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Determination of carbon sources in fermentation media using high-performance anion-exchange liquid chromatography and pulsed amperometric detection

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Abstract

The technique of high pH anion-exchange chromatography method with pulsed amperometric detection (HPAEC–PAD) has been adapted for the rapid determination of common carbohydrates present in fermentation broths. Simple water dilution of filtered fermentation broth samples was the sole sample preparation step required. The samples were analyzed using a Dionex CarboPac PA1 column with 150 m*M* NaOH as the mobile phase at a flow-rate of 1 ml/min and a total run time of 20 min. A gradient method was also developed to resolve species which exhibited similar t_R in the isocratic procedure. Among the analytes examined in this study were ethanol, glycerol, galactose, glucose, mannose, fructose, raffinose, ribose and lactose. Examples from several microbial fermentations using chemically defined or complex medium are presented.

1. Introduction

Fermentations are complex processes designed to control the growth and productivity of microorganisms or cultured cells. The productivity of the fermentation process is directly influenced by environmental parameters such as pH, temperature, dissolved oxygen, and other nutritional factors.

Thus, definition of the nutrient composition of fermentation medium is essential to the understanding of the physiology of fermentation processes. Carbohydrates are one of the most important classes of nutrients that can affect microbial growth. An understanding of carbon catabolism is critical to optimization of culture conditions. The concentration of the preferred carbohydrate utilized by the microorganism can vary widely depending upon the type of fermentation as well as the stage of culture growth (e.g., from 1–5 mg/l to 100 g/l in batch cultures). Clearly, analysis of carbohydrates is a critical element of medium optimization and for productivity improvements of fermentation processes.

Many analytical techniques have been used to quantitate carbohydrate concentration in fermentation broths. Historically, enzyme-based or chemical identity assays have been used to detect individual saccharides [1]. However, sample throughput is limited, the technique is cumbersome, and the assays are specific for a single saccharide. More recently, flow injection analysis has been tested as an on-line process monitoring

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technique [2]. Some drawbacks of this technology are the limitation of one analyte per channel and the extensive training necessary to allow the use of this technology. HPLC analysis affords a more comprehensive picture of the carbohydrate constituents in media. This technology has been used extensively for analysis of defined and complex fermentation media [3]. Commonly, refractive index (RI) detection is utilized for carbohydrate determination as direct UV detection is not practical [4]. However, since gradient methods cannot be used with RI detection, resolution may be compromised. To overcome this problem, post-column derivatization has been investigated by some groups [5,6]. Bell and Newman [7] found that solid-phase extraction of the fermentation sample was necessary to remove broth matrix interferences prior to HPLC–RI analysis.

To the authors' knowledge, an alternative HPLC technology that has not been applied to fermentation broth analysis is high-performance anion-exchange chromatography (HPAEC) in combination with pulsed amperometric detection (PAD). HPAEC–PAD has been widely applied as a sensitive detection system for alcohols, monosaccharides and oligosaccharides in biological samples such as soil [8], dairy products [9], peat samples [10] and fruit juices [11]. The successful application of HPAEC–PAD for the determination of carbohydrates in numerous biological samples led us to investigate and develop this technique for ion chromatographic determination of common carbohydrates used in fermentation. Carbon sources of wide interest in fermentation technology include, but are not limited to, the following compounds: ethanol, glycerol, galactose, glucose, mannose, fructose, raffinose, ribose and lactose.

This report describes the method development, optimization and validation of chromatographic methods to monitor carbohydrates in fermentation broth without the need for excessive sample pretreatment. The utility of this analysis was documented by application to a variety of microbial fermentations employing chemically defined media or complex nutrient sources, several examples of which are illustrated.

2. Experimental

2.1. Chemicals

Sodium hydroxide (50%, w/w) was obtained from Fisher Scientific (Malvern, PA, USA). HPLC-grade glacial acetic acid was obtained from J.T. Baker (Phillipsburg, PA, USA). All stock carbohydrate standards were prepared from analytical-grade material obtained from either Pfanstiehl (Waukegan, IL, USA) or Fluka (Ronkonkoma, NY, USA).

2.2. Carbohydrate chromatographic system and eluents

Isocratic

Component analysis was performed using a Dionex ion chromatography system (Sunnyvale, CA, USA) that contained a gradient pump, PAD system, autosampler and data-handling system. The actual separation was accomplished using a CarboPac PA1 analytical column (Dionex, 250 × 4 mm) and a CarboPac PA1 guard column (Dionex, 50 × 4 mm) with a 150 m*M* NaOH mobile phase. The method was isocratic with a flow-rate of 1 ml/min. A PAD system equipped with a gold electrode was used for detection. The PAD settings were: $E1 = 0.05$, $E2 = 0.65$, $E3 = -0.95$; $T1 = 2$, $T2 = 2$, $T3 = 5$ and range = 2. A 50-μl sample was injected using the filled-loop mode. The total run time was 20 min.

Gradient

The aforementioned analytical system was used for a gradient elution method by changing appropriate conditions. The mobile phase components were distilled water (A) and 50 m*M* NaOH–2 m*M* acetic acid (B). The gradient conditions were: A–B (94:6) held constant for 13.8 min; varied linearly to 100% B over the next 11.2 min; and then returned to A–B (94:6) over an additional 16 min. The PAD settings were: $E1 = 0.05$, $E2 = 0.60$, $E3 = -0.65$; $T1 = 5$, $T2 = 2$, $T3 = 1$ and range = 2.

Data system

A Dionex advanced computer interface (ACI) Model III was used for data transfer to an AST

Premium 486/33TE computer. Data reduction and processing were accomplished using Dionex AI-450 software (version 3.20).

Preparation of standards and samples

Stock carbohydrate standards were prepared at a concentration of 10 mg/ml using Milli-Q water and were stored at −70°C. Dilutions of stock standards to prepare 10, 25, 50, 100, 250 and 500 μg/ml working standards were made daily. Fermentation samples were prepared by diluting 0.22-μm-filtered broth with Milli-Q water; dilutions of at least 1:50 were used.

3. Results and discussion

The objective of the study was to develop an HPAEC–PAD method for the determination of carbohydrate concentration in fermentation broths. While establishing the sensitivity and linear range of the assay, we were especially concerned with the wide range of carbohydrate concentrations (1 mg/l to 100 g/l) that can occur during the course of a microbial fermentation. To accommodate this magnitude of change, we employed the least sensitive PAD setting that allowed for a broad range of sensitivity. The working limit of detection for most analytes was 10 μg/ml under these conditions. These detection limits could be reduced to ca. 10 pg/ml by adjusting the PAD settings appropriately, as reported by other investigators [12,13]. The concentration of these analytes in fermentation media usually exceeds this range (10 μg/ml) by several orders of magnitude. It was determined that simple dilution with water (commonly 10- to 400-fold) was required to perform the analysis, thus, method sensitivity was not stressed.

To determine if the isocratic method could be adapted for fermentation broth analysis, analytes of interest were evaluated. These included ribose, trehalose, fructose, galactose, mannose, glucose, rhamnose, sucrose, lactose, maltose, raffinose, ethanol, glycerol, ribitol, galactitol and sorbitol. Because of the wide variety of fermentation media that were potentially available and to simplify assay protocol, a common matrix for the standards was necessary. Water was chosen as the first approximation for the matrix because the starting concentrations of most carbohydrates of interest in fermentation broths were in the g/l range and considerable dilution with water was necessary for analysis. To define the HPLC parameters for separation and quantitation, mixtures of known standards in water were examined.

Fig. 1 illustrates the resolution of a mixture of components (each present at 50 μg/ml) using the isocratic method. Hardy et al. [14] reported that the order of elution of simple carbohydrates and polyols was affected by the accessibility of polyanions to functional groups attached to the stationary phase; thus, there is not a strict relationship between t_R and molecular mass. Alcohols exhibited the shortest t_R followed by monosaccharides, disaccharides, then trisaccharides. Chromatographic parameters, including t_R, response factor (1/sensitivity), sensitivity (peak area/concentration), column capacity factor (k') and working range of linearity are listed in Table 1.

For some applications, glucose and galactose

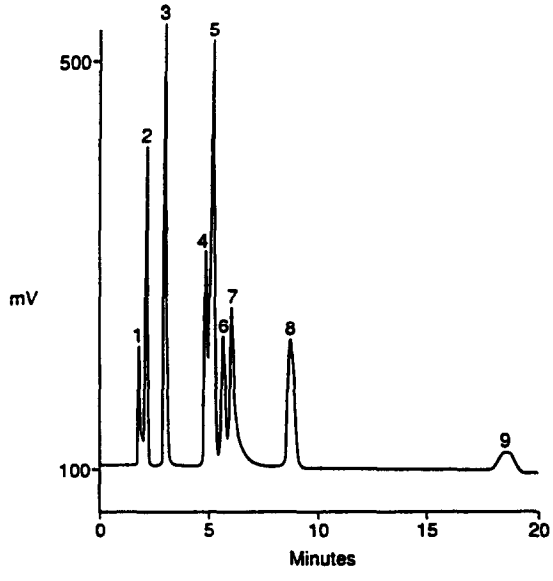

Fig. 1. Chromatogram of standard mixture of alcohols and carbohydrates: separation by the isocratic method. Injection volume, 50 μl. Peaks: 1 = ethanol (1000 μg/ml); 2 = glycerol; 3 = sorbitol; 4 = mannose; 5 = galactose; 6 = fructose; 7 = ribose; 8 = lactose; 9 = maltose (all at 100 μg/ml).

Table 1
HPLC parameters for eleven carbohydrates and five alcohols generated using the isocratic carbohydrate method

Carbohydrate	M_r	t_R (min)[a]	Response factor	Sensitivity	k'	Range of linearity (μg/ml)
D-Ribose, C.P.	150.13	5.57	0.000091	1105	2.71	10–500
Trehalose dihydrate, C.P.	179.90	3.28	0.00078	1280	1.19	10–500
D-Fructose, C.P.	180.16	5.20	0.00078	1288	2.47	10–500
D-Galactose, C.P.	180.16	4.78	0.00070	1422	2.19	10–500
D-Mannose, C.P.	180.16	4.43	0.00061	1632	1.95	10–500
Glucose	180.16	4.72	0.00061	1649	2.15	10–500
L-Rhamnose monohydrate, C.P.	182.17	3.20	0.00047	2117	1.13	10–500
Sucrose	342.30	10.03	0.00448	223	5.69	25–500
Lactose monohydrate	360.31	8.25	0.00181	553	4.50	10–500
Maltose monohydrate, C.P.	360.31	19.57	0.00713	140	12.05	25–500
Raffinose, C.P.	594.52	17.93	0.00463	216	10.95	10–500
Ethanol	46.06	1.65	0.03264	31	0.10	500–5000
Glycerol	92.09	1.80	0.00024	4091	0.20	10–250
Ribitol	152.15	2.63	0.00038	2647	0.75	10–250
Galactitol	182.17	2.62	0.00038	2644	0.75	10–500
Sorbitol, N.F.	182.17	2.62	0.00039	2566	0.75	10–500

Data were obtained using 100 μg/ml standards; ethanol at 1000 μm/ml. C.P. = Chemically pure, N.F. = national formulary.
[a]Based on chromatograms from 100 μg/ml samples.

separation was important. As these analytes cannot be resolved using the isocratic method, a gradient method was optimized to resolve these two species which have the same molecular mass (Fig. 2). In practical terms, this did not hamper analysis of fermentation media since most samples could be analyzed using the isocratic method. Chromatographic parameters for the gradient separation are listed in Table 2. The data listed in Tables 1 and 2 are comparable to values reported by other investigators [14,15].

The linearity of response was investigated for all analytes listed in Tables 1 and 2. Representative plots for seven saccharides and two polyols are depicted in Fig. 3. Using either method, the linearity of response for most analytes was between 10 and 500 μg/ml (Tables 1 and 2). This relatively large standard curve range was selected to accommodate the large variability in analyte concentrations expected to occur in fermentation broths. A cubic fit of the data was used to obtain a better quantitation of standards.

To ensure that water was a good approximation of the medium background, and that

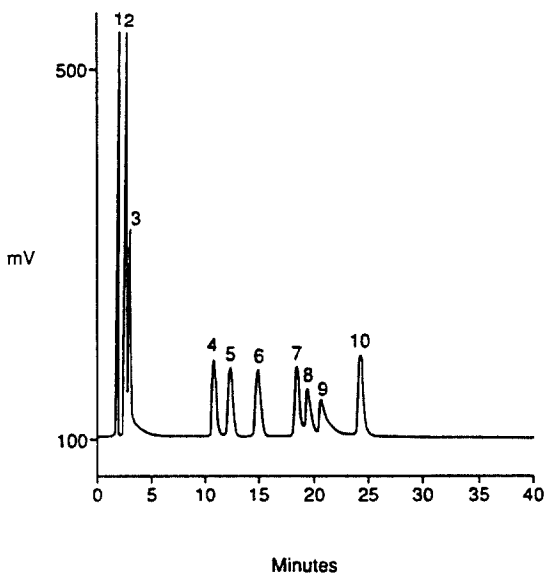

Fig. 2. Chromatogram of standard mixtures of alcohols and carbohydrates: separation by the gradient method. Injection volume, 50 μl. Peaks: 1 = ethanol (1000 μg/ml); 2 = glycerol; 3 = sorbitol; 4 = mannose; 5 = galactose; 6 = fructose; 7 = ribose; 8 = lactose; 9 = maltose; 10 = lactose (all at 100 μg/ml).

Table 2
HPLC parameters for ten carbohydrates and five alcohols generated using the gradient carbohydrate method

Carbohydrate	M_r	t_R (min)[a]	Response factor	Sensitivity	k'	Range of linearity (μg/ml)
D-Ribose, C.P.	150.13	22.30	0.00007	13 662	13.87	10–500
Trehalose dihydrate, C.P.	179.90	2.83	0.00008	12 164	0.89	10–500
D-Fructose, C.P.	180.16	19.38	0.00006	15 508	11.92	10–250
D-Galactose, C.P.	180.16	11.45	0.00005	19 952	6.63	10–500
D-Mannose, C.P.	180.16	17.95	0.00005	19 444	10.97	10–500
Glucose	180.16	13.85	0.00005	19 248	8.23	10–500
L-Rhamnose monohydrate, C.P.	182.17	10.08	0.00005	19 019	5.72	10–500
Sucrose	342.30	13.25	0.00018	5 480	7.83	25–500
Lactose, monohydrate	360.31	22.98	0.00007	13 608	14.32	10–500
Raffinose, C.P.	594.52	21.75	0.00009	10 619	13.50	10–500
Ethanol	46.06	1.62	0.03175	31	0.08	250–5000
Glycerol	92.09	1.73	0.00003	38 776	0.15	10–500
Ribitol	152.15	2.47	0.00003	28 739	0.65	10–500
Galactitol	182.17	2.40	0.00004	22 341	0.60	10–500
Sorbitol, N.F.	182.17	2.52	0.00005	20 963	0.68	10–500

Data were obtained using 100 μg/ml standards; ethanol at 1000 μg/ml.
[a]Based on chromatograms from 100 μg/ml samples.

standards exhibited the same characteristics in fermentation medium, potential interferences from medium components were examined. Interferences from compounds with ionizable groups should be minimized by dilution. Concentrations for most analytes (2.5 μg/ml), other than the predominant carbohydrate species, were below the limit of detection with the PAD settings used. When an alkaline pH mobile phase is used, a PAD system equipped with a gold electrode is sensitive to analytes that contain oxidizable functional groups such as hydroxyls, amines and sulfides [16,17]. Carboxylic acids and inorganic species are transparent in such a system. Other organic compounds that would be converted to anions under the alkaline conditions used for chromatography were also potential sources of interference. Thus, certain amino acids, nucleosides, and choline (all with relatively high dissociation constants, $pK_a > 10.5$) were tested as potential interfering compounds by spiking water with 250 μg/ml standards. At these levels only proline, arginine and lysine exhibited any noticeable detector response (data not shown). These compounds did not present an interference problem because the normal concentration of these amino acids was <2–5 μg/ml and at these levels they were undetectable. To confirm that medium components posed no interference, spike recovery studies in various complex media were performed. Equivalent analyte concentrations were detected in water spiked with analyte, media spiked with analyte then diluted, and diluted media spiked with analyte when either a representative alcohol, pentose, hexose or disaccharide was used (data not shown).

The intraday accuracy of the method was tested by assaying a galactose standard six times. The relative standard deviations (R.S.D.s) of retention times or peak heights were 1.8 and 0.2% for galactose at 10 and 500 μg/ml, respectively. Interday accuracy has been monitored for over two years with R.S.D.s of 5.6 and 0.5% for the 10 and 500 μg/ml galactose standard, respectively. Intraday and interday reproducibility studies have also been performed for five additional analytes (glucose, ribose, ethanol, glycerol and lactose) with similar results (data not shown).

To ensure assay performance during fermentation sample analysis, quality control (QC) samples were analyzed approximately every ten

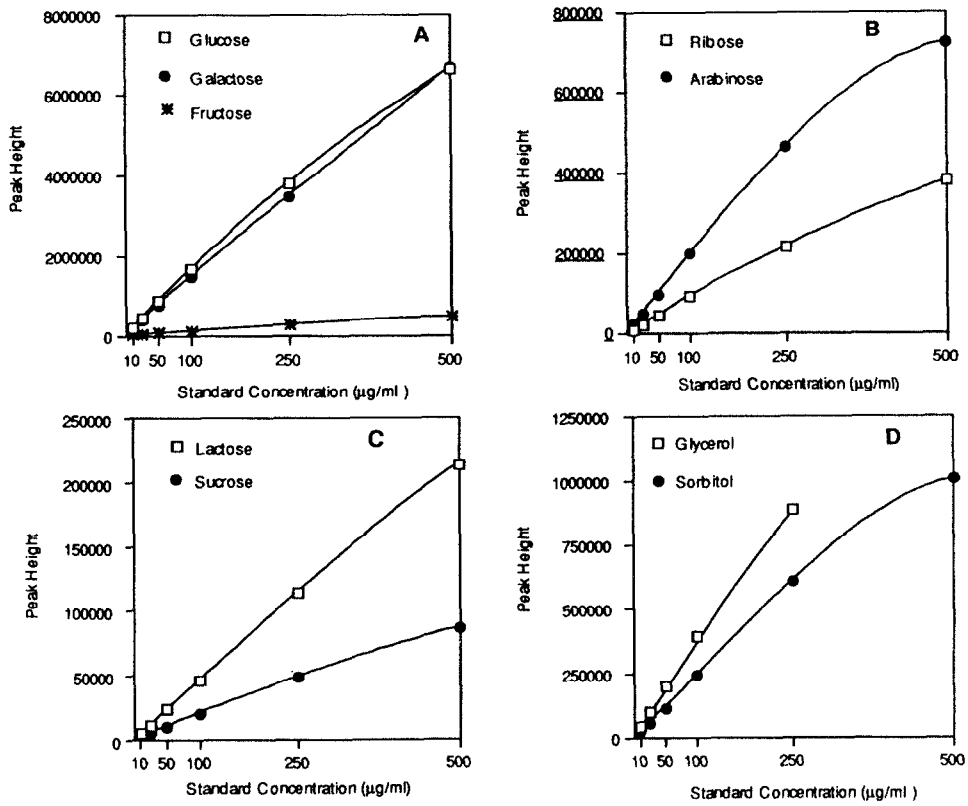

Fig. 3. Typical calibration curves for (A) three hexoses (glucose, galactose, fructose), (B) two pentoses (ribose, arabinose), (C) two disaccharides (lactose, sucrose) and (D) two alcohols (glycerol, sorbitol). Standard curve ranges: 10 to 500 μg/ml for hexoses, pentoses, disaccharides and sorbitol; 10 to 250 μg/ml for glycerol. $R^2 > 0.9999$ for all analytes.

fermentation samples. QC samples were 100 μg/ml galactose in water. For each fermentation sample set, the variability of the QC samples was determined. Typical variation for QC samples was R.S.D. $< 5\%$ with actual values being $< \pm 5\%$ of the nominal QC value. The limiting factor for run size was the sample schedule (software limitation), not QC variation. Accuracy was observed to vary with the type of fermentation medium used, thus the minimum recommended dilution was established for each medium.

The utility of the system for analyzing fermentations broths was demonstrated by monitoring carbohydrate profiles of several different fermentations. Several examples are depicted in Figs. 4–6. Fig. 4 depicts the chromatograms from two recombinant *Escherichia coli* fermentations generated using the isocratic method. The upper panel is an analysis of a filtered broth sample from a chemically defined fermentation medium with glucose (peak 1) as the sole carbon source; the lower panel is a sample from a complex medium formulation with glycerol (peak 2) as the primary carbon source. In both cases a simple 1:50 dilution with water was the only sample pretreament. The chromatogram generated from the chemically defined medium sample shows that except for the glucose peak, virtually no other components are detectable. While several minor peaks were detected in the complex medium, the major peak was glycerol (peak 2), the principal carbon source.

Fig. 5 presents a representative time course of the chromatograms from a fermentation of *Haemophilus influenzae* b cultivated in complex medium with glucose as the major carbon

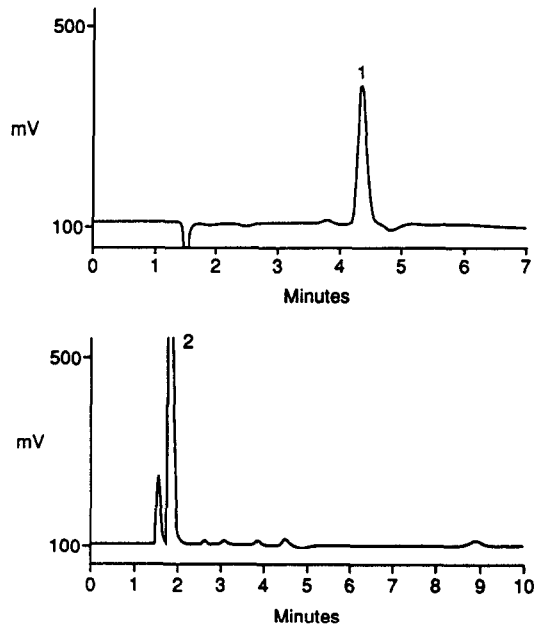

Fig. 4. Representative chromatograms showing isocratic analysis of carbohydrates in an *E. coli* fermentation using chemically defined medium with glucose (peak 1) as the major carbon source (top) or complex medium with glycerol (peak 2) as the major carbon source (bottom).

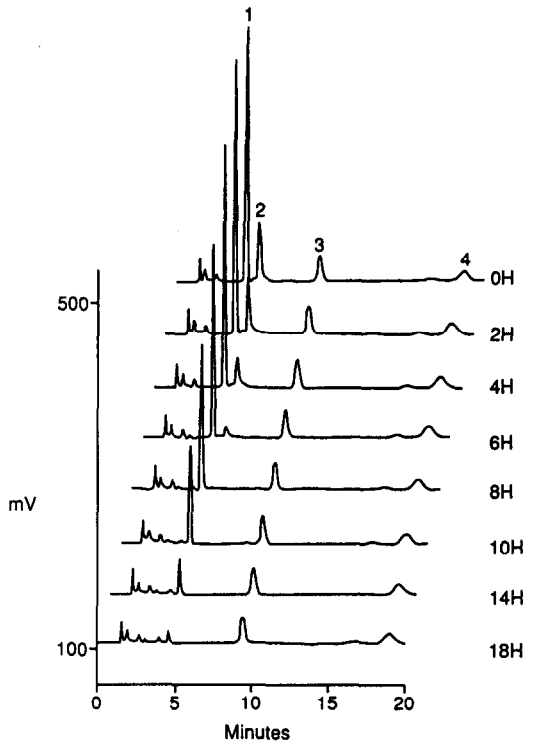

Fig. 5. A time course of representative chromatograms showing isocratic analysis of carbohydrates in a *H. influenzae* fermentation using complex medium with glucose as the major carbon source. Peaks: 1 = glucose; 2 = fructose; 3 = ribose; 4 = maltose. H = Hours.

source. These were generated using the isocratic method. Two major monosaccharides are reduced in concentration during the course of the fermentation. One can infer that glucose (peak 1) and ribose (peak 2) are coordinately metabolized during the period of 18 and 8 h, respectively. Note also the relative stability of the minor peak such as sucrose (peak 3) over the same time course.

