Journal of Chromatography, 546 (1991) 251–257
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2095

Cation analysis of fermentation broth by high-performance liquid chromatography utilizing ion interaction

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ABSTRACT

Nutrient information concerning the specific needs of microorganisms in fermentation broth is crucial to the potency and production of an antibiotic. Nutrient analysis is hindered by an array of fermentation broth matrix interferences. These matrix interferences can be effectively removed by ashing the sample, although most of the major interferences can be removed by solid-phase extraction. The ashed sample was extracted using 10^{-3} *M* nitric acid and filtered through a 0.45-μm filter. The sample extract was chromatographed on an Adsorbosphere HS C_{18} column with conductivity detection. The mobile phase consisted of 40 m*M* tartaric acid, 2 m*M* sodium octanesulfonate in 5% methanol adjusted to pH 4.0 with sodium hydroxide. The flow-rate was 0.8 ml/min. Under these conditions, calcium, copper, lead, zinc, nickel, cobalt, cadmium, iron(III), magnesium and manganese are separated in less than 35 min. All analytes, with the exception of calcium and iron, are baseline-resolved. The response is linear for the range 1–1000 ppm. This method is able to monitor the microorganisms' cation consumption from the starting raw materials such as the flours and water used in producing a suitable starting culture to the final fermentation products. The use of post-column derivatization using ultraviolet detection allows for greater minimum detectable quantities (approximately 10^{-9} g) with a larger linear dynamic range.

INTRODUCTION

Information concerning the nutritional requirements of a microorganism in fermentation broth is crucial to the potency and production of an antibiotic. Their shifting nutrient consumption of carbohydrates, amino acids, vitamins, minerals and medium additives need to be monitored in order to promote optimal growth of the microorganism. All nutrients can limit the antibiotic production rate by being present in concentrations that imbalance the microorganisms' metabolic processes. The concentration ranges which enhance or inhibit fermentation activity vary with each microorganism, chemical species and growth conditions. The conditions and medium required for the optimal growth phase may differ somewhat from the conditions required for the production phase.

Concentrations of specific elements are vital as co-factors to the optimal metabolism of commercially important compounds. Optimal bacitracin production requires manganese at 0.7 μM but is inhibited by a manganese concentration of 40 μM [1]. *Streptomyces griseus* requires five times as much iron to produce streptomycin as

0021-9673/91/$03.50

it does for growth [2]. *Corynebacterium diphtheriae* only produces its toxin when iron levels are below 0.8 ppm [2].

Analysis of the trace elements is hindered by an array of fermentation broth matrix interferences. Industrial fermentation broth contains a host of natural ingredients, such as soy and cottonseed flours, peanut and corn meals, molasses, starch, beef extract and lard oil, as well as the starting culture. Through aerobic respiration, these starting materials undergo enzymatic and chemical degradation which result in numerous metabolic by-products which can interfere with chromatographic analysis. These matrix interferences can be effectively removed by ashing the fermentation broth sample, although most of the major interferences can be removed by solid-phase extraction, enabling trace mineral measurement by high-performance liquid chromatography (HPLC) utilizing an ion-interaction or ion-pairing reagent (IC).

HPLC–IC is a version of ion-exchange chromatography where ionizable solutes can be separated by differences in the electrostatic interaction with an ionizable stationary phase. The conductivity of the mobile phase has to be low in order to attain instrumental sensitivity if conductivity detection is used. This is usually attained by either suppressed conductivity or with the use of low-conductivity eluents.

An alternative to ion-exchange and ion-suppression analysis of ionic samples is the technique referred to as ion-pair chromatography. The pH of the eluent is adjusted to optimize ionization of the sample. The chromatographic retention is altered by including a counter ion in the mobile phase. This technique has the ability to separate both ionic and neutral materials. The detection of the separated species can be done with conductivity detection unless post-column derivitization is performed to enable UV–visible or fluorescence detection. In this paper, techniques for sample preparation and HPLC–IC analysis for several trace metals in fermentation broth are described.

