Waters™

Application Note

Introducing a Rapid Throughput LC-MS Method for Cell Culture Media Nutrient and Metabolite Analysis Supporting Upstream Bioprocessing

Yun Wang Alelyunas, Josh Gray, Mark D. Wrona, Patrick Boyce

Waters Corporation

Abstract

A nine-minute rapid and direct liquid chromatography and mass spectrometry (LC-MS) analysis method for cell culture media (CCM) nutrient and metabolite covering 220+ compounds is described. The method is based on reversed phase chromatography where amino acids are directly detected without derivatization. This rapid method reduces turn-around time for monitoring and decision making using previously established user-friendly data review workflows and access to multivariate data analytics (MVDA). The method has been applied for qualitative and quantitative determination of cell culture media nutrient and metabolite analysis in commercial cell culture media and spent media from process optimization in cNISTmAb production using NISTCHO cells. Monitoring of glucose directly from media using this method was also investigated and described.

Benefits

- Rapid nine-minute analysis for cell culture media nutrient and metabolite analysis with 220+ compound coverage in the provided library

- Superior chromatographic separation using ACQUITY™ Premier based technology and HSS™ T3 bonding chemistry offering robust repeatability and extended column lifetime
- LC-MS analysis using negative electrospray ionization (ESI-) mode of acquisition provides info of key nutrients and metabolites, such as glucose, lactate, glutamine, and glutamic acid
- Complete and comprehensive workflow for sample preparation, analysis, and reporting, offering deeper understanding of media used in incubation

Introduction

Correct composition and concentration of critical components in culture media are vital for optimal cell growth, quality, and yield in biotherapeutic cell culturing. Timely in-process monitoring of these compounds during incubation optimization is highly desirable. Recently, we described a LC-MS method and workflow for cell culture media (CCM) analysis based on the BioAccord™ LC-MS System.[1] In this application note, we describe an updated method, where the acquisition time is significantly reduced from 20 minutes to nine minutes while maintaining method robustness, data integrity, quality, and compound coverage. Figure 1 shows a schematic representation of the analysis method, from sample preparation using Andrew+™ Pipetting Robot, LC-MS analysis using BioAccord, to data review and report using waters_connect informatics™. This short run time and rapid analysis enables bioprocess engineers to quickly monitor metabolite changes and support faster decision making.

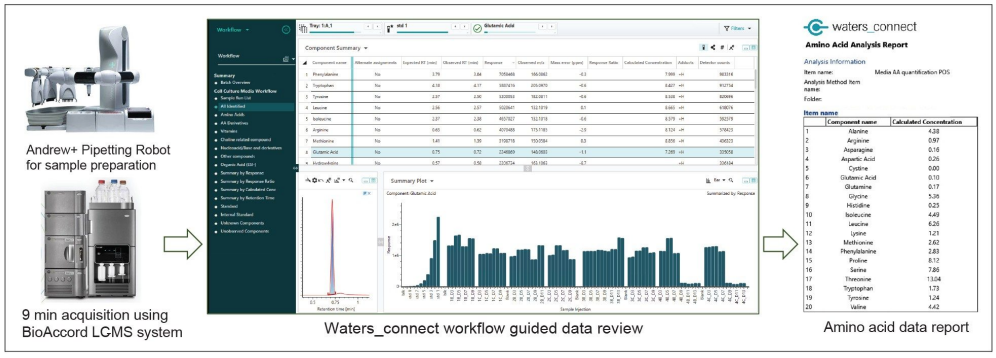

Figure 1. Schematic overview of sample preparation, LC-MS analysis, and report for cell culture media nutrient and metabolite analysis based on Andrew+ Pipette Robot and BioAccord LC-MS platform.

