ZipChip

Bioreactor Monitoring via ZipChip CE-MS: Quantifying Amino Acids in Spent Growth Media

INTRODUCTION

Manufacturing of biotherapeutic proteins often relies on expressing the drug product in large scale bioreactors. Monitoring the components of the cell growth media in the bioreactors can provide important insight into the growth and nutrient consumption of the cell culture. This information can be vital for maintaining the health of a bioreactor and maximizing production of the drug product. Many of the media components included to promote and sustain cell growth are small, polar analytes that can be difficult to analyze using conventional techniques. To achieve acceptable separation performance and detection these analytes are often derivatized to increase either their hydrophobicity, volatility, or UV absorbance properties for analytical techniques such as liquid and gas chromatography. The reliance on derivatization increases the time, complexity, and cost associated with the analysis making the assay impractical for routine use in many environments.

The separation mechanism used by ZipChip is capillary zone electrophoresis (CZE). CZE is based on charge and hydrodynamic radius rather than retention on a stationary phase, making this technique well suited to analyzing small, polar analytes. ZipChip integrates CZE separations with on-line electrospray ionization, directly connecting high efficiency separations with MS characterization. By combining CZE separations with MS detection the derivatization step can be eliminated and the only sample preparation necessary is dilution.

Here we demonstrate a simple and rapid method for quantifying small molecules in spent growth media samples. In this work amino acids are specifically targeted as indicators of cell metabolism to assess cell growth and the general health of the bioreactor. However, this method could easily be extended to include other classes of analytes, such as vitamins or biogenic amines. For this experiment samples were drawn from three different bioreactors over the course of 14 days. The bioreactors were set up to represent different growth conditions. One bioreactor simulated standard growth conditions while the other two simulated growth in a stressed environment through the addition of ammonia. By tracking the levels of amino acids over the course of cell growth it was observed that differences in amino acid levels and trends were detectable before changes in cell density or cell viability were detected between the three bioreactors. This suggests that monitoring the media composition directly could lead to earlier fault detection in a bioreactor and provide the potential to remedy the fault and preserve production output.

METHODS

Sample preparation. Bioreactor cultures of mammalian cells expressing an IgG type monoclonal antibody were grown using an ambr® 250 high throughput system (ambr Bioreactor System, Sartorius). Three different bioreactor conditions were explored: Bioreactor A represents standard growth conditions, Bioreactor B contained 10 mM added ammonia, and Bioreactor C contained 30 mM added ammonia to simulate stressed cell growth. All bioreactors were supplemented with two different feed stocks during cell growth: Feed A and Feed B. Samples were derived from time points taken over the course of a 14-day growth cycle. The collected spent media time points were stored at -20 °C until analysis. Samples were prepared following the ZipChip protocol *Quantitation of Amino Acids in Growth Media*. Spent media samples were first diluted 1000-fold with LC/MS grade water to bring the concentration of media components into the linear dynamic range of the system. The diluted samples were then diluted an additional 10-fold with 80/20 methanol/water containing 100 mM ammonium acetate and 1 µM stable isotope labeled amino acids to serve as internal standards (Cambridge Isotope Laboratories, Inc.).

For quantitation, a calibration curve was prepared containing the 20 essential amino acids. The curve was created using 4 points at 0.5 µM, 5 µM, 50 µM, and 500 µM which were then diluted 10-fold with the solution containing internal standards, for a final concentration of 0.05 µM, 0.5 µM, 5 µM, and 50 µM.

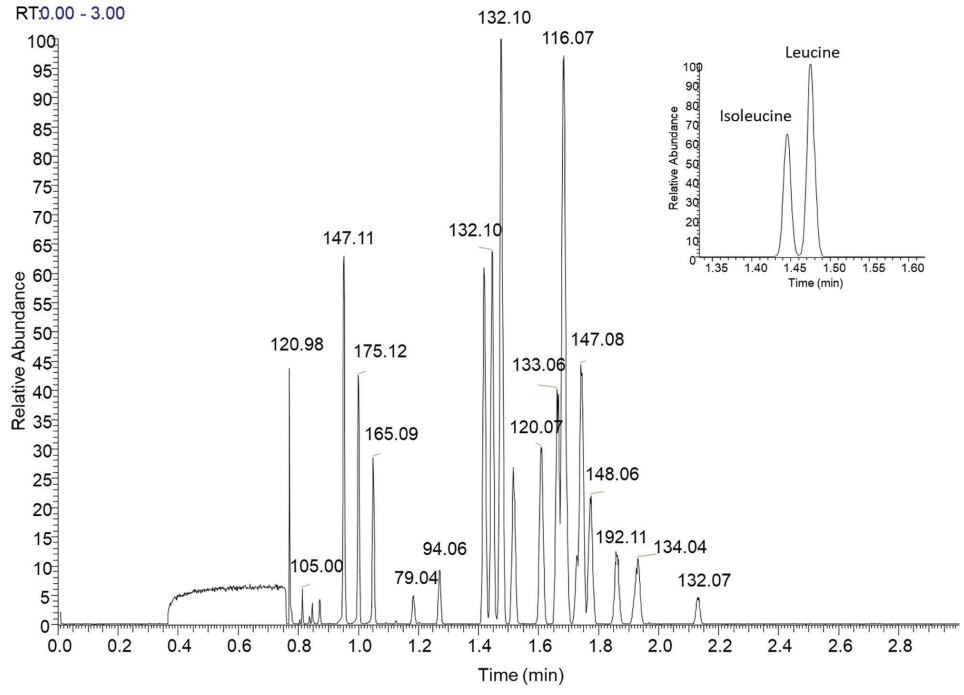
Analysis. The autosampler, ZCI, and an HS chip were primed with Metabolite background electrolyte from the ZipChip Metabolite Kit. 20 µL of the prepared growth media samples were loaded into the sample well and a 5 nL on-chip injection was performed. Analytes were separated with a field strength of 1000 V/cm and pressure assist was set to enable at 2.0 minutes after the start of the run. A Thermo Exactive Plus EMR mass spectrometer was used for data collection. The instrument was operated in Normal Mode and set to acquire data over a 70-500 m/z range. The prepared growth media samples and calibration curve points were analyzed in triplicate.

Data processing. Data were viewed using Xcalibur QualBrower. Data was processed using Skyline (University of Washington) to generate calibration curves and calculate the concentration of target analytes in the growth media samples. Calibration curves were created by taking the ratio of the amino acid to its internal standard and plotting the ratio with respect to the known concentration. For amino acids without a heavy isotope internal standard, the heavy isotope of its nearest neighbor was used for quantitation. Responses for the amino acids was linear over four orders of magnitude with all R^2 values greater than 0.98. For each growth media time point the concentration of the amino acids was calculated via the linear fit of the calibration curve and the peak area ratio of the amino acid and internal standard.

RESULTS AND DISCUSSION

Figure 1 shows the separation of the t_0 timepoint from Bioreactor A. The amino acids in the sample migrate through the chip in less than 3 minutes. A beneficial feature of the ZipChip analysis is the ability to separate isobaric species. This facilitates accurate and unequivocal identification of target analytes that exist as isomers. As seen in the inset of Figure 1, isoleucine and leucine, which have the same mass, are baseline separated and easily identified.

Table 1 shows the measured amino acid content of the starting growth media and both feeds. The starting growth media contains all 19 amino acids quantified while the feed stocks contain only select amino acids. When tracking the levels of the amino acids across the



Analyte	m/z	Migration Time (min)
Lysine	147.11	0.95
Lysine heavy	155.13	0.95
Arginine	175.12	0.99
Arginine heavy	185.13	0.99
Histidine	156.08	1.05
Histidine heavy	165.09	1.05
Glycine	76.04	1.18
Glycine heavy	79.04	1.18
Alanine	90.06	1.27
Alanine heavy	94.06	1.27
Valine	118.09	1.42
Valine heavy	124.10	1.42
Isoleucine	132.10	1.44
Isoleucine heavy	139.12	1.44
Leucine	132.10	1.48
Leucine heavy	139.12	1.48
Serine	106.05	1.52
Serine heavy	110.06	1.52
Threonine	120.07	1.61
Threonine heavy	125.08	1.61
Methionine	150.06	1.61
Methionine heavy	156.07	1.61
Asparagine	133.06	1.66
Proline	116.07	1.68
Proline heavy	122.08	1.68
Phenylalanine	166.09	1.69
Phenylalanine heavy	176.11	1.69
Tryptophan	205.10	1.73
Glutamine	147.08	1.74
Glutamic Acid	148.06	1.77
Glutamic Acid heavy	154.07	1.77
Tyrosine	182.08	1.86
Tyrosine heavy	192.11	1.86
Aspartic Acid	134.04	1.93
Aspartic Acid heavy	139.06	1.93

Figure 1. ZipChip separation of the t0 timepoint from Bioreactor A. The amino acids migrate through the chip in less than 3 minutes. As shown in the inset, isomers such as isoleucine and leucine are resolved and detected as separate peaks for confident identification and quantitation.

