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Simultaneous determination of 2-keto-L-gulonic acid and 2-keto-D-gluconic acid in fermentation broth by HPLC

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ABSTRACT

A simple and fast HPLC method for simultaneous determination of 2-keto-L-gulonic acid and 2-keto-D-gluconic acid in fermentation broth was described. Separation and quantitation were achieved on a shim-pack CLC-NH_2 column (150mm×6mm, 5μm) using 0.015mol/L ammonium dihydrogen phosphate solution (pH 4.1 adjusted by phosphoric acid) as mobile phase. Detection was by UV absorbance at a wavelength of 210nm, and the flow rate was 1mL/min. The elution of the analytes was achieved in less than 19.0min. The linearity, accuracy and precision of the method were found to be good and acceptable over the concentration ranges from 10 to 600μg·mL-1 for both 2-keto-L-Gulonic acid and 2-keto-D-Gluconic acid.

KEYWORDS

Fermentation broth;
HPLC;
2-keto-L-gulonic acid (2-KLG);
2-keto-D-gluconic acid (2-KDG);
Biochemistry analysis;
Vitamin C.

INTRODUCTION

Vitamin C is a kind of essential vitamin and antioxidant in human body and it can be broadly used in the medical and food industry. In the commercial production of Vitamin, only 2-keto-L-gulonic acid (2-KLG) is a key intermediate. The most popular process for synthesizing 2-KLG is the 70-year-old Reichstein's method, in which glucose is transformed to 2-KLG in five chemical steps[1]. Since Gray's description of microbial methods for the conversion of L-sorbose to 2-KLG, the use of microbial methods has become attractive and gradually replaced the chemical method for lower industrial cost and less ecological problems[2,3]. "2, 5-diketo-D- gluconic acid(2, 5-DKG) pathway" is one of the most commercially promising methods for microbial production of 2-KLG[4], during the course of preparing 2-KLG, 2-keto-D-gluconic acid (2-KDG), the stereoisomer of 2-KLG might be accumulated simultaneously with 2-KLG from other organic acids in the fermentation broth(see figure 1). Thus, simultaneous identification and quantitative determination of 2-KLG and 2-KDG in fermentation broth was very important in industrial production and scientific research of vitamin C.

In the past years, the identification of 2-KLG was usually performed by paper chromatography method[5], but this method could not be used for the quantitative determination of 2-KLG. The quantitative determination of 2-KLG was usually performed by iodometry

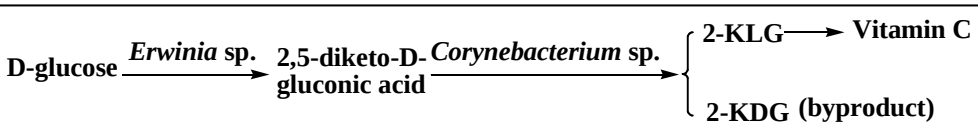

Figure 1 : Simple scheme about the transformation of glucose to vitamin C

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method, but the method could not be used for the identification of 2-KLG. In addition, both above methods were complicated to be operated.

A recent literature survey revealed that literatures about the simultaneous determination of 2-KLG and 2-KDG were very less to our knowledge. Eero[6] had applied HPLC with a column packed with sulfonated polystyrene copolymer resin to the analysis of 2-KLG and its related organic acids, this method had a lower theoretical plates yielding relatively overlapping peaks, long run time and poor resolution, which was not good enough for quantitative analysis. Qiao et al.[7] demonstrated a HPLC method to determine 2-KLG, in which the refractive index detector was employed and the results obtained presented poor sensitivity, resolution and low recovery. The best method reported was that presented by Choi et al.[8], which described a capillary zone electrophoresis method to determine 2-KLG and its related organic acid simultaneously. In our paper, a simple and fast HPLC method was described, which could be used for the simultaneous determination of 2-KLG and 2-KDG that were formed in the fermentation process for synthesis of ascorbic acid using an ordinary NH_2 column and a simple mobile phase at detection wavelength 210nm.

EXPERIMENTAL

Apparatus and reagents

Apparatus

The chromatographic system consisted of a Shimadzu LC-6A liquid delivery pump(Japan), Shimadzu SPD-6AV variable UV/VIS detector(Japan), Shimadzu CR-3A data processor(Japan) and Rheodyne 7125 model injector(USA). PERKIN-ELMER AD-6 autobalance(USA) was used for weighing substances.

