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Fermentanomics: Monitoring Mammalian Cell Cultures with NMR Spectroscopy

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Abstract: As the number of therapeutic proteins produced by mammalian cell cultures in the pharmaceutical industry continues to increase, the need to improve productivity and ensure consistent product quality during process development activities becomes more significant. Rational medium design is known to improve cell culture performance, but an understanding of nutrient consumption and metabolite accumulation within the medium is required. To this end, we have developed a technique for using 1D ^{1}H NMR to quantitate nonprotein feed components and metabolites in mammalian cell cultures. We refer to the methodology as "Fermentanomics" to differentiate it from standard metabolomics. The method was found to generate spectra with excellent water suppression, signal-to-noise, and resolution. More importantly, nutrient consumption and metabolite accumulation was readily observed. In total, 50 media components have been identified and quantitated. The application of Fermentanomics to the optimization of a proprietary CHO basal medium yielded valuable insight regarding the nutrient levels needed to maintain productivity. While the focus here is on the extracellular milieu of CHO cell cultures, this methodology is generally applicable to quantitating intracellular concentrations and can be extended to other mammalian cell lines, as well as platforms such as yeasts, fungi, and *Escherichia coli*.

The number of therapeutic proteins produced by mammalian cell cultures in the pharmaceutical industry continues to increase. Improved protein productivity will not only lower production costs but also increase manufacturing flexibility. Therefore, improving yield and ensuring consistent product quality are high priorities during process development activities for protein therapeutics. One way to improve cell culture performance is through rational medium design, but this requires an understanding of nutrient consumption and metabolite accumulation within the medium.[1−4] Traditional HPLC analysis of amino acids[5] and mass spectrometry-based profiling[6−8] are costly and require significant sample preparation. Consequently, the only entities regularly monitored are glucose, glutamine/glutamate, lactate, and ammonium.

To this end, we have developed a method for the measurement of cell culture components with Nuclear Magnetic Resonance (NMR) spectroscopy. NMR is widely known to be nondestructive and noninvasive, but there are three additional characteristics that make it particularly suitable for this application. First, unlike mass-spectrometry-based methods, NMR does not require excessive sample manipulation to remove matrix material prior to analysis; thus, the risk of altering analyte concentrations and losing volatile compounds is avoided while saving analyst time. Second, it uniformly observes all organic species in solution without requiring a chemical tag like those used for traditional HPLC amino acid analysis. Finally, it provides detailed structural information on metabolites as they appear so that identification is possible without further experimentation. These advantages have led to many examples of using ^{1}H NMR to study biological matrices, such as body fluids (aka metabolomics) and plant cells.[9−13] Nevertheless, the application of ^{1}H NMR to mammalian cell cultures has not been reported, although ^{31}P and ^{13}C NMR have been used to monitor cellular physiology and metabolic pathways.[14,15] Proton NMR is more suitable for our purposes because very few nutrients contain phosphorus and natural-abundant ^{13}C lacks the ability for high-throughput analysis due to its low sensitivity. In this communication, we define our technique for using 1D ^{1}H NMR to rapidly quantitate nonprotein feed components and metabolites in mammalian cell cultures and demonstrate its value with one case study. Since the focus is on cell culture fermentations, we refer to the described method as "Fermentanomics" to differentiate it from standard metabolomics.

A representative 1D ^{1}H NMR spectrum of spent medium from a mammalian cell culture is shown in Figure 1A. This particular sample is from day 12 of a 250-L, fed-batch culture of Chinese hamster ovary (CHO) cells engineered for an antibody currently in development. In keeping with one of the stated benefits of NMR, sample preparation was kept to a minimum. It consisted solely of diluting one part centrifuged spent medium with three parts of a D_2O solution containing 30 mM phosphate buffer (Medicago premixed pH 6.5 phosphate buffer, P/N 12-9184-10), ca. 0.4 mg/mL DSS-d_6, and 0.02% NaN_3. The D_2O improves solvent suppression, the buffer minimizes shifting of NMR peaks due to pH changes that occur during the course of culture, the DSS-d_6 serves as an internal standard for chemical shift referencing and quantitation, and the NaN_3 prevents bacterial contamination. The spectrum was recorded on a Varian 600 MHz NMR with a modified first-increment NOESY pulse sequence.[16] For the sake of sample throughput, we chose not to acquire the spectra under true quantitative conditions (i.e., recycle times >5 times the T_1 relaxation times), but rather to determine the response factors of each analyte to our conditions via standard addition experiments. As can been seen in Figure 1A, the method generates spectra with excellent water suppression, signal-to-noise, and resolution. The overall experiment time, including preacquisition routines, was 20 min, enabling high throughput on systems equipped with an autosampler.

