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ARTICLE *in* ANALYTICAL CHEMISTRY · FEBRUARY 2014

Impact Factor: 5.64 · DOI: 10.1021/ac4033966 · Source: PubMed

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Profiling Convoluted Single-Dimension Proton NMR Spectra: A Plackett–Burman Approach for Assessing Quantification Error of Metabolites in Complex Mixtures with Application to Cell Culture

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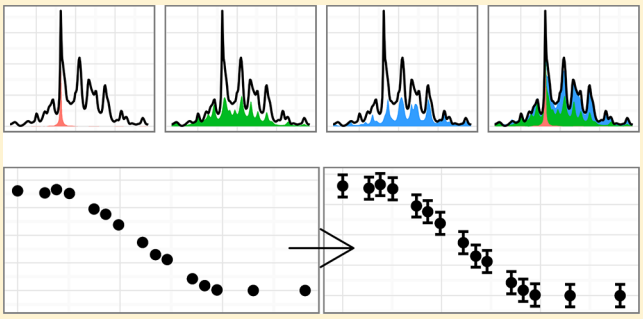
Supporting Information

ABSTRACT: Single-dimension hydrogen, or proton, nuclear magnetic resonance spectroscopy (1D-^{1}H NMR) has become an attractive option for characterizing the full range of components in complex mixtures of small molecular weight compounds due to its relative simplicity, speed, spectral reproducibility, and noninvasive sample preparation protocols compared to alternative methods. One challenge associated with this method is the overlap of NMR resonances leading to "convoluted" spectra. While this can be mitigated through "targeted profiling", there is still the possibility of increased quantification error. This work presents the application of a Plackett–Burman experimental design for the robust estimation of precision and accuracy of 1D-^{1}H NMR compound quantification in synthetic mixtures, with application to mammalian cell culture supernatant. A single, 20 sample experiment was able to provide a sufficient estimate of bias and variability at different metabolite concentrations. Two major sources of bias were identified: incorrect interpretation of singlet resonances and the quantification of resonances from protons in close proximity to labile protons. Furthermore, decreases in measurement accuracy and precision could be observed with decreasing concentration for a small fraction of the components as a result of their particular convolution patterns. Finally, the importance of a priori concentration estimates is demonstrated through the example of interpreting acetate metabolite trends from a bioreactor cultivation of Chinese hamster ovary cells expressing a recombinant antibody.



In recent years, metabolomics has become an attractive experimental tool for generating multivariate quantification data from systems featuring complex mixtures of small molecular weight compounds, with applications in cell culture bioprocessing,[1] biofluid analysis,[2] environmental toxicology,[3] and pharmacology,[4] as well as food and nutrition,[5] among others. Analytical methods employed in this field are derived from classical analytical chemistry methodologies, such as mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR), and high- and ultraperformance liquid chromatography (HPLC and UPLC).

While mass spectrometry is capable of metabolite quantifications in the picogram range, offering the greatest sensitivity of available metabolomics methods, this sensitivity varies as a function of sample characteristics such as acidity and hydrophobicity.[6] Furthermore, sample preparation and processing can be relatively time-consuming and multiple MS methods may need to be employed for final determination of unknown species.

Single-dimension hydrogen, or proton, nuclear magnetic resonance spectroscopy (1D-^{1}H NMR) offers numerous competitive advantages over MS and HPLC. NMR spectra are highly reproducible and can be rapidly acquired; furthermore, sample-preparation is simple, noninvasive, and nondestructive.[7−9] Metabolite detection occurs uniformly, providing detailed structural information for compound identification.[7,8] Challenges associated with employing 1D-^{1}H NMR for global metabolite analysis include a lower sensitivity (typically in the micromolar range), the overlap or "convolution" of spectral resonance peaks of different metabolites, and differences between samples due to matrix effects such altered pH and ionic strength.[7] The latter of these may cause shifts in the positions of metabolite peaks, hampering tools such as automated spectral binning.[10]

Received: October 18, 2013
Accepted: February 20, 2014
Published: February 20, 2014

dx.doi.org/10.1021/ac4033966 | *Anal. Chem.* 2014, 86, 3330−3337

To overcome these challenges, 1D-^{1}H NMR spectra can be analyzed through "targeted-profiling", a method whereby metabolite peaks of NMR spectra are directly matched against pure compound spectra from a database by a human "profiler".[10] This method is tolerant to peak shifts from matrix effects and also allows for "deconvolution" of overlapping peaks.[10] With respect to our work, identification and quantification of metabolites through targeted profiling was achieved by using Chenomx NMR Suite 7.5 (Chenomx Inc., Edmonton, Alberta). While multidimensional NMR has been argued as a possible solution to the problem of convolution, 1D-^{1}H NMR remains an attractive option for quantification on the basis of sensitivity and scan time.[11]