Reagents

2-KLG, 2-KDG standards were purchased from sigma(St. Louis, MO, USA), ammonium dihydrogen phosphate, phosphoric acid and other reagents were all analytical grades. The fermentation broth was kindly provided by Agricultural University of Hebei. Triple-distilled water was used to prepare all the standard and sample solutions.

Chromatographic conditions

Final chromatographic conditions were an isocratic elution with 0.015 mol/L $NH_4H_2PO_4$ solution(pH 4.1, adjusted by phosphoric acid) on a Shim-pack CLC-NH_2 chromatographic column(150mm×6mm, 5μm). The flow rate was 1.0mL·min^{-1}. The detective wavelength was selected at 210nm. The mobile phase was filtered through a 0.45μm membrane filter and degassed before use. Separation was at ambient temperature. Under these chromatographic conditions described above, the retention times of 2-KLG and 2-KDG were 14.3min and 16.2min respectively. The chromatogram of standards for them was shown in figure 2.

Preparation of standard solutions

The standard stock solution(1mg/mL) was prepared by dissolving 25.0mg 2-KLG and 25.0mg 2-KDG in a 25mL volumetric flask and make up to volume with mobile phase. A series of standard solutions were prepared by quantitatively transferring suitable stock solution to 10mL volumetric flasks and made up to the volume with mobile phase.

Preparation of sample solutions

The fermentation broth provided was taken and mixed with $ZnSO_4$(72.0mg/mL) and $K_4Fe(CN)_6 \cdot 3H_2O$ (36.0mg/mL) according to the proportion of 1:1:1(v/v/v), and centrifugal precipitation for 10min by 13000rpm. Then the above clear solution was filtered through a 0.45μm Millipore membrane and diluted to three times volume with mobile phase, and then the sample solution was obtained and stored in a refrigerator to be used in the experiments.

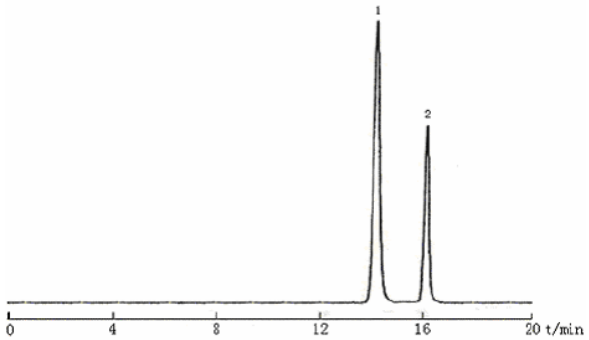

Figure 2 : Chromatogram of standards of 2-KLG and 2-KDG 1, 2-KLG; 2, 2-KDG

RESULTS AND DISCUSSION

The treatment of the fermentation broth

As we all know, the composition of the fermentation broth was very complex, which not only strongly influenced the separation of tested components, but also damage the chromatographic column to some degree. According to the experiences and some related references[5,7], the flocculants($ZnSO_4$ and $K_4Fe(CN)_6 \cdot 3H_2O$) was added into the fermentation broth to eliminate the interference of the impurity, varying concentration and proportion of the flocculants was investigated in our experiments, and the results obtained showed that the volume rate of the fermentation, $ZnSO_4$(72.0mg/mL) and $K_4Fe(CN)_6 \cdot 3H_2O$(36.0mg/mL) was 1:1:1 as a result of optimization.

The selection of column and the mobile phase

We have been engaging in the separation work by HPLC, a lot of experiences was collected in this way. Considering the solubility and the properties of 2-KLG, 2-KDG, we began with CLC-NH_2 column and a mobile phase of ammonium dihydrogen phosphate solution as mobile phase with phosphoric acid adjusted to keep the mobile phase in acidic condition. Varying concentrations of $NH_4H_2PO_4$ solution (0.05-0.4mol/L) were studied in our experiments, and the results showed that the retention times of 2-KLG, 2-KDG were gradually shortened with the concentration of $NH_4H_2PO_4$ solution increased and the baseline resolution between them could also be obtained before the concentration of $NH_4H_2PO_4$ solution increased to 0.015mol/L, but when the concentration of $NH_4H_2PO_4$ solution was higher than 0.015mol/L, the resolution between 2-KLG and 2-KDG was not very good. Besides above, the effect of pH(from 2.9 to 4.9 in increments of 0.4, adjusted with phosphoric acid) on the separation was also investigated, and the results showed that the retention time of both organic acids was obviously shortened with the pH value decreased, so lower pH value was in favor of fast elution of the tested materials, therefore, reduced the total analytical time. However, the baseline resolution between 2-KLG and 2-KDG could not be obtained if the pH value was lower than 4.1. Therefore, taking all things into consideration, 0.015mol/L $NH_4H_2PO_4$ solution with phosphoric acid adjusted to keep pH 4.1 was selected finally as the optimum mobile phase for the separation of the organic acids.

The selection of detection wavelength

The refractive index detector had ever been used to determine 2-KLG, but poor sensitivity was obtained[7]. In this paper, UV/VIS detector was used to determine them. Initial studies were carried out at several wavelengths (190 nm, 193 nm, 205 nm, 210nm, 215nm, 220nm.). It was found that both 2-KLG and 2-KDG had strong absorbance at 210nm, so a detection wavelength of 210 nm was selected finally.

System suitability test

System performance parameters of the suggested method were determined by analyzing standard working solutions. Chromatographic parameters such as number of theoretical plates(N), resolution(Rs), and capacity factor(k) were determined. The number of theoretical plates were 5438 for 2-KLG and 3890 for 2-KDG, the resolution between 2-KLG and 2-KDG was 3.28, and the capacity factor determined from the retention time relative to the dead time(t_M=2.2min) were 5.50 for 2-KLG and 6.36 for 2-KDG, respectively. Also, the resolutions between the peak of analyte and the adjacent peak of impurity were all >2.0. From the above data, it could be seen that the described method showed adequate column efficiency, well-separated peaks and good resolution from other peaks and the void volume. Besides above, the repeatability of the system was also determined by six replicate injections of the standard solutions and the RSD calculated from the peak areas were 1.69% for 2-KLG and 1.87% for 2-KDG(n=6), indicating that the described method showed good repeatability.

Linearity

The linearity studies were generally performed by preparing standard solutions of six different concentration levels. A proper amount of the standard stock solution was accurately transferred to a 10mL volumetric flask and diluted with mobile phase to volume, then varying concentration of 2-KLG and 2-KDG(10μg/mL,50μg/mL, 100μg/mL, 200μg/mL, 400μg/mL and 600μg/mL) was obtained. 10μL of those above solutions were injected in sequence from lower concentration solution to higher concentration solution and the

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TABLE 1 : Linear regressive equations, linear ranges and correlation coefficients (r) of the two components

Components	Linear regressive equations	Linear ranges (μg/mL)	r
2-KLG	Y=2301.2x-355.5	10.0-600.0	0.9999
2-KDG	Y=2202.1x-1321.5	10.0-600.0	0.9998

TABLE 2 : The average recoveries of 2-KLG and 2-KDG(n=3)s

The added mass of 2-KLG and 2-KDG (μg/mL)		2-KLG		2-KDG	
2-KLG	2-KDG	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
32.8	65.6	95.7	2.73	96.5	3.04
49.2	82.0	96.4	2.49	95.2	2.68
65.6	98.4	96.8	3.01	95.4	2.87

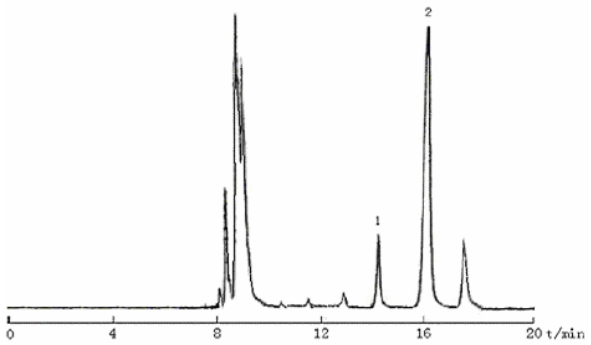

Figure 3 : Chromatogram of sample solution 1, 2-KLG; 2, 2-KDG

corresponding peak area of both analysts was recorded, each concentration was analyzed three times. Then the regressive curves with the peak area of the tested components as ordinate(Y) and the corresponding concentration(μg/mL) as abscissa(x) were achieved. The results were listed in TABLE 1. From TABLE 1, it could be seen that those parameters showed a good linearity with correlation coefficients >0.999 for each component.

