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Short communication

Simultaneous determination of glucose, D-gluconic, 2-keto-D-gluconic and 5-keto-D-gluconic acids by ion chromatography-pulsed amperometric detection with column-switching technique

Zuoyi Zhu, Lingling Xi, Qamar Subhani, Zhongping Huang, Yan Zhu*

Department of Chemistry, Xixi Campus, Zhejiang University, Hangzhou 310028, Zhejiang, PR China

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ABSTRACT

A simple ion chromatographic (IC) method for simultaneous determination of glucose, D-gluconic acid (DGA), 2-keto-D-gluconic acid (2-KDG) and 5-keto D-gluconic acid (5-KDG) was proposed, with pulsed amperometric detection (PAD) and column-switching technique. Using this technique, the four compounds were detected simultaneously in a short time with strongly retained compounds (2-KDG and 5-KDG) eluted out prior to weakly retained compounds (glucose and DGA). Under the optimized conditions, the method showed good linearity in the range of 0.01–20 mg L^{-1} with determination coefficients (R^2)≥99.84%. Low detection limits (LODs) in the range of 0.87–2.59 μg L^{-1} and good repeatability (RSD < 3%, $n=6$) were obtained. The proposed method has been successfully applied to the analysis of the four compounds in the fermentation broth, in which *Gluconobacter oxydans* was used to produce gluconic acids from glucose.

1. Introduction

Gluconobacter oxydans is a Gram-negative bacterium belonging to the family, *Acetobacteraceae*. This organism can be used industrially to produce gluconic acids from glucose [1]. *G. oxydans* first oxidizes D-glucose to D-gluconic acid (DGA) [2], and then DGA can be converted to 2-keto-D-gluconic acid (2-KDG) [3,4] and 5-keto D-gluconic acid (5-KDG) [5,6]. Extensive fermentation studies have been carried out to study the direct glucose oxidation by *G. oxydans*. Conditions such as strains, carbon sources and salt content can greatly affect the kinds and yields of the oxidation products, so they should be optimized to achieve selective production of these gluconic acids. Furthermore, establishing a fast and simple method to determine the results of the products in the fermentation process is especially important.

Enzymatic assay [7], spectrophotometry [8] and thin-layer chromatography [9] have been carried out to determine gluconic acids. However, these methods are time-consuming and laborious. Several reports applied high performance liquid chromatography (HPLC) [10,11] and capillary electrophoresis (CE) [12,13] with UV or refractive index detection to the analysis of gluconic acids, but low sensitivity was obtained. Anion-exchange chromatography (AEC) coupled with pulsed amperometric detection (PAD) is nowadays recognized as a very selective and sensitive technique for the analysis of neutral and acidic carbohydrates [14–20]. It has been employed in the studies of various kinds of carbohydrates and related compounds. However, to the best of our knowledge, very little work has been performed on the determination of gluconic acids by direct AEC–PAD.

Column-switching technique is one of the most used techniques in liquid chromatography. Several column-switching models have been exploited in IC for eliminating the matrices and preconcentration of targets [21–28], as well as simultaneous determination of cations and anions [29]. We have also applied the column-switching technique for the fast determination of hexafluorophosphate and common inorganic anions [30]. With the above technique, ions strongly retained are expected to be eluted out into the detector prior to ions weakly retained. It is well known that carbohydrates behave as weak anions in basic solution [31]. Compared with common inorganic anions and some organic acids, monosaccharides and disaccharides are weakly retained, while as the derivatives of glucose with carboxyl and keto functionalities, 2-KDG and 5-KDG show relatively strong retention.

In the present work, in order to shorten the analytical time and achieve simultaneous detection of glucose and gluconic acids, a simple and sensitive IC method was proposed, with PAD and column-switching technique. A column with low exchange capacity was used to separate strongly retained 2-KDG and 5-KDG, and a high-capacity column was used to separate weakly retained glucose and DGA in a single run. By carrying out column-switching, 2-KDG

* Corresponding author. Tel./fax: +86 571 882 73637.
E-mail address: zhuyan@zju.edu.cn (Y. Zhu).

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and 5-KDG were eluted out to the detector prior to glucose and DGA. Simultaneous analysis of the four compounds was accomplished within a short time. Under the optimum conditions, satisfactory results were obtained from the method validation. The method has been successfully applied for the simultaneous determination of glucose, DGA, 2-KDG and 5-KDG in the fermentation broth.

