HPLC Methods for Determination of Dihydroxyacetone and Glycerol in Fermentation Broth and Comparison with a Visible Spectrophotometric Method to Determine Dihydroxyacetone

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Abstract

High-performance liquid chromatographic (HPLC) methods were respectively developed for the quantitative determination of dihydroxyacetone (DHA) and glycerol in the fermentation broth. Validation parameters such as linearity, precision, accuracy, and specificity, limit of detection (LOD), and limit of quantitation (LOQ) were determined. Both HPLC methods were carried out on a Lichrospher 5–NH_2 column with a mobile phase constituted of acetonitrile and water (90:10, v/v). The linearity range for DHA was 2.00–12.00 mg/mL with a correlation coefficient (*r*) of 0.9994. The LOD and LOQ were 0.06 and 1.20 mg/mL, respectively. The linearity range for glycerol was 0.50–20.00 mg/mL with a correlation coefficient of 0.9998. The LOD and LOQ were 0.22 and 0.50 mg/mL, respectively. Also, the HPLC method to determine DHA was compared with an existing visible spectrophotometric method. Statistical analysis by F-test and *t*-test showed no significant difference at 95% confidence level between the two methods when applied to low DHA concentrations while a large deviation existed in the determinations of high DHA concentrations. The HPLC method was more accurate to determine high DHA concentrations.

Introduction

1,3-dihydroxyacetone (DHA), an important raw material in the cosmetic and food industry, is the simplest member of the family of ketoses. DHA is now obtained by biotransformation wherein glycerol is converted to DHA by bacteria with glycerol dehydrogenase (1,2) such as Acetobacter suboxydans ATCC 621 (3). Various fermentation technologies such as multi-vessel system (4) and semi-continuous two-stage repeated-fed-batch have been reported (5).

Several analytical methods for the determination of DHA have been described. Gas chromatography (6) requires derivatization of DHA, the pretreatment is extensive and easily influenced by water. Thin-layer chromatography (7) is relatively simple, however exact quantitation could hardly be reached. Liquid chromatography using a calcium column (8) is a better choice to assay DHA, but this kind of column is not commonly used.

For glycerol quantitation, oxidation by periodic acid is the most widely used method. The procedure is tedious and existing DHA in the fermentation broth could also be oxidized by periodic acid, which will dramatically affect the result. The disadvantage of thin-layer chromatography is that the limit of quantitation is usually above the residual glycerol concentration at the later stage of fermentation process. In enzymatic determination (9) of glycerol, the amount of NADH (nicotinamide adenine dinucleotide, reduced form) produced after reaction with glycerol dehydrogenase presents a proportional relationship with glycerol concentration. Highly specific as this method is, the high cost limits its application.

The aim of this work was to develop efficient HPLC methods to determine DHA and glycerol. Also, the HPLC method to determine DHA was compared with an existing visible spectrophotometric method (10) using diphenylamine solution to quantitate DHA.

Experimental

Reagents, chemicals, and standards

Dihydroxyacetone (99.8% purity) was purchased from Sigma. Acetonitrile (HPLC grade), glycerol (analytical grade), acetic acid, and sulphuric acid (98%) were obtained from Nanjing Wanqing Chemical Reagent Ltd. Ultrapure water was used to prepare all the solutions for the proposed HPLC methods. Distilled water was used to prepare all the solutions for the visible spectrophotometric method to determine DHA and to prepare the fermentation media.

Instrumentation and analytical conditions

The HPLC methods for both DHA and glycerol were performed on a liquid chromatograph (Agilent 1000) with a UV–vis detector as well as a refractometer. Both DHA and glycerol were analyzed at a flow rate of 1.0 mL/min using a

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mobile phase constituted of acetonitrile–water (90:10, v/v). A Lichrospher 5–NH_2 column obtained from Jiangsu Hanbon Sience & Technology Co. Ltd. was used and operated at 30°C. DHA was detected with the UV detector at 271 nm under room temperature while glycerol was detected with the Aligent refractometer at 35°C.

In the visible spectrophotometry method for the determination of DHA, chromogenic agent was used. 60 mL of such chromogenic agent contained 0.6 g diphenylamine, 54 mL acetic acid, and 0.6 mL of sulphuric acid (98%). 0.5 mL DHA sample was added into 4.5 mL chromogenic agent, mixed, and allowed to stand in boiling water for 20 min. The resulting solution took on blue color that was detected at 615 nm. Pure water was used as blank.

Preparation of standards

In the HPLC method for DHA, 1200.0 mg of DHA was accurately weighed and transferred into a 10-mL volumetric flask and dissolved in the mobile phase to result in stock solution (I) of 120.00 mg/mL. This solution was diluted with mobile phase to yield 12.00, 10.00, 8.00, 6.00, 4.00, and 2.00 mg/mL working standard solutions, each in triplicate. For the precision determination, solution of 6.00 mg/mL was prepared from the stock solution.

In the HPLC method for glycerol, 1000.0 mg of glycerol was accurately weighed and transferred to a 50-mL volumetric flask and dissolved in water to result in stock solution (II) of 20.00 mg/mL. Other solutions of 15.00, 10.00, 5.00, 1.00, and 0.50 mg/mL were obtained by dilution of the stock solution with ultrapure water, each in triplicate. For the precision determination, solution of 10.00 mg/mL was prepared from the stock solution.

In visible spectrophotometry for DHA, 30.0 mg of DHA was accurately weighed and transferred to a 10-mL volumetric flask and dissolved in distilled water to result in stock solution (III) of 3.00 mg/mL. Other concentrations of 0.25, 0.20, 0.15, 0.10, 0.05, and 0.04 mg/mL were obtained by dilution of the stock solution with distilled water, each in triplicate. For the precision determination, solution of 0.10 mg/mL was prepared in triplicate from the stock solution.

Preparation of sample solution

The producing strain belonging to Acetobacter suboxydans was cultivated in a 500 mL shaken flasks with 100 mL fermentation medium containing yeast extract (1%,w/v), tryptone (1%, w/v), and glycerol (8%, w/v) as substrate. Biotransformation was carried out with an agitation rate of 220 rpm at 28°C. The fermentation broth was sampled at the 11th, 26th, 35th, 46th, 52nd, 59th, 76th, and 80th of the time-course. All samples were centrifuged for 10 min at 8000 rpm to remove the cells.

After filtration through 0.22-µm membrane filter, the supernatant fluid was diluted to appropriate concentration within the calibration range.

Method validation

The proposed methods were validated according to the guidelines for validation of analytical methods in Chinese Pharmacopoeia (11).

Linearity

The calibration curves for each method were obtained at 6 concentration levels, each with triplicate determinations. The calibration curves of both HPLC methods were constructed by plotting the peak areas of the analytes (y) against concentration of the analytes (x) by linear regression analysis. The calibration curve of the visible spectrophotometry for DHA was constructed by plotting the absorbance (y) against the concentration of DHA (x) by linear regression analysis.

Precision

The precision of each method was evaluated by analyzing solutions of certain concentrations within the linearity range (6.00 mg/mL for HPLC method to determine DHA, 10.00 mg/mL for HPLC method to assay glycerol and 0.10 mg/mL for visible spectrophotometry to quantitate DHA). RSD was obtained from six determinations of the same concentration on the same day.

Accuracy

The accuracy was determined by recovery test. A known amount of DHA or glycerol was added to the blank fermentation media (1% yeast extract, 1% tryptone and distilled water, w/v) and analyzed by the proposed methods.

As for the HPLC method for DHA, aliquots of 0.2, 0.4, and 0.9 mL DHA stock solution (I) were transferred into 10-mL volumetric flasks, respectively, and diluted with a mixture constituted of 90% absolute acetonitrile and 10% blank fermentation media in order to make the solvent in correspondence with the mobile phase. Concentrations of 2.40, 4.80, and 10.80 mg/mL were obtained.

In the HPLC method for glycerol, aliquots of 1.5, 6.0, and 9.0 mL glycerol stock solution (II) were transferred into 10-mL volumetric flasks, respectively and diluted with blank fermentation media, giving final concentrations of 3.00, 12.00, and 18.00 mg/mL.

As for visible spectrophotometry to assay DHA, aliquots of 0.2-, 0.4-, and 0.9-mL of stock solution (III) were transferred into 10-mL volumetric flasks respectively and diluted with blank fermentation media to form final concentrations of 0.06, 0.12, and 0.27 mg/mL.

Specificity

Apart from residual glycerol and DHA other contents in the fermentation broth mainly included water, yeast extract, and tryptone that were not totally utilized by the bacteria, and also some metabolites secreted from the cells. In order to evaluate interferences or overlaps of system responses from these factors, fermentation medium without the analyte was analyzed.

LOD and LOQ

For HPLC methods, LOD was defined by the lowest detectable concentration yielding a signal-to-noise ratio of three and LOQ was defined by the lowest detectable concentration yielding a signal-to-noise ratio of ten according to Chinese Pharmacopoeia.

The LOD of visