Journal of Chromatography A, 1192 (2008) 187–190

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Determination of organic acids in the presence of inorganic anions by ion chromatography with suppressed conductivity detection

Xiumei Geng, Sufang Zhang, Qian Wang, Zongbao (Kent) Zhao *

Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

ARTICLE INFO

Article history:
Received 4 October 2007
Received in revised form 24 March 2008
Accepted 27 March 2008
Available online 30 March 2008

Keywords:
Ion chromatography
Conductivity detection
Organic acids
Culture media
Peptone
Yeast extract

ABSTRACT

Simultaneous separation of 19 organic acids and 10 inorganic anions has been demonstrated using ion chromatography with a high capacity anion exchange column and the suppressed conductivity detector under an auto-suppression external sulfuric acid mode. Quantitative merits of this method were examined for analysis of nine organic acids of potential significance in cell culture broth. External calibration curves for these analytes were linear with correlation coefficients exceeding 0.999, and the relative standard derivations of observed analyte concentrations were less than 3.0% in both inter- and intra-day evaluations of aqueous standards. Developed methodology was subsequently applied to obtain organic acid profiles of Luria–Bertani liquid media, yeast extract, peptone, and the culture broth of a mutant *Escherichia coli* strain. Analytes recoveries observed for triplicate analysis of LB media spiked at two concentration levels ranged from 88% to 105% with less than 7% RSD. These data demonstrate quantitative accuracy for LB media and suggest that the report method may also be applicable to complex samples such as fermentation mixture and lignocellulose hydrolysate.

1. Introduction

Fermentation and cell culture media are routinely prepared by mixing carbon sources such as glucose, and other complex materials including yeast extract and peptone. These materials are used to supply nutrients for cell growth. However, detailed compositional data at the molecular level are largely unknown. Very recently, cellular metabolism and flux analysis have been studied extensively [1–3], for which a quantitative analysis of the compositional constituents presented in the media is essential. Therefore, it is important to detect individual organic acid, so that the metabolic networks can be precisely elucidated. Similarly, studies examining organic acid production via fermentation processes also require profiling of organic acids in the culture in order to rationally design the acid-producing microorganism, as well as the bioprocess [4].

A number of methods for organic acid analysis are available. Reversed-phase high-performance liquid chromatography (HPLC) incorporated with an ultraviolet (UV) detector [5,6], a refractive index (RI) detector [7], or photo-diode array detection [8] has been generally employed. In a representative report, 32 species, including aliphatic acids, aromatic acids, and phenolic compounds were identified from a biomass pretreatment hydrolysate sample [6]. However, UV detection at approximately 210 nm for carboxylic acids lacks selectivity and is sensitive to other interfering compounds, while the HPLC system is known to fluctuate under a lower UV wavelength close to 200 nm. Additionally, rapid quantification of organic acids during microbial fermentation using ultraperformance liquid chromatography–tandem mass spectrometry has been reported with detection limits around 0.6–1.0 mg/l, although the instrument system is quite expensive [9]. Ion chromatography (IC) with conductivity detection has also been introduced to quantify organic acids in complex samples such as tobacco [10], corn stover hydrolysate [11], mammalian cell culture [12], and by-products of aluminium production [13]. However, direct analysis of organic acids in the presence of common inorganic anions remains an analytical challenge [9].

In the present study, we demonstrated an IC protocol for separation of 29 potential analytes including mono-, di-, and tri-carboxylic acids and inorganic anions under a suppressed conductivity detection and an auto-suppression external sulfuric acid mode. We successfully profiled organic acids in biologically relevant samples including Luria–Bertani (LB) liquid media, yeast extract, peptone, and the fermentation broth of a gene-knockout *Escherichia coli* strain. When compared to other published methods [5,6,9,11], our procedure offers an advantage in terms of resolution of target analytes in some cases, sensitivity in others, and in cost compared to the UPLC–MS/MS approach [9]. This procedure can be applied to analysis other complex samples such as food products, beverages, biomass hydrolysates and fermentation broth, where strongly hydrophilic compounds and anions are also present.

* Corresponding author. Tel.: +86 411 84379211; fax: +86 411 84379211.
E-mail address: zhaozb@dicp.ac.cn (Z. Zhao).

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doi:10.1016/j.chroma.2008.03.073

2. Experimental

2.1. Chemicals

Sodium formate (99%), isocitric acid lactone (97%) and malic acid (99%) were purchased from J&K Chemical (Beijing, China). Sodium acetate (98%), succinic acid (98%), fumaric acid (98%), 2-ketoglutaric acid (98%), and pyruvic acid (98%) were purchased from ABCR (Karlsruhe, Germany). Sodium lactate (99%) and citric acid (99.5%) was obtained from Sigma (St Louis, MO, USA), sodium oxalate (98%), sodium propionate (99%) and glutaric acid (99%) were from Guangfu Fine Chemical Research Institute (Tianjin, China). Peptone (batch: 20050510) and yeast extract (batch: 20060526) were products of Aoboxing Biotech. (Beijing, China). HPLC-grade solvents were from Merck (Darmstadt, Germany). Ultrapure water used for the mobile phase and the stock solutions of the analytes was generated by Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Chromatography system

The IC system and components were from Dionex (Sunnyvale, CA, USA). The hardware consisted of an ICS-2500 IC system equipped with a GP50 gradient pump, an ED50A conductivity detector, a guard column (IonPac AG11-HC, 50 mm × 4 mm), a high capacity anion exchange analytical column (IonPac AS11-HC, 250 mm × 4 mm), a 25 μl sample loop and an anion self-regenerating suppressor (ASRS, 4 mm) which was operated in the auto-suppression external sulfuric acid (5 mM) mode. Eluents were degassed via sonication and protected with nitrogen, and were further purified by an anion trap (ATC-3, 9 mm × 24 mm) placed between the pump and the injection valve. Data acquisition and instrument control were performed using the Chromeleon software installed on a personal computer.