Experimental

Sample Preparation

Commercial media solutions were purchased from Millipore Sigma according to Table 1. Spent media samples were generated by Waters™ Immerse Delaware center where a fed-batch bioprocess experiment was carried out using NISTCHO cell line to produce cNISTmAb product in CHO Fed-batch medium (entry 5, Table 1). NISTCHO cell line (RGTM 10971 NISTCHO Test material) was obtained from nist.gov (https://www.nist.gov/programs-projects/nistcho <https://www.nist.gov/programs-projects/nistcho>). All sample preparation from clarified media solution and standards were performed using the Andrew+ Pipetting Robot. The spent media samples were diluted with 1:400 (V:V) using 0.1%FA containing 0.1 µM stable isotope labeled Tyrosine (Tyrosine $^{13}C_9{}^{15}N$) as the internal standard. Standard calibration solutions were prepared by serial dilution of Waters amino acid cell culture standards (entry 1, Table 1) from 10 µM to 0.01 µM using 0.1%FA solution containing 1:400 diluted Earl's Balanced Salt solution (Millipore Sigma E2888) and 0.1 µM Tyrosine SIL.

Method Conditions

LC-MS System	BioAccord LC-MS system with ACQUITY Premier BSM	
Sample prep system:	Andrew+ Pipetting Robot	
LC conditions:	ACQUITY Premier HSS T3 Column 1.8 µm, 2.1 × 100 mm (P/N 186009468, or with guard P/N 186009471)	
	Mobile phase	(A) 0.1% FA in H_2O (B) 90%ACN/ 10%IPA/ 0.1%FA
	Injection volume	2 µL
	Run time	9 min, gradient elution
MS conditions:	Acquisition mode	Full scan or full scan with fragmentation, ESI+ or ESI-
	Mass Range	Small molecules (50–800 *m/z*)
	Scan rate	5 Hz
	Lockmass correction mode	Standard
LC-MS software:	waters_connect 3.1 or higher, preinstalled with UNIFI	

Results and Discussion

Description of the Rapid Throughput LC-MS Method

A rapid throughput nine-minute method for cell culture nutrient and metabolite analysis is described. The nine-minute method run time represents 50 percent reduction compared to the previously published 20-minute methods while maintaining the same compound coverage.[1] The method employs the ACQUITY Premier HSS T3 Column with dimensions of 2.1 x 100 mm and detected using BioAccord LC-MS System. Similar to the 20-minute method, amino acids are detected directly without derivatization. For spent media monitoring, a simple 1:100 to 1:400 (V:V) dilution of clarified media samples using the 0.1% formic acid aqueous mobile phase is the only sample preparation needed. The dilution ratio is determined by the concentration/fortification of media formulations. An automated and downloadable sample preparation protocol based on Andrew+ Pipetting Robot for the dilution is used. The compound library has been updated to include 220+ compounds, representing the most comprehensive coverage for cell culture media analysis currently available. The available workflow for data processing using waters_connect, including easy data review, elucidation of unknown compounds in the spent media, and MVDA using EZInfo, is the same as has been described previously in the 20-minute method.[1] The method's broad applicability was assessed by analyzing representative commercial media solutions, including Waters amino acid cell culture standard solution, DMEM, IMDM, CHO Fed-batch media, HEK293 viral vector media, and microbial growth media (see Table 1). For all media samples, the nine-minute method produced the same detection results as for the 20-minute method. Extracted ion chromatogram (XIC) of the 26 amino acids in

Waters amino acid cell culture standard kit is summarized in Figure 2. In all, the method produces sharp and symmetrical peaks as well as baseline separation of the isobaric compound pairs; isoleucine/leucine and 2-aminobutyric acid/4-aminobutyric acid.

Entry	Source	Name
1	Waters 186009300	Amino acid cell culture standard kit - has 26 amino acids
2	Millipore Sigma M4530	Medium 199
3	Millipore Sigma D6046	Dulbecco's Modified Eagle's Medium - low glucose
4	Millipore Sigma I3390	Iscove's Modified Dulbecco's Medium
5	Millipore Sigma 14366C	EX-CELL Advanced CHO Fed-batch Medium
6	Millipore Sigma 14385c	EX-CELL Advanced CD HEK293 Viral Vector Media
7	Millipore Sigma L2542	LB Broth (Miller), Liquid microbial growth media
8	Millipore Sigma T5574	Terrific broth, Liquid microbial growth media

Table 1. Summary of commercial solutions analyzed using the nine-minute method.

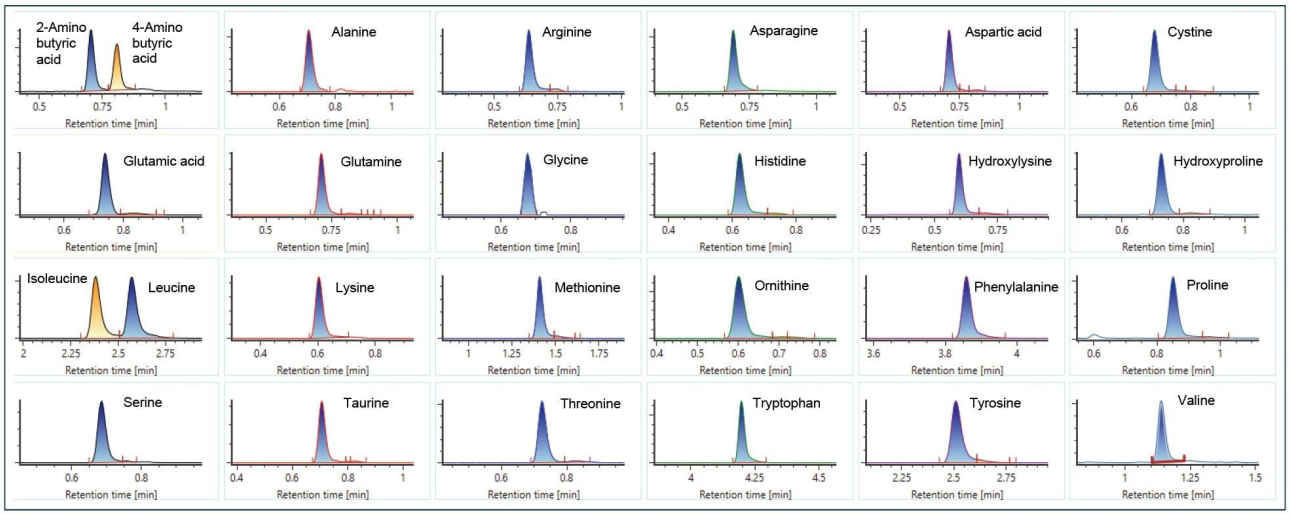

Figure 2. Extracted ion chromatogram (XIC) of 26 compounds in the amino acid cell culture standard kits. Two isobaric compound pairs, isoleucine/leucine and 2-amino/4-amino butyric acid are baseline separated.

The method performance is determined using Waters amino acid cell culture kit (p/n: 186009300 < https://www.waters.com/nextgen/global/shop/standards--reagents/186009300-amino-acid-cell-culture-standard-kit.html>) containing 26 amino acids. A separate calibration solution of glucose was also prepared.

Extracted ion chromatogram for each of the amino acid is shown in Figure 2. Method linearity was determined using linear regression with $1/x^2$ fitting and a 20% deviaton exclusion criteria. Method precision and accuracy were determined based on 6 replicate injections at two concentrations, 0.5 µM and 2.5 µM respectively. Data summarized in Table 2 showed excellent reproducibility and accuracy for all compounds measured and comparable to the 20-minute method.[2] These results suggest that the rapid throughput method can provide both quantitative and qualititive results. In the absence of standard, the relative reponse can be highly informative for analyzing changes in media nutrients and metabolites in process optimization experiment and subsquent MVDA analytical approaches.

Entry	Component name	Expected RT (min)	Neutral mass (Da)	Range (uM)	R^2	QC conc = 0.5 µM (n=6)		QC conc = 2.5 µM (n=6)	
						%Accuracy	%Precision	%Accuracy	%Precision
1	4-Aminobutyric acid	0.62	103.0633	0.025–10	0.99	94	3.6	98	1.7
2	Alanine	0.67	89.0477	0.05–10	0.99	108	4.3	103	2.3
3	Arginine	0.63	174.1117	0.01–5	0.99	92	5.6	107	1.4
4	Asparagine	0.68	132.0535	0.025–10	0.99	98	3.3	97	2.0
5	Aspartic Acid	0.66	133.0375	0.25–10	0.98	101	5.6	108	5.1
6	Cystine	0.64	240.0239	0.01–5	0.99	92	5.3	96	1.7
7	Glutamic Acid	0.75	147.0532	0.01–10	0.99	97	1.6	103	2.8
8	Glutamine	0.72	146.0691	0.01–10	0.99	98	3.6	105	1.4
9	Glycine	0.64	75.0320	0.5–10	0.99	113	5.5	108	4.0
10	Histidine	0.62	155.0695	0.1–10	0.99	96	5.9	102	1.6
11	Hydroxylysine	0.57	162.1004	0.25–10	0.99	107	3.1	102	2.6
12	Hydroxyproline	0.62	131.0582	0.025–10	0.99	96	4.4	104	2.5
13	Isoleucine	2.37	131.0946	0.01–10	0.99	90	3.7	104	2.0
14	Leucine	2.56	131.0946	0.025–5	0.99	89	3.8	108	3.9
15	Lysine	0.58	146.1055	0.025–10	0.99	95	3.0	106	2.5
16	Methionine	1.41	149.0511	0.01–5	0.99	91	3.5	107	2.7
17	Ornithine	0.58	132.0899	0.025–10	0.99	99	5.1	104	2.8
18	Phenylalanine	3.79	165.0790	0.01–10	0.99	91	5.4	103	3.3
19	Proline	0.83	115.0633	0.05–10	0.99	97	3.6	111	2.8
20	Serine	0.65	105.0426	0.05–10	0.99	98	4.3	100	2.0
21	Taurine	0.66	125.0147	0.05–2.5	0.99	116	9.0	95	3.6
22	Threonine	0.69	119.05824	0.025–10	0.99	100	3.6	100	2.3
23	Tryptophan	4.18	204.08988	0.01–10	0.99	88	2.8	103	2.3
24	Tyrosine	2.57	181.07389	0.025–10	0.99	92	3.9	103	2.1
25	Valine	1.12	117.07898	0.025–10	0.99	93	2.7	110	11.8
26	Glucose	0.76	215.0323 [+Cl]	0.1–5	0.99	118	2.3	105	2.3

Table 2. Summary of linear range, accuracy, and precision of amino acids and glucose quantification using the nine-minute method. Amino acid standard solution used in the analysis is the amino acid cell culture standard kit (p/n: 186009300). The linear range is derived from linear regression with $1/x^2$ fitting, 20% deviation was used as the exclusion criteria. Amino acids data were acquired using ESI+ and glucose using ESI- mode of acquisition.

Application to Spent Media Analysis

The method has been applied to spent media analysis of a CHO cell cultivation to produce cNISTmAb. Spent media solutions under different glucose feeding conditions were sampled during a 14-day incubation. After centrifugation, the supernatant solutions were diluted 1:400 with 0.1%FA and analyzed using both the nine-minute fast method and the 20-minute method. Figure 3 shows trending plots of MS response versus sampling day of different reaction flasks and comparing two analysis methods. Three representative amino acids are included in Figure 3, aspartic acid as an example of early chromatographically eluting compound, leucine as an example of mid eluting compound, and phenylalanine as an example of late eluting compound. Results showed excellent correlation where same trends were obtained using either method, indicating high quality data obtained in the 20-minute method is maintained using the rapid nine-minute method.

