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On-Bead Quantitation of Resin Bound Functional Groups Using Analogue Techniques with Vibrational Spectroscopy

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This report introduces two novel methodologies for the on-bead, functional group quantitation of solid phase synthesis using vibrational spectroscopy. "Analogue bleed" quantitation is an infrared spectroscopic technique in which combinatorial library bead spectral data are regressed against solution phase functional analogue spectra of a ligand in question. "Dual Analogue" quantitation is introduced as an infrared or Raman technique in which ligand-to-styrene band ratios of solution phase ligand/styrene monomer standards are used to generate an internally path length referenced calibration which can then be applied to solvent swollen beads. These technologies have application across a wide range of functional groups and can be applied throughout each step of a resin bound synthesis.

Introduction

The introduction of resin supported solid phase synthesis (SPS) places many new demands on the analytical technologies which support the effort. The primary analytical tools supporting solution phase synthesis (HPLC, NMR, and MS) are of minimal use in on-bead analysis of resin supported systems. Vibrational spectroscopy has stepped up in an effort to fill the analytical void, but it too has associated problems. Qualitative aspects of the vibrational experiments for resin bound entities have been dealt with extensively.[1,2] However, quantitative aspects of the on-bead vibrational experiments are still in the very early stages of development. To date, we are aware of only one publication reporting absolute functional group quantitation for resin bound functional groups. In that report, infrared spectroscopy was used in conjunction with deuterium labeled protecting groups.[3]

There are two factors primarily responsible for the very limited developments in on-bead quantitation. The first limitation is the lack of primary standards. Presently, there are no resin bound standards for quantitative functional group analysis available. The second problem is poor control of sample quantity for each analysis. In infrared microscopy experiments, a sampling volume can be accurately defined via aperture and cell path length; however, the absolute quantity of resin within that volume can vary widely as a function of resin functionalization, cell pressure, and most importantly, solvent swelling. Functional group concentration per unit volume in solvent swollen beads can be almost an order of magnitude less than that for unswollen beads. Sampling swollen vs unswollen beads in a fixed path length cell where the bead is allowed to expand in the plane perpendicular to the sampling axis can lead to order of magnitude variations in absorbance intensity, due to the reduced absolute quantity of swollen resin within the beam path volume sampled by the spectrometer.

In this report, two methodologies have been developed to circumvent the hurdles inherent to on-bead infrared quantitation. The two methodologies respectively designated "analogue bleed" and "dual analogue" are each shown to be simple yet robust technologies for quantitation of an extremely wide range of pendant functional groups on a solid phase support.

Part 1: "Analogue Bleed" Quantitation

Throughout discussion of the theory and derivation of calibration mathematics, the chloromethyl functionality of chloromethylstyrene[4] is used as the target functional group. In the "analogue bleed" experiment, calibration was obtained by collection of a spectrum from a "dry" bead using the infrared flow cell[1] in conjunction with the infrared microscope. The microscope was then focused to an open region of the cell in which no beads were present, solutions of analogue standards were bled into the cell, and spectra were collected. Using the microflow cell, identical spectral path lengths are obtained from the bead and subsequent analogue solutions. A detailed account of the procedure is presented in the "analogue bleed" calibration procedure section of this document.

According to Beer's law, the relationship of absorbance and molar concentration of the chloromethyl functional group in both the beads and solution standards can be calculated as follows:

$$A = ebc \tag{1}$$

where A = absorbance, e = molar extinction coefficient, b = path length, and c = molar concentration. Since the resin bound chloromethyl and the benzyl chloride analogue standard are very similar with respect to chloromethyl functionality, it is a good assumption that e is constant and eq 2 is rendered valid. This assumption can be supported by analysis of band shape and frequency for solid vs solution phase analogues. Lack of discernible differences in these

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parameters for solution vs resin bound analyte was a strong indication that minimal perturbation of the vibration mode was imparted by the variant matrixes.

$$\left(\frac{A}{bc}\right)_{-CH_2Cl\_bead} = \left(\frac{A}{bc}\right)_{-CH_2Cl\_solution} \quad (2)$$