	Growth Media		Feed A		Feed B	
	Average [c]		Average [c]		Average [c]	
	mM	%RSD	mM	%RSD	mM	%RSD
Lysine	2.3	4.7%	53.1	2.6%		
Arginine	1.2	1.3%	36.3	3.0%		
Histidine	1.0	3.6%	15.3	2.7%		
Glycine	1.3	6.3%				
Alanine	0.1	23.8%	21.8	3.7%		
Valine	3.2	1.5%	77.0	2.9%		
Isoleucine	2.7	1.8%	44.6	4.7%		
Leucine	4.3	1.8%	76.8	3.2%		
Serine	5.1	3.1%	110.5	4.2%		
Threonine	2.6	1.9%	56.6	6.0%		
Methionine	1.3	1.6%	18.0	3.3%		
Proline	5.4	1.9%	38.0	3.8%		
Phenylalanine	1.3	1.5%	44.5	3.5%		
Glutamic Acid	2.0	5.3%	100.6	5.7%		
Tyrosine	0.8	1.8%			294.7	4.2%
Aspartic Acid	1.5	1.8%	62.3	3.5%		
Asparagine	6.1	5.9%				
Tryptophan	0.8	3.9%			112.3	14.2%
Glutamine	4.0	6.7%				

Table 1: Quantified amino acid levels in the starting growth media and feed stocks.

measured time points, distinct trends are apparent. Many amino acids follow a trend of steadily increasing in concentration over time. Figure 2 plots the normalized concentration of these amino acids during cell growth in Bioreactor A. A measurement of cell density is overlaid on this plot. As cell density increases, the amino acid levels increase at a moderate rate. Once the cell density reaches the apex at ~228 hrs of growth and begins to decrease, the amino acid levels begin to increase at a slightly higher rate. This suggests that the cells are not consuming the amino acids as quickly after they have reached maximum cell density. A second subset of amino acids show little change in concentration over time. Figure 3 plots the levels of these amino acids overlaid with the cell density curve. The concentration of these amino acids remains stable, dipping slightly near the apex of cell density before returning to levels similar to their starting concentration. This suggests a balanced rate of feed supply and consumption for these amino acids. Finally, several amino acids change in concentration much more drastically throughout cell growth. Figure 4 plots the normalized concentration of these amino acids overlaid with the cell density curve. Alanine, glycine, glutamic acid, and aspartic acid all initially increase in concentration before decreasing near the apex of cell density and then increasing again as cell density begins to decrease. It is interesting to note that alanine, glutamic acid, and aspartic acid are present in Feed A, but glycine is not present in either feed stock and is not supplied to the bioreactor in another way. Despite this,

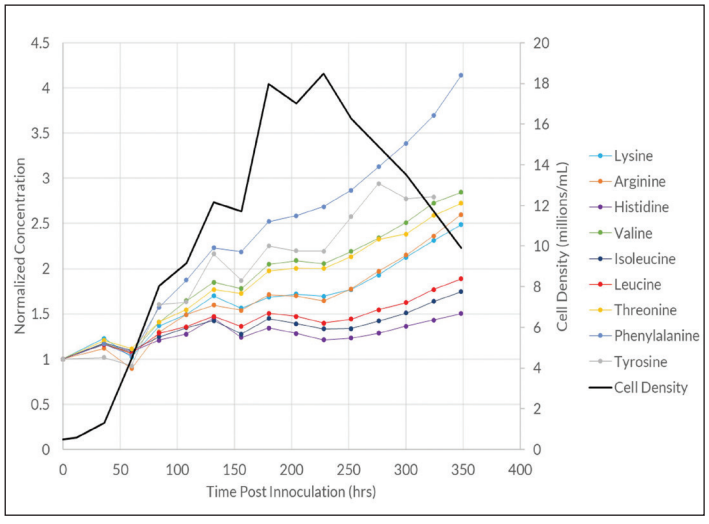


Figure 2. Normalized concentration of select amino acids over time from Bioreactor A (left axis) overlaid with cell density measurements (right axis). The amino acids plotted in this figure all displayed a similar trend where concentration increased steadily over time.