Despite the appearance of automated or semiautomated deconvolution methods over 10 years ago[12] and their growing application to cell culture,[1,13] little in depth validation has been done to assess their applicability to particular complex mixtures. A lot of work has focused on quantifying the error associated with the integration of well-dispersed peaks,[14−18] but these studies treated spectral overlap as an occasional problem rather than the norm, making their conclusions only loosely relevant to analysis of cell culture supernatant and other complex spectra, where overlap is highly prevalent. NMR spectra reproducibility has also been examined in the context of a large-scale split-sample study.[19] However, the focus was on binning, meaning that metabolites found at low concentrations, whose resonances are almost entirely hidden, would not have had a significant impact on the analysis, despite their possible importance. In more recent years, at least two studies tackled the issue of metabolite quantification with significant convolution with the use of Chenomx software.[20,21] Unsurprisingly, the general level of precision was much lower than the ∼1.5% standard deviation reported for the integration of well-dispersed peaks.[14] Slupsky et al.[20] also found that mean concentrations could systematically deviate from theoretical values by as much as 22% for the nine compounds tested. In our work, we have found that relative standard deviation can vary from 1% to 70% depending on a compound's concentration and the environment around its NMR resonance.[22] These studies make it clear that to determine the precision and accuracy of NMR quantification, convolution must be considered explicitly. Unfortunately, the nature of convolution is that it is entirely dependent on the metabolic makeup of a tested sample, meaning that its effect must be considered on an application basis.

In this work, we apply 1D-^{1}H NMR to a typical animal cell culture media to determine the metabolic profiles NMR analysis can reveal. More importantly, we make use of a common Plackett−Burman design to rigorously probe the observed data for quantification accuracy and precision through the analysis of synthetic metabolite mixtures. The chosen design assesses orthogonal (independent) combinations of compound concentrations, allowing the estimation of accuracy and precision of a given compound considering possible variation in other (convoluting) compounds.[23] Consistent with the idea of tailored media compositions and rational nutrient feed regimes, the metabolic profile results are specific to the cell line and media under consideration and cannot be generalized. However, we show how a simple, 20−30 sample synthetic mixture design is able to reveal a wealth of useful information about 1D-^{1}H NMR quantification, necessary for understanding application-specific results.

EXPERIMENTAL SECTION

Experimental Design. The purpose of this investigation was to determine the impact of spectral overlap on the precision and accuracy of metabolite quantification in cell culture supernatant by 1D-^{1}H NMR. The hypothesis was that the concentration of one compound may, by spectral overlap, hinder the accuracy and precision in the quantification of another. A more pronounced effect was expected for resonances whose peaks were found exclusively in overlapping regions corresponding to multiple metabolites, particularly in circumstances of significant concentration differences between the chemical species. In order to achieve an accurate measure of quantification error, it was necessary to simultaneously vary multiple compounds at known concentrations. Therefore, synthetic media formulations were constructed with defined two-level component concentrations and varied according to a Plackett−Burman design, allowing for a realistic amount of variability in an experiment of practical size.

One limitation of this design (as compared to the full or fractional factorial design) was the convolution of main effects with interaction terms. However, Plackett and Burman[23] have previously argued that interaction terms can be validly neglected if they are of the same order of magnitude as main effects. As spectral convolution is summative in nature, the presence of multiple convoluting compounds was not expected to produce an effect that is substantially greater than the sum of their individual effects. Thus, there was no reason to suspect that factor interactions, if present, would exceed the magnitude of the main effects, allowing the use of the more efficient Plackett−Burman design rather than the full or fractional factorial approach. More information about the Plackett−Burman design as well as steps to reproduce it can be found in many textbooks on statistical design, such as "Statistical Design and Analysis of Experiments: With Applications to Engineering and Science" by Mason et al.[24]

Synthetic Media Formulation. The compound concentration levels used in the synthetic media formulations were generated from commonly observed metabolic time course data collected in our lab using Chenomx NMR Suite, excluding those that were not biologically relevant or were identified inconsistently. The remaining compounds were divided into two groups—compounds whose concentration remains approximately constant during fermentation and those whose concentration changes with respect to time. Compounds that were found to have a significant correlation to time (using a Spearman rank correlation coefficient at a 95% confidence level) were added at two concentration levels equal to the minimum and maximum observed concentration values extended by 25% of the minimum to maximum range. The lower level concentrations were not prevented from reaching zero. For these compounds, it was deemed more important to assess the possibility of false detection, to determine if a true zero concentration would actually be observed. Compounds without a significant correlation to time were added at a single constant concentration level equal to their median observed value. pH was not controlled other than through the use of a pH buffer. Thus, compound composition was allowed to dictate natural pH variation, as it would during a fermentation process, e.g., higher lactate concentration resulting in increased acidity.