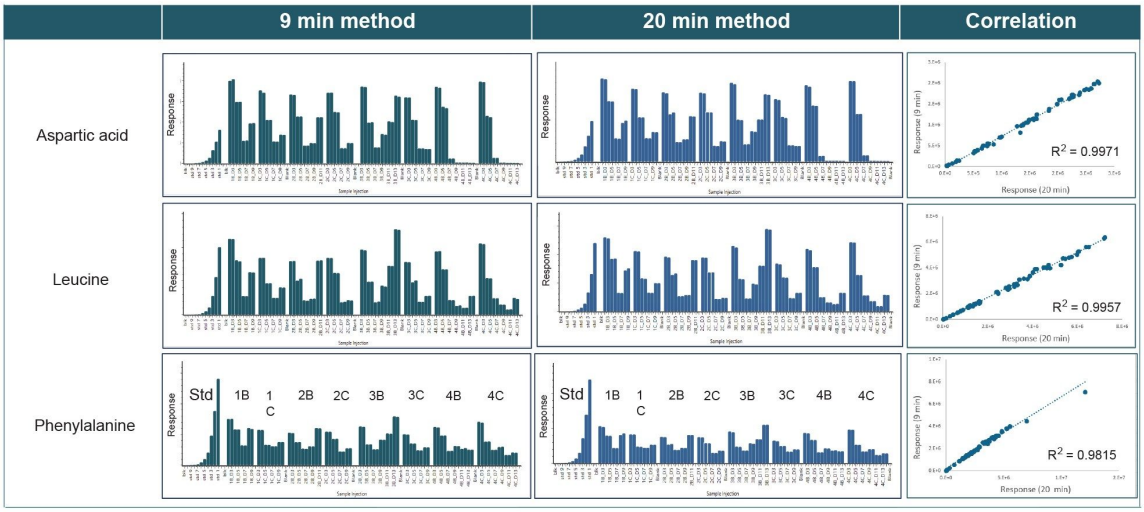

Figure 3. Trending plot of compound showing observed response as function of incubation condition and sampling time. Green graphs on the left are data from nine-minute method, blue graphs in the middle are from 20-minute method. On the right is correlation plot of response obtained from nine-minute method vs 20-minute method. All spent media samples were injected in duplicate.

Figure 4 is a plot of glucose reponse versus sampling time overlaying different glucose feeding conditions and comparing nine-minute (A) with 20-minute method (B). Figure 4 also included glucose data obtained from Nova Flex2 (C), a popular cell culture analyzer. In the experimental design, four glucose feeding conditions were tested. Flasks 1, 2, and 3 used reduced glucose feeding conditions. In flask 4, glucose were fed to maintain a constant 6

g/L concentration throughout the incubation. Results showed that at reduced glucose feeding conditions (flask 1–3), glucose was rapidly consumed and depleted during the exponential cell growth period (5–7 days); in comparison, glucose concentration was maintained during the same period using the maintanance dose conditions (greenline, flask 4). Results of cell viability and protein titer showed high cell viability and higher protein titer using the maintenance dose (see Waters application note for intact protein analysis in cell culture media using BioAccord[3]). Figure 4 shows that data from nine-minute method is comparable and of similar high quality to 20-minute LC-MS method, and there is good correlation between data obtained using either LC-MS method with the Nova Flex2 analyzer. In addition to glucose which was detected under negative ionization conditions, several amino acids including glutamine and glutamic acid are also detectable. Hence when monitoring key nutrient and metabolite, such as glucose, lactate, glutamine, and glutamic acids, is desired, results can be obtained from a single injection under negative ionization conditions.

