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# 2,5-Dimethylfuran (DMFu): An Internal Standard for the "Traceless" Quantitation of Unknown Samples via $^{1}$H NMR†

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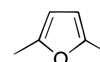
The use of 2,5-dimethylfuran (DMFu) as an internal standard for the quantitation of unknown samples by $^{1}$H NMR is described. DMFu was selected based upon its low chemical reactivity, good solubility in standard NMR solvents, appropriate volatility (bp 92 °C), and the chemical shifts of its two singlets (5.80 and 2.20 ppm). In particular, the widely separated singlets of DMFu offer two advantages over more traditional "single peak" internal standards (e.g., hexamethyldisiloxane): (1) they reduce the possibility for overlap between analyte and internal standard signals; and (2) they enable the chemist to assess the accuracy of integration (and ergo, the quantitation) by calculating the ratio of the two singlets and comparing the observed ratio to the theoretical value. Our experiments have shown that the precision of the ratio of DMFu singlets strongly correlates to the precision of sample quantitation, an observation which greatly simplifies the assessment of the quality of the quantitation. Utilizing this technique, we have developed straightforward conditions for $^{1}$H NMR experiments which consistently provide quantitative results with less than 5.0% error.

The widespread application of parallel synthesis techniques for the generation of solid[1] and solution phase[2] chemical libraries has not only produced dramatic increases in synthetic efficiency but also underscored the paucity of high throughput methods for sample quantitation.[3] In particular, the quantitation of crude samples in vials or 96-well plates has been problematic because traditional methods such as gravimetric analysis do not readily afford reliable results. Quantitation is critically important in medicinal chemistry, where the biological activity of a sample is directly related to the quantity of material tested; in the absence of good quantitation, it is difficult to have confidence in the structure−activity relationships that are the foundation of drug discovery. As a result of our own efforts in the parallel synthesis of biologically active compounds, we became interested in identifying rapid, high throughput methods for the quantitation of library samples.

In general, qualitative analytical techniques have kept pace with the throughput gains achieved by parallel synthesis through the use of high pressure liquid chromatography in conjunction with UV spectroscopy and mass spectrometry (LC/MS).[4] While LC/MS facilitates the rapid *qualitative* analysis of unknown samples, it is not conducive to *quantitation*. Recently, sample quantitation using an LC/MS equipped with a chemiluminescent nitrogen detector (CLND) was reported,[5] but the method requires each component to contain at least one nitrogen atom and the operator must know the exact number of nitrogens per compound. Despite these limitations, CLND is a useful technique because of its low sample requirements and compatibility with existing HPLC equipment. Quantitation of samples using $^{1}$H NMR and an internal standard is a well established and reliable method,[6] but sample handling and quantity requirements have been problematic until recently. The advent of high throughput flow injection NMR,[7] in which the analyte is directly sampled from a vial or plate into a flow cell, prompted us to revisit the use of an internal standard for quantitation via $^{1}$H NMR.

Virtually any proton-containing organic molecule could serve as an internal standard for quantitation via $^{1}$H NMR. From our perspective, the ideal internal standard would have the following chemical properties: (1) low chemical reactivity/high stability to long-term storage; (2) adequate solubility in typical NMR solvents such as methanol, dimethyl sulfoxide, and chloroform; (3) appropriate volatility (boiling point between 60 and 100 °C) for removal on standard evaporatory equipment; (4) a simple $^{1}$H NMR spectrum (preferably two singlets—vide infra) affording peaks with a low probability of overlap with the analyte. Recently, hexamethyldisiloxane (HMDS) has been reported as an $^{1}$H NMR internal standard for the quantitation of chemical libraries.[8] We have found HMDS to be a useful internal standard, but it does not meet the aforementioned "two singlets" requirement. After a careful search, we identified 2,5-dimethylfuran (**1**) as a potentially useful internal standard




**1**

for $^{1}$H NMR quantitation. In concordance with our specifications, 2,5-dimethylfuran (which we abbreviate as DMFu) has

† Dedicated to Professor Satoru Masamune on the occasion of his 70th birthday.