A portion of the 1D ^{1}H NMR spectra of spent media from multiple days of a CHO cell culture are shown overlaid in Figure

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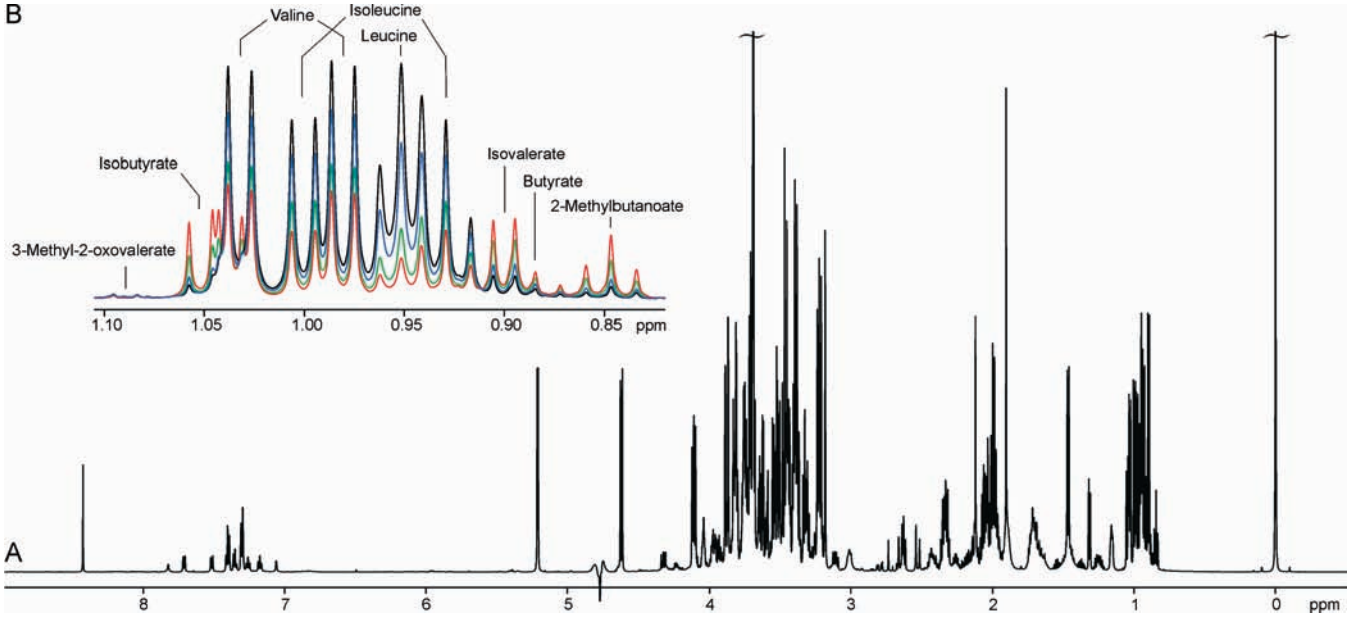

Figure 1. 1D ^{1}H NMR spectra of spent medium from CHO cell cultures on [A] day 12 of a 250-L, fed-batch culture and [B] days 4 (black), 6 (blue), 8 (green), and 10 (red) of a 50-mL batch culture. The spectra were acquired on a 600 MHz instrument equipped with a cold probe using 128 scans, a 4.5-s acquisition time, and a 3-s presaturation/relaxation delay for an overall experiment time of 16 min. The FIDs were zero-filled 2-fold, multiplied by an exponential window function and/or reference deconvoluted to bring DSS to 1.5 Hz fwhh, Fourier transformed, and spline baseline corrected.

