Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1043 (2004) 3–7
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Potential of fermentation profiling via rapid measurement of amino acid metabolism by liquid chromatography–tandem mass spectrometry

Joseph J. Dalluge[a,*], Sean Smith[a], Fernando Sanchez-Riera[b], Chris McGuire[a], Russell Hobson[a]

[a] *Scientific Resources Center, Cargill, Inc., P.O. Box 5702, Minneapolis, MN 55440-5702, USA*
[b] *Biotechnology Development Center, Cargill, Inc., P.O. Box 5702, Minneapolis, MN 55440-5702, USA*

Available online 20 March 2004

Abstract

Monitoring amino acid metabolism during fermentation has significant potential from the standpoint of strain selection, optimizing growth and production in host strains, and profiling microbial metabolism and growth state. A method has been developed based on rapid quantification of underivatized amino acids using liquid chromatography–electrospray tandem mass spectrometry (LC–MS–MS) to monitor the metabolism of 20 amino acids during microbial fermentation. The use of a teicoplanin-based chiral stationary phase coupled with electrospray tandem mass spectrometry allows complete amino acid analyses in less than 4 min. Quantification is accomplished using five isotopically labeled amino acids as internal standards. Because comprehensive chromatographic separation and derivatization are not required, analysis time is significantly less than traditional reversed- or normal-phase LC-based amino acid assays. Intra-sample precisions for amino acid measurements in fermentation supernatants using this method average 4.9% (R.S.D.). Inter-day (inter-fermentation) precisions for individual amino acid measurements range from 4.2 to 129% (R.S.D.). Calibration curves are linear over the range 0–300 μg/ml, and detection limits are estimated at 50–450 ng/ml. Data visualization techniques for constructing semi-quantitative fermentation profiles of nitrogen source utilization have also been developed and implemented, and demonstrate that amino acid profiles generally correlate with observed growth profiles. Further, cellular growth events, such as lag-time and cell lysis can be detected using this methodology. Correlation coefficients for the time profiles of each amino acid measured illustrate that while several amino acids are differentially metabolized in similar fermentations, a select group of amino acids display strong correlations in these samples, indicating a sub-population of analytes that may be most useful for fermentation profiling.

Keywords: Metabolomics; Process monitoring; Amino acids

1. Introduction

Fermentation technology is playing an increasingly important role in the commercial production of chemicals, as metabolic pathway engineering and biotechnology make significant inroads into the global chemical industry. The current interest in metabolic engineering and bioprocess optimization [1–4] necessitates the development of highly sensitive and selective methods for the measurement of key intracellular and extracellular metabolites and intermediates. Data compiled from such methods can be employed in modeling and optimization of production processes. Because amino acids represent a significant nitrogen source for microbial cells in industrial fermentations, methods capable of rapid measurement of amino acid levels during fermentation has significant potential from the standpoint of strain selection, optimization of media formulations, and profiling microbial metabolism and growth state.

Methods of choice for the measurement of amino acids have traditionally been based on liquid chromatography (LC) combined with ultraviolet (UV) absorbance detection of amino acid derivatives obtained after pre- or post-column derivitization. However, these methods can be confounded by reagent interference, derivative instability, and time-consuming preparation and analysis [5,6]. A number of alternative detection methods for the determination of underivatized amino acids have been described [7] that overcome these limitations, including electrochemical, UV–fluorometric, and evaporative light scattering detection, but these approaches have not been widely practiced due to

* Corresponding author. Tel.: +1-952-742-3038; fax: +1-952-742-3010.
E-mail address: joseph_dalluge@cargill.com (J.J. Dalluge).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.02.010

limitations including low sensitivity, incompatibility with gradient elution, and the inability of analyzing complex biologically-derived mixtures. Recently, the determination of underivatized amino acids has been achieved using combined LC–tandem mass spectrometry (LC–MS–MS) [7–9], and this approach overcomes all limitations described above, although analysis times for the reported MS-based methods are still in the range 20–40 min, and reported applications of these methods to biological measurements are limited.

