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Determination of inorganic cations in fermentation and cell culture media using cation-exchange liquid chromatography and conductivity detection

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Abstract

Reliable methods to quantitate inorganic cations in biological culture media are often helpful for medium optimization and comprehensive process monitoring and control. Analysis of inorganic cations found in culture media was accomplished by ion chromatography for several types of microbial fermentations as well as mammalian and insect cell culture samples. An isocratic method consisting of a Dionex IonPac CS12 analytical column and 4 m*M* methanesulfonic acid mobile phase was used with suppressed conductivity detection. Sample preparation was a simple dilution of filtered broth with water. The analyses time was 20 min. This analysis accurately monitored eight inorganic cations, several of which are commonly present in fermentation media (Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}).

1. Introduction

While often overlooked as macronutrients, inorganic cations are nevertheless essential for cellular growth and fulfill specific metabolic and structural roles. In this regard, improvements in process performance may be achieved by supplementation with organic [1] or inorganic [2] nutrients. Some cations are required in millimolar concentrations to satisfy cellular growth requirements (macronutrients such as Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}).

The relationship of H^+, Na^+, and K^+ is especially critical in the maintenance of membrane physiology and in the role of various cytoplasmic membrane ATPases in the uptake of nutrients mediated by proton antiport or symport. The optimal concentrations vary depending upon the species and cell density. For example, some halophilic bacteria require molar concentrations of Na^+ to survive, levels that are inhibitory to other microbial species [3,4]. Other cations are necessary in only trace amounts (micronutrients such as Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}). Quite often, the concentrations of micronutrients can be difficult to assess due to chelation of these species by various components in the culture medium (amino acids, proteins) [3]. Throughout the course of a fermentation, a wide range of concentrations for some inorganic cations may be observed. For example, ammonium ion can be established at concentrations ranging from trace amounts to several grams per liter during the course of a mircobial fermentation [4]. One cannot supply a priori, elevated levels of all cations suspected to be required for

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cellular growth, as excess levels of macro- or micronutrients can be deleterious to biological systems; however, these levels are rarely a matter of record [5]. Often high cell density fermentations are desirable in industrial fermentations and medium composition becomes a critical factor in achieving a successful high cell density process. Thus, analytical tools to determine the concentrations of inorganic species in fermentation media are essential for industrial culture medium development.

Atomic absorption, UV–Vis spectrophotometry, flow injection chemiluminescence, and HPLC [6,7] have been used for the analysis of inorganic cations and metals in biological samples. Increasingly, HPLC analysis is being applied to monitor cation and trace element composition of fermentation broths. Bell [8] used reversed-phase chromatography with conductivity detection to analyze ashed fermentation samples for ten cations highlighting the importance of micronutrient cations in industrial antibiotic fermentations. In addition, Joergensen et al. [9] have reported the use of ion chromatography coupled with conductivity detection to quantitate five cations present in a methanotropic bacterial fermentation. A previous report from this laboratory [10] described an ion chromatography method for the determination of five common inorganic cations in fermentation broths from several sources. Included in that report was information about the selectivity, sensitivity, and reproducibility of a cation method using a Dionex CS 10 separator column.

The present paper describes the development of an isocratic ion chromatography method using a Dionex CS12 column to quantitate eight inorganic cations with emphasis on five principal macronutrient cations commonly present in culture media as freely soluble species. Sample preparation was a simple dilution of filtered broth with water before injection and had analysis time of 20 min. Chemically defined and complex culture media for various microbial, mammalian, and insect cell cultures were investigated. This assay was an improvement over the previously described method in that there were six additional cations which were addressed; better selectivity was observed for Na^+, NH_4^+, and K^+; and the assay was simplified by using a methanesulfonic acid (MSA) mobile phase with a cation self-regenerating suppressor.

2. Experimental

2.1. Chemicals

Methanesulfonic acid (MSA) was obtained from Sigma (St. Louis, MO, USA). All inorganic cation standards were prepared from chloride salts (Purity >97%) (Sigma). All solutions were prepared with doubly distilled, chemically purified water (Millipore, Bedford, MA, USA).