By design, path length (b) was identical for bead and analogue standard solution spectra and may be eliminated to give eq 3.

$$\left(\frac{A}{c}\right)_{-CH_2Cl\_bead} = \left(\frac{A}{c}\right)_{-CH_2Cl\_solution} \quad (3)$$

Least squares regression analysis of spectral data obtained from benzyl chloride analogue standards are used to evaluate the solution phase based standard expression $(A/c)_{-CH_2Cl\_solution}$. Substitution of regression parameters into the eq 3 yields eq 4 after rearrangement.

$$(c)_{-CH_2Cl\_bead} = \frac{A_{-CH_2Cl\_bead} - \text{intercept}}{\text{slope}} \quad (4)$$

The chloromethyl molar concentration of the bead can then be converted to mmol/g through division of the eq 4 result by bead density (eq 5). Bead density (D) can be calculated using vendor supplied values for the number of beads per gram and the average bead diameter. However, best results were experimentally obtained by estimating bead density through buoyancy tests in a series of increasingly dense solvents. The wide range of readily available chlorinated and dichlorinated hydrocarbons provides good coverage of density ranges incurred. While solvent mixtures can be used to obtain very fine density increments, this approach was found to be unsuitable due to selective solvent partitioning[5] into the bead and its effect on the density measurement.

$$\text{loading (mmol/g)} = \left(\frac{A_{-CH_2Cl\_bead} - \text{intercept}}{\text{slope}}\right)/D \quad (5)$$

Finally, eq 6 includes an internal styrene absorbance band intensity term to compensate for variant path lengths of future experiments. In eq 6, RS represents the average absorbance area of the internal styrene reference band obtained from the dry bead sample as described in the calibration procedure. R represents the individual absorbance area of that styrene band for the bead of each subsequent cell loading and analysis.

$$\text{loading (mmol/g)} = \frac{\left(\frac{A_{-CH_2Cl\_bead} - \text{intercept}}{\text{slope}}\right)}{\left(D \times \frac{RS_{\text{internal\_styrene\_PeakAREA}}}{R_{\text{internal\_styrene\_PeakAREA}}}\right)} \quad (6)$$

Calibration Procedure

A Nicolet 750 infrared spectrometer fitted with a NicPlan infrared microscope was used for collection of all single bead infrared data. The spectral background was collected through a 2 mm BaF_2 window. Bead(s) were then mounted in the microflow cell using the 2 mm BaF_2 window and a drilled 2 mm ZnSe window separated by a nominal 0.030 in. Teflon spacer. During assembly, the beads were sufficiently pliable to "cleanly" mate to the cell walls without undue force. Spectra from three beads were collected from their respective centers according to Figure 1A and prior to introduction of any solvent. The baseline corrected absorbance area of the $-CH_2Cl$ peak (1286 to1242 cm^{-1}) and a polystyrene backbone absorbance band (1473 to 1404 cm^{-1}) were calculated for each spectrum.

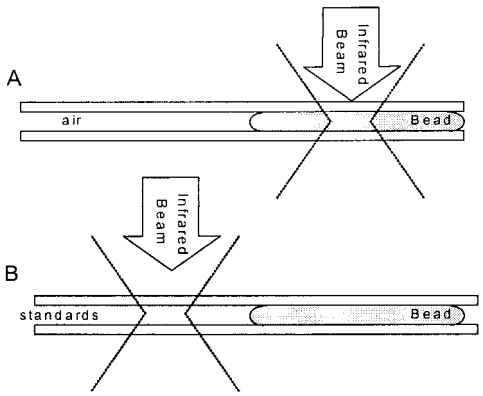

Figure 1. Sampling configuration.