1B. The samples were pulled from a 50-mL batch culture of CHO cells engineered for another antibody in development and prepared as described above. These representative spectra demonstrate the excellent resolution and chemical shift stability of the method. More importantly, the desired information—nutrient consumption and metabolite accumulation—is readily observed. The ^{1}H chemical shift assignments for common feed components and metabolites are detailed in Table S1 of the Supporting Information for our experimental conditions. The assignments were made using a combination of public databases, 1D NMR, 2D homo- and heteronuclear NMR experiments, and spiking of authentic purchased material. The metabolites that regularly appear in our CHO cultures represent the citric acid cycle, amino acid degradation, glycerolipid metabolism, and glycolysis pathways. The most surprising discovery was the accumulation of the volatile metabolites acetate and formate, which have not previously been reported for mammalian cell culture and would not have been detected by HPLC- or MS-based assays. We confirmed that these primarily arise metabolically and not merely by chemical degradation via 14-day stability experiments on the media in the absence of cells.

For routine sample analysis, the concentrations of each component is extracted from the NMR spectra of the spent media using Chenomx NMR Suite 5.1 or 6.0 (Chenomx, Inc., Edmonton AB, Canada) or MestreNova Global Spectral Deconvolution (GSD)[17] and plotted as a function of culture duration. The limits of quantitation (LOQ) for our experimental conditions are given in Table S1. With the exception of those species marked with a superscript, these values were determined by measuring the peak intensity for a resolved NMR resonance (or part of a resonance) of known concentration and extrapolating to a concentration where the signal/noise$_{peak-peak}$ ratio was 10. This was done on 1D spectra of mixtures containing >85% of the listed components to mimic the actual spectral complexity. The values were then multiplied by four to reflect the concentrations in the media and not the NMR tube. As is shown in the table, the method is capable of quantitating media components that are above a concentration of 3−200 μM, provided they have at least one resolved signal. Analytes whose resonances are completely overlapped can be quantitated from 2D

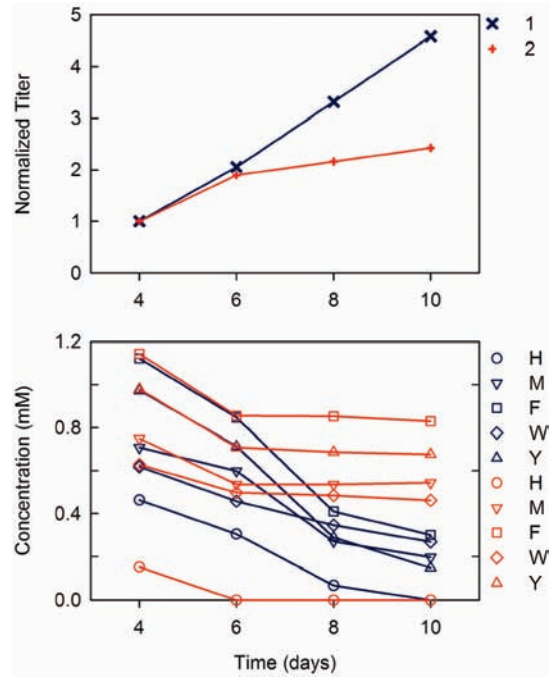

Figure 2. Titer (top) and extracellular concentrations of a subset of nutrients (bottom) for two CHO cell cultures.

spectra (TOCSY or HSQC) if so desired, but at the expense of sample throughput. To ensure that potential binding between the analytes and proteins/antibody were not adversely effecting the quantitation, we incubated samples in denaturing conditions (e.g., 6 M urea). No significant changes in analyte peak areas were observed.