We presently report on the development of a method based on LC–electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS) for the rapid quantification (less than 4 min per analysis) of underivatized amino acids to monitor the metabolism of 20 amino acids during microbial fermentation. Data visualization and correlation techniques for constructing semi-quantitative fermentation profiles of nitrogen source utilization have also been developed and implemented. General illustrations of the application of this methodology to fermentation profiling are presented herein.

2. Experimental

2.1. Reagents

HPLC-grade methanol (MeOH) was obtained from Mallinkrodt Baker (Paris, KY, USA). Glacial acetic acid was obtained from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade water (18 mΩ), prepared using a Barnstead E-Pure purification system (Barnstead International, Dubuque, IA, USA), was used to prepare all solutions. L-Amino acids were purchased from Sigma (Sigma LAA-21, St. Louis, MO, USA). Deuterated internal standards [$^2H_3$]alanine, [$^2H_3$]methionine, [$^2H_3$]glutamic acid, [$^2H_4$]lysine, and [$^2H_5$]tryptophan were purchased from Cambridge Isotope Labs. (Andover, MA, USA).

2.2. Directly combined LC–ESI-MS–MS

Determination of amino acids in standards and fermentation broths was carried out using a Waters/Micromass liquid chromatography–tandem mass spectrometry system consisting of a Waters 2795 liquid chromatograph directly coupled to a Micromass Quattro Ultima triple quadrupole mass spectrometer. LC separations were made using a 50 mm × 4.6 mm Advanced Separation Technologies Chirobiotic T column at room temperature. The LC mobile phase consisted of (A) water containing 0.25% acetic acid; (B) methanol containing 0.25% acetic acid. The isocratic elution was at 50% B, 0–5 min. The flow rate was 0.6 ml/min. All parameters of the ESI-MS–MS system were optimized and selected based on optimal in-source generation of the protonated molecular ions of the 20 amino acids measured and the internal standards [$^2H_3$]alanine, [$^2H_3$]methionine, [$^2H_3$]glutamic acid, [$^2H_4$]lysine, and [$^2H_5$]tryptophan, as well as collision-induced production of amino acid-specific fragment ions for multiple reaction monitoring (MRM) experiments (Table 1). The following instrumental parameters were used for LC–MS–MS analysis of amino acids in the positive ion multiple reaction monitoring mode: capillary, 3.5 kV; cone, 20 V; hex 1, 15 V; aperture, 1 V; hex 2, 0 V; source temperature, 100 °C; desolvation temperature, 350 °C; desolvation gas, 500 l/h; cone gas, 40 l/h; low mass resolution (Q1), 12.0; high mass resolution (Q1), 12.0; ion energy, 0.2; entrance, −5 V; collision energy, 14; exit, 1 V; low mass resolution (Q2), 15; high mass resolution (Q2), 15; ion energy (Q2), 0.5; multiplier, 650. MRM parameters: interchannel delay, 0.03 s; interscan delay, 0.03 s; dwell, 0.05 s.

Table 1
MRM transitions and internal standards employed for the 20 amino acids measured in this study

Amino acid	Parent $[M+H]^+$	Daughter $[M+H]^+$	Internal standard
Gly	76	30	[$^2H_3$]alanine
Ala	90	44	[$^2H_3$]alanine
[$^2H_3$]Ala	93	47	
Ser	106	60	[$^2H_3$]methionine
Pro	116	70	[$^2H_5$]tryptophan
Val	118	72	[$^2H_3$]alanine
Thr	120	74	[$^2H_3$]methionine
Cys	122	76	[$^2H_3$]methionine
Ile/Leu[a]	132	86	[$^2H_3$]alanine
Asn	133	74	[$^2H_3$]glutamic acid
Asp	134	88	[$^2H_3$]glutamic acid
Gln/Lys[b]	147	84	[$^2H_3$]glutamic acid/[$^2H_4$]lysine
[$^2H_4$]Lys	151	88	
Glu	148	102	[$^2H_3$]glutamic acid
Met	150	104	[$^2H_3$]methionine
[$^2H_3$]Glu	151	105	
[$^2H_3$]Met	153	107	
His	156	110	[$^2H_4$]lysine
Phe	166	120	[$^2H_5$]tryptophan
Arg	175	116	[$^2H_4$]lysine
Tyr	182	136	[$^2H_5$]tryptophan
Trp	205	146	[$^2H_5$]tryptophan
[$^2H_5$]Trp	210	151	

[a] Leucine and isoleucine have identical mass spectrometric fragmentation behavior and are not separated using the methodology described herein. Concentrations reported herein for Leu/Ile are a composite value.
[b] Glutamine and lysine have identical mass spectrometric fragmentation behavior, but are separated chromatographically, allowing their independent determination.