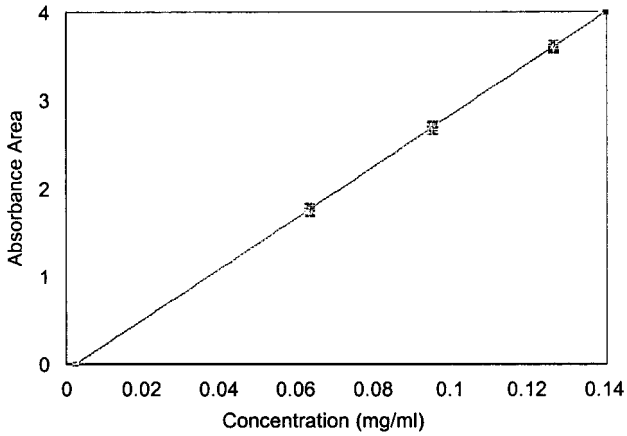

Figure 2. Chloromethyl regression analysis.

The microscope stage was then repositioned to a clear area of the cell in which no bead was present (Figure 1B). Then, 0.5, 1.0, and 1.5 M benzyl chloride in CH_2Cl_2 analogue standards were sequentially bled into the cell, and spectra were collected at three positions in the cell for each standard. The on-bead/off-bead procedure provided "dry" bead and solution phase analogue spectra from identical spectral path lengths.

The baseline corrected absorbance area of the $-CH_2Cl$ peak (1286 to 1242 cm^{-1}) was calculated for each standard. Figure 2 illustrates the plot of $-CH_2Cl$ absorbance area vs concentration obtained from the solution phase standards. An R^2 correlation coefficient of 0.999 was obtained from the illustrated regression. Slope and intercept values obtained from the regression constitute the needed variables for eqs 4−6 in the theory section above.

Results and Discussion

Table 1 illustrates the value and precision data obtained from the "analogue bleed" procedure for a series of beads analyzed using the data collection and analysis routine

Table 1. Quantitation Data

	$-CH_2Cl$ area 1286−1242 cm^{-1}	styrene area 1473−1404 cm^{-1}	$-CH_2Cl$ (mmol/g)
bead 1	2.55	25.52	0.67
bead 2	2.56	24.87	0.69
bead 3	2.68	26.61	0.67
average	2.60	25.67	0.68
% RSD			0.016

described below. From Table 1, "analogue bleed" analysis yielded a $-CH_2Cl$ concentration value of 0.68 mmol/g versus the vendor supplied value of 0.7 mmol/g. Void of additional supporting methodology for $-CH_2Cl$ determination and/or reliable on-bead $-CH_2Cl$ standards, the data are accepted on the rationality of the methodology and the correlation of the data with the manufacturer's specification for bead loading. The relative precision of this analysis is outstanding in the overall scope of the experiment.

Automation Procedure

Using the Nicolet Macros Basic macro programming system, a macro was written to automate the entire process of data collection, manipulation, calibration, and reporting. Subsequent analyses were performed by collecting a spectral background as described above, mounting a bead in the cell, and initiating a macro with the appropriate calibration parameters. Ligand content in mmol/g are automatically reported to the screen. The entire process including mounting takes less than 5 min.

"Analogue Bleed" Limitations

Although "analogue bleed" experiments have proven very useful in our laboratory, the technique does possess inherent limitations of which the experimenter needs to be cognizant of throughout the application.

(1) Single Bead Experiment. Necessitated by design, the analogue bleed experiment is a single bead experiment requiring an infrared microscope and flow cell. In single bead experiments, representative sampling issues are always a concern.

(2) Large Quantities of Standards. For high molecular weight analogues, hundred milligram quantities of analogue compound may be required for each standard in order to obtain the 0.25 mL volume solutions required by the flow cell/pump system.

(3) "Analogue Bleed" Experiments Are Limited to Infrared Spectroscopy. Analogue bleed is only applicable to infrared flow cell experiments conducted in the absorbance mode. Although infrared is a very powerful technique, introduction of more exotic resins (i.e., TentaGel) can produce severe spectral interference throughout the infrared spectrum. While spectral background produced by these resins presents little challenge to the selectivity of Raman experiments, Raman spectroscopy is an emission experiment which does not yield a valid path length term in the sense required by the analogue bleed calculations and therefore cannot be used.

