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Analysis of Fructose 1,6-Diphosphate in Fermentation Broth Using Ion Chromatography

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Abstract

In this study, a new method for rapid and accurate detection of fructose 1,6-diphosphate (FDP) present in fermentation broth was established using ion chromatography. With an AS11-HC anion column, suppressed conductivity detection, and 50 m mol/L KOH elution, qualitative and quantitative analyses of FDP in the fermentation broth can be completed in 5 min. The minimum detection limit of the method for FDP was 0.032 μmol/L (S/N=3). Within the concentrations ranging from 3.3 to 211.5 μmol/L a significant linear relationship (r=0.9999, p<0.0001), good recovery (99.0%~100.3%), and measurement precision (≤0.04%, n=5) were obtained. The method can qualitatively analyze PO_4^{3-}, fructose-6-phosphate, and glucose 6-phosphate in the fermentation broth simultaneously. The results obtained by this method had no significant difference from those obtained using the enzymatic analysis method.

Keywords: Fructose 1,6-diphosphate; Ion chromatography; The detection limit; Recovery; Precision

Introduction

Fructose 1,6-diphophate (FDP) is an important intermediate of glycolysis. It can regulate activities of a variety of glycolytic enzymes [1]. Harden and Young were the first to isolate the compound and clarify its physical and chemical properties in 1908 [2]. Subsequently, great efforts were made to study FDP physiological functions. It is known that FDP can enhance cell metabolism and cardiac myocyte nutrients, build up the resistance to convulsions, improve anoxic organ performance [3-9]. Now it is primarily used as a rescue drug for shock patients, in addition to, or used for angina pectoris, heart failure, myocardial infarction secondary treatment, in the cardiovascular acute and chronic disease treatment and prevention with an important role.

For FDP fermentation production, a lot of studies have been performed on fermentation process, yeast cell cultures, FDP separation and purification, correlation between yeast cell permeability and FDP yield [10-14]. The method of Leisola and Linko using the enzymes of beer fermentation yeast cells to bio-convert sugar and phosphate into FDP for the industrial FDP production is widely used now [12]. In the 1980s, the industrial production of FDP was first accomplished by Foscama, an Italian company and the FDP powder for injection was produced. In 1992, the state-level appraisal of a pilot production of FDP trisodium salt was implemented in China and the production was industrialization in the late 1990s.

With regard to the analysis of FDP, several studies have been performed. In 1954, Roe and Papadopoulos completed the determination of FDP and fructose-6-phosphate with color-reaction of resorcinol and furan ring from FDP or fructose-6-phosphate [15]. However, this method was not applicable to the qualitative analysis of FDP and the results were easily influenced by many factors of a complex course. Subsequently, the analysis of FDP was performed employing under-mentioned enzymatic reaction. The dihydroxyacetone phosphate, a decomposition product of FDP, can oxide nicotinamide adenine 2 nucleotide (NADH) into nicotinamide adenine dinucleotide (NAD) under the action of glycerol-3- phosphate dehydrogenase. Changes in the NADH absorbance value at 340nm were correlated with the changes in FDP contents [16]. This method is very specific and accurate, but it has multiple steps and is time consuming. Additionally it requires controlled temperatures, reaction mixture pH, and other experimental conditions. It cannot be used for a rapid and on-line quantification of FDP levels in industry.

Over the years, ion chromatography, as a rapid and simple method for the analysis of hydronium, has been widely used in the analyses of trifluoroacetic acid, oxalate, sodium cyclamate and other organic compounds [17-19]. In this study, a simple, rapid ion chromatographic method was established for FDP qualitative and quantitative analyses.