2.2. Chromatographic system and eluents

Isocratic inorganic cation analysis was performed using a Dionex DX-100 chromatography system consisting of a conductivity detector, an autosampler, and a data handling system (Dionex Corp., Sunnyvale, CA, USA). The separation was achieved using an IonPac CS12 analytical column (Dionex, 250 × 4 mm I.D., P/N 44020) and an IonPac CG12 guard column (Dionex, 50 × 4 mm I.D., P/N 44019) with a 20 m*M* MSA mobile phase at a flow-rate of 1 ml/min. Cation suppression was achieved using a Dionex self-regenerating suppressor (SRS) with a Dionex SRS controller (setting = 3). A range setting of 30 was used with the conductivity detector. The injection method was a 10-μl filled loop. Total run time for the analysis was 20 min.

2.3. Data system

A Dionex Advanced Computer Interface (ACI), Model III was used to transfer data to an AST Premium 486/33TE computer. Data reduction and processing were accomplished using Dionex AI-450 software, version 3.3.

2.4. Preparation of standards and samples

Stock inorganic cation standards were prepared at a concentration of 1 mg/ml for NaCl

and KCl, and 2 mg/ml for NH_4Cl, LiCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, RbCl, CsCl, $SrCl_2$, and $BaCl_2$ in water. All standards were stored in 1.8-ml aliquots at −70°C in 2-ml Wheaton vials (Wheaton, Millville, NJ, USA) with screw caps. Standards were not sensitive to these storage conditions (data not shown). Dilutions of stock standards were made daily to prepare either 0.5, 1.25, 2.5, 5, 12.5 and 25 μg/ml standards for NaCl and KCl or 1, 2.5, 5, 10, 25, and 50 μg/ml standards for NH_4Cl, $MgCl_2$, and $CaCl_2$. Diluted stock standards were used to generate standard curves.

Mircobial fermentation and cell culture samples were prepared by making dilutions of 0.22-μm filtered fermentation broth in water. A dilution of at least 1:50 was used for all samples.

3. Results and discussion

A cation-exchange method was developed that can baseline resolve and quantitative eight inorganic cations in fermentation broths although up to eleven cations can be monitored if baseline resolution is not required. This report focuses on five principal cations (sodium, ammonium, potassium, magnesium, and calcium) which were important for our specific applications. The cation content of fermentation broth samples was monitored with minimal sample handling and with reliable, precise isocratic chromatography. Previous investigations in this area relied upon the use of a Dionex CS10 column and conductivity detection with an external regeneration source for the suppressor [9,10]. This report describes the use of a new column (Dionex IonPac CS12) that provided an improvement in the chromatography by allowing for the detection of eleven inorganic cations, baseline resolution of eight cations, and better selectivity between Na^+, NH_4^+, and K^+. Also, because of a change in the mobile phase composition to MSA, a self-regenerating suppressor was used which simplified the method.

Minimizing sample dilution was a consideration when the sensitivity and linear range of the assays were established. A mid range setting (30) was used for the conductivity detector which allowed a larger dynamic range for the standard curves. The working limit of detection (LOD) for the inorganic cations under these conditions was either 0.5 or 1 μg/ml. Detection limits can be reduced by adjusting the conductivity settings [10,11]. The concentration of inorganic cations in fermentation media usually exceeded the assay LOD by several orders of magnitude so simple dilutions with water of 100- to 2000-fold were typically required to perform the analyses.

Several inorganic cations, including the five of major interest for fermentation media analysis, can be measured using this method. Standard curve ranges and system parameters were established using inorganic cation standards in water. A chromatogram showing the baseline resolution of eight inorganic cation standards, at concentrations of either 5 or 10 μg Cl^- salt/ml, is illustrated in Fig. 1A. This isocratic cation analysis allowed baseline resolution and quantitation of monovalent (sodium, ammonium, potassium, lithium) and divalent (magnesium, calcium, strontium, barium) inorganic cations. In addition, calcium (peak 8) and cesium (peak 9) can also be partially resolved in a mixture of the eleven cations (Fig. 1B). Rubidium (peak 6) can likewise be monitored as a single species but co-elutes with magnesium (peak 5) using this system. Separation of these compounds was performed in 20 min with monovalent cations eluting first. Retention time increased as the size of the ion in the hydrated state increased [12]. Although several of these species are not normally vital nutrients for cellular growth (lithium, rubidium, cesium, barium), their presence can confound medium development due to competition with uptake systems for essential cations [3].

Concentration ranges and chromatographic parameters were established using inorganic cations (10 μg Cl^- salt/ml) in water. Table 1 lists the chromatographic parameters for eleven inorganic cations and includes retention time (t_R), sensitivity (peak area/concentration), capacity factor (k'), and working range of linearity. These data are comparable to values reported by other investigators [9–11]. The linearity of response for these inorganic cations varied depending

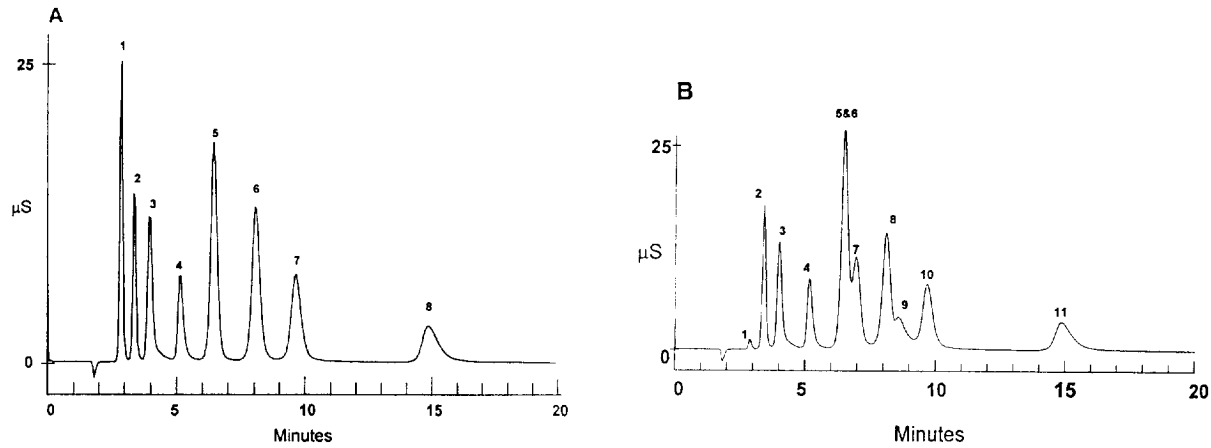

Fig. 1. (A) Chromatogram of a mixture of eight inorganic cation standards separated using an isocratic method. Injection volume 10 µl. Peaks: 1 = Li^+ (5 µg/ml), 2 = Na^+ (5 µg/ml), 3 = NH_4^+ (10 µg/ml), 4 = K^+ (5 µg/ml), 5 = Mg^{2+} (10 µg/ml), 6 = Ca^{2+} (10 µg/ml), 7 = Sr^{2+} (10 µg/ml), 8 = Ba^{2+} (10 µg/ml). (B) Chromatogram of a mixture of eleven inorganic cation standards separated using an isocratic method. Injection volume 10 µl. Peaks: 1 = Li^+ (5 µg/ml), 2 = Na^+ (5 µg/ml), 3 = NH_4^+ (10 µg/ml), 4 = K^+ (5 µg/ml), 5 = Mg^{2+} (10 µg/ml), 6 = Rb^+ (10 µg/ml), 7 = Mn^{2+} (10 µg/ml), 8 = Ca^{2+} (10 µg/ml), 9 = Cs^{2+} (10 µg/ml), 10 = Sr^{2+} (10 µg/ml), 11 = Ba^{2+} (10 µg/ml).

upon the cation. For the five cations of interest in fermentation, the linearity of response range was 0.5 to 25 µg/ml for sodium and potassium and 1 to 50 µg/ml for ammonium, magnesium and calcium. Typical standard curves for these five cations are found in Fig. 2. A cubic fit of the data was used to obtain a better quantitation of the analyte over the large standard range used. This large range was selected so that sample repeats due to inappropriate sample dilution would be minimized when analyzing culture medium samples.