4. Density. The density term, while extremely important, is indirectly estimated through isobuoyancy tests.

Despite these concerns, the "analogue bleed" method has repeatedly demonstrated utility and remains a useful analytical tool. The "dual analogue" methodology presented below does not supersede "analogue bleed" techniques; it is, however, a very powerful complementary tool.

Part 2: The "Dual Analogue" Quantitation Method

"Dual analogue" quantitation is based upon simulation of base resin to pendant functional group band ratios, using solution phase analogue standards for both the resin and pendant group. Two advantages of the "dual analogue" over "analogue bleed" experiments are elimination of bead density from the quantitation calculations and that it can be implemented using either infrared or Raman spectroscopy with its associated benefits.

In an experiment conducted for quantitation of the previous chloromethylstyrene resin, 0.7 mmol/g chloromethylstyrene was simulated using mixtures of benzyl chloride and un-cross-linked polystyrene dissolved in CCl_4. Specifically, standards were prepared by weighing 0.0, 0.05, 0.07, and 0.09 mmol of benzyl chloride into discrete vials. Sufficient un-cross-linked polystyrene was then added to each vial to achieve a total weight of 100 mg. The mixtures were then dissolved in minimal CCl_4 to provide homogeneous benzyl chloride/styrene solutions. The CH_2Cl vs polystyrene band ratios obtained from these solutions provided a calibration against which spectra of CCl_4 swollen, candidate beads were quantified. Although quantities of CCl_4 do not enter the calculation equations, use of minimal solvent is important since signal-to-noise attenuates with reduced analyte concentration. Spectral differences due to cross-linking and molecular weight variations between the styrene standards and the bead itself are effectively nonexistent for methylene bands resulting from the polystyrene backbone.

"Dual Analogue" Quantitation of Chloromethylstyrene by Infrared Spectroscopy

Initial quantitation studies were conducted for a 0.7 mmol/g chloromethylstyrene resin.[10] Table 2 presents the actual weights used for generation of 0, 0.5, 0.7, and 0.9 mmol/g benzyl chloride/polystyrene[6] "dual analogue" standards.

Table 2. Dual Analogue Standards

nominal standard (mmol/g)	0.5	0.7	0.9
benzyl chloride (g)	0.0062	0.0090	0.011
polystyrene (g)	0.0922	0.0938	0.0883
mmol $(-CHCl)/g(-CH_2Cl$ + polystyrene)	0.498	0.692	0.875
benzyl chloride band frequency (cm^{-1})	1264	1264	1264
benzyl chloride baseline frequency (cm^{-1})	1304−1218	1304−1218	1304−1218