2.3. Sample preparation and analysis

The method involved addition of precisely known amounts of five isotopically labeled amino acid internal standards to a 10:1 dilution of filtered fermentation samples, followed by direct LC–MS–MS analysis. For standardization, four calibration mixtures were prepared for each amino acid by mixing known amounts of the amino acids and appropriate internal standards to achieve four different mass ratios for each of 20 amino acids in the mixtures. These solutions were then analyzed by LC–MS–MS, and the data were subjected to a linear least squares analysis.

For simplicity, as no interfering responses were observed in blank samples, and because the method was applied here to semi-quantitative profiling of amino acid metabolism, intercepts for all calibration curves were forced through the origin. Fermentation samples were prepared as described above. The peak area ratios (amino acid/selected deuterated internal standard, Table 1) were then used in conjunction with the calibration curves to derive the concentration of individual amino acids in the starting material. For quantification, internal standards were grouped with amino acids based on complementary side-group functionality (Table 1).

2.4. Data correlation and visualization

All amino acid profile visualization and correlation data and figures were completed using Matlab v6.5 (Mathworks, Natick, MA, USA).

3. Results and discussion

The specific aims of this study were to develop a rapid (<5 min per sample) method based on LC–MS–MS that would allow simultaneous identification and quantification of 20 underivatized amino acids in complex biological mixtures such as fermentation supernatants or cell extracts, and to apply the developed methodology to fermentation profiling by measurement of microbial amino acid (nitrogen source) metabolism. The method developed here is similar to those described previously by Petritis et al. [8] and Qu et al. [9]. However, the method described herein is capable of quantifying 20 amino acids in less than 4 min per analysis, the only limitation of which is the inability to separate isoleucine and leucine, whereas analysis times for the previously reported methods are in the range 20–40 min. For the application described in this report, high-throughput capability was deemed more important than the ability to differentiate ile and leu. The method developed here is applicable also to the measurement of non-proteinogenic amino acids, and the separation and measurement of chiral amino acids (not shown). A description of the preliminary application of this LC–ESI-MS–MS method to fermentation profiling via measurement of microbial amino acid metabolism follows.

A typical MRM chromatogram for the analysis of four representative amino acids is illustrated in Fig. 1. MRM transitions monitored for each amino acid and internal standard, as well as the identities of the internal standards used for quantification of each amino acid, are tabulated in Table 1. The most abundant daughter ion monitored for the amino acid population was $[M-46+H]^+$, the only exceptions being Asn, Arg, and Trp that produced high-abundance daughter ions at $m/z = [M - 59 + H]^+$and Glu and Lys that produced high-abundance daughter ions at $m/z = [M - 63+H]^+$. Cross-channel interference from co-eluting amino acids has not been observed. To determine intra-sample and inter-day (inter-fermentation) precisions for the analyses of amino acids in fermentation broths, three identical *Rhodobacter sphaeroides* fermentations were run on three separate days, and time zero and harvest fermentation broth samples were collected for each run. Calibration curves were linear over the range 0–300 μg/ml, and detection limits calculated for the amino acids were in the range 50–450 ng/ml. Intra-sample precisions were very good, as reflected in a mean R.S.D. value of 4.9% (average R.S.D. of all triplicate sets, data not shown). Inter-day (inter-fermentation) precisions for time zero samples were in the range 4.2–19.0% R.S.D. (mean R.S.D. = 9.6%). Inter-day precisions for harvest collections were less reproducible, ranging from 27.6 to 128.9%. This scatter is due at least in part, to the very low concentrations (low parts-per-billion) measured for the amino acids at harvest, and may also be attributed to other minor between-day variations in fermentation conditions. If higher degrees of precision and accuracy are desired for a given amino acid, the corresponding isotopically-labeled analyte can be added to standards and samples to effect more accurate and precise measurements. The degree of scatter observed here, however, should not affect visualization of an overall fermentation amino acid profile, and this preliminary statistical data suggest that the method is capable of profiling amino acid metabolism in fermentation samples.