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**Table 1.** Effect of Relaxation Time on the DMFu Integration Ratio

| entry | relaxation time, s | DMFu ratio[a] |
|---|---|---|
| 1 | 0.1 | 3.7 |
| 2 | 1.0 | 3.4 |
| 3 | 10.0 | 3.1 |
| 4 | 30.0 | 3.0 |

[a] Ratio of DMFu peak areas at 2.2 and 5.8 ppm.

a boiling point of 92 °C and an $^1$H NMR spectrum comprising singlets at 2.2 and 5.8 ppm. In particular, the singlet at 5.8 ppm occurs in a region of the NMR spectrum that is generally devoid of signal,[9] yet is close to the aromatic region which is usually where the most convenient sample comparison peaks are located. This proximity provides for easier processing and display of expanded spectral regions for quantitation. Having selected DMFu as a removable internal standard for quantitation via $^1$H NMR, we conducted a series of experiments to demonstrate its utility.

Quantitation of samples via NMR often requires time and expertise that the average organic chemist does not possess. Because we were interested in developing a quantitation method which could be used routinely by a bench chemist, we attempted to simplify the procedure by making the following assumption: *insufficient relaxation time is a common and significant source of experimental error in NMR quantitation*. Determining sufficient relaxation time for the analyte can be estimated by simply comparing the areas of integrated peaks, where the area of each peak should be an integer multiple of the average peak area for a single proton. Obviously, multiple peaks are required to carry out this straightforward analysis. In cases where an internal standard gives rise to only a single peak (e.g., HMDS), the longitudinal relaxation time ($T_1$) must be measured with an inversion−recovery experiment, a nonroutine procedure for most synthetic chemists. In this respect, DMFu has a significant advantage because its two singlets serve as an "internal relaxation standard" and an appropriate $T_1$ can be easily estimated.[10] Indeed, as described in Table 1, the ratio of the peak areas of the DMFu singlets at 2.2 and 5.8 ppm (a ratio which should equal 3.0) is quite sensitive to the relaxation time. In particular, increasing the relaxation time from 0.1 to 10.0 s has a pronounced effect on the ratio. Although a relaxation time of 30.0 s provided the most precise ratio, time considerations prompted us to select 10.0 s as the relaxation time for further quantitation experiments. Because the $T_1$ of a small molecule (MW < 1 kDa) generally decreases with increasing molecular weight,[6b] the low molecular weight of DMFu (MW = 96.13 g/mol) ensures that most samples will have a high molecular weight and thus a shorter $T_1$. Consequently, the DMFu integration ratio can also serve as an indicator of sufficient relaxation for the analyte, assuming its molecular weight exceeds that of DMFu.

Having established a strong relationship between the DMFu integration ratio and relaxation time, we next investigated the effect of relaxation time on the quantitation of $\beta$-naphthyl-L-phenylalanine (**2**) at two concentrations. The samples were quantitated by first calculating the average peak area for a single proton in **2** and in DMFu, and then applying eq 1 to calculate the concentration of **2**. As shown in Table 2, the quantitation error is directly related to relaxation time.

**Table 2.** Effect of Relaxation Time on the Quantitation of Analyte **2** Using 5.0 mM DMFu as an Internal Standard

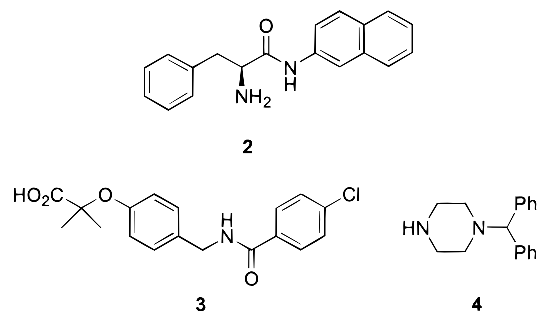
| entry | known [analyte], mM | relaxation time, s | DMFu ratio[a] | calculated [analyte], mM[b] | quantitation error, % |
|---|---|---|---|---|---|
| 1 | 15.2 | 0.1 | 3.3 | 18.1 | 19.4 |
| 2 | 15.2 | 10.0 | 3.1 | 15.4 | 1.3 |
| 3 | 76.2 | 0.1 | 3.3 | 97.1 | 27.4 |
| 4 | 76.2 | 10.0 | 3.0 | 76.5 | 1.0 |

[a] Ratio of DMFu peak areas at 2.2 and 5.8 ppm. [b] Calculated using eq 1.