The concentration of the maltose (peak 4) did not change during this period but in this series of chromatograms, maltose exhibited an t_R of ca. 19.2 min which does not agree with the 19.6 min t_R as listed in Table 1. This finding illustrated a notable feature of the method. Over time, with repeated injections of complex medium samples, the t_R of disaccharides such as maltose and raffinose tended to decrease. The column performance was restored by washing the column with 1 *M* NaOH for 30 min. This phenomenon was attributed to the loss of column binding sites due to interference from complex medium components [18]. An altered t_R of other analytes that elute sooner than 15 min was not observed.

Fig. 6 illustrates the fermentation time course of a recombinant *Saccharomyces cerevisiae* grown in chemically defined medium containing galactose (peak 1) and glucose (peak 2) at starting concentrations of 89 and 20 g/l, respectively. As shown, the concentration of galactose in the broth decreased by 86% during the fermentation, while glucose is virtually depleted from the broth by 16 h.

A gradient chromatography method, which has an extended run time, was required to resolve these two main analytes. From a practical viewpoint, to maximize ample throughput the isocratic method was utilized for analysis of fermentation samples after glucose exhaustion (e.g., >16 h, data not shown).

HPAEC–PAD can be used for direct detection

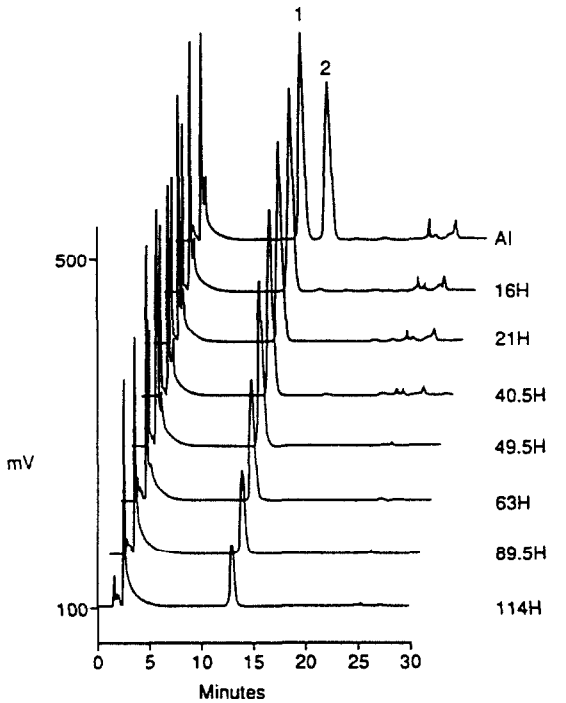

Fig. 6. A time course of representative chromatograms showing gradient analysis of carbohydrates in a *S. cerevisiae* fermentation using chemically defined medium with two principal carbon sources, galactose (peak 1) and glucose (peak 2). H = Hours. AI = After inoculation.

of common carbon sources in fermentation media with only 0.22-μm filtration of fermentation broths and ca. 100-fold dilution of the filtered broth as the sole sample preparation steps. The system can be used for fermentation broths of various composition (either chemically defined or complex) with neither extraction, sample derivatization, nor ashing required. As fermentation broths can contain a variety of components, we were especially concerned about interferences from high ion levels, complex organic components and metabolic by-products. The majority of the components in fermentation media have relatively low pK_a values; thus, the only analytes which would be ionized in alkaline mobile phases are those with high pK_a values such as certain amino acids and nucleoside bases. No interference was observed due to media components. The system proved to be rugged, with greater than 1000 injections prior to column cleanup (defined as change in t_R of standards by 10%). The system has been in continuous use for three years with no major problems in over 10 000 injections. Samples from multiple fermentations constituting different media formulations have been analyzed without compromising the accuracy of the system. In comparison to other analytical methodologies for obtaining carbohydrate concentration in fermentation broth, this system is straightforward and simple. Biologically relevant information about carbohydrate metabolism is critical to the understanding of microbial physiology. Moreover, the central role of carbon metabolism in microbial fermentation processes is the basis for many on-line process control strategies. Thus, the dilute and shoot feature of this method makes on-line sampling/analysis an attractive option for future studies.

Acknowledgements

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