EXPERIMENTAL

Materials

The mobile phase components were prepared with HPLC-grade methanol and water (Burdick and Jackson, Muskegon, MI, U.S.A.), sodium octanesulfonate and tartaric acid (Sigma, St. Louis, MO, U.S.A.). A system suitability solution containing calcium, cadmium, cobalt, copper, iron, lead, maganese, magnesium, nickel and zinc as the chloride salts was prepared. The system suitability solution provides a measure of performance of the chromatographic systems' ability to reproducibly resolve the ions of interest.

Chromatographic system

A chromatograph equipped with a constant-flow pump (Wescan 2815, Alltech Assoc., Deerfield, IL, U.S.A.) was used with a conductivity detector (Wescan 215), a variable-wavelength UV–visible detector (Shimadzu SPD-6AV, Kyoto, Japan), an autosampler (Shimadzu SIL-9A) and integrator (Shimadzu CR-501). The separations were performed on an Adsorbosphere HS C_{18} column (250 mm × 4.6 mm, 7 μm particle size, Alltech Assoc.). The mobile phase consisted of 40 m*M* tartaric acid and 2 m*M* sodium octanesulfonate in 5% methanol. The pH of the mobile phase was adjusted to 4.0 with sodium hydroxide. The mobile phase was filtered and degassed.

The flow-rate was 0.8 ml/min. The system temperature was maintained at 30°C. Injection volume was 50 μl.

Sample preparation

Approximately 10–20 g of fermentation broth were placed in a porcelain ashing crucible and placed in an ashing oven (Neytech 85P, Ney Corp., Yucaipa, CA, U.S.A.). The oven was programmed to heat to 160°C for 1 h, then increased at 10°C/min to 600°C and hold at that temperature for 3 h. After ashing was complete, the sample was dissolved in 100 ml of $1.0 \cdot 10^{-3}$ *M* nitric acid, shaken, then stirred for 30 min. The extract was filtered and prepared for HPLC–IC analysis by filtering an aliquot through a 0.45-μm Nalgene syringe filter (Nalge, Rochester, NY, U.S.A.). An alternative to ashing would be solid-phase sample preparation which was used for the post-column reaction. This involves a 1:1 (v/v) dilution of the broth sample with a 1:1 (v/v) methanol–water mixture. The sample was centrifuged and filtered. Approximately 5–10 ml of the sample were passed through a Maxi-Clean IC-RP (Alltech Assoc.) cartridge (previously conditioned with methanol) using a luer-lok syringe at a flow-rate of approximately 1 ml/min. The first 1 ml of solution was discarded. The remaining eluent was collected for analysis.

The solid-phase sample preparation method was more reproducible and less time-consuming than ashing. Fermentation media for erythromycin (starch, soybean meal and lard oil) and cephalosporin C (cottonseed flour, peanut meal, lactose and methionine) were spiked with zinc chloride. Six samples of each fermentation media were ashed or solid phase-extracted. The ashing technique had a 95% recovery (94.87 ppm ± 2.11% Zn). The solid-phase extraction had greater than 99% recovery (99.58 ppm ± 0.917% Zn) with better reproducibility. The choice of sample preparation technique depends on the concentration of analyte to be determined and the type of detection [3]. In many instances in fermentation, a large amount of broth (20–100 ml) would have to be extracted in order to attain analyte concentrations needed for conductivity detections, making ashing the preferred choice. If a post-column reactor is available, solid-phase extraction is the preferred method.

Standard and system suitability preparation

Standards were prepared from the individual chloride salts by dissolving an accurately weighed amount in a 100-ml volumetric flask and diluting with $1.0 \cdot 10^{-3}$ *M* nitric acid. The concentrations of the stock solutions were 1000 ppm of the aforementioned ions. Dilutions were made accordingly. The system suitability solution contained calcium, cadmium, cobalt, copper, iron(III), lead, manganese, magnesium, nickel and zinc. The concentration of these salts were approximately 100 ppm.