Precision

The precision study mainly included two parts: intra-assay precision and inter-assay precision. The former was determined by repeatedly analyzing ten aliquots of the standard solution(10μL the standard solution with 200μg/mL 2-KLG and 200μg/mL 2-KDG) in one day, and the RSD(n=10) for 2-KLG and 2-KDG were 1.71% and 1.84%, respectively. The latter was performed the same experiments as the inter-assay on different days(3 days) with newly prepared mobile phase and samples, and the RSD(n=3) values obtained were 2.58% for 2-KLG and 3.04% for 2-KDG. From the results, it could be seen that a good precision was achieved in this method.

Limits of detection and quantitation

Limit of detection(LOD) was the lowest concentration of analyte in a sample that could be detected under the stated experimental conditions: typically three times of the noise level. The LOD of each active gradient was experimentally determined by six injection of it at its LOD concentration, and the results were found to be 2.7 μg/mL for 2-KLG, 3.1 μg/mL for 2-KDG Meanwhile, Limits of quantitation (LOQ), which was the lowest concentration of the tested components in a sample that could be determined with acceptable precision and accuracy, was established at a singal-to-noise ratio of 10 and experimentally verified by six injections of each organic acid at its LOQ concentration, the results obtained was 7.8μg/mL for 2-KLG and 9.1μg/mL for 2-KDG respectively.

Solution stability

The stability of both standard and sample solutions was determined by monitoring the peak area of the standard solution of 2-KLG and 2-KDG and sample solution over a period of three days. The results indicated that peak area of 2-KLG and 2-KDG of these solutions remained almost unchanged and no degradation was observed within the given period if these solutions were stored in a refrigerator at about 4^0C, indicating that these solutions were stable for at least three days.

Determination of sample solution

The sample solution was obtained according to "the preparation of the sample solution" in experimental, 10μL of above solution was injected and the peak area was recorded, the results were shown in figure 3. The concentration of 2-KLG and 2-KDG were calculated to be 82.0μg/mL and 417.3μg/mL respectively according to the linearity regression equations.

ACCURACY

The accuracy of a method was the closeness of the measured value to the true value for the sample. In our experiments, an amount of standards of 2-KLG and 2-KDG were accurately weighed and added into above

known concentration sample solution, and then shaken up. 10μL of those solutions were injected in sequence and the peak areas were recorded, then the percent recovery and RSD values were calculated, each concentration was analyzed three times, and the mean values for both were listed in TABLE 2. From TABLE 2, it could be seen that the accuracy of this method for determination of both products in the fermentation was satisfactory.

CONCLUSION

An isocratic HPLC method has been developed for the determination of 2-KLG and 2-KDG in fermentation broth in less than 19.0min. All the parameters obtained from the experiments for validation show that the proposed method here is reliable and accurate, therefore it not only can be used in the analysis of 2-KLG and 2-KDG in the fermentation process synthesizing ascorbic acid, but also can be extended to the quality control and the monitoring of fermentation broth in biological and biosynthetic laboratories.

REFERENCES

[1] T.Reichstein, A.Gruessner, R.Oppenauer; Helvetica Chimica Acta, **(16)**, 1019 **(1933)**.
[2] J.Boundrant; Enzyme and Microbial Technology, **12(5)**, 322 **(1990)**.
[3] S.Makover, G.B.Ramsey, F.M.Vane (etc.); Biotechnology and Bioengineering, **17(10)**, 1485 **(1975)**.
[4] T.Sonoyama, H.Tani, K.Matsuda (etc); Applied and Environmental Microbiology, **43(5)**, 1064 **(1982)**.
[5] Ch.H.Qiao, C.Sh Chen, F.Chen (etc.); Industrial Microbiology, **30(1**), 9 **(2000)**.
[6] R.Eero; Journal of Chromatography, **218**, 695 **(1982)**.
[7] Ch.H.Qiao, G.L.Yin; Modern Scientific Instruments, **71(4)**, 18 **(2000)**.
[8] O.K.Choi, C.G.Kim, Y.D.Kim (etc.); Journal of Chromatography A, **745**, 249 **(1996)**.