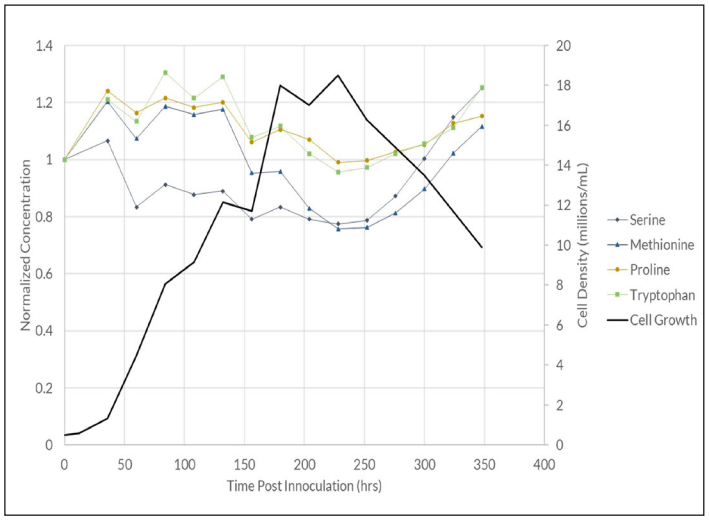


Figure 3. Normalized concentration of select amino acids over time from Bioreactor A (left axis) overlaid with cell density measurements (right axis). The concentration of these amino acids remained relatively stable throughout cell growth suggesting a balanced rate of consumption and supplementation from the feed stocks.