**Table 3.** Comparison between Known and Calculated Analyte Concentrations Using 5.0 mM DMFu as an Internal Standard

| entry | analyte | known [analyte], mM | calculated [analyte], mM[a] | quantitation error, % |
|---|---|---|---|---|
| 1 | **2** | 0.86 | 0.85 | 0.7 |
| 2 | **3** | 4.14 | 4.23 | 2.3 |
| 3 | **2** | 13.09 | 12.38 | 5.4 |
| 4 | **4** | 22.98 | 22.06 | 4.0 |
| 5 | **3** | 28.47 | 28.42 | 0.2 |
| 6 | **2** | 70.26 | 72.63 | 3.4 |

[a] Calculated using eq 1.




$$[\text{analyte}] = [\text{DMFu}] \times \frac{\text{average_peak_area}_{\text{analyte}}}{\text{average_peak_area}_{\text{DMFu}}} \quad (1)$$

Entries 1 and 3 utilized a 0.1 s relaxation time and grossly overestimate the concentration of **2** in the solution. It is noteworthy that the large variance from the ideal DMFu ratio in entries 1 and 3 correlates strongly with the quantitation error. By contrast, entries 2 and 4 used a 10.0 s relaxation time and both the DMFu ratio and the calculated concentration of **2** are very accurate. These experiments support our assumptions that relaxation time has a significant effect on the quantitation error and that the DMFu ratio can be used as an "indicator" of quantitation accuracy.

To measure the quantitation accuracy in some real world situations, we conducted a series of experiments in which both the composition and concentration of the analyte were varied, while holding the DMFu concentration constant. As summarized in Table 3, the quantitation error never exceeded 5.5% over a range of concentrations and there appears to be no relationship between the quantitation error and either the identity or concentration of the analyte. From a practical

standpoint, we found it difficult to quantitate samples which were present in concentrations lower than 0.5 mM because the signal-to-noise ratio was too low. All of the samples in Table 3 were concentrated in vacuo and redissolved in MeOH-$d_4$ (sans DMFu) to ensure that DMFu could be removed in this manner. In all cases, no trace of DMFu was observed by NMR. It is also noteworthy that DMFu has a low extinction coefficient at 254 nm, and so it is not observable under typical HPLC conditions.

In summary, we have described the use of DMFu as a practical internal standard for the quantitation of unknown samples via $^1$H NMR. While this method is particularly relevant for the quantitation of final products prior to screening in a biological assay, it should be noted that yield information is useful at every stage of a chemical synthesis. In addition, the volatility of DMFu makes possible the quantitation and subsequent recovery of a sample which, following in vacuo removal of DMFu, can be directly used in an assay. Our studies which describe the use of DMFu and flow-injection NMR for the high throughput quantitation and recovery of samples will be reported in due course.

## Experimental Section

All reagents and solvents were obtained from commercial sources and used without further purification. $^1$H NMR experiments were conducted on a Varian Unity Inova 500 MHz spectrometer and processed using the Varian VNMR (version 6.1b) software.

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**Supporting Information Available.** NMR spectra for compounds **1**−**4** as reported in Tables 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Clearly, some peak overlap is inevitable, but a careful examination of "general regions of chemical shifts" (as found in Gordon, A. J.; Ford, R. A *The Chemist's Companion*; John Wiley and Sons: New York, 1972; pp 252−255) suggested to us that the 5.5−6.0 ppm region of the NMR spectrum offers the best compromise between signal overlap and proximity to the aromatic region of the spectrum.

(10) We measured the longest $T_1$ for DMFu to be 6.2 s.

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