Concentrated stocks were made from pure chemical stock (Supporting Information Table S-1) dissolved in PBS buffer. For powder stock, the minimum amount of chemical added was

dx.doi.org/10.1021/ac4033966 | Anal. Chem. 2014, 86, 3330−3337

Table 1. Measurement Errors and Standard Deviations for All Compounds Included in the Synthetic Media[a]

compound	level	theoretical concentration (mM)	measurement error (%)	measurement SD (%)	error significance
glucose	high	19.944	−1.941	3.801	
	low	9.650	−4.064	4.404	***
*lactate	high	6.222	7.576	3.809	***
	low	0.834	16.029	11.638	***
*glutamine	high	3.966	−7.289	5.345	***
	low	0	NA	NA	***
alanine	high	2.348	−4.945	3.066	***
	low	0	NA	NA	***
proline	high	2.047	−10.796	8.793	***
	low	1.261	−11.796	10.468	***
*isoleucine	high	1.403	−14.149	1.639	***
	low	0.314	−13.849	9.236	***
valine	high	1.238	−4.876	2.181	***
	low	0.534	−5.28	2.996	***
*leucine	high	1.154	−10.604	5.979	***
	low	0.248	−3.843	2.823	***
*glycine	high	1.038	−13.802	5.588	***
	low	0.033	−16.152	12.121	***
threonine	constant	0.888	−8.979	3.941	***
lysine	constant	0.814	−10.452	4.791	***
arginine	constant	0.692	−4.425	10.838	
glutamate	high	0.560	−5.819	16.071	
	low	0.049	192.952	242.857	***
*formate	high	0.57	6.021	2.632	***
	low	0	NA	NA	***
pyruvate	constant	0.497	−4.736	2.616	***
tyrosine	constant	0.454	18.676	8.590	***
phenylalanine	constant	0.431	3.741	5.104	***
serine	constant	0.420	−14.107	4.286	***
methionine	high	0.360	2.178	4.444	
	low	0.211	2.587	4.739	
pyroglutamate	constant	0.269	22.204	24.907	***
asparagine	constant	0.144	14.362	9.028	***
*tryptophan	high	0.118	1.772	5.085	
	low	0.063	18.042	12.698	***
myo-inositol	constant	0.111	4.611	10.811	
arabinose	high	0.101	4.873	17.822	
	low	0.035	48.647	54.286	***
acetate	constant	0.087	21.168	11.494	***
*choline	high	0.059	−11.113	1.695	***
	low	0.015	−14.595	13.333	***
aspartate	constant	0.057	17.912	17.544	***
succinate	high	0.045	−6.223	4.444	***
	low	0.002	NA	0	***

[a]An error significance of *** represents 95% confidence that the mean observed deviation from theoretical concentration is significantly different from 0 as determined via a *t* test. Compounds marked with * have significantly different percent standard deviations between their high and low concentration levels as judged by an *F*-test at a 95% confidence level.

kept at a level sufficient to achieve 99.9% weighing accuracy. The amount of PBS added was determined by the solubility of each compound. The formulation of individual mixtures was performed by volumetric addition of the concentrated stocks with the further addition of PBS to ensure accurate total volume measurement. The use of PBS was a significant simplification when considering the more complex salt and buffer composition of most media. While this ignored the effect of NMR-detectable buffers such as HEPES, the impact on quantification had been previously assessed as minimal (data not shown), in that nonmetabolite resonances did not overlap with metabolite resonances to a significant degree. A full list of compound concentrations and measured pH values for all mixtures can be seen in Supporting Information Table S-2.

NMR Analysis. An amount of 630 μL of each clarified medium sample was mixed with 70 μL of internal NMR standard composed of 99.9% D_2O with 5 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) serving as a chemical shape indicator (CSI) and 0.2% w/v sodium azide (NaN_3) to inhibit bacterial growth (Chenomx Inc., Edmonton, Canada). The solution was vortexed, and 700 μL was pipetted into a 5 mm NMR tube (NE-UL5-7, New Era Enterprises Inc., Vineland, NJ) for scanning.