2. Experimental

2.1. Equipment

All chromatographic operations were performed on a Thermo Scientific (Sunnyvale, CA, USA) ICS 2100 ion chromatograph. The system consists of a dual-piston serial pump, a column heater, a Rheodyne (Cotati, CA, USA) six-port valve with a 25 μL sampling loop, a Rheodyne (Cotati, CA, USA) 10-port valve and an EGC-KOH eluent generator. Thermo Scientific CarboPac PA10 guard column (50 mm × 4 mm) was used as the high-capacity column in this system. Thermo Scientific IonPac AG11 (50 mm × 4 mm) and AS11 (250 mm × 4 mm) were used as low-capacity column. ED40 Electrochemical Detector from Thermo Scientific was equipped with a gold working electrode, an Ag/AgCl reference electrode and a stainless steel counter electrode. Chromeleon 6.8 chromatography data management software (Thermo Scientific, Sunnyvale, CA, USA) was used for system control and data process. The cell of the pulsed amperometric detector was kept at the temperature of 35 °C. The time and voltage parameters for the pulsed amperometric detector were +0.10 V from 0.00 to 0.40 s, −2.00 V from 0.41 to 0.42 s, +0.60 V at 0.43 s, −0.10 V from 0.44 to 0.50 s, using the Ag/AgCl reference electrode mode with current integrated between 0.20 s and 0.40 s for detection. The system was operated under gradient mode as follows: 20 mmol L^{-1} KOH from 0 to 8 min and 80 mmol L^{-1} KOH from 8 to 20 min, with a flow rate of 1.0 mL min^{-1}. Column temperature was set at 30 °C. The injection volume was 25 μL.

2.2. Reagents

Glucose, D-gluconic acid, 2-keto-D-gluconic acid hemicalcium salt hydrate and 5-keto-D-gluconic acid potassium salt were purchased from Sigma (St. Louis, MO, USA). The fermentation broths were supplied by College of Life Sciences of Zhejiang University (China) and were diluted 1000-fold with deionized water before sampling. All other chemicals used in this investigation were of analytical grade. Deionized water employed in all experiments was obtained from a Millipore-Milli-Q system. Stock solutions containing 1000 mg L^{-1} of glucose, DGA, 2-KDG and 5-KDG were prepared using deionized water and stored at 4 °C. Standard working solutions were prepared every day and diluted with deionized water.

2.3. System operation procedure

The schematic diagram of the chromatographic instrument for this column-switching system is shown in Fig. 1. First, the sample was loaded into the 25 μL sample loop. Second, the sample was transported to the low-capacity column (AG11-HC/AS11-HC) by switching the six-port valve and 10-port valve to inject position (Fig. 1A). Weakly retained compounds (glucose and DGA) were washed out through the low-capacity column almost without any retention, and then they were eluted into the high-capacity column (PA10). However, strongly retained compounds (2-KDG

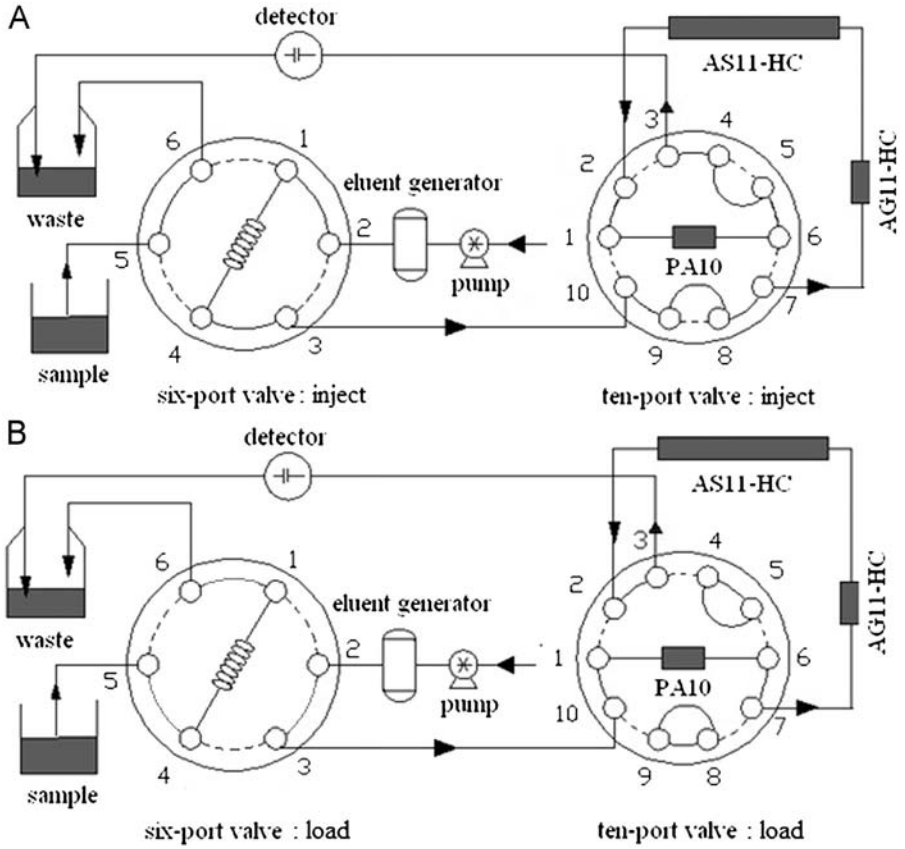

Fig. 1. Chromatographic instrument configuration of the column-switching system. (A) Separating weakly retained compounds from strongly retained compounds (0–3.1 min) before column-switching. (B) Analyzing the four compounds after column-switching.

and 5-KDG) were still retained on the low-capacity column. After that, both the valves were switched to load position, and the flow path was changed (Fig. 1B). With the positions of the columns exchanged, strongly retained compounds only passed through the low-capacity column and were eluted out first, while weakly retained compounds were separated again by the subsequent elution through the high-capacity column. A gradient separation condition was used to achieve satisfactory resolution and short analysis time.

3. Results and discussion

3.1. Selection of chromatographic parameters

An EGC-KOH eluent generator was utilized to generate high-purity and contaminant free potassium hydroxide on-line. To investigate the chromatographic retention behaviors of the four compounds, a series of experiments were performed. Thermo Scientific CarboPac PA10 guard column and analytical column, which are designed for carbohydrates analysis and have a relatively high exchange capacity, were preliminarily employed for the elution of the four compounds. However, 2-KDG and 5-KDG could not be eluted out even from the PA10 guard column with 80 mmol L^{-1} NaOH as eluent. Glucose and DGA could be separated easily within a short time (shown in Fig. 2). Then, Thermo Scientific Ionpac AG11-HC and AS11-HC columns, which have a much lower exchange capacity compared with CarboPac PA10, were used for separation. It turned out that glucose showed no retention even when the concentration of the eluent was as low as possible. DGA also showed rather weak retention. With 20 mmol L^{-1} NaOH as eluent, 2-KDG and 5-KDG could be eluted out from the Ionpac column in a reasonable time with good resolution (shown in Fig. 3). Thus, a column-switching technique was employed to separate 2-KDG and 5-KDG through the Ionpac column, while glucose and DGA were separated through the CarboPac guard column. An isocratic elution was initially performed using 20 mmol L^{-1} NaOH as eluent. The retention time for DGA was 31.58 min, which was lengthy. Moreover, a poor peak shape of DGA was obtained. To further shorten the analysis time and improve the peak shape of DGA, gradient elution was implemented. First, an eluent of 20 mmol L^{-1} NaOH was used to analyze 2-KDG and 5-KDG; after the column-switching, gradient elution was implemented with 20 mmol L^{-1} NaOH again for eluting glucose, and then increased to 80 mmol L^{-1} NaOH at 8 min for eluting DGA.

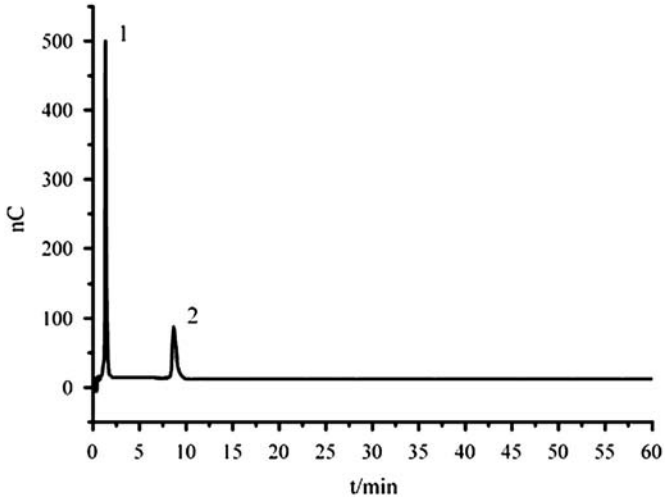

Fig. 2. Separation of 5 mg L^{-1} glucose and DGA on the CarboPac PA10 guard column. Eluent: 80 mmol L^{-1} KOH. Flow rate: 1.0 mL min^{-1}. Peaks: (1) glucose; and (2) DGA.

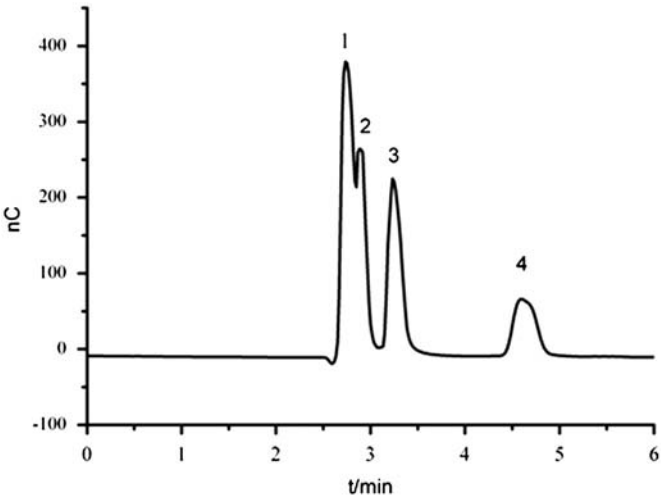

Fig. 3. Separation of the four compounds (5 mg L^{-1} each) on the IonPac AG11-HC and AS11-HC column. Eluent: 20 mmol L^{-1} KOH. Flow rate: 1.0 mL min^{-1}. Peaks: (1) glucose; (2) DGA; (3) 2-KDG; and (4) 5-KDG.

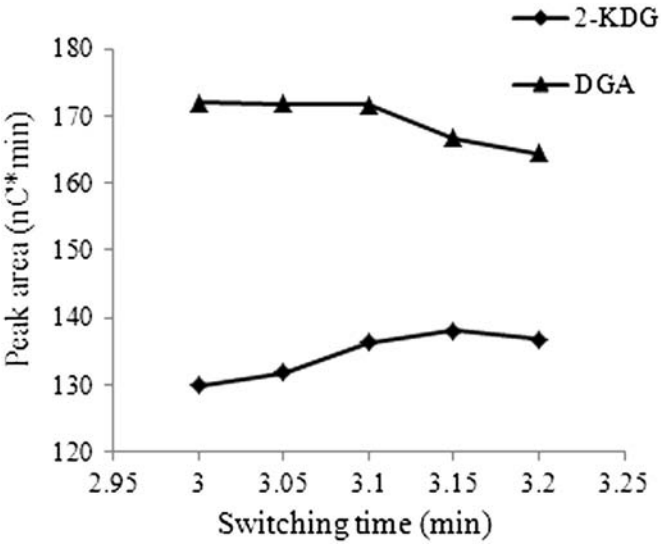

Fig. 4. The relationship of peak areas of 20 mg L^{-1} DGA and 2-KDG with different column-switching times. Gradient eluent: 20 mmol L^{-1} KOH from 0 to 8.0 min; 80 mmol L^{-1} KOH from 8.0 to 20.0 min. Flow rate: 1.0 mL min^{-1}.

In this system, the column-switching time should be adjusted when the flow rate and concentration were fixed. It should be set at the point when the weakly retained compounds were all eluted out from the Ionpac column while the strongly retained compounds still remained. According to Fig. 3, different switching times around 3.1 min were studied. Fig. 4 shows the relationship of the peak areas of DGA (20 mg L^{-1}) and 2-KDG (20 mg L^{-1}) with different switching times ranging from 3.0 to 3.2 min. Here the switching time was set at 3.1 min to ensure complete separation of DGA and 2-KDG. Under the optimal conditions, a standard solution containing 5 mg L^{-1} glucose, DGA, 2-KDG and 5-KDG was analyzed with good resolution (shown in Fig. 5). As the implement of gradient elution, the background signal increased when the concentration of the eluent increased from 20 to 80 mmol L^{-1} at 8 min, which resulted in a baseline drift. However, the baseline drift had no effect on the determination of DGA due to the high stability and reproducibility of the AEC–PAD with column-switching system.

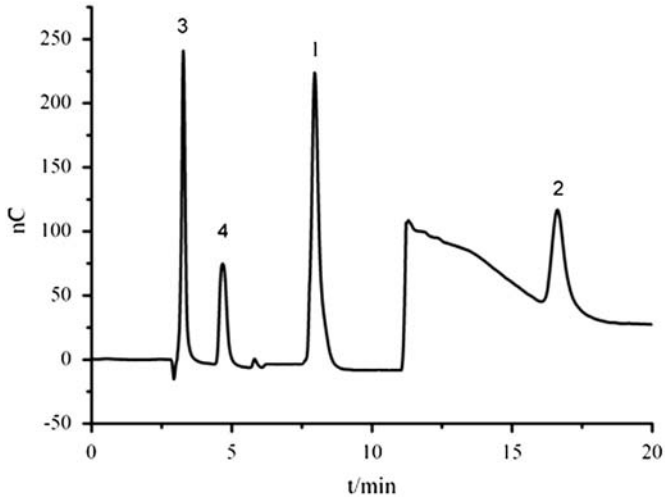

Fig. 5. Chromatogram of the four compounds (5 mg L^{-1} each) separated by the column-switching system. Switching point: 3.1 min. Peaks: (1) glucose; (2) DGA; (3) 2-KDG; and (4) 5-KDG. Other conditions were the same as in Fig. 4.