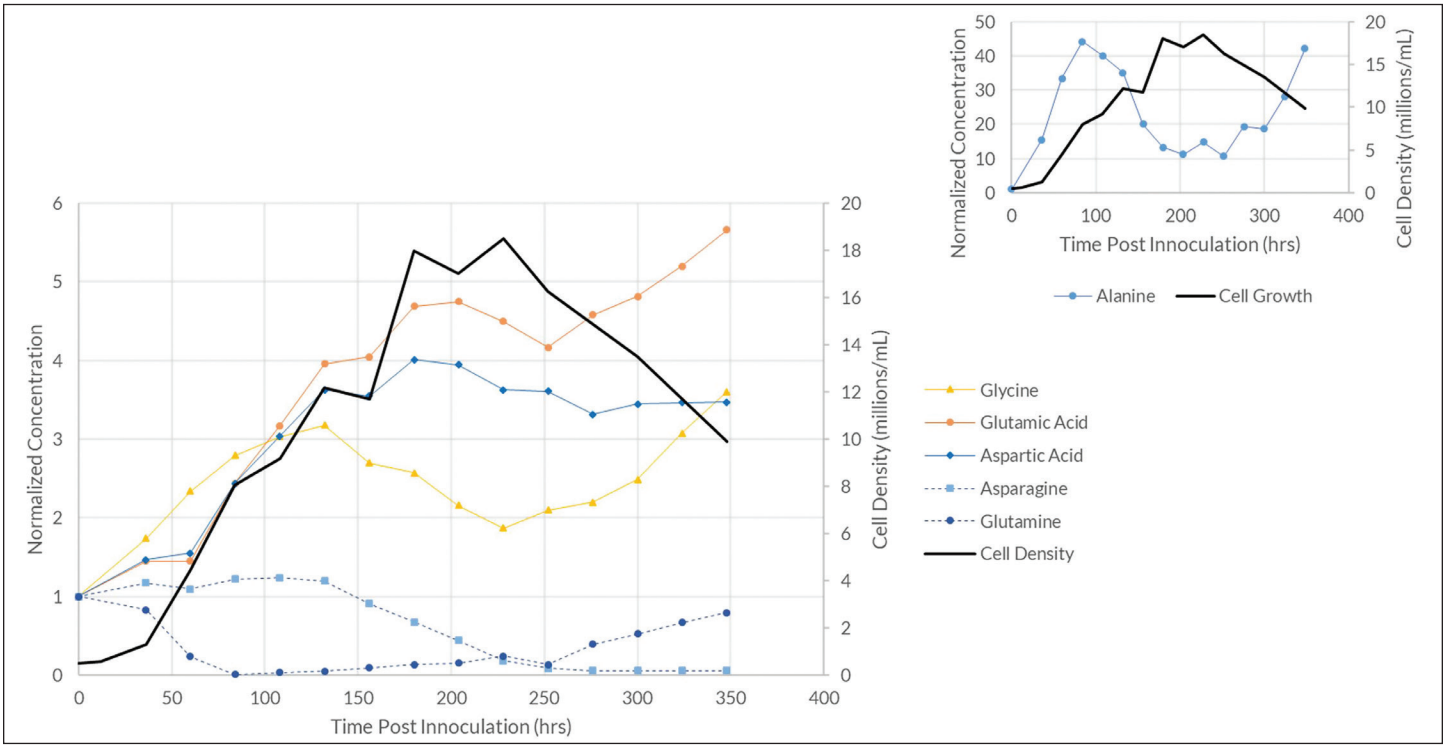


Figure 4. Normalized concentration of select amino acids over time from Bioreactor A (left axis) overlaid with cell density measurements (right axis). These amino acids changed in concentration more significantly over time and in a much more varied way. The inset shows the normalized plot of alanine, which was present in low amounts to start but increased significantly during incubation.

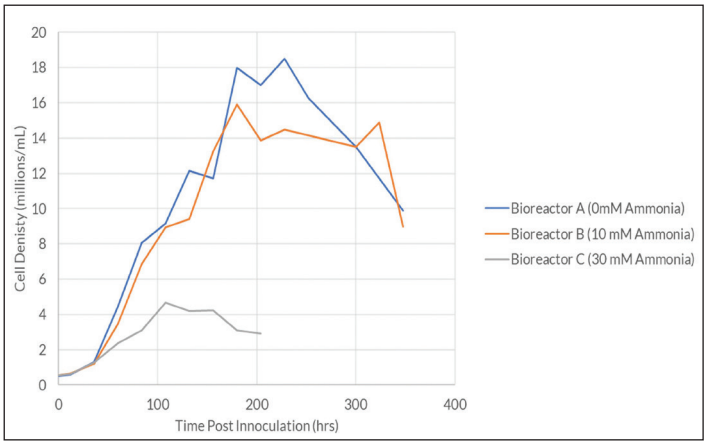


Figure 5. Viable cell density for Bioreactors A, B, and C monitored over the course of cell growth. Time point measurements were taken approximately 12 hours apart.

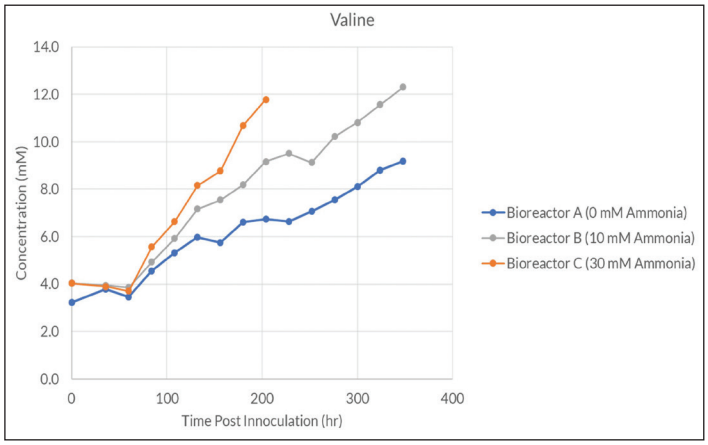


Figure 6. Plot of valine concentration over time for each bioreactor. Although initial starting concentrations are similar, the levels clearly diverge after 60 hours of growth.

it follows a similar trend to the other 3 amino acids. This is in contrast to the other two amino acids that are not present in either feed: glutamine and asparagine. Both of those molecules decrease in concentration as cell density increases indicating that they are steadily consumed in the bioreactor.

When comparing Bioreactors A, B, and C (0 mM, 10 mM, and 30 mM ammonia added) differences are apparent. Figure 5 plots the cell density measurements for all three bioreactors. Cell density measurements for Bioreactors A and B appear similar though lower for B, but growth in Bioreactor C with 30 mM ammonia added is clearly inhibited. Differences between the three bioreactors are even more apparent when viewing the time dependence of amino acid levels in the growth media. Figure 6 plots the concentration of valine over time for Bioreactors A-C. Though the initial concentration is comparable, the