Water was used as the dilution matrix for the standards since extensive dilution of fermentation samples with water (typically 1:100 or greater) was necessary to reduce the analyte concentration of the samples to an appropriate range. Potential interferences from the medium

Table 1
HPLC parameters for inorganic cations generated using the isocratic cation method

Inorganic cation	Retention time (min)	Sensitivity	k'	Range (µg/ml)
Lithium	2.90	2695955	0.5263	1.0–25
Sodium	3.43	749095	0.8053	0.5–25
Ammonium	4.03	537809	1.1211	1.0–50
Potassium	5.23	454344	1.7526	0.5–25
Magnesium	6.58	1457864	2.4632	1.0–50
Rubidium	6.63	232476	2.4895	1.0–100
Manganese	7.08	543288	2.7263	0.5–25
Calcium	8.27	909716	3.3526	1.0–50
Cesium	8.82	130935	3.6421	1.0–100
Strontium	9.92	409332	4.2211	1.0–100
Barium	15.30	239078	7.0526	1.0–100

Data was obtained using either 5 (LiCl, NaCl and KCl) or 10 (NH_4Cl, $MgCl_2$, RbCl, $MnCl_2$, $CaCl_2$, $CsCl_2$, $SrCl_2$, $BaCl_2$). µg/ml standards.

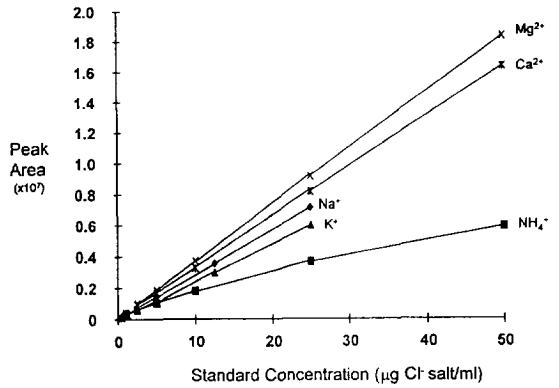

Fig. 2. Calibration curves for sodium, ammonium, potassium, magnesium, and calcium ions generated using the isocratic cation analysis. Standard curve range from 0.5 to 25 μg/ml for sodium and potassium and from 1 to 50 μg/ml for ammonium, magnesium and calcium. $[Na^+] = 1.891484 \cdot 10^{-18}y^3 - 1.026815 \cdot 10^{-12}y^2 + 4.245989 \cdot 10^{-5}y - 0.0424$, $R^2 = 0.999998$; $[NH^{3+}] = 1.628513 \cdot 10^{-16}y^3 + 3.860538 \cdot 10^{-10}y^2 + 7.243507 \cdot 10^{-5}y - 0.0928$, $R^2 = 0.999996$; $[K^+] = 1.503837 \cdot 10^{-16}y^3 - 6.839330 \cdot 10^{-11}y^2 + 9.434817 \cdot 10^{-5}y + 0.1007$, $R^2 = 0.999873$; $[Mg^{2+}] = 7.695368 \cdot 10^{-19}y^3 + 4.195556 \cdot 10^{-12}y^2 + 7.142409 \cdot 10^{-5}y - 0.1233$, $R^2 = 0.999999$; $[Ca^{2+}] = 7.193543 \cdot 10^{-18}y^3 + 1.190153 \cdot 10^{-11}y^2 + 1.045722 \cdot 10^{-4}y - 0.2464$, $R^2 = 0.999997$.

components were expected to be minimal because of the mode of separation and the detection method. Because separation was based upon cation exchange, anionic compounds were not retained. (Anionic species present in fermentation samples may include inorganic anions, organic acids, many proteins, and neutral and basic amino acids.) Conductivity detection also contributes to method specificity. Carbohydrates and alcohols which are retained on the cation-exchange resin are essentially invisible to the detector because they are not dissociated at the eluent pH [13,14]. Compounds that can be retained by the column and detected by suppressed conductivity, besides cations, are amines and a few transition metals. Biological molecules containing amines that were examined as potential interferences included ethanolamine, triethanolamine, nicotinamide, thiamine, and various forms of nucleosides or nucleotides. Other transition metals tested included cobalt, copper, iron, and zinc. Only cobalt, choline, and ethanolamine were detected but were resolved from analytes of interest (see Table 2).