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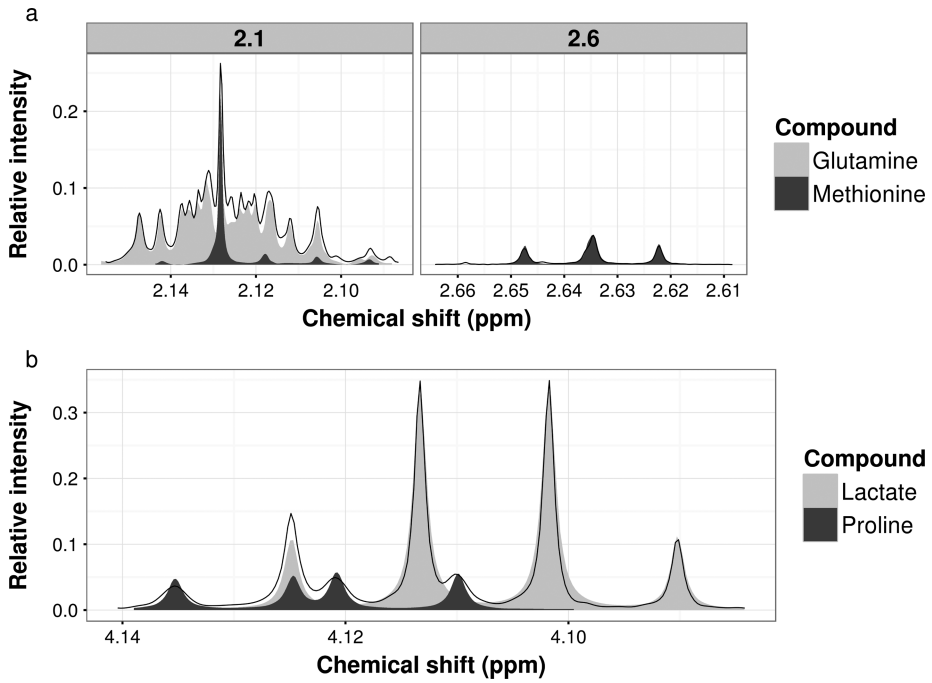

Figure 1. Examples of convolution between methionine and glutamine (a) as well as proline and lactate (b). Shaded areas represent pure compound spectra generated from the Chenomx library, with the observed spectra superimposed (thin black line). The differences in peak width of observed spectra when compared to the generated spectra of proline (b) are also an example of the shape distortion observed for some peaks associated with a labile hydrogen.

NMR spectra were acquired using the first increment of a nuclear Overhauser effect spectroscopy (NOESY) pulse sequence with a 1 s presaturation pulse (10 ms relaxation delay, 990 ms water suppression), 100 ms mixing time, and a 4 s acquisition time on a Bruker Avance 600.13 MHz spectrometer with a triple resonance probe (TXI 600). Following acquisition, spectra were imported into Chenomx NMR Suite 7.5 (Chenomx Inc., Edmonton, Canada). Phase and baseline corrections were carried out manually. Line asymmetry correction (reference deconvolution[25]) was performed automatically on each sample by the software based on a manual comparison of ideal and observed DSS peaks. Compounds were quantified ("profiled") by a single person using the software's built-in 600 MHz compound library by comparison to a known amount of DSS as the internal standard (see http://www.chenomx.com/ or Weljie et al.[10] for more information on targeted profiling). Briefly, all resonances were considered in the fitting of a compound's concentration. Isolated resonances were given preference, with concentrations confirmed based on the overall fit of overlapping regions.

General Statistical Analysis. All data manipulation and analysis were carried out using the R programming language.[26] All plots were generated with the ggplot2 package.[27] Unless otherwise noted, the significance of a difference between an observed sample mean and a theoretical value was determined with the use of a two-tailed *t* test at a significance level of 0.05. Likewise, the significance of a difference between the variability of two observed samples was determined using a two-tailed *F*-test at a significance level of 0.05.

Modeling the Effect of Convoluting Compounds on the Mean of Individual Compounds. The experimental design allowed the estimation of spectral overlap effect on the observed concentrations in the form of the following model:

$$\mathbf{y}_i = \mu_i + \sum_{j \in J, j \neq i} a_{ij}\lambda_j + \boldsymbol{\epsilon}_i$$

where $\mathbf{y}_i$ is the column vector of concentrations of compound i from all samples where compound i is at a single concentration level, μ_i is the mean concentration of compound i across those samples, λ_j is the concentration level of compound j whose resonance overlaps with that of compound i (1 for high, 0 for low), a_{ij} is the regression coefficient, J is the set of all varied compounds, and $\boldsymbol{\epsilon}_i$ is a column vector of residuals. In this way, it was possible to determine if the concentration level of one compound resulted in an increased or decreased observed concentration of another when the two have overlapping resonances. The effect was quantified using iterative linear regression. When no effect is observed, the model breaks down to the base case of mean estimation. For each compound, a list of compounds with overlapping resonances was compiled based on the spectra obtained in the experiment (Supporting Information Table S-3). Any compounds with overlapping resonances large enough to have a possible effect on the quantification of a target compound were added to the list (judged subjectively—corresponding to the presence of resonance sections with more than ~5% overlap), as were any compounds whose resonances could be mistaken for those of the target compound, or vice versa.

Linear models of each compound's concentration mean were generated by iteratively considering the effect of overlapping compounds' concentration level. Overlapping compound levels were included in the model one at a time, and any whose level was found to have a significant effect on the measured concentration of a target compound (as judged by an *F*-test with a significance level of 0.05) was added to a model shortlist. From this list, the compound whose inclusion in the model resulted in the greatest increase in adjusted R^2 value was kept in the model. The process was then repeated to test if further

dx.doi.org/10.1021/ac4033966 | Anal. Chem. 2014, 86, 3330−3337

compound terms should be added. Once no improvement could be made to the adjusted R^2 value, the iteration was terminated with the selection of current best model. A schematic of this procedure is presented in Supporting Information Figure S-1.

RESULTS AND DISCUSSION

Overall, 28 biologically relevant compounds were used in the synthetic formulation, with the bulk consisting of amino acids,

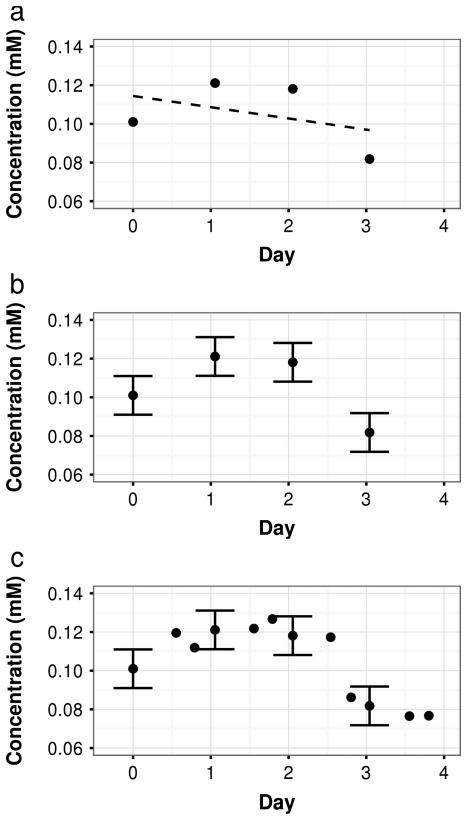

Figure 2. Application of variability estimates to acetate concentration data from a 3 L bioreactor (Applikon Biotechnology Inc., Foster City, U.S.A.). Cells were seeded at a concentration of 3×10^5 cells/mL with a working volume of 1.5 L. Supernatant samples were taken three times a day and stored at −80 °C until NMR analysis 1 week later. The process parameters were set to a pH of 7.4, 50% dissolved oxygen (DO), 37 °C, and an agitation speed of 120 rpm. (a) Daily samples with the possibility of high variability around an approximately constant or decreasing concentration. (b) Error bars in the form of standard deviations added to the observed data, allowing the use of *t* tests to compare acetate concentrations at days 2 and 3 to those on day 0 and day 3. (c) High-frequency sampling from the same cultivation reinforcing the trends determined with the use of variability estimates.

making the formulation typical of a generic animal cell culture process. Of the 28, 16 compounds were included at two levels (corresponding to a significant change with respect to time), while the rest were kept at constant concentrations. With two levels per varying compound, this corresponded to a 20 run Plackett−Burman experiment. While synthetic complex mixtures have previously been used to probe different aspects of NMR-based analysis;[28−30] this application of synthetic formulations is the first to account for the significant amount of variation in the metabolite composition of cell culture supernatant. The following is presented as a case study to illustrate the accuracy and precision that can be achieved with the application of 1D-^{1}H NMR to the observation of cell culture, explicitly considering realistic resonance overlap.

Measurement Accuracy. A comparison of observed means to theoretical concentrations is presented in Table 1 (a box-plot representation can be found in Supporting Information Figure S-2). The level of accuracy was found to vary considerably between different compounds as well as for different concentrations of the same compound. Overall, the absolute percent differences between observed and theoretical concentrations ranged from a low of 1.8% to a singular high of 193.0%, with a median deviation of 9.7%.

On the basis of the results presented in Table 1, the consistency of measurement accuracy across concentration levels depended in large part on the compound in question. Three of the compounds—glutamate, tryptophan, and arabinose—had considerable spikes in percent measurement error at lower concentrations. In all three cases, the magnitude of percent measurement error at the higher concentration level was below or at the median error, in the range of 1.8−10.0%. At lower concentration levels, the lowest percent error for measurement was tryptophan at 18.0%. Analysis of the profiled spectra revealed that the particularly high percent error for glutamate (193.0%) was due primarily to its significant spectral overlap as both of the other compounds had nonconvoluted resonances at 7.5 and 7.7 ppm for tryptophan and 4.5 ppm for arabinose, which could be used for quantification. However, the presence of isolated resonances did not guarantee perfect accuracy. With a lower concentration of approximately 0.05 mM, the resonances of both tryptophan and arabinose were very close to the baseline and proved difficult to quantify accurately. For many of the other compounds, the general trend was of constant percent error. Proline, isoleucine, valine, glycine, methionine, and choline were all observed with similar percent error, whether at high or low concentrations. Glucose is singular in that it actually had a relatively constant absolute error (0.39 mM) with a corresponding change in percent error at different concentration levels. As the experimental conditions included the variation of all compounds whose concentrations were previously observed to vary with time, the measurement errors presented in Table 1 are good general estimates for the expected bias in the concentration measurement of the above compounds. Overall, a compound's concentration was not an effective predictor of its quantification error, as highlighted by the fact that the compound found at one of the lowest concentrations, succinate, was observed with an absolute percent error of only 6.2%. This compound-dependent nature of the observations reinforces the need for validation experiments to include as many relevant compounds as possible and at realistic concentrations.

Depending on the application, consistent biases in metabolite quantifications with 1D-^{1}H NMR may not be problematic. Often, it is not the absolute concentration values that are being analyzed, but correlations (or lack thereof) between time course trends.[8,31,32] For these types of applications, a constant deviation of a reasonable magnitude (~10−20%) is unlikely to result in disputed conclusions. As we have been able to identify, however, the accuracy of some compounds is not consistent over all concentrations. As compound concentrations are generally correlated with time, this can result in biased time trends whether looking at the trends of compound concentrations[8,31,32] or their rates of change.[32]

dx.doi.org/10.1021/ac4033966 | *Anal. Chem.* 2014, 86, 3330−3337

False Positive Identification. Three of the compounds included in the formulation—glutamine, alanine, and formate—had a theoretical concentration of 0 mM as their low concentration level. The use of a 0 mM lower level prevented their comparison to other compounds as they could only be overestimated at this level. It was judged more important to know the prevalence of false positives for these compounds rather than get an estimate of their precision at a low concentration. At low concentration levels, glutamine and formate were both "observed" despite a theoretical concentration of 0 mM; alanine was correctly identified as not being present. The measured concentrations were between 0.005 and 0.015 mM for formate and between 0.01 and 0.05 mM for glutamine, making these values a good estimate of their detection limits. Observations in these ranges can therefore be said to be likely the result of noise and indistinguishable from 0 mM. While false positives in this range are unlikely to influence general time trends, a good understanding of quantification limits can help distinguish between the cases when a metabolite has been entirely depleted versus when its uptake has been down-regulated.

Improving Measurement Accuracy. The use of synthetic compound mixtures with known compound concentrations allowed a direct assessment of profiling technique. Analysis of the deconvoluted spectra revealed peak-specific strategies that can be used to improve accuracy. Tredwell et al.[21] have approached this subject already by recommending group overview of profiled spectra for the generation of a master profiling list as a way to avoid inconsistent metabolite identification. Our results go a step further and illustrate how a validation experiment can provide actionable information for the improvement of quantification accuracy.

In Chenomx NMR Suite 7.5 software, fit quality is generally determined with the use of a "subtraction line", the difference between the observed spectra and the fitted curve. While the ideal is to get the subtraction line all the way to zero, the reality is that the line may be high in some parts of the curve and low in others, resulting in a considerable amount of profiling ambiguity. How much the sum line tends to vary across the span of a curve is often resonance-dependent. This was found to be particularly problematic for compounds whose spectra consisted of only one or two narrow peaks, such as choline, formate, glycine, and lactate, resulting in inaccurate quantification (for a comparison of peak width, see Supporting Information Figure S-3). The knowledge of true concentrations from the synthetic mixture study made it possible to adjust the profiling pattern to achieve better accuracy by identifying the specific shape of the subtraction line that resulted in the most accurate fit. In particular, glycine and choline concentration accuracy was improved when the total area between the subtraction line and the baseline was set close to zero (treating sections of the subtraction line below the baseline as having a negative area), ignoring poor fitting at the peak itself. The concentrations of formate and lactate, on the other hand, were more accurately fit by using the peaks alone (1.3 ppm doublet for lactate). For lactate, quantification of the 1.3 ppm doublet is preferred to that of the quartet at 4.1 ppm due to the latter's proximity to the suppressed water peak. It should be noted, however, that these techniques may not be valid for different sample compositions.

It was also found that less reliable quantification was obtained for resonances resulting from labile protons, more specifically, hydrogen atoms bonded to nitrogen atoms, or protons vicinal to labile protons. While this configuration is technically found in all amino acids, such resonances were more characteristically problematic for proline, lysine, and isoleucine, where they caused considerable underestimation of the true concentration of the compounds. Even in cases where there was no significant underestimation, these problematic resonances were frequently wider than the pure-compound spectra of the Chenomx NMR Suite 7.5 library (an example can be seen in Figure 1). While this would not be a problem for scans of pure compounds, where all resonances are available for quantification and problematic ones can be ignored, complex mixtures such as cell culture supernatant do not always allow the luxury of resonance comparison due to significant spectral overlap from a myriad of metabolites. While the above strategies pertain only to Chenomx software, the synthetic mixture design proposed in this work can be used to identify compound- or peak-specific issues regardless of the software used for quantification.

Effect of Spectral Overlap on Accuracy. From the results of this experiment, two effects of spectral overlap were identified. As mentioned in the Measurement Accuracy section, overlap can have an indirect impact on the accuracy of compound quantification by masking quantifiable resonances. This can occur when the relative intensity of a compound resonance is large enough to completely absorb visible signs of smaller overlapping compound peaks. As expected for animal cell culture supernatant, this effect was observed most prominently for (but not exclusive to) glucose, due to its high concentration. If alternative quantifiable resonances of a compound are available, the net effect may be negligible. However, it can also force a profiler to rely on less dependable resonances, such as those corresponding to hydrogen atoms near an amine group, or simply more overlapping regions, leading to a loss in precision. As long as the masking compound resonance stays significantly larger than the masked compound, the overall effect will not be dependent on the exact concentrations of the compounds involved.

A more direct impact of spectral overlap was also identified. When overlapping compound resonances were of similar size, resonance peaks could be misattributed, resulting in a measurement bias that was dependent on the concentration of overlapping compounds. This was tested by modeling the effect of overlapping compound concentration level on the measured concentration of a given compound with linear regression (described in the Modeling the Effect of Convoluting Compounds on the Mean of Individual Compounds section), corresponding to main effect estimation generally performed on Plackett–Burman data. Unlike in general practice, however, the effects had to be calculated separately for each level of a target compound as measured concentration distributions were not consistent between levels of the target compound (described in greater detail in the Measurement Variance section, below).

Of all the compound relationships tested, three were found to have been statistically significant. Two of these involved methionine and proline quantification. At high levels of methionine, high glutamine levels corresponded to a statistically significant increase in the observed concentration of methionine. Of methionine's five resonances, three are of sufficiently large relative intensity to allow quantification—one at 2.6 ppm and two at 2.1 ppm. The resonances at 2.1 ppm generally overlapped with glutamine (Figure 1a). Following the results of the analysis, it was found that when the methionine

dx.doi.org/10.1021/ac4033966 | Anal. Chem. 2014, 86, 3330–3337

concentration level was high, high levels of glutamine resulted in a slight (but significant) overestimation of methionine. At low methionine concentrations, a similar effect was also observed, but it was not found to be statistically significant. The results suggest that some of glutamine's resonances at 2.1 ppm were being incorrectly attributed to methionine, which is in agreement with the general pattern of glutamine underestimation. A similar relationship was observed between proline and lactate, whose resonances overlap considerably at 4.1 ppm (Figure 1b). At low proline concentration levels, high lactate levels corresponded to significantly lower proline concentrations, despite the fact that proline has at least four other resonances suitable for verification. A similar but nonsignificant trend could also be observed for high proline concentrations. Similarly to the issues described in the context of direct profiling improvements (Improving Measurement Accuracy section), these biases can be avoided by a human profiler once identified.

The third identified relationship was also the most directly relevant to cultures supplemented with glutamine. Pyroglutamate, present at only one level, was observed to be approximately 30% higher when the glutamine concentration level was high. An analysis of the spectra revealed that this was not an example of spectral overlap but, rather, evidence of glutamine breakdown, a phenomenon that has been previously observed in literature.[33] An increase in glutamine concentration from 0 to approximately 4.0 mM resulted in an increase of pyroglutamate concentration of approximately 0.1 mM and is responsible for most of the overestimation seen in Table 1. Similar forms of degradation, if present, could also be detected with this form of analysis.

Measurement Variance. Standard deviation values (Table 1), like the differences between observed mean and theoretical concentrations, did not have a single general trend. While there was a slight tendency for compounds found at lower concentrations to have a higher relative standard deviation, the results for choline, tryptophan, and methionine are clear counterexamples. When looking at the concentration levels of each compound individually, however, there did appear to be an indication that lower concentrations translate into higher relative variability. Of all the varied compounds, only leucine had a significantly lower standard deviation at lower concentrations for reasons that have not been identified. The rest of the compounds that had a nonzero lower concentration level either showed no change in standard deviation (glucose, proline, valine, glutamate, and methionine) or a significant increase of 2-fold or more (lactate, isoleucine, glycine, tryptophan, arabinose, and choline). While the increase in the standard deviation of glutamate was not technically significant, as it was already high to begin with, its magnitude suggests that this compound belongs more with the latter group than the former. At lower concentrations, the relative standard deviations of these compounds frequently increased to a magnitude of 10% or more. As a 95% confidence interval around an observed concentration with a standard deviation of 10% translates into a range of ±20%, required precision levels should be carefully considered when dealing with low concentrations of these compounds.

The applications of estimating standard deviation values for metabolite quantification may not be as easily apparent as those of bias estimation, but they are just as important. A priori information about the confidence of a metabolite observation can be used to confirm the applicability of 1D-^{1}H NMR observation for a given task. The particular overlap patterns of specific media or cell type can render some metabolites (such as glutamate at low concentrations in the case of certain animal cell culture media) practically unquantifiable. While general glutamate levels can still convey a considerable amount of information, it should be clear that studies focusing on glutamate in particular should seek more tenable methods of quantification.

Application to Cell Culture Results. One example of how these results can be applied relates to the monitoring of acetate concentration in Chinese hamster ovary (CHO) culture. A mammalian bioprocess is typically sampled only once per day, resulting in four or more sample points per compound, depending on the length of cultivation. Acetate concentrations from the first four days of culture can be seen in Figure 2a. Due to the sparseness of the data, it was difficult to conclude whether the concentration fluctuations corresponded to a parabolic trend or were the result of measurement noise around a nearly constant value. Adding the variability estimates from the Plackett–Burman results (Figure 2b) suggested that the acetate concentrations on days 1 and 2 may be different from those on days 0 and 3, a hypothesis that could be confirmed with the use of *t* tests. For this particular cultivation, sampling was performed three times a day, rather than only once. Adding the extra data points to the plot (Figure 2c) confirmed the calculated trend. While acetate is not typically seen as a particularly important metabolite in mammalian cell culture, its concentration profile can be important in monitoring pyruvate metabolism. This application serves to demonstrate how the generation of a priori variability allows better data interpretation while diminishing the need for high-frequency or repeated sampling.

■ ASSOCIATED CONTENT

Supporting Information

Extra details concerning the experimental procedure described in the manuscript, including the chemical 1D data (Table S-1), final concentrations and pH values of the prepared synthetic mixtures (Table S-2), a breakdown of compound NMR spectra convolution (Table S-3), and a schematic of the algorithm used for modeling the effect of convoluting compounds on the mean of individual compounds (Figure S-1), extra graphical representations to complement the Results and Discussion section of the main text (Figures S-2 and S-3), and spectra files from Plackett–Burman experiment in the form of JCAMP files (the file names correspond to sample numbers in Table S-2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Dr. David Chang is an employee of Chenomx Inc.

■ ACKNOWLEDGMENTS

This work was supported in part by NSERC ENGAGE and NSERC Strategic Network (MabNet) Grants to M.G.A., NSERC Canada Graduate Scholarship to S.S., and a King Abdullah Scholarship (Saudi Arabia) to N.A.

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