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Chemiluminescent Nitrogen Detection for HPLC: An Important New Tool in Organic Analytical Chemistry

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Abstract: Chemiluminescent nitrogen detection for HPLC is an important new high throughput technique for measuring yields and purities in synthetic organic chemistry. This destructive detector measures the total nitrogen content of a chromatographic peak, can be calibrated with any nitrogen containing compound, and thus allows quantitative analysis when authentic reference standards are not available. The application to solid phase synthesis is illustrated with a diketopiperazine synthesis.

The widespread application of combinatorial chemistry techniques[1] leads to increased pressure on all aspects of organic analytical chemistry.[2] With new automated instrumental methods of organic synthesis, especially those based on solid phase synthesis (SPS), a single chemist can prepare tens or hundreds of organic compounds in a day. New analytical methods are needed to follow reactions and to allow for the routine, high speed measurement of structure, yield and purity.

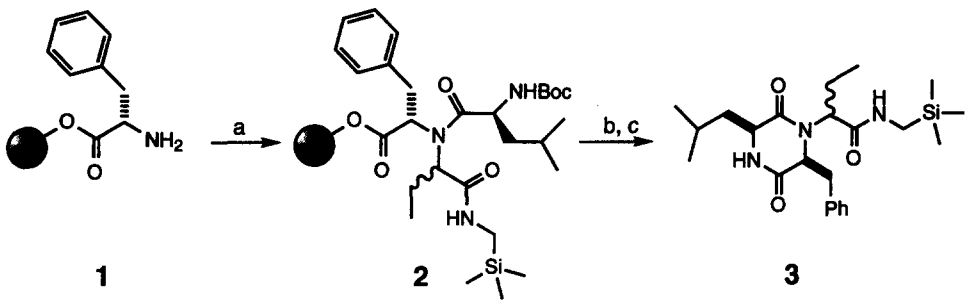
Measurement of yield is a critical part of successful organic synthesis. In traditional solution phase organic chemistry using a weighed quantity of starting material, gravimetric yield is calculated after purification of the product. In SPS, a starting material is attached to a solid support via a cleavable linker. The solid can be weighed, and the loading, or amount of ligand per unit of solid support, is known (but low, 10% or less). After reaction, the product can be cleaved but gravimetric yield measurement is difficult for convenient, small scale reactions. The rigorous SPS chemist will scale up a few representative reactions solely for the purpose of documenting reaction yields.[3] But gravimetric methods require the laborious purification of the product; it would be preferable to have a convenient, high throughput method to measure the yields of parallel reactions.

Combustion elemental analysis is the gold standard for establishing the purity of organic compounds, especially for publication of new structures.[4] In the working chemistry laboratory, purity is usually estimated by subtraction techniques[5] using ^{1}H NMR, GC/FID or HPLC/UV making the assumption that all of the impurities are detectable. Because of its relative speed and the simplicity of data interpretation, HPLC/UV has become the analytical tool of choice for purity estimation in the modern organic synthesis laboratory. But UV cannot detect many salts, aliphatic reagents and solvents, and the unpredictability of UV response makes it a questionable choice for relative quantitative analysis. Relative peak heights in electrospray mass spectra have been proposed for purity assessment in peptide synthesis.[6] A new development is the evaporative light scattering detector[7] which is more universal than a UV detector; it detects all but volatile compounds. However the response of this detector is not linear nor is it predictable.[8]

We now propose the use of the chemiluminescent nitrogen detector (CLND) as a unique method for directly measuring the yield and purity of solid and solution phase reactions. This detector, which oxidizes nitrogen compounds to NO, converts the NO to excited NO_2 by reaction with ozone and detects the emitted light in a photomultiplier, has recently been developed for use with HPLC flows up to 300 μL/min. The application of this detector to peptide mixture analysis has been described.[9] All nitrogen compounds tested to date give equivalent response/mole of N.[10] A nitrogen detector is a remarkably universal detector for

pharmaceuticals. Of the compounds in the MDDR,[11] a database of 67,483 developmental and marketed drugs, 91% contain nitrogen. The ACD, a database of 177,700 compounds in general commerce, has a 65% rate of compounds containing nitrogen. Many of the scaffolds which Affymax has described for SPS of combinatorial libraries contain nitrogen.[12]

As an example application of the CLND for measuring purity and yield in a SPS reaction, the diketopiperazine structure **3** can be prepared from resin-bound Ugi reaction product **2** by mild acid or base catalyzed cleavage.[13] We have developed this chemistry as a general method for synthesizing diketopiperazines in very high purity; only the desired product is released from the solid support. In a typical case, resin bound intermediate **2** was prepared from amino acid on Pam resin **1** of loading 0.4 mmol/g. A 150 mg sample of **2** (nominally 60 μmoles) was subjected to the reaction sequence[13] and the product was diluted with methanol[14] to 1 mL final volume for a nominal concentration of 60 mM.