The benefit of Fermentanomics has been realized during media optimization work. In one example, nutrient concentrations in a proprietary CHO basal medium were studied with the goal of increasing productivity. Figure 2 demonstrates the importance of one media component, histidine, by comparing a control culture

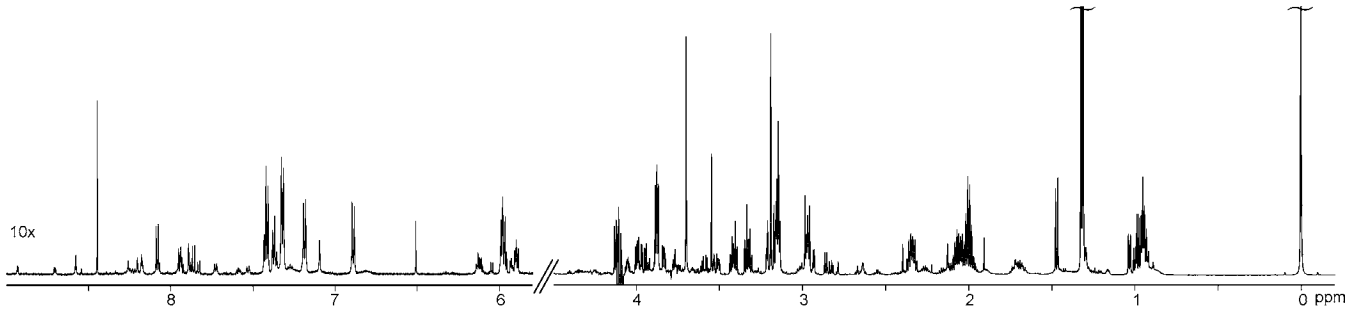

Figure 3. 1D ^{1}H NMR spectrum of 10 million washed and lysed CHO cells from a culture. The spectrum was acquired on a 600 MHz instrument equipped with a cold probe using a CPMG pulse sequence with presaturation. The parameters included 256 scans, a 4.5-s acquisition time, and a 5-s presaturation/relaxation delay for an overall experiment time of 41 min. The T_2-filter consisted of 200 repetitions of a 500-μs delay for an overall time of 100 ms. The FID was zero-filled twofold, multiplied by an exponential window function and/or reference deconvoluted to bring DSS to 1.5 Hz fwhh, Fourier transformed, and spline baseline corrected.

(**1**) to one where the concentration of histidine was reduced (**2**). There were no other differences in nutrient levels or process conditions between the two cultures. As can be seen from the top plot, **2** experienced a drop in productivity near day six even though the growth profiles for each culture were similar. A few of the extracellular concentrations that were measured by NMR are shown in the bottom plot. The data revealed that histidine was depleted from the medium near the time when productivity dropped, demonstrating the need to sustain an adequate concentration of this nutrient over the duration of the culture to maintain productivity. Moreover, the slowdown of cellular processes was evidenced by a drop in consumption of nearly all of the other nutrients.

In addition to measuring the concentration of components in the extracellular media, the method described here can easily be extended to the intracellular media as well. A representative 1D ^{1}H NMR of the intracellular milieu is shown in Figure 3. This sample was obtained by lysing approximately 10 million washed CHO cells via a quick freeze−thaw procedure (Supporting Information) and then mixing 1 part of the centrifuged supernatant with 3 parts of the previously described buffer. The spectrum was recorded using a Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence instead of the NOESY to reduce the broad background signals associated with matrix-related debris and proteins. While it is certainly more difficult to harvest the cells and prepare the samples, the information afforded is complementary to the extracellular concentrations and allows uptake rates to be calculated. This can be particularly beneficial for nutrients that appear to deplete quickly from the extracellular milieu.

In conclusion, we have described a rapid and robust NMR method for monitoring mammalian cell cultures. The spent medium analysis of nutrients and metabolites across varying process conditions enables improvements in productivity and potential product quality, in addition to enabling the development of a robust production process. This approach also provides significant analytical data that can be used to ensure culture consistency during scale-up from lab 5 L scale to 2000+ L manufacturing scale fermentations. While the focus here is on the extracellular and intracellular milieu of CHO cell cultures, this methodology is generally applicable to other mammalian cell lines, as well as platforms such as yeasts, fungi, and *Escherichia coli*. We have even successfully applied the technique to media containing hydrolysates from yeast and soy. Further details and more case studies will be presented in a subsequent report.

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Supporting Information Available: Table of chemical shift assignments and limits of quantitation. Description of quick freeze−thaw procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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