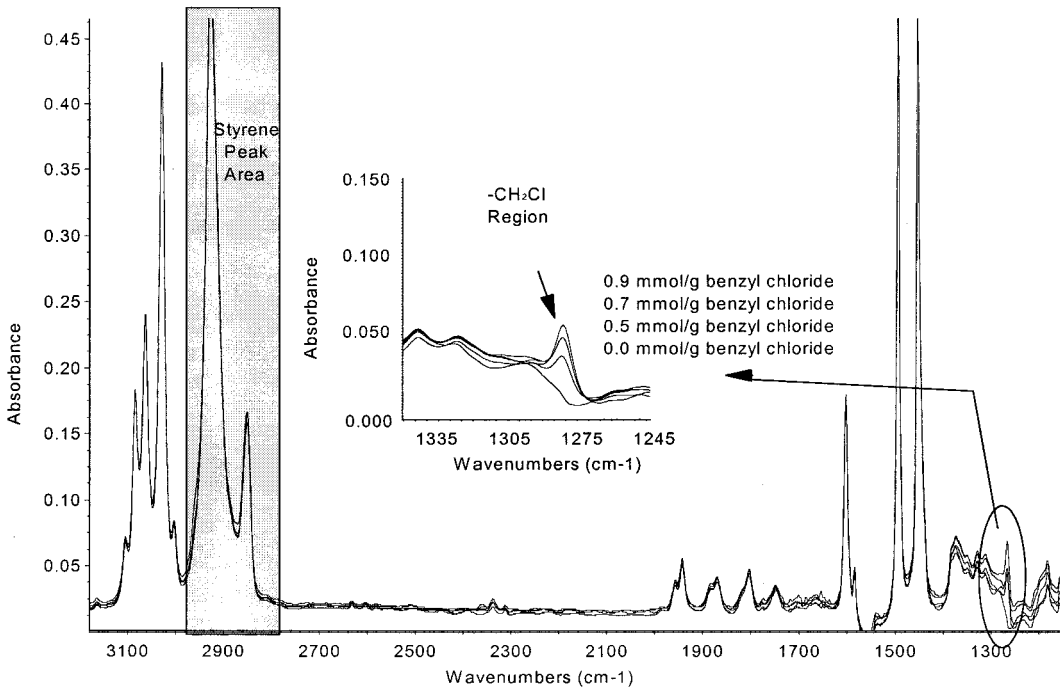

Figure 3. Chloromethylstyrene calibration infrared spectra.

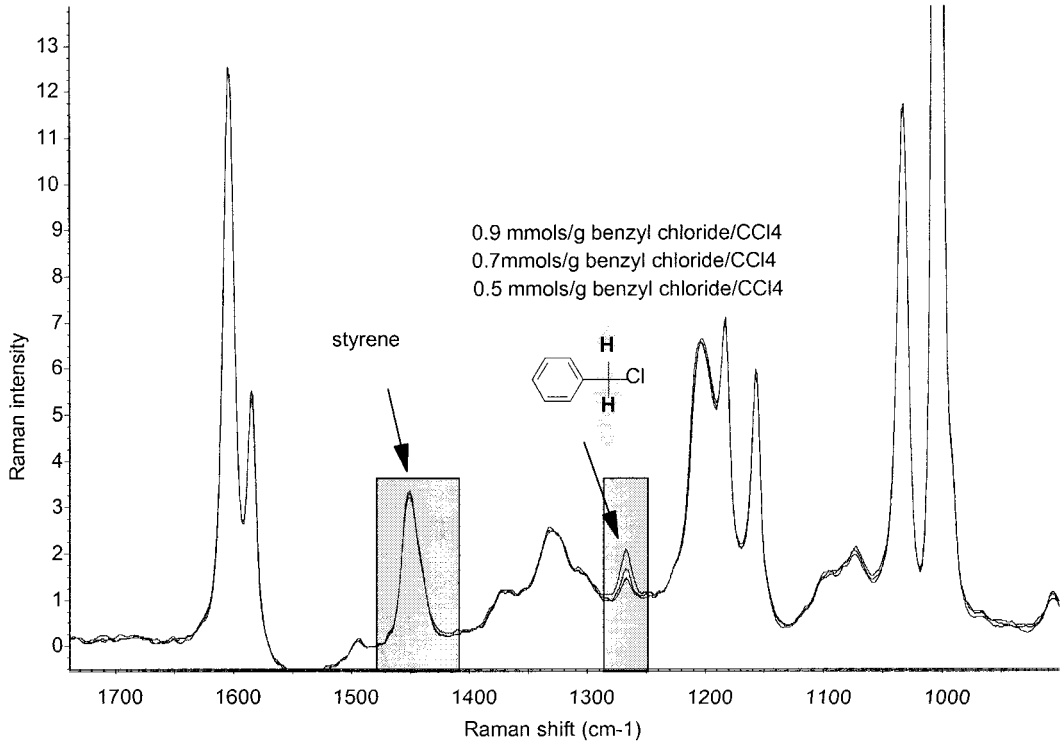

Figure 4. Raman spectral data.

Infrared quantitation was conducted by mounting a bead in the diamond flow cell. The bead was then swollen in CCl_4 to match the bead matrix with that of the synthetic standards, and the spectrum was collected and stored to disk. The infrared microscope was then focused on a portion of the cell in which no bead was present, standards were introduced, spectra were collected, and data were stored accordingly. Spectra are presented in Figure 3.

