Analysis of Sugars and Organic Acids in Cell Culture Fermentation Broths using Cation Exchange HPLC

Authors

A. Rattenholl, A. C. Braun Streb, F. Gudermann, D. Lütkemeyer

Fachhochschule Bielefeld, Fachbereich Ingenieurwissenschaften und Mathematik, Apparative Biotechnologie, Kurt-Schumacher-Str. 6, 33615 Bielefeld, Germany

I. Garaguso

Omnilab-Laborzentrum GmbH & Co.KG, Robert-Hooke-Str. 8, 28359 Bremen, Germany

FH Bielefeld
University of
Applied Sciences

OMNILAB

Abstract

A Hamilton HC-75 H^+ column was used to separate sugars and organic acids present in cell culture supernatants. An evaporative light scattering detector (ELSD) was used for detection.

Introduction

Cell culture media are complex mixtures of carbon sources such as glucose and other compounds including yeast extract and peptone. For cellular metabolism and flux analysis a quantitative analysis of the compositional constituents present in the media is essential. Moreover, it is important to detect individual components including organic acids and carbohydrates, so that the metabolic networks can be precisely elucidated. A number of methods for analysis are available. Reversed-phase high-performance liquid chromatography (HPLC) incorporated with an ultraviolet (UV) detector, a refractive index (RI) detector, or photo-diode array detection has been generally employed. In addition, ion chromatography (IC), mostly with conductivity detection, has also been applied for the separation and detection of organic acids and sugars in complex samples. However, direct analysis of these analytes in the presence of common inorganic anions remains an analytical challenge.

The HC series columns from Hamilton are gel-type cation exchange columns which separate compounds through size exclusion and ligand exchange. In the present study, we demonstrated an IC protocol for separation of several potential analytes including organic acids and sugars with evaporative light scattering detection (ELSD) using an HC-75 H^+ form column. We successfully profiled organic acids and sugars in biologically relevant samples including cell culture media. This procedure can be applied to the analysis of other complex samples such as food products, beverages, biomass hydrolysates and fermentation broths, where strongly hydrophilic compounds and anions are also present.

Material & Methods

Fermentation

Cell culture was carried out in a 2 L glass bioreactor (Biostat Aplus, Sartorius Stedim Biotech, Göttingen, Germany). 1.5 L MAMP-F2 medium (BioConcept, Allschwil, Switzerland) supplemented with 2 mM Gln was inoculated with CHO cells to an initial cell concentration of 1.95*10E5 cells/mL. Cells were subsequently cultured for 7 days at 37°C. Samples were drawn daily. Supernatants were collected after centrifugation for 5 min at 800xg. They were then split in half for subsequent analysis. One half was analyzed with a CuBiAn XC bioanalyzer (Optocell, Bielefeld, Germany) to monitor glucose and lactate contents. The other half was analyzed by HPLC/ELSD as described below.

HPLC/ELSD

Separation of analytes was carried out on a Varian HPLC system consisting of two ProStar 210 pumps, a ProStar 410 autosampler, a 510 Mistral column oven and a 380-LC evaporative light scattering detector (ELSD) (Agilent, Darmstadt, Germany). Data recording and processing was done using Galaxie software (Agilent). Separation was achieved with a Hamilton HC75 H^+ form column (250 x 4.1 mm; 9 µm; pressure limit: 27 bar). The mobile phase consisted of water/TFA pH 2 (isocratic elution). Standards were dissolved or diluted in water and the pH was subsequently adjusted to 2 with TFA. Cell culture supernatant samples were prepared as follows: 400 µL sample was heated to 99°C for 15 min to denature proteins and subsequently acidified with 25 µL 2 M HCl to pH 2.

HAMILTON®

Then samples were filtered through a 0.45 µm filter and injected onto the column. Samples were separated by isocratic elution at 0.2 mL/min and 50°C oven temperature. Peaks were detected using a nebulizing temperature of 60°C and an evaporation temperature of 30°C. The nitrogen flow was set to 1.9 standard litres per min. The acquisition rate was set to 10 Hz. The column was stored in 0.5 mM succinic acid.

