured by DBU deprotection and subsequent HPLC quantitation and compared to the loading given by the manufacturer[a]

Resin[b]	Loading by manufacturer (mmol/g)	DBU/HPLC loading (mmol/g)	SD[c]	n[d]	Difference between expected and measured loading (%)
Wang-Ile-FMOC	0.46	0.486	0.021	11	+4.3
Rink Amide HMBA	0.59	0.610	0.022	3	+3.4
Sieber Resin	0.38	0.386	0.013	3	+2.6
Novasyn TG Sieber	0.15	0.157	0.007	3	+4.7

[a] Resins were treated with 2% DBU for 5 min followed by HPLC analysis of the supernatant.
[b] All resins obtained from Novabiochem, structures of the groups on the surfaces are depicted on Fig. 3.
[c] SD=Standard deviation.
[d] n=Number of replicates for DBU deprotection/HPLC quantitation.

is proven to be extremely reliable, however, labor intensive and time consuming.

For us it was very important to analyze the available amino groups on different amino polystyrenes, which can be used for solid-phase reagent or scavenger applications. These resins usually have high functional group loading (measured by elemental analysis). Recently, highly crosslinked macroporous polystyrene was introduced by Argonaut Technologies (ArgoPore) for solid-phase synthesis since these resins can be used in a wide range of solvents as long as the solvent wets the polymer surface. Due to the very high level of crosslinking and low swelling it is conceivable that part of the amino groups may not be available for reactions, which is very important to know for the accurate calculation of the yields.

It is a widely used method for functional group measurement (amino, hydroxyl, chloromethyl, etc.) to modify a resin with FMOC-amino acid and then measure the released DBF or DBF/piperidine adduct. We have used FMOC-alanine as ligand and measured DBF by our validated HPLC method. Table 2 summarizes the results for three different resins obtained from Argonaut Technologies. It can be seen that even on the relatively low loading ArgoPore NH only the 80% of the amino groups available. In the case of the very high loading trisamine resin (which is a gel with 2% crosslinking) only 40% of the amines are reactive while in the case of the ArgoPore NH-HL about 70% of the amines are available. This tandem coupling of FMOC-amino acid/DBU deprotection can be easily automated and give vital information for planning solid-phase library synthesis.

4. Conclusion

A high throughput HPLC method for the analysis of the FMOC release has been developed. A fast reversed-phase method combined with an optimized deprotection step can provide an accurate information in less than 10 min. Using low concentration of DBU in combination with very short deprotection time ensures the minimization of side reactions.

Table 2
Comparison of total amine loading with the available amines using tandem reaction with FMOC-Ala and DBU/DMF deprotection (for the experimental details see Materials and Methods)

Resin[a]	Total amine loading by the manufacturer (mmol/g)	Available amines based on released DBF (mmol/g)	SD[b]	n[c]	Difference between the total and available amines (%)
AP-NH	0.72	0.59	0.03	3	−18
AP-NH-HL	1.13	0.77	0.04	3	−32
AG-Trisamine	3.8	1.56	0.05	3	−59

[a] All resins obtained from Argonaut Technologies, AP-NH=ArgoPore-NH, AP-NH-HL=ArgoPore-NH High Loading, AG=ArgoGel.
[b] SD=Standard deviation.
[c] n=Number of replicates for DBU deprotection/HPLC quantitation.

Using tandem FMOC-amino acid coupling/DBU deprotection in situ allows an accurate assessment of the density of the available functional groups as opposed to relying on elemental analysis data. This information is vital for developing solid-phase reagents as well as scavengers.

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