The band area of the 2926−2733 cm^{-1} region (corresponding to methylene functionality of polystyrene) was measured for each spectrum. A blank spectrum (polystyrene in CCl_4) was subtracted from each of the standard and sample spectra to provide a cleaner spectral baseline. Peak heights of the 1264 cm^{-1} bands (baseline corrected at 1304 and 1218 cm^{-1}) were measured from each resultant spectrum.

Regression analysis of the standard spectral data in Table 2 provided a slope of 132.2, a *y*-intercept of −0.0117, and an R^2 correlation of 0.997. Finally, application of calibration data to the quantitation of the chloromethylstyrene sample

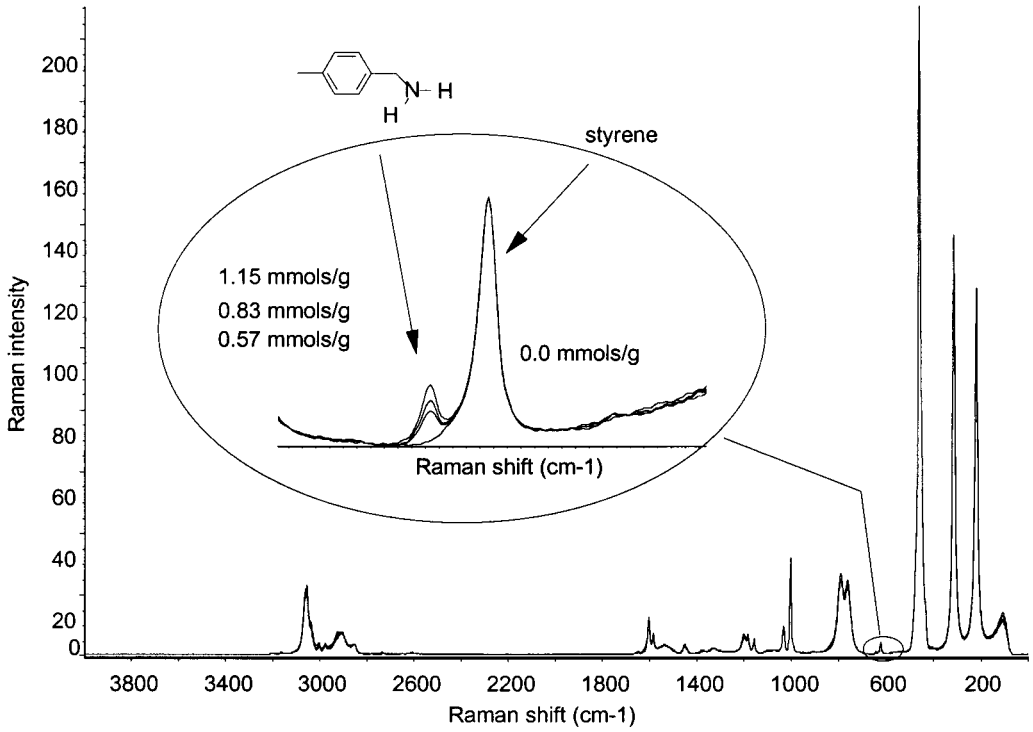

Figure 5. *p*-Methylbenzylamine Raman quantitation data.

yielded a value of 0.70 mmol/g. This value is identical to that specified by the manufacturer.

"Dual Analogue" Quantitation of Chloromethylstyrene by Raman Spectroscopy

In the Raman experiment, Table 2 standards were placed in NMR tubes and analyzed directly. Aminomethylstyrene beads were also placed in a separate NMR tube, and a few drops of CCl_4 were added to permit swelling prior to spectral acquisition. After spectral acquisition, the CCl_4 spectrum was subtracted from each standard to provide the Raman spectral data presented in Figure 4. Regression analysis for the Raman experiment yielded an R^2 correlation of 0.996, a value virtually identical to that of the infrared based regression. Application of the calibration regression to quantitation of the chloromethylstyrene sample yielded a value of 0.71 mmol/g compared to the vendor supplied value of 0.7 mmol/g.