Materials and Methods

Experimental instruments

DX-600 Ion Chromatography (Dionex Corporation, USA) including EG40 automatic eluent generation system, ED50 Conductivity Detector, AS50 auto-sampler, ATC-I anion trap column and an ASRS-Ⅱ (2mm) anion self regenerating suppressor operating in the auto-suppression recycle mode, using a Dionex IonPac AS11-HC analytical column(2×250 mm) and AG11-HC guard column (2×50 mm). The detector sampling rate was 0.2s. Dionex PeakNet6.0 software was used for hardware control, detector signal acquisition and chromatographic peak integration. 0.22 μm filter membrane (Millipore Corporation, USA); ultra-pure water system (Millipore Corporation, USA).

Experimental methods

Preparation of an FDP fermentation broth: The fermentation

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mixture (200 mL) contained 19.64 g glucose, 6.24 g NaH_2PO_4, 14.32 g Na_2HPO_4, 0.552 g $MgCl_2$, 20 g dry yeast powder, and distilled water. The mixture was well mixed and shaken at 37°C and 160 rpm for 2.5 h. Then the fermentation broth was incubated for 10 min in boiling water and then centrifuged at 10,000 g for 5 min. The precipitation was discarded and the supernatant was collected and kept at 4°C for future analysis.

Sample processing: Ultrapure water (18.2 MΩ) was used to prepare the following solutions:

FDP stock solution: 2.5 mg $FDPNa_3 \bullet 8H_2O$ (Sigma) in 25 mL H_2O.

Solution I: 2.5 mg $FDPNa_3 \bullet 8H_2O$, 3.0 mg D-fructose-6-phosphate sodium (Sigma) in 25 mL H_2O.

Solution II: 10.0 mg $FDPNa_3 \bullet 8H_2O$ and 1.0 mg D-glucose -6 -phosphate sodium (Sigma) in 25 mL H_2O.

Solution III: 8.0 mg $FDPNa_3 \bullet 8H_2O$ and 3.0 mg $Na_2HPO_4.7H_2O$, in 25 mL H_2O.

Solution IV: 2.5 mg $FDPNa_3 \bullet 8H_2O$ and 3.0 mg D-fructose in 25 mL H_2O.

Solution V: 2.5 mg $FDPNa_3 \bullet 8H_2O$ and 3.0 mg D-glucose in 25 mL H_2O.

Solution VI: 0.25 mg FDPCa in 100 mL H_2O.

Solution VII: 1.0 mg FDPCa in 100 mL H_2O.

Solution VIII: 2.0 mg FDPCa in 100 mL H_2O.

SolutionIX: 4.0 mg FDPCa in 100 mL H_2O.

Solution X: 6.0 mg FDPCa in 100 mL H_2O.

Solution I, II, III, IV, V, VI, VII, VIII and the supernatant of the FDP fermentation (2.2.1, kept at 4°C) were diluted with ultrapure water (18.2 MΩ) to the desired concentrations, filtered through 0.22 μm membrane, and then directly injected into the DX-600 Ion Chromatography.

Chromatographic conditions: 50 mmol/L KOH eluent was used as mobile phase and the flow rate was set at 0.38 mL/min. The suppressor current was operated at 124 mA and the conductivity detector cell was set at 30°C with 5 min of running time. The column temperature was set at 30°C. 20 μL of a sample was injected.

Determination of inorganic phosphorus contents: The assay was conducted according to Biologic et al. [20]. 0.493 mmol/L of D-fructose-1,6-phosphate trisodium ($FDPNa_3$) solution was prepared using 40 mmol/L or 50 mmol/L of KOH solutions, and stirred for 30 min at 25°C. The contents of inorganic phosphorus in $FDPNa_3$ solutions added with or without KOH were measured using this method.

Enzymatic determination of FDP: The assay was conducted according to Yin [16]. In brief, 3 ml of 100 mmol/L TEA, 0.01 ml of 17 μmol/L NADH, 0.1 ml of FDP at defined concentration was mixed, then OD_{340} was detected and defined as A_1. Then, 0.01 ml of 130 u/L Glyceraldehyde-3-phosphate dehydrogenase (GDH) was added for reaction, OD_{340} was detected after 15 min and defined as A_2. Then, 0.01 ml of 830 u/L Triosephosphate isomerase (TIM) was added for reaction, OD_{340} was detected after 15 min and defined as A_3. At last, 0.01 ml of 150 u/L aldolase (ALD) was added for reaction, OD_{340} was detected after 15 min and defined as A_4. All reactions were carried out at 25°C unless otherwise noted. The absorption reduction of NADH was A_3- A_4. The concentration of the FDP could be calculated by the reduction of NADH, and the total equation was showed below.