RESULTS AND DISCUSSION

Fig. 1 illustrates the ability of the chromatographic system to separate the cations present in the system suitability mixture. There is excellent resolution for all the cations except for calcium and iron(III). The chromatrographic system was specific for the ions examined. There were no interfering fermentation components after ashing or solid-phase extraction. Monovalent cations in this system elute at the void. A linearity test for zinc, cobalt and maganese was conducted (Fig. 2). There was

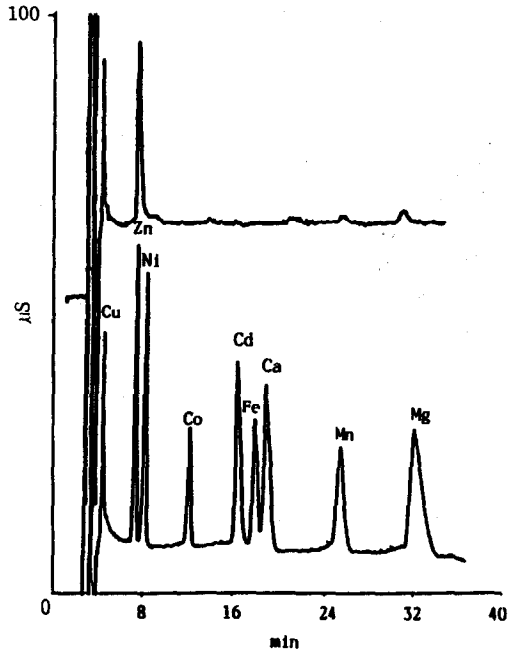

Fig. 1. Chromatogram of system suitability cations using conductivity detection. Chromatogram insct of 23-h fermentation broth sample.

excellent linear correlation ($r = 0.999$) for these cations in the concentration range 1–1000 ppm. The chromatographic method and system were precise and accurate (within 2%, $n = 6$) for repeated analysis of samples and standards.

The purpose of ion pairing is to add a second ion to the eluent which will combine with the sample ions, effectively binding them to create a neutral ion pair which then will undergo the normal partitioning or other distribution process between the stationary and mobile phase. From this point on, changes in retention time are accomplished by using standard reversed-phase techniques such as adjusting the pH, organic/inorganic solvent ratio or buffer concentrations. If the pH of the mobile

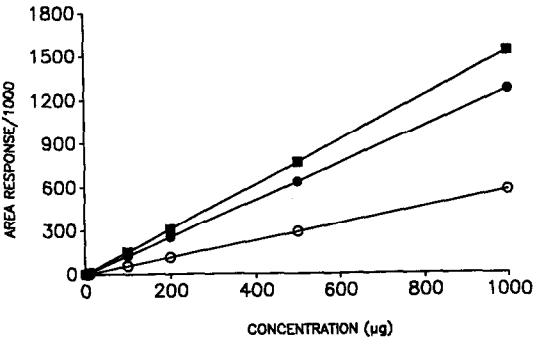

Fig. 2. Calibration curves of peak areas *versus* concentrations of zinc (■), manganese (●) and cobalt (○).

phase is changed from 4.0 to 3.5, the retention time for the cations examined doubled. The conditions used in this analysis can separate and elute the above cations in approximately 35 min. The inset in Fig. 1 represents a chromatogram of a 23-h fermentation broth sample. Zinc is the most abundant cation present (55 ppm ± 1.84%) with smaller amounts (less than 20 ppm) of copper, iron and magnesium. These chromatograms illustrate that these cations can be monitored in fermentation broth for the lifetime of the microorganism, which is approximately 24 h. This method is applicable to other forms of fermentation media such as beverage production [4]. Chromatographic monitoring of these elements can also reflect the varying seasonal composition of starting materials as well as elucidate incidental metal contaminants from the metal-based fermentation tanks or other contact materials which can lead to low antibiotic titer production.

This procedure provides valuable information for fermentation monitoring. It, however, has several limitations. A large amount of sample is needed in order to obtain sufficient quantities of the trace elements. Conventional ashing requires 4–6 h, thus increasing the time requirement for this analysis. If ashing is the preferred method of sample preparation, one might consider microwave ashing to reduce the sample ashing time. Solid-phase sample preparation is a viable alternative to ashing,