2.3. Standard solution and sample preparation

The concentrations of the standard mixtures ranged from 1.0 to 30.0 mg/l in ultrapure water. The concentrations for peptone and yeast extracts were 1.0 and 0.5 g/l, respectively, which were injected to the IC system without further dilution. LB liquid medium was prepared by dissolving 1.0 g peptone, 0.5 g yeast extract, and 1.0 g glucose in 100 ml ultrapure H_2O, and was autoclaved at 121 °C for 15 min. This media was diluted 100-fold before analysis.

Culture broth was obtained by growth of a mutant *E. coli* strain in LB liquid medium at 37 °C and 200 rpm for 24 h. One milliliter of the culture broth was centrifuged at 13,000 × *g* for 10 min, and then diluted 50-fold before sampling.

2.4. Procedures

All experiments were performed at a flow rate of 1.0 ml/min and with a 30 °C oven temperature. The samples were injected through a 0.22 μm filter before entering the IC system. Data collection was carried out in triplicate. Ultrapure water was injected before the unknown samples.

2.5. Quantification

The concentration of each component was obtained by direct interpolation of the peak area from the corresponding regression equation. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated experimentally as a signal-to-noise ratio of 3 and 10, respectively.

Recovery was obtained by means of the standard addition procedure. Two sets of recovery experiments, each with three replicates, were conducted. In the first set, LB media was supplemented with lactate (2.0 mg/l), acetate (0.73 mg/l), propionate (0.08 mg/l), formate (0.34 mg/l), pyruvate (0.10 mg/l), succinate (0.05 mg/l), malate (0.02 mg/l), oxalate (0.13 mg/l), and citrate (0.40 mg/l). In the second set of experiments the concentration for each compound was reduced to 50% of that of the first set. The experimental recovery of each compound was calculated by dividing the determined amount by the supplemented amount and multiplying by 100%.

3. Results and discussion

Yeast extract is the total water-soluble portion of autolyzed *Saccharomyces cerevisiae* cells that were grown in a carbohydrate-rich plant medium. Yeast extract is considered one of the most complete and versatile fermentation nutrients, and is used extensively in non-animal formulations for bacterial, fungal, mammalian and insect cell culture. Peptone is the peptic digests of animal tissue prepared under carefully defined digestion parameters, and is widely used in culture medium for a variety of applications including production of substances from the culture of bacteria, fungi and mammalian cells. Yeast extract and peptone are assumed to contain various organic acids, amino acids, vitamins, and inorganic salts; however, no detailed compositional data are defined. It is important to note that separation of inorganic anions from organic acids is critical for analysis of these samples, as they are inevitably mixed with salts in order to promote cell growth or to establish a proper osmotic pressure.

In the present study, we established an IC method utilizing a degassed mobile phase including solvent B (100 mM NaOH), solvent C (methanol), and solvent D (1 mM NaOH). The gradient conditions are tabulated in Table 1. Using this gradient procedure, a typical chromatogram for separation of 29 analytes was recorded (Fig. 1). These carboxylic acids and inorganic anions including F^-, Cl^-, Br^-, I^-, SCN^-, NO_2^-, NO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, and PO_4^{3-} were well separated, as the resolutions for all analytes were higher than 1.5.

Many experiments targeting optimal separation for all compounds were performed. *Cis*-aconitate routinely gave two well-separated peaks, at 59.07 min (peak 27) and 61.55 min (peak 28), respectively (Fig. 1), likely resulting from rapid isomerisation between *cis*-aconitate and *trans*-aconitate in the presence of NaOH [14]. Similarly, itaconate and citraconate were also found interconverted under a higher concentration of NaOH (25 mM); however, they could be separated at a low concentration of NaOH in the absence of methanol. The quantification of pyruvate in the presence of oxalacetate with NaOH as a mobile phase is also important [15], as decarboxylation of oxalacetate could occur in strong basic conditions, leading to an α-hydroxyacrylate intermediate that ketonizes to pyruvate [16]. Methanol as a co-solvent played an important role in the baseline separation of the 29 analytes. For instance, succinate and malate could not be well separated

Table 1
Gradient elution profile

Time	Solvent B (100 mM NaOH)	Solvent C (MeOH)	Solvent D (1 mM NaOH)
0	0	0	100
8	0	0	100
15	2	5	93
18	4	7	89
20	8	10	82
25	12	17	71
30	16	17	67
40	25	16	59
60	65	16	19
67	68	16	16