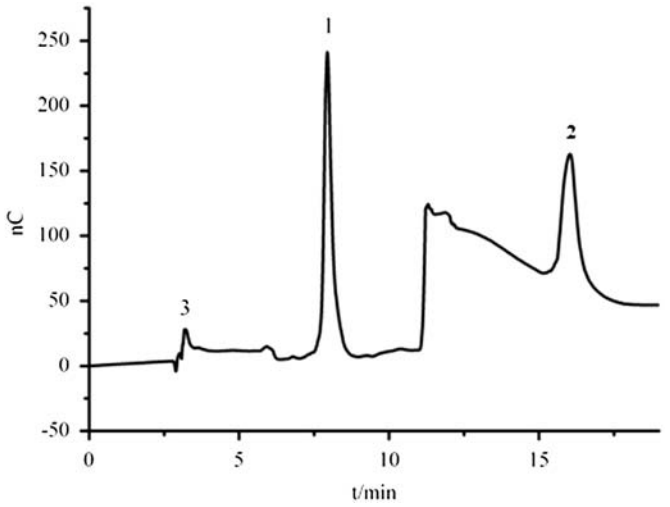

Fig. 6. Analysis of the diluted fermentation broth by the column-switching system. Peaks: (1) glucose; (2) DGA; and (3) 2-KDG. Other conditions were the same as in Fig. 5.

Table 1
Figures of merit of the proposed method.

Analyte	Linear range (mg/L)	Coefficient, R^2 (%)	LOD ($S/N=3$) (μg/L)	RSD ($n=6$) %		
				Retention time	Peak area	Peak height
Glucose	0.01–20	99.87	0.87	0.42	2.89	1.41
DGA	0.1–20	99.84	2.30	0.80	2.26	1.71
2-KDG	0.01–20	99.86	0.89	0.27	2.44	2.45
5-KDG	0.05–20	99.89	2.59	0.36	1.03	2.09

Table 2
Results for the analysis of the four compounds in 1000-fold diluted fermentation broth.

Analyte	Found [a](mg L^{-1})	Added (mg L^{-1})	Recovery (%)
Glucose	5.46	5.00	107.35
DGA	6.79	5.00	95.28
2-KDG	0.38	0.50	104.17
5-KDG	ND [b]	0.50	103.96

[a] Each represents the mean value of three determinations.
[b] Not detected.

3.2. Validation

Using the conditions above, a series of standard solutions consisting of the four compounds of various concentrations from 0.01 to 20 mg L^{-1} were analyzed. Each compound exhibited satisfactory linearity with determination coefficients (R^2) ≥99.84%. The detection limits (LODs), based on the signal-to-noise ratio of 3 ($S/N=3$), were in the range of 0.87–2.59 μg L^{-1}. The coefficients of determination (R^2), LODs and linear ranges are all shown in Table 1. Repeatability was evaluated by injecting six times the same standard solution on the same day. The relative standard deviations (RSDs) of retention time, peak height and peak area (shown in Table 1) were all less than 2.89% and proved to be satisfactory.

3.3. Application

The method was applied to the determination of glucose, DGA, 2-KDG and 5-KDG in the fermentation broth, in which *G. oxydans* was employed for the oxidation of glucose. Normally DGA is the main product, and small quantities of 2-KDG and 5-KDG were produced as side products. The chromatogram of the 1000-fold diluted fermentation broth is shown in Fig. 6. As shown, a part of glucose was converted to DGA and 2-KDG. Concentrations of the three compounds in the fermentation broth were calculated by referring to the calibration equation of working solutions. The contents of the analytes in the diluted fermentation broth and recovery data are shown in Table 2.

4. Conclusion

A simple and sensitive IC method with PAD and column-switching technique was proposed for simultaneous determination of glucose, DGA, 2-KDG and 5-KDG. The proposed method allowed fast measurements of the four compounds in a single running. The results showed good reproducibility and linearity over a wide range of concentrations. The proposed method here can be used for the quality control and the monitoring of fermentation broth in biological and biosynthetic laboratories.

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