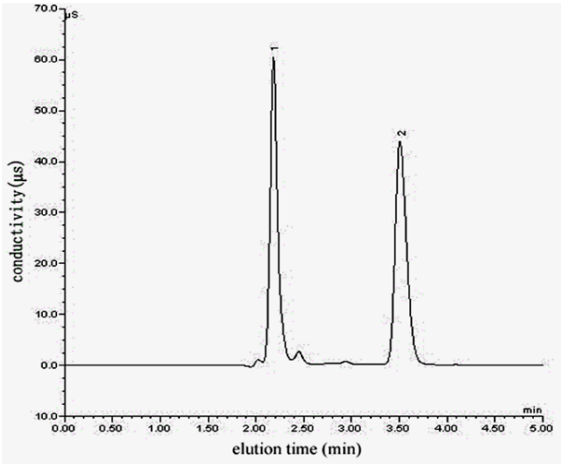
$$FDP + 2NADH + 2H^+ \rightleftharpoons 2glycerol\text{-}3\text{-}p + 2NAD^+$$

Data analysis: All data analysis was conducted using the software Statistica 6.0.

Results

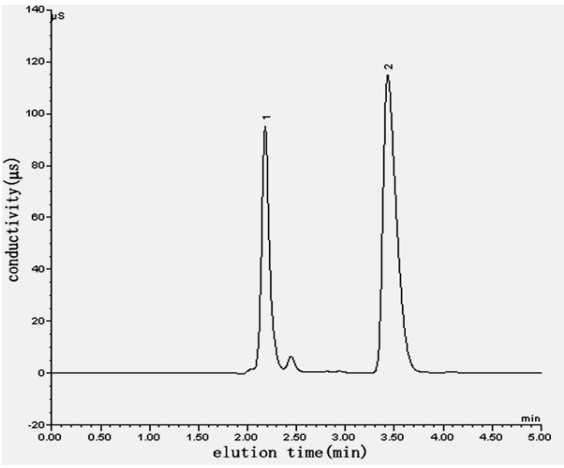
FDP qualitative analysis

The separation and retention time of FDP, phosphate, D-fructose-6-phosphate, D-glucose-6-phosphate on an AS11-HC-type separation column were studied using different eluent KOH solutions (5 mmol/L, 10 mmol/L, 20 mmol/L, 30 mmol/L, 40 mmol/L, 50 mmol/L). Our results showed that FDP was strongly retained on the column and not eluted out within 1 h if the eluent concentration ≤30 mmol/L. When the eluent concentrations were increased to 40 and 50 mmol/L, FDP and phosphate, D-fructose-6-phosphate, D-glucose-6-phosphate were all separated and eluted down in 10 min and 5 min, respectively (Figures 1-3).



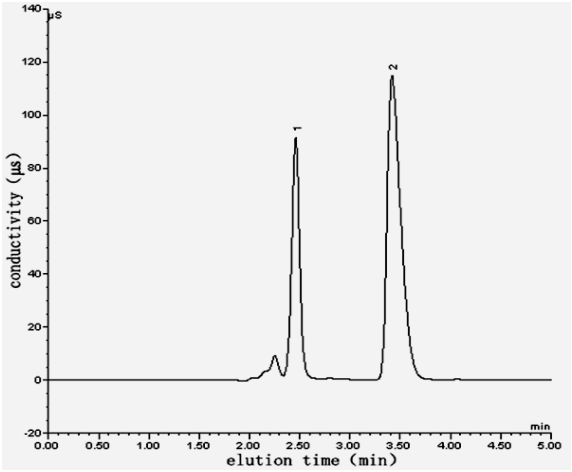
1. Fructose-6-phosphate sodium, 0.375 mmol/L;
2. Fructose-1, 6-diphosphate trisodium, 0.232 mmol/L

Figure 1: Separation of Fructose-1, 6-diphosphate trisodium and Fructose-6-phosphate sodium.



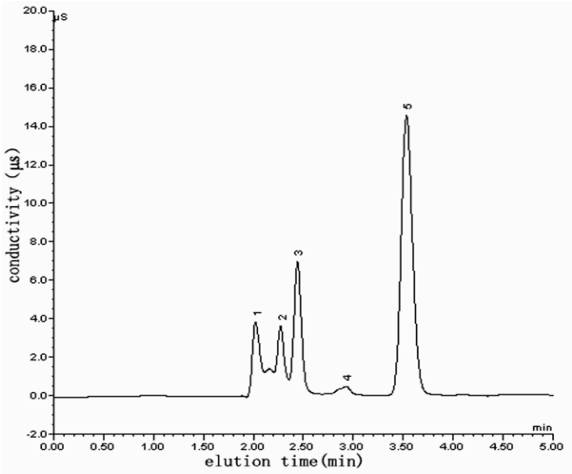
1. Gluctose-6-phosphate sodium, 0.125 mmol/L;
2. Fructose-1, 6-diphosphate trisodium, 0.695 mmol/L.

Figure 2: Separation of Fructose-1, 6-diphosphate trisodium and Gluctose-6-phosphate sodium.



1. Sodium phosphate, 0.425 mmol /L;
2. Fructose-1, 6-diphosphate trisodium, 0.683 mmol/L.

Figure 3: Separation of Fructose-1, 6-diphosphate trisodium and Sodium Phosphate.



1. Cl^-; 2. NO_3^-; 3. PO_4^{3-}; 4. Unknown; 5. FDP

Figure 4: Chromatogram of FDP fermentation with a dilution of 2000 fold (2.25 h).

The determination data of inorganic phosphorus in $FDPNa_3$ solutions added with or without KOH using the method described in Section 4.2.4 showed almost no increase in the inorganic phosphorus contents in the KOH-treated $FDPNa_3$ solutions remained unchanged compared to those in the solution not treated, suggesting that dissociation of phosphate group from FDPNa3 was not significantly enhanced due to the presence of the tested concentrations of KOH in the eluents. This result was confirmed in the follow-up experiments.

To identify the peaks eluted at 3.53 min using 50 mmol/L KOH eluent, Solution Ⅰ, Ⅱ, III, Ⅳ and Ⅴ were injected after filtered as described in Section 4.2.2. The results are presented in Figures 1-3, revealing that there was no peak of D-fructose or D-glucose (Solution IV and V) (data not shown) under the conditions described in Section 4.4. Additionally, the result, no increase in the peak area of PO_4^{3-} in Figures 1-3, again confirmed that dissociation of phosphate group from $FDPNa_3$ did not occur when eluted with 50 mmol/L KOH. Thus, the peak at 3.53 min was indeed FDP. The resolutions of chromatographic peaks between FDP and D-fructose-6-phosphate, D-glucose-6-phosphate or sodium dihydrogen phosphate were 7.487 (usp), 6.373 (usp) and 4.863 (usp), respectively, all being greater than 1.5, indicating that the target FDP was completely separated from the three above compounds. With the 50 mM KOH eluent, the samples of the FDP fermentation broth (diluted 2000-fold) were analyzed and FDP was also completely separated from other compounds (Figure 4). These results suggest that identification of FDP in the fermentation broth can be conducted using 50 mmol/L KOH solution as the eluent, with which the analysis resulted in better peak parameters and much shorter of running time than with 40 mmol/L KOH(data not shown). Therefore, 50 mmol/L KOH solution was used in future analyses.

Standard curve and detection limit

The reproducibility of retention time, chromatographic peak height and chromatographic peak area: esults concerning reproducibility of peak retention time, peak height and peak area obtained from 5 repeated analyses of 2000-fold diluted FDP fermentation samples using the methods described in Section 4.2.3 were presented in Table 1. The relative standard deviation (RSD) of the peak retention times was quite low, suggesting that the retention times of the compounds were highly consistent. This is basis for FDP qualitative analysis. The RSD of peak areas was smaller than that of peak heights, indicating that peak areas had higher reproducibility than peak heights, thus, the values of chromatographic peak areas were chosen for FDP standard curve plotting.

FDP standard curve for quantitative analysis: Standard $FDPNa_3$ solutions of 0.0033, 0.0066, 0.0132, 0.0264, 0.0529, 0.1058, and 0.2115 mmol/L were prepared and analyzed as described in Section 4.2.2 and 4.2.3. The FDP concentration was plotted against peak areas to get a stand curve (Table 2). Within the range from 0.0033 to 0.2115 mmol/L, FDP peak areas were positively correlated with the concentrations ($r=0.9999$, $p<0.0001$) and the resulting regression equation could be used for FDP quantitative analysis.

The limit of detection (LOD) and the lowest limit of quantification (LOQ): In this study, the concentration of FDP sample resulted in the ratio S (peak height)/N (baseline noise)=3:1 is defined as the minimum detection limit and the lowest concentration of FDP plotted in the standard curve is defined as the lowest limit of quantification. Our study revealed that the values of LOD and LOQ were 0.032 μmol/L and 3.3 μmol/L, respectively.

Precision

Analysis precision was obtained from quantification results of

Factors studied	Mean	SD (%)	RSD (%)
Elution (min)	3.539	1.26	0.36
Peak height (μs)	12.457	4.65	0.37
Peak area (μs*min)	1.650	0.36	0.22

Table 1: Reproducibility studies of retention time, peak height and peak area (n=5).

Sample	FDP
Linear range (mmol/L)	0.0033-0.2115
Linear regression	
Slope	26.523 ± 0.0119
Intercept	-0.0286 ± 0.0103
Regression coefficient	0.9999
Equation	y^a=26.523 x^b-0.0286

Table 2: Results of linearity tests.

[a] y : peak area; [b] x : the concentration of FDP

FDP concentrations (mmol/L)	Mean [a] (mmol/L)	RSD [b] (%)
0.0066	0.00658	2.50
0.0264	0.02664	1.01
0.0529	0.05324	0.63
0.1058	0.10606	0.39
0.1587	0.15834	1.09

[a]The data was based on five replicates;
[b]The data was figured basing on five FDP determination replicates.

Table 3: Results of precision tests (n=5).

FDP fermentation (mmol/L)	FDP added (mmol/L)	FDP measured [a] (mmol/L)	Recovery [b] (%)
0.0066	0.0054	0.0118	96.7%
	0.0870	0.0937	100.1%
	0.1969	0.2039	100.2%
			99.0% ± 1.99% ± 2.01%[c]
0.0670	0.0054	0.0723	98.1
	0.0217	0.0886	99.5
	0.0870	0.157	103.4
			100.3% ± 2.75% ± 2.74%[c]
0.1477	0.0027	0.1504	99.3%
	0.0217	0.1695	100.6%
	0.0435	0.1914	100.4%
			100.1% ± 0.7% ± 0.69%[c]

[a] The data was based on five replicates;
[b] Recovery was figured basing on the mean of five FDP determination replicates;
[c] Mean ± SD ± RSD of recovery.

Table 4: Recovery studies of FDP added in fermentation (n=5).

FDP fermentation (mmol/L)	Method [a]	FDP measured [b] (mmol/L)	RSD [c]	F	P	t
0.0033	1	0.00328	4.48%	0.182	0.6811	0.4264
	2	0.00332	3.51%			
0.0132	1	0.01322	1.24%	0.930	0.3641	0.9623
	2	0.01332	1.23%			
0.0264	1	0.02664	1.01%	0.100	0.7103	0.1491
	2	0.02662	0.49%			
0.1058	1	0.10606	0.39%	1.000	0.461	0.775
	2	0.10590	0.19%			
0.2115	1	0.21146	0.53%	0.000	1.000	0.000
	2	0.21146	0.09%			

[a] "1": ion chromatographic method, "2": enzymatic method;
[b] The data was the mean of five replicates;
[c] The data was figured basing on five FDP determination replicates

Table 5: Comparison between enzymatic method and chromatographic method on FDP determination.

5 $FDPNa_3$ samples with different concentrations using the methods described in Section 4.2.2 and 4.2.3 (Table 3). The values of RSD of 5 replicates from the same sample were all ≤2.50%, suggesting high consistency among the measured results. In particular the RSD values of the $FDPNa_3$ samples with the concentrations corresponding to the middle of the standard curve were lower than those of the samples with lower or higher FDP concentrations.

This clearly indicated that our method resulted in precise and reproducible quantitative data.

Recovery

Different amount of $FDPNa_3$ were added into the fermentation samples with 0.0066, 0.0670, and 0.1477 mmol/L FDP concentrations. The quantitative analysis of FDP levels in each sample was performed and recovery was obtained as shown in Table 4. The recoveries for the three tested fermented samples spiked with different amount $FDPNa_3$ were 100.1%, 100.03% and 99.0%, respectively and all had low RSD, indicating that the results were highly repeatable.

Comparison between ion chromatographic method and enzymatic determination of FDP

The ion chromatography method established in this study was compared with the enzymatic quantitative method described in Section 4.2.5 to quantify FDP levels in five different samples. The results were statistically analyzed using STATISTICA 6.0 software (Table 5).

The F tests showed that F values for five different samples using two analytic methods were 0.182, 0.930, 0.100, 1.000, and 0.000, all smaller than the $F_{4,\ 4,\ (0.95)}$=6.388, indicating that the difference in analytic precision was not significant between the two. The t tests indicated that when P=0.90, t values were 0.4264, 0.9623, 0.1491, 0.7752, and 0.0000, all less than $t_{0.10,\ 8}$=1.860, suggesting that there was no significant difference between the two methods.

Discussion

This study established a new method for FDP qualitative and quantitative analyses. Compared with the enzymatic analytic method, it has the following advantages:

(1) It is rapid. Enzymatic analysis process took about 1 h [16] and this method took only 5 min.

(2) It is highly sensitive. The minimum detection limit of FDP using this method was 0.032 μmol/L and FDP quantification limit was 3.3 μmol/L while the quantitative limit of the enzymatic method was reportedly higher than 9 μmol/L [21]. No reports on FDP minimum detection limits have been found so far. The detection limit using the ion chromatography method could be further reduced if the sample injection volume was increased and a thin analytical column or on-line sample enrichment are employed.

(3) It is known that changes in ambient temperature have a higher degree of impact on the results of enzymatic determination [16]. Therefore the enzymatic assays have to be performed under well controlled temperature conditions. Whereas in this method, an ion-chromatography conductivity detector can detect the temperature of the sample right out of the sample cell so that an automatic normalization of measurements are performed to eliminate the impact of temperature variation. Thus, the requirement for a controlled ambient temperature during the course of measurements is not critical.

Using the ion chromatography method established in this study, separation of FDP from PO_4^{3-}, fructose-6-phosphate and glucose 6-phosphate in a sample was achieved with the selected elution conditions. However, fructose-6-phosphate and glucose 6-phosphate cannot be separated (data not shown). The separation of the two substances could be realized by optimizing elution conditions, using an effective gradient elution procedure or a highly effective column. Nevertheless, separation of the two compounds does not affect the quantitative analysis of FDP using ion chromatography.

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