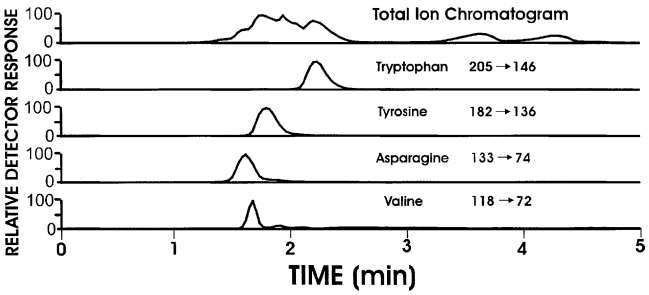

Fig. 1. LC–ESI-MS–MS multiple reaction monitoring (MRM) for the determination of amino acids during fermentation. The total ion chromatogram for the 20 MRM transitions monitored, and individual MRM transitions for four selected amino acids are illustrated.

To demonstrate further, the feasibility of the method described herein for generating and visualizing amino acid profiles, amino acids were measured over the full course of two *Moniliella pollinis* erythritol fermentations, each of which utilized a unique carbon source (C1, beet sugar syrup; C2, concentrated beet syrup). Note that care was not taken to minimize oxidation of the fermentation samples analyzed. As such, measured free cysteine levels were very low (parts-per-billion) and imprecise, and were therefore not included in the profiles illustrated in Fig. 2. The values used to generate the two plots are the respective means of duplicate runs for each sample time point. The concentration of each amino acid over the course of the run is normalized to its maximum concentration (defined as 1.0), and then mapped to a pseudocolor scale from deep blue (0) to deep red (1). Included with each graph as a visual reference is the actual color scale. Also noted is the maximum concentra-

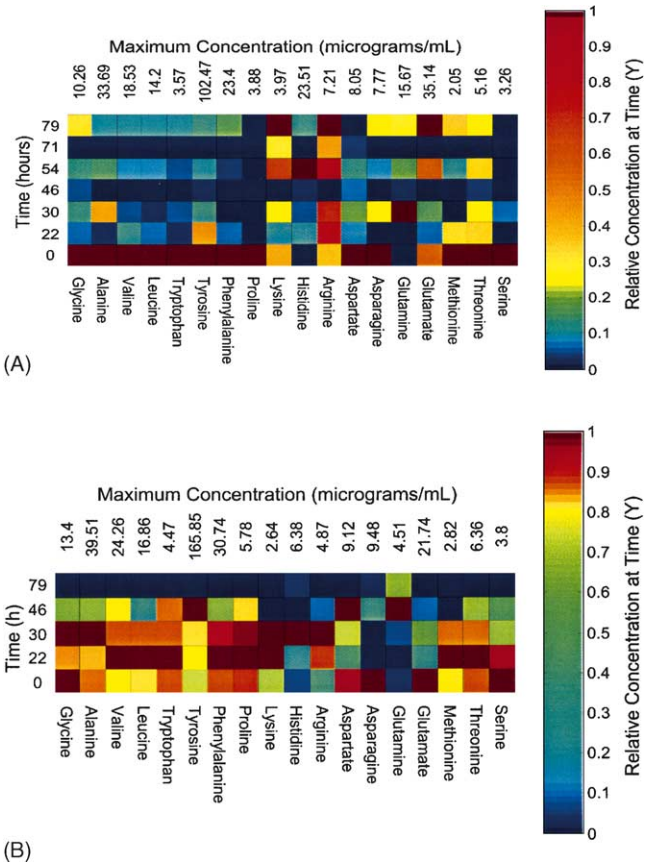

Fig. 2. Amino acid profiles for *Moniliella pollinis* erythritol fermentations employing different carbon sources. (A) Fermentation C1, carbon source: beet syrup; (B) fermentation C2, carbon source: concentrated beet syrup.