Results & Discussion

Disaccharides (maltose and lactose), monosaccharides (glucose, galactose and fructose) as well as the small molecule glycerol were well separated by the HC-75 H^+ column. Disaccharides elute before monosaccharides. However, disaccharides and monosaccharides among themselves were not well resolved (Fig. 1). Table 1 shows the retention times of standards achieved under the elution conditions described above.

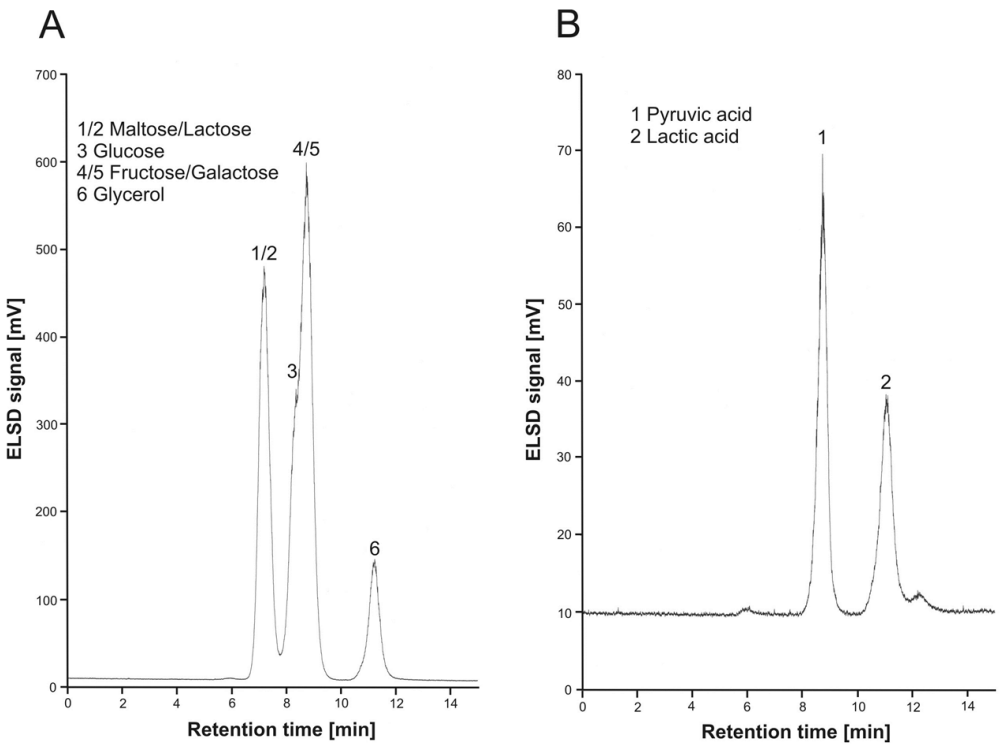

Fig. 1: Separation of some sugars (A) and organic acids (B) present in fermentation broths.

Standard	Quantity [µg]	Retention time [min]	Peak height [mV]	Area [mV*min]
Injection peak	-	5.88	-	-
Maltose	5	7.06	195.0	70.6
Lactose	5	7.14	175.5	61.6
Glucose	5	8.16	187.6	70.3
Galactose	5	8.65	172.9	60.6
Fructose	5	8.72	189.0	68.3
Pyruvic acid	50	8.68	127.9	52.8
Lactic acid	50	11.00	118.0	58.4
Glycerol	5	11.14	101.0	36.7

Tab. 1: Retention times of some sugars and organic acids on HC-75 H^+.

Note that the peak signals of the organic acids were roughly ten times smaller compared to those of the sugars analyzed. Moreover, it was important to lower the pH of the mobile phase below the pKs value of the acids. Otherwise, they deprotonated, became charged and did not elute (not shown). TFA was chosen to acidify the mobile phase because it is a strong acid that only slightly enhanced baseline noise. If only uncharged molecules (e.g. sugars) were analyzed, pure water was sufficient for elution and did not adversely affect data accuracy (not shown). In Figure 2, calibration curves for representative analytes are presented. The relationship between the concentration of the analyte and the ELSD signal displayed an exponential behaviour [1]. Figure 3 shows chromatograms obtained from a CHO batch fermentation process. Glucose and pyruvate were consumed over time whereas lactate was produced. A quantitative analysis of the analytes glucose, pyruvate and lactate is given in Figure 4.

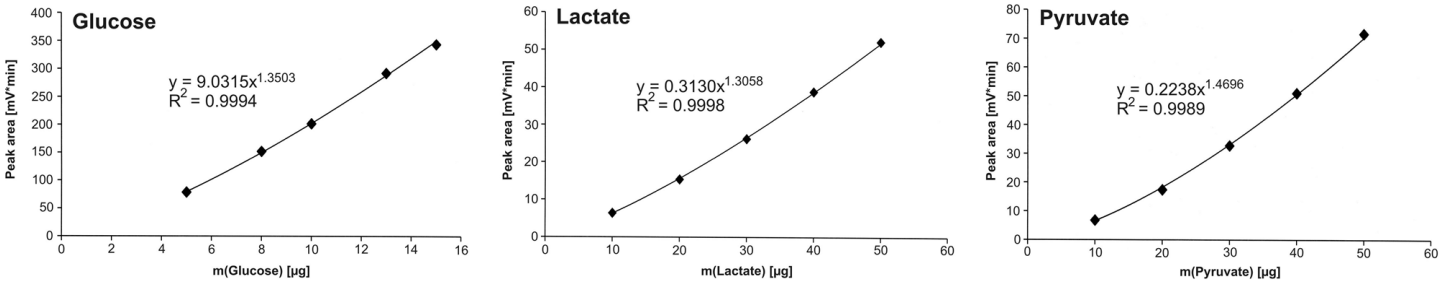

Fig. 2: Calibration curves of some important metabolites that are monitored during eucaryotic cell culture processes. The response curves were fitted to a 2nd order polynomial using Excel software.

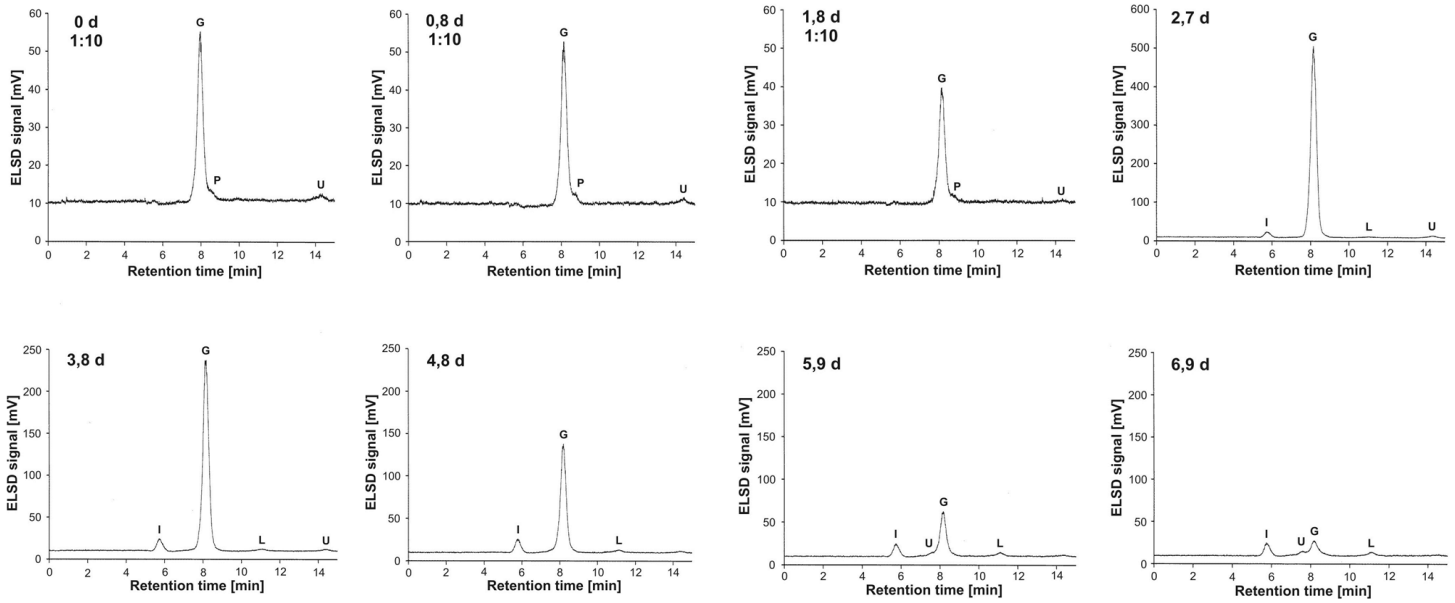

Fig. 3: Chromatograms of samples drawn from a CHO batch fermentation process. Note that samples 1-3 had to be diluted due to the large glucose signal. G: glucose, P: pyruvate, L: lactate, I: injection peak, U: unknown species.