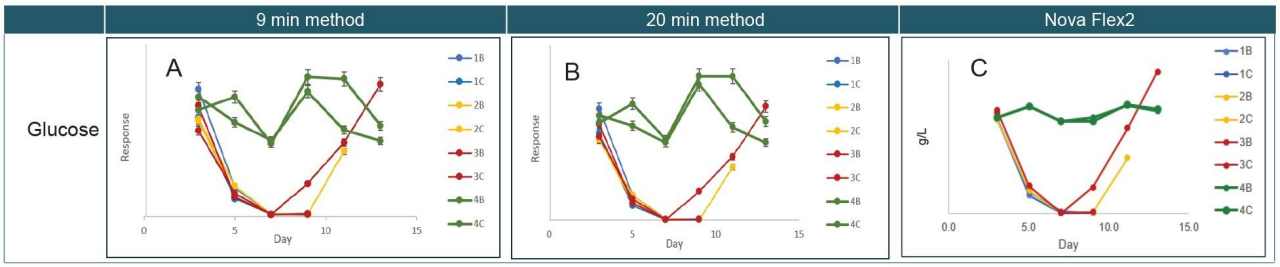

Figure 4. Plots of glucose response versus incubation time overlaying all feeding conditions. (A) data from nine-minute method, (B) data from 20-minute method, and (C) data from Nova Flex2 analyzer. Four glucose feeding conditions, represented by different colors, were used using four separate flasks in duplicate. Error bars in the graph represent duplicate measurements. Glucose chlorine adduct (+Cl) in LC-MS was used for its selective detection.

Conclusion

A rapid throughput method is described for cell culture media nutrient and metabolite monitoring. The method consists of a rapid nine-minute data acquisition, 220+ compound library screen, ease of data review, data report along with compound elucidation and batch comparison (MVDA) capabilities. Reducing the analysis time to

nine-minute allows rapid data generation in bioprocess laboratories. The method uses less than 10 µL of spent media sample and employs a simple dilution of clarified spent media sampled from bioreactors using aqueous mobile phase. Automated sample preparation of the media and standard solutions are readily carried out using the Andrew+ Pipetting Robot. Extensive data comparison of the rapid nine-minute method with the previously published 20-minute method suggests the same high-quality data and method robustness are obtained even when the analysis time is shortened by 50%. In conclusion, combination of the rapid throughput method with available waters bioprocess walk-up solution can enable analytical scientist and bioprocess engineers to obtain high quality data easily and routinely for upstream bioprocess optimization.[4] The full featured BioAccord LC-MS System provides routine monitoring as well as in-depth analysis of media samples for fully understanding your process.

References

1. YW Alelyunas, MD Wrona, W Chen, Monitoring Nutrients and Metabolites in Spent Cell Culture Media for Bioprocess Development Using the BioAccord LC-MS System with ACQUITY Premier, Waters Application note. 720007359. September 2021.
2. YW Alelyunas, MD. Wrona, YQ Yu, Quantification of Underivatized Amino Acids in Cell Culture Media Using the BioAccord LC-MS System, Waters Application note. 720007766. October 2022.
3. YW Alelyunas, C Prochaska, C Kukla, C Hanna, MD Wrona, Monitoring Intact Glycoprofiles and Spent Media Metabolites in Samples from Sartorius Ambr 250 High Throughput Bioreactor System to Support Upstream Process Development, Waters Application note. 720008042. September 2023.
4. YW Alelyunas, E Embrey, L Collins, A Pegaz-Blanc, G Mignard, MD. Wrona, Simplifying Bioreactor In-Process Monitoring with Waters Bioprocess Walk-Up Solutions, Waters Application note. 720008062. August 2023.

Featured Products

ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>

BioAccord LC-MS System for Biopharmaceuticals <https://www.waters.com/135005818>

Screening Platform Solution with UNIFI <https://www.waters.com/waters/nav.htm?cid=134682903>

UNIFI Scientific Information System <https://www.waters.com/134801648>

720008170, January 2024