tion of each amino acid (at the top of its respective *y*-scale), allowing one to semi-quantitatively compare amino acid concentrations, one with another over time, in addition to the ability to follow overall trends for specific amino acids. When the data are visualized in this way, differences in nitrogen source (amino acid) metabolism in the fermentations employing different carbon sources can be seen and tend to correlate with observed growth profiles (Fig. 3). For instance, fermentation C1 (Fig. 2A) represents the profile calculated for *M. pollinis* during a "normal" fermentation in

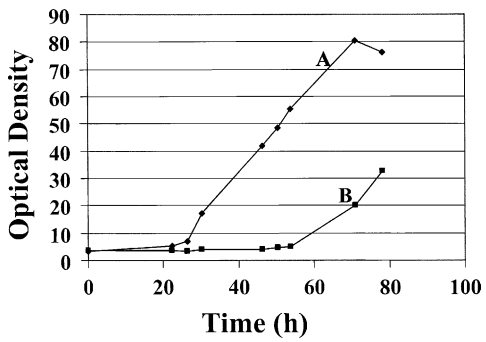

Fig. 3. Overlaid growth profiles of *Moniliella pollinis* fermentations C1 (A) and C2 (B).

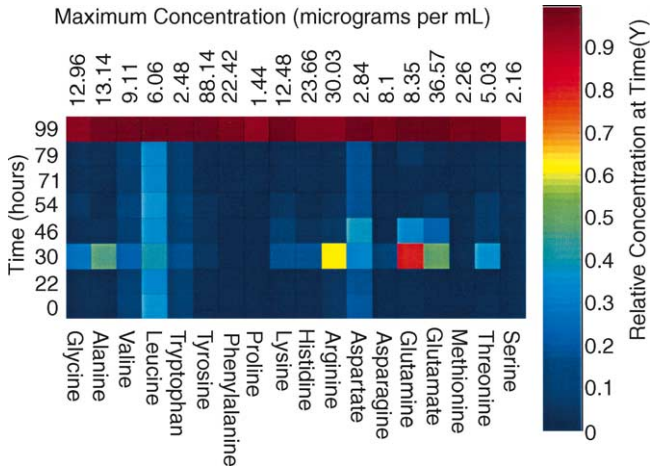

Fig. 4. Amino acid profile of a *Moniliella pollinis* fermentation demonstrating detection of cell lysis between 79 and 99 h.

which product accumulation was as expected, and no unusual growth events occurred. By contrast, *M. pollinis* in fermentation C2 had an extensive lag phase prior to growth (Fig. 3), and the comparatively static profile of C2 shown in Fig. 2B reflects this. When an amino acid profile for a similar fermentation includes the 99 h time-point (Fig. 4), a dramatic spike in free extracellular amino acid concentration due to cell lysis is observed, illustrating the potential of this profiling method in identifying key growth events during fermentation. Determination of cell lysis time for a fermentation can be valuable during process development and optimization. It is important to note the caveat in interpreting these differences at a preliminary stage as the fermentations profiled here (Fig. 2) were run in singlicate. As such, more data needed to be collected on control fermentations to be confident that differences in amino acid profiles such as those illustrated here, correlate in a definitive way with differences in fermentation feedstocks, and to understand better what differences may be due to uncontrolled variables present in the system.