Table 2
HPLC parameter for interfering compounds in the isocratic inorganic cation method

Interfering compound	Retention time	Sensitivity	k'
Cobalt	7.32	434819	2.75
Choline	12.82	83784	5.57
Ethanolamine	4.33	196341	1.22
Copper	ND[a]		
Iron	ND		
Zinc	ND		
Triethanolamine	ND		
Nicotinamide	ND		
Thiamine	ND		
Adenosine	ND		
Cytosine	ND		
Cytidine	ND		
Thymine	ND		
Thymidine	ND		
Uracil	ND		
Uridine	ND		
Guanosine	ND		

Data was obtained using 10 μg/ml standards.
[a] ND = Not detectable at 10 μg/ml concentrations.

Spike recovery studies were performed using sodium, ammonium, potassium, magnesium, and calcium ions (Table 3) in chemically defined and complex media formulations [15]. Dilutions from 1:2500 to 1:10 were made using double distilled water. To each dilution, either 5 (Na^+ and K^+) or 10 (NH_4^+, Mg^{2+}, and C^{2+}) μg/ml of each individual inorganic cation was added. Differential recoveries for individual cations were observed. For detection of all five cations, a 1:1000 and a 1:2500 dilution was necessary for complex media and the chemically-defined medium examined, respectively. Recoveries of 0.92 to 1.01 were seen using complex medium with dilutions of 1:1000 or greater. Higher dilutions were necessary with the chemically defined medium. Spike recovery studies should be performed for each fermentation broth tested to establish the minimum dilution required.

The intra-day accuracy of the method was tested by assaying sodium, ammonium, potassium, magnesium, and calcium ions six times. Intra-day and inter-day validation data for inorganic cation analyses can be found in Table 4.

Table 3
Spike recovery studies for sodium, ammonium, potassium, magnesium, and calcium ions in complex and chemically defined media

Dilution	Complex media					Chemically defined media				
	Sodium	Ammonium	Potassium	Magnesium	Calcium	Sodium	Ammonium	Potassium	Magnesium	Calcium
1:2500	1.13	1.04	1.15	1.11	1.09	1.04	1.08	1.14	1.13	1.12
1:1000	0.93	0.92	1.01	0.99	1.00	0.71	1.02	0.98	0.97	0.96
1:500	0.93	0.89	1.02	1.00	0.99	ND	1.00	0.97	0.96	0.97
1:250	0.98	0.81	1.09	1.01	1.01	ND	0.97	0.93	0.96	0.91
1:100	ND	0.64	0.68	0.98	0.99	ND	0.89	1.03	1.01	1.02
1:50	ND	0.46	ND	0.99	0.99	ND	0.83	0.98	0.94	0.95
1:25	ND	0.32	ND	1.03	1.02	ND	0.67	1.00	0.95	0.96
1:10	ND	0.22	ND	ND	0.91	ND	ND	1.11	1.00	1.00

Spikes of either 5 (Na^+ and K^+) or 10 (NH_4^+ Mg^{2+}, and Ca^{2+}) μg/ml were used for all experiments.
ND: the peak was off scale or merged with another peak so no quantitation for this cation peak was possible.

The relative standard deviation (R.S.D.) of the retention times or peak areas was <5.2% for all inorganic cations at their lowest standard curve concentration (0.5 or 1 μg/ml) and <1% for all inorganic cations at their highest standard curve concentration (25 or 50 μg/ml). The inter-day validation occurred over seven months and was generated using one separator column. Inter-day

Table 4
Intra-day and inter-day validation data for sodium, ammonium, potassium, magnesium, and calcium ions generated using the isocratic cation analysis

Std Conc (μg/ml)	n	Relative standard deviation				
		Sodium	Ammonium	Potassium	Magnesium	Calcium
Intra-day						
0.5	6	1.55	–	4.79	–	–
1	6	–	2.43	–	4.52	5.19
1.25	6	1.56	–	3.14	–	–
2.5	6	0.88	1.39	1.13	1.12	2.23
5	6	0.74	1.06	0.36	3.10	0.84
10	6	–	0.52	–	0.55	2.63
12.5	6	1.20	–	0.61	–	–
25	6	0.71	0.92	0.59	0.51	0.47
50	6	–	1.11	–	0.16	0.25
Inter-day						
0.5	12	5.13	–	3.85	–	–
1	12	–	6.74	–	3.37	2.80
1.25	12	1.88	–	2.17	–	–
2.5	12	2.15	2.31	2.14	1.74	1.66
5	12	0.68	2.51	0.54	1.11	0.80
10	12	–	1.41	–	0.35	0.24
12.5	12	0.05	–	0.04	–	–
25	12	0.00	0.22	0.00	0.02	0.02
50	12	–	0.02	–	0.00	0.00

R.S.D. (area) was <6.8% for all inorganic cations at their lowest standard curve concentration and 0.1% for all inorganic cations at their highest standard curve concentration.

To ensure assay performance while analyzing fermentation samples, quality control (QC) samples with 5 μg/ml sodium and potassium and 10 μg/ml ammonium, magnesium, and calcium in water were analyzed approximately every eight fermentation samples. Typical intra-run variation for QC samples was <2% R.S.D. The range of values observed was ±0.1% of the nominal QC value for all five standards.

Utility of this method was demonstrated by analyzing fermentation broths of microbial and cell culture samples. Fig. 3 shows a series of chromatograms from a recombinant *Saccharomyces cerevisiae* fermentation time course where five inorganic cation levels were measured in a chemically defined medium. The time course shows that some cation concentrations change dramatically; potassium (peak 3) becomes undetectable and then reappears at the end of the fermentation, while others, such as magnesium (peak 4), retain a constant concentration throughout the fermentation. Despite changing biomass concentrations, the magnesium ion content is in excess throughout the course of the fermentation. It is unknown if this apparently high level of magnesium affects the uptake of other components by the cell. Conversely, greater biomass yields might be expected if the potassium limitation is addressed.

Analyses of a filtered broth samples from a complex medium fermentation for *Escherichia coli* can be found in Fig 4. The time course shows that ammonium (peak 2) and potassium (peak 3) decreased by 16–20% and magnesium (peak 4) decreased by 60–65% of the original concentrations over the course of the fermentation. In contrast, calcium (peak 5) levels increased by 10% relative to the starting concentration. Levels of sodium (peak 1) remain virtually unchanged throughout the fermentation.

Fig. 5 shows a time course from an insect cell culture using a complex medium. The time course shows that levels of sodium (peak 1), potassium (peak 3), magnesium (peak 4), and calcium (peak 5) remained virtually unchanged throughout the fermentation. Ammonium ion (peak 2) was the only inorganic cation measured that changed in concentration and increased approximately 2-fold over the course of the fermentation. A similar situation was observed with the mammalian cell culture example (Fig.

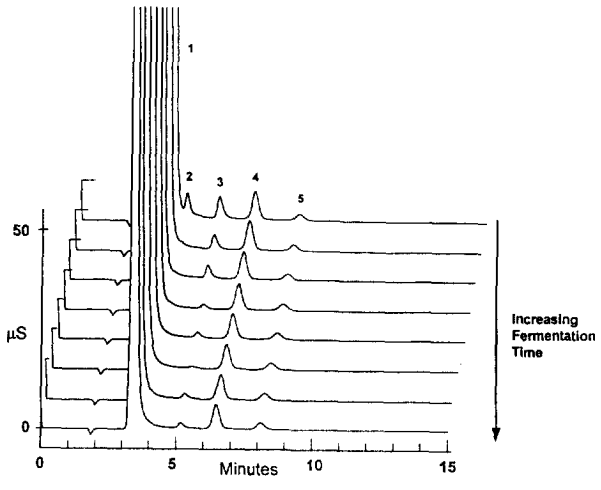

Fig. 3. A time course of representative chromatograms show inorganic cation analyses of fermentation broth samples for *S. cerevisiae* grown in chemically defined medium with galactose as the primary carbon source. Peaks: 1 = sodium, 2 = potassium, 3 = magnesium, and 4 = calcium.