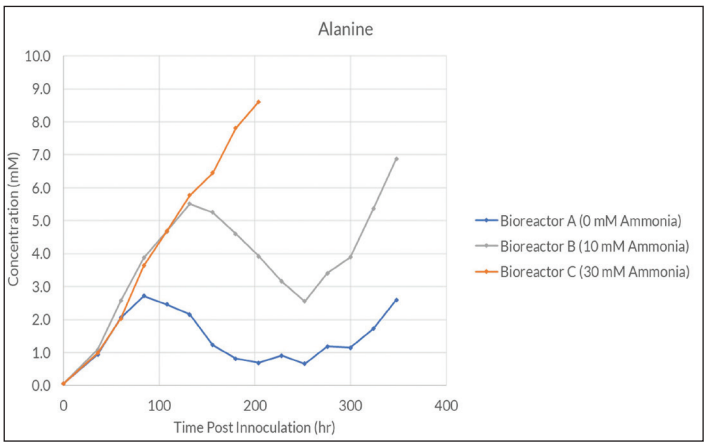


Figure 7. Plot of the concentration of alanine over time. For Bioreactors A and B the concentration reaches a maximum before decreasing and increasing again, but for Bioreactor C the concentration continually increases without reaching a maximum before that bioreactor was terminated due to insufficient cell growth.

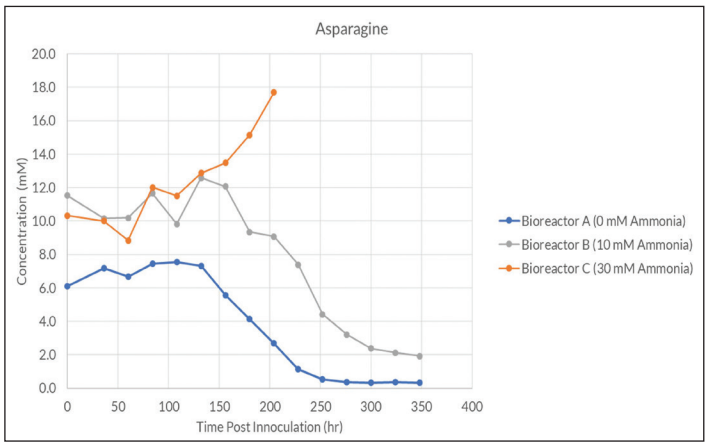


Figure 8. Plot of the concentration of asparagine over time. This amino acid is not supplied in either of the feed stocks and for Bioreactors A and B the concentration decreases over time as it is consumed by the cells. For Bioreactor C, where significant growth inhibition was observed, the concentration increases over time.

levels very clearly begin to diverge after approximately 60 hours of growth. For the bioreactors treated with ammonia the concentration of valine is consistently higher than that of Bioreactor A which represents standard growth conditions. This pattern is seen for all other amino acids following a similar trend to valine as described above. More drastic differences between the bioreactors are observed for other amino acids, such as alanine and asparagine. As seen in figure 7, the concentration of alanine increases for bioreactors A and B before reaching a peak, decreasing in concentration, and then increasing again. The time at which this peak is reached varies for each bioreactor. For Bioreactor A the concentration peak occurs at 84 hours of growth. For Bioreactor B this peak is reached at 132 hours of growth and is also higher in magnitude. Bioreactor C does not reach a peak before the cell growth was deemed too poor and no further time points were taken. For asparagine, as observed in Figure 8, the concentration in Bioreactor A reaches a minimum at 276 hours of growth and the concentration remains consistent at that minimum value for the rest of the culture time. The asparagine trace for Bioreactor B follows a similar trend but does not reach a steady minimum in the timeframe of the experiment. Bioreactor C on the other hand follows a very different trajectory where the concentration of asparagine increases over time rather than decreases, implying a difference in how the amino acid is consumed in that bioreactor. The observable differences in amino acid trends between the 3 bioreactors show that for a well characterized system, changes in amino acid levels could be indicative of a fault in the bioreactor. Additionally, the fault could be detected earlier in the process via amino acid monitoring than by monitoring just cell density alone.

CONCLUSIONS

With ZipChip, amino acids in growth media can be quickly and easily monitored. The method demonstrated here does not require any sample prep more extensive than simple dilution. Run times for spent media samples can be as fast as 3 minutes with all isobaric species resolved in the ZipChip separation prior to MS analysis. For the bioreactors analyzed here, distinct trends in the amino acids over time were observed. When comparing stressed versus unstressed bioreactors additional differences were apparent and could be used to detect faults in the growth process.

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