To address this, two *Propionibacter* sp. P18 propionic acid fermentations (run side-by-side on the same day in separate fermentors) were profiled to correlate amino acid profiles and understand better the "noise level" of extracellular amino acid concentrations over the course of a fermentation. Correlation coefficients (R values) were calculated for each of the measured amino acids between the two fermentations according to the following relation:

$$R = \frac{\sum(N156_i - \mu_{N156})(N356_i - \mu_{N356})/3}{\sigma_{N156}\sigma_{N356}}$$

where μ is the mean of the individual amino acid concentrations measured over four time points, and σ is the standard deviation of these values [10]. This analysis allows identification of highly correlated amino acids, which, when measured subsequently for comparison of similar fermentations, will be most suitable for identifying growth trends

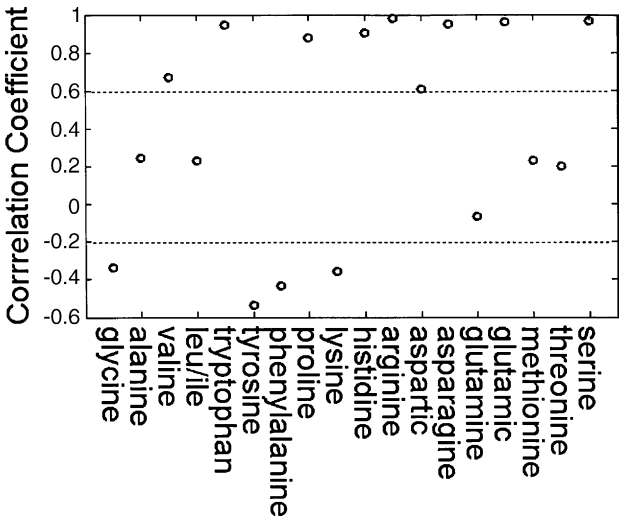

Fig. 5. Correlation of individual amino acid profiles in two *Propionibacter* sp. P18 propionic acid fermentations. Standard univariate correlation coefficients (R) are plotted for each of the 19 amino acids measured in the two side-by-side fermentation runs.

and physiological events. The results of this experiment are illustrated in Fig. 5. It is observed that nine amino acids (valine, tryptophan, proline, histidine, arginine, aspartic acid, asparagine, glutamic acid, and serine) display strong positive correlations between the two fermentations, and represent the best candidates for amino acid profiling in fermentation/process optimization studies. By contrast, five amino acids illustrate very little correlation, and four actually appear to be anti-correlated in the fermentations studied here. The latter amino acids (glycine, alanine, leucine/isoleucine, tyrosine, phenylalanine, lysine, glutamine, methionine, and threonine) may therefore represent less appropriate candidates for amino acid profiling in fermentation/process optimization studies, as their measured concentrations can vary widely due to environmental and organismal factors that cannot be controlled.

In conclusion, a rapid method for the simultaneous measurement of underivatized amino acids in complex biological mixtures has been developed and applied to profiling the metabolism of extracellular amino acids in microbial fermentations. This methodology has the potential to provide valuable information for optimization of media formulations, nitrogen and carbon source selection, and detection of significant host strain physiological events. In addition, the developed methodology has been demonstrated for the determination of non-proteinogenic amino acids, and for the separation and determination of chiral amino acids (not shown). This technology should find widespread use in the burgeoning areas of metabolic engineering and bioprocess optimization.

References

[1] M.C. Coleman, K.K.S. Buck, D.E. Block, Biotechnol. Bioeng. 84 (2003) 274.
[2] S.V. Patil, V.K. Jayaraman, B.D. Kulkarni, Appl. Biochem. Biotechnol. 102–103 (2002) 119.
[3] R. Berkholz, D. Rohlig, R. Guthke, Enzyme Microbiol. Technol. 27 (2000) 784.
[4] G. Chotani, T. Dodge, A. Hsu, M. Kumar, R. LaDuca, D. Trimbur, W. Weyler, K. Sanford, Biochim. Biophys. Acta 1543 (2000) 434.
[5] H. Godel, P. Seitz, M. Verhoef, LC–GC Int. 5 (1992) 44.
[6] D. Fekkes, J. Chromatogr. B 682 (1996) 3.
[7] K. Petritis, C. Elfakir, M. Dreux, J. Chromatogr. A 961 (2002) 9.
[8] K. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, J. Chromatogr. A 896 (2000) 253.
[9] J. Qu, Y. Wang, G. Luo, Z. Wu, C. Yang, Anal. Chem. 74 (2002) 2034.
[10] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building, Wiley, New York, 1978, p. 61.