Quantitation of Aminomethylstyrene by "Dual Analogue" Raman Spectroscopy

Aminomethylstyrene resin[7] is another well-characterized commercially available resin with which to test the "dual analogue" methodology. Characterization of this resin further illustrates the utility of the "dual analogue" Raman experiment for samples in which there are no suitable bands for infrared quantitation.

In these experiments, *p*-methylbenzylamine was used as the functional analogue. Due to price and availability, Aldrich PN 33,165-1 with a bimodal molecular weight distribution (ca. 200 000 and 4000) was selected as the un-cross-linked styrene reference analogue. Variations in molecular weight of the un-cross-linked styrene analogue demonstrated no measurable difference in the Raman spectrum. In this

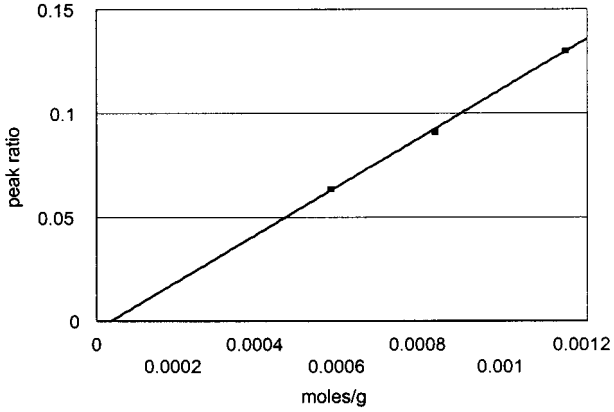

Figure 6. *p*-Methylbenzylamine Raman calibration data.

experiment, standards were prepared by adding *p*-methylbenzylamine to prediluted solutions of styrene in CCl_4 and running immediately.

Figure 5 presents the Raman spectra collected from 0.0, 0.57, 0.83, and 1.15 mmol/g standards. The inset illustrates the very subtle 643 cm^{-1} Raman band of *p*-methylbenzylamine within the comprehensive Raman spectrum. This band is not visible in the full scale expansion plot, further reinforcing that great care must be taken to prevent underutilization of the information available in defining a quantifiable spectral feature. The adjacent 622 cm^{-1} band was used as the polystyrene reference.

Figure 6 illustrates calibration data obtained for the *p*-methylbenzylamine/polystyrene standards. Regression calculations yield an R^2 correlation coefficient of 0.999. The negative *y*-intercept obtained for the calibration results from the concave curvature of the polystyrene spectral baseline in the *p*-methylbenzylamine analytical region. This intercept was included in the calibration calculations. The calculated

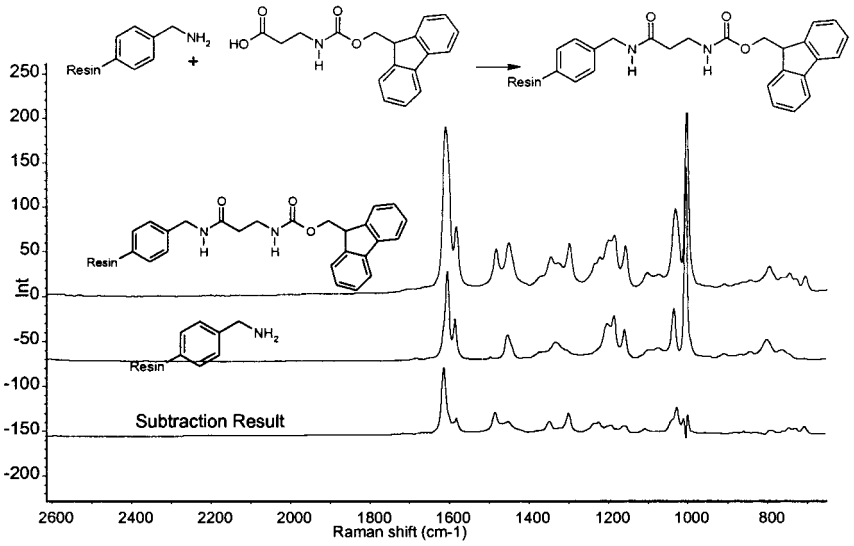

Figure 7. Aminomethylstyrene/Fmoc-β-Ala coupling spectra.

aminomethylstyrene loading for the NovaBiochem resin was 0.71 mmol/g versus the 0.75 mmol/g vendor supplied value.