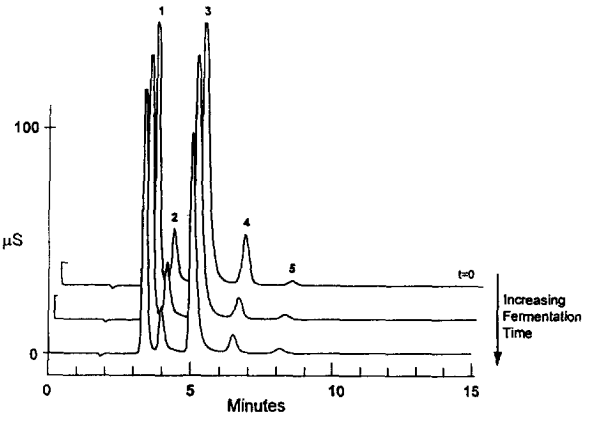

Fig. 4. A time course of representative chromatograms show isocratic cation analyses of fermentation broth samples for *E. coli* grown in complex medium containing glucose as the primary carbon source. Peaks: 1 = sodium, 2 = ammonium, 3 = potassium, 4 = magnesium, and 5 = calcium.

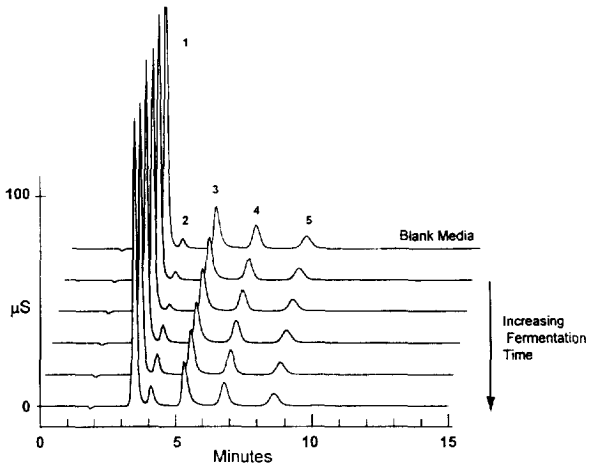

Fig. 5. A time course of representative chromatograms show cation analyses of fermentation broth samples from insect cell culture grown in complex medium with glucose and sucrose as the primary carbon sources. Peaks: 1 = sodium, 2 = ammonium, 3 = potassium, 4 = magnesium, and 5 = calcium.

6) with ammonium ion not detected in the culture media but elaborated during the growth of the culture. All other cations remained unchanged in this example.

Cation-exchange chromatography with suppressed conductivity detection was shown to be a reliable, rugged method for inorganic cation monitoring in fermentation media. Sample preparation for this method required only a ca. 1000-fold dilution of 0.22-μm filtered fermentation broth in water. The method was evaluated for chemically defined and complex fermentation media and was shown to be useful for various microbial and cell culture samples. With the exception of ethanolamines, major physiological components were invisible to this system and no interferences were observed. In comparison to other analytical methodologies for obtaining inorganic cation concentrations, this method is accurate, simple, and allows the detection and quantitation of multiple cations simultaneously. Because of the ease of sample preparation and the short analyses times, this method is potentially useful for on-line monitoring or process control.

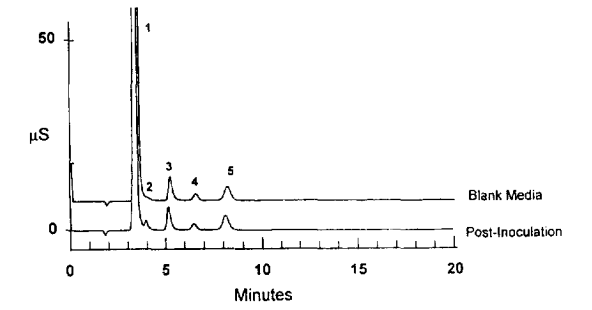

Fig. 6. Representative chromatograms show cation analyses of fermentation broth samples from mammalian cell culture grown in complex medium with glucose as the primary carbon sources. Peaks: 1 = sodium, 2 = ammonium, 3 = potassium, 4 = magnesium, and 5 = calcium.

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