a. propanal, BocLeu, trimethylsilylmethylisocyanide, MeOH b. 50% TFA/DCM c. toluene, 4% TEA

To validate the HPLC/CLND concept, an authentic standard of **3** was synthesized on a larger scale, purified to homogeneity and characterized by the usual methods. This standard was then used as HPLC/UV external reference standard to measure the concentration of **3** in the unknown reaction mixture.[15] Alternatively, the concentration was determined by HPLC/CLND using the reference standard or by HPLC/CLND using an external standard of benzamide.[16] Table 1 compares the results obtained by the three methods. The three quantitative methods yield essentially identical results.

Table 1. Comparison of Quantitation Methods

Method	Reference Standard	Measured Concentration(mM)	Calculated Yield(%)
external standard HPLC/UV	pure **3**	48.1	80.2
external standard HPLC/CLND	pure **3**	46.7	77.8
external standard HPLC/CLND	benzamide	47.6	79.3

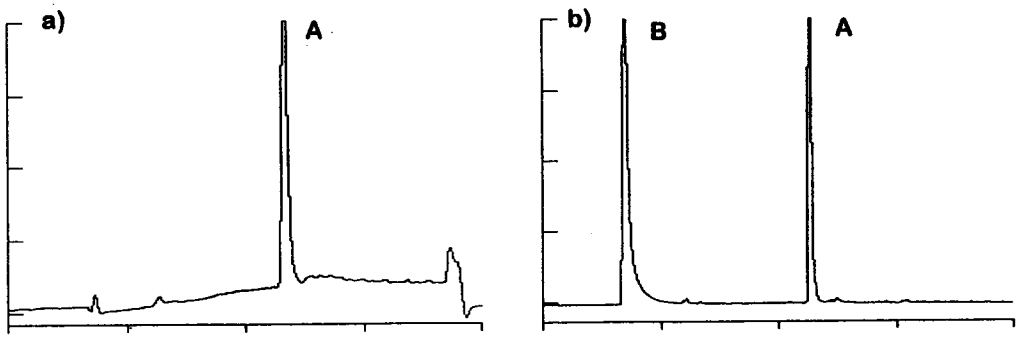


Fig. 1. HPLC chromatogram of crude **3** using a) UV detection and b) CLND detection

For direct purity estimation of a reaction mixture, CLND is complementary to UV. Many reaction byproducts or starting materials do not contain nitrogen; others lack a UV chromophore. In the present case the crude material looks to have only the desired component (Peak A), when observed by UV (Fig. 1a). But CLND shows the presence of a major impurity (Peak B), and the product peak of Fig 1b only represents 20.5 mg when the total crude sample weighed 100 mg. The unretained impurity is triethylammonium trifluoroacetate.

After isolation, the CLND can also be used to do a quantitative destructive nitrogen analysis on a weighed sample, analogous to a combustion analysis, but with the added value that the chromatographic separation assures that the nitrogen is due only to the desired compound. In the case of the purified sample of **3**, we measured[13] a combustion nitrogen percentage of 9.73 which is 100% of the theoretical. The corresponding percentage by HPLC/CLND is 9.57% nitrogen and 98.5% of the theoretical.

We have demonstrated the measurement of yield as the premier application of HPLC/CLND quantitative analysis, but other applications are clear. In the early stages of a lead development project, weighable quantities of authentic pure samples of a compound are not available, and yet quantitative measurements such as IC_{50}, solubility or plasma stability need to be made. HPLC/CLND can be used to calibrate solutions made from sub-milligram synthetic samples. In the split/pool method of combinatorial synthesis, mixtures of compounds are made which are difficult to characterize. The HPLC/CLND of a nominally equimolar pool (based on nitrogen) should yield equal sized peaks. The CLND will be especially useful in conjunction with splitting to a mass spectrometer. The electrospray mass spectrometer is likely to detect many of those peaks which do not contain nitrogen and offer structural proof for all components as well as confirm the purity of each peak. HPLC with chemiluminescent nitrogen detection is an important new technique to add to the arsenal of the organic analytical laboratory.

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14. A limitation of the CLND is that CH_3CN cannot be used in the mobile phase.
15. Chromatography was done under standard conditions, as described previously.[9a]
16. Benzamide was chosen for its chromatographic properties and its availability in 99.9% purity(Aldrich). Linearity over the range of 0-.598 μg of N injected was demonstrated.

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