Analogous to the "analogue bleed" experiment, Nicolet instrumentation permits insertion of all required calibration parameters into a macro program which entirely automates the "dual analogue" quantitation process. Once calibrated, quantitation consists of collecting a spectrum of beads swollen in CCl_4 and running the macro. Millimoles per gram are directly printed to the comment field of the spectrum. Since the calibration is based on internal band ratios, no recalibration is required for future experiments using this analogue.

To further illustrate the utility of this technique for quantitation of subsequent reaction products, the previous aminomethylstyrene resin was reacted with 0.1 M Fmoc-β-Ala-OH for 2 h at 20 °C in DMF. HOBt (0.5 M) and diisopropylcarbodiimimide (0.3 M) were added as coupling agents. Fmoc-β-Ala-OH was obtained from Bachem; all other reagents were from Aldrich.

Figure 7 illustrates the initial and reaction product spectra, along with the scaled difference spectrum obtained by spectral subtraction. Dual analogue quantitation of the reaction product was obtained using 0.6, 0.8, and 1.0 mmol/g Fmoc-β-Ala-OH/polystyrene analogue mixtures. Due to the limited solubility of Fmoc-β-Ala-OH in CCl_4, DMF was used as the diluent solvent.

In this case, quantitation was performed using the vendor supplied quantitation package, TurboQuant Analyst. Classical least squares regression analysis was used in conjunction with the first derivative spectra from the 1314.3 to 1275.2 cm^{-1} region. Internal path length standardization was performed using the 637.47 to 606.58 cm^{-1} baseline corrected polystyrene band area. The resultant Fmoc concentration was 0.72 mmol/g of starting resin. Again, these results are in excellent agreement with the previous aminomethylpolystyrene styrene values. Through implementation of the TurboQuant analysis procedure, the need for development of manual, or macro based, peak measurement and regression analysis was eliminated.

Conclusion

Quantitation methodologies via analogue simulation methods provide a simple yet robust technology for quantitation of an extremely wide range of pendant functional groups throughout polystyrene based solid phase syntheses. With no apparent statistical difference between infrared and Raman results, Raman spectroscopy presents itself as a very useful method of quantitation. The Raman method requires little sample preparation, permits multiple bead sampling, requires greatly reduced quantities of standard relative to the infrared flow experiments, and can be conducted using standard NMR tube sampling, thus eliminating the need for infrared compatible optics. These technologies break down previous barriers to on-bead quantitation through what has developed into trivial experiments. Finally, the technology has broad application across a wide range of functional groups.

References and Notes

(1) Pivonka, D. E.; Russell, K.; Gero, T. *Appl. Spectrosc.* **1996**, *50*, 1471−1478.
(2) Yan, B.; Fell, J. B.; Kumaravel, G. *J. Org. Chem.* **1996**, *61*, 7467−7472.
(3) Russell, K.; Cole, D. C.; McLaren, F. M.; Pivonka, D. E. *J. Am. Chem. Soc.* **1996**, *118*, 7941−7945.
(4) Advanced ChemTech, chloromethylstyrene, 0.7 mmol/g, 200−400 mesh, 1% DVB, NB# 8336-83-0.
(5) Pivonka, D. E.; Palmer, D. L. *J. Comb. Chem.*, **1999**, *1*, 294−296.
(6) DuPont Instruments, polystyrene standard, PS4, MW = 9000, PN 850673-904.
(7) NovaBiochem, aminomethylstyrene, 0.75 mmol/g, 200−400 mesh, 1% DVB.

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