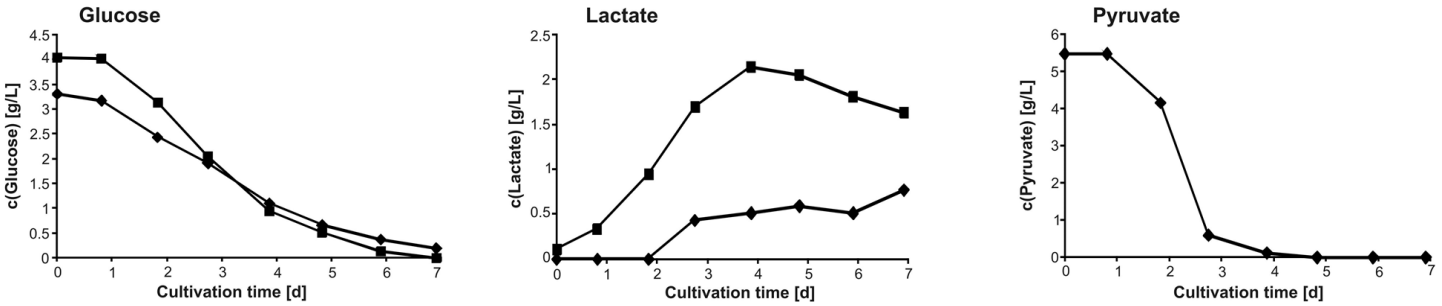

Fig. 4: Quantification of glucose, pyruvate and lactate concentrations of the CHO fermentation process using cation exchange HPLC/ELSD (diamonds). In addition, glucose and lactate contents were analyzed with a colorimetric reference method (bioanalyzer, squares).

Glucose and lactate were also analyzed with a CuBiAn XC bioanalyzer as reference measurement. A comparison of both data shows that quantification using the HPLC/ELSD method is limited. Whereas glucose concentrations up 2 g/L could be monitored well, the data related to higher glucose concentrations were not so reliable. In addition, quantitation of lactate and pyruvate was difficult to achieve since signals of organic acids were smaller than those of sugars when using the ELSD detection method. Moreover, the pyruvate peak was not well resolved since this analyte had a similar retention time as glucose.

Conclusion

With many automated bioanalyzers, glucose and lactate can be easily monitored. However, most modern cell culture media contain galactose and pyruvate. It is often very difficult to analyze these analytes with the mentioned analyzers. With the same Hamilton HC-75 H^+ column, both analytes can be monitored. However, a quantification of analytes might be limited when using light scattering for detection. For reliable quantitative analysis, other detectors might be more appropriate, e.g. UV absorbance or refraction index measurements. Nevertheless, the HPLC/ELSD method is appropriate for qualitative analysis: one can see that pyruvate and glucose are consumed after five days of cultivation. Thus, both nutrients have to be fed after this time period.

References

[1] R. Lucena, S. Cárdenas, M. Valcárcel: Evaporative light scattering detection: trends in its analytical uses. Anal. Bioanal. Chem. (2007) 388:1663-1672.

Lit. No. 695147/00 — 04/2013

HAMILTON®

Web: www.hamiltoncompany.com
USA: 800-648-5950
Europe: +41-81-660-60-60

Hamilton Americas & Pacific Rim
4970 Energy Way
Reno, Nevada 89502 USA
Tel: +1-775-858-3000
Fax: +1-775-856-7259
sales@hamiltoncompany.com

Hamilton Europe, Asia, & Africa
Via Crusch 8
CH-7402 Bonaduz, Switzerland
Tel: +41-81-660-60-60
Fax: +41-81-660-60-70
contact@hamilton.ch

To find a representative in your area, please visit hamiltoncompany.com/contacts.