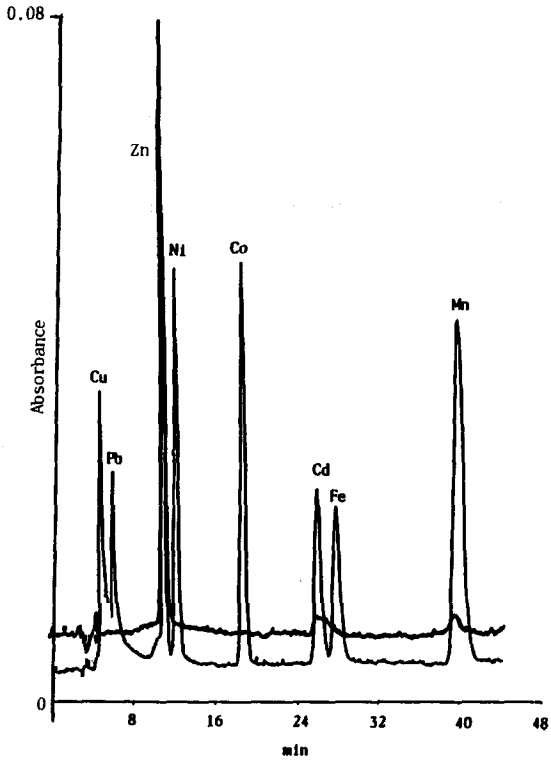

Fig. 3. Chromatogram of system suitability cations using post-column derivatization with UV–visible detection. Chromatogram inset of fermentation broth sample at 1 h using post-column derivatization with UV–visible detection.

although a large amount (20–100 ml) of fermentation broth needs to be eluted through the cartridge in order to obtain the amounts needed for conductivity detection. Conductivity detection is usually limited to approximately 1 ppm of analyte. Many elements can inhibit antibiotic metabolic pathways at concentrations well below 1 ppm [1].

A modification of this procedure can be accomplished by using a post-column derivitization technique. The sensitivity of the trace metal analysis can be increased dramatically by using a post-column reaction combined with a UV–visible detection. The sample preparation would involve dilutions, filtrations and solid-phase extraction (as describe in Experimental) to reduce the hydrophobic components that exist in the sample matrix.

The separation conditions are the same except for the addition of a post-column reactor. Although commercial units are available, the equipment needed to perform post-column reactions is relatively simple. A low dead volume tee for adding the post-column reagent to the column effluent is connected to a single-bead string reactor (Supelco, Bellefonte, PA, U.S.A.) and a 3-m delay tube. The apparatus is temperature-controlled by either a water jacket or a column heating box. The post-column reactant, 0.2 m*M* 4-(2-pyridylazo)resorcinol (PAR) (Sigma) in 3 *M* ammonium hydroxide and 1 *M* acetic acid was used to derivatize the cations. Detection was by visible at 520 nm [5]. Fig. 3 depicts the system suitability solution at a concentration of approximately 1 ppm. The response and baseline stability are improved considerably in comparison to the non-derivatized conductivity detected cations. Although calcium and magnesium are retained on the column, they do not react with PAR, and are not detected. The inset of Fig. 3 is a fermentation broth sample at 1 h. Comparisons of Figs. 1 and 3 illustrate the sensitivity enhancement of the post-column technique over conductivity detection. The post-column technique is 10–500 times more sensitive in detecting inorganic ions than conventional conductivity detectors.

CONCLUSIONS

HPLC–IC provides a means in which to analyze multiple trace elements in fermentation media with a single analysis. Ashing of the sample will eliminate many of the matrix interferences although a large amount of sample is needed in order to concentrate the trace elements and conventional ashing is rather time-consuming. Solid-phase sample preparation is a viable method for sample preparation and clean up, although a large amount of sample needs to be eluted through the cartridge in order to obtain the amounts needed for conductivity detection. The chromatographic system is capable of separating and detecting calcium, cadmium, cobalt, copper, iron(III), lead, manganese, magnesium, nickel and zinc at levels close to 1 ppm using conductivity detection. Sensitivity can be increased at least 100 fold over conventional conductivity detection by the use of a post-column derivitization reaction, enabling visible detection of metal complex formed. HPLC–IC is capable of monitoring the ions present in the starting raw materials, such as the flours and water used for the production of a suitable starting culture or trouble shooting media contamination by metal fermentation tanks or incidental additives.

ACKNOWLEDGEMENTS

Technical assistance and expertise was provided by R. Saari-Nordhaus, J. M. Anderson, Jr. and I. K. Henderson of Alltech Assoc. (Deerfield, IL, U.S.A.), Chem-Sultants (Steger, IL, U.S.A.), Quantitative Technologies (Bound Brook, NJ, U.S.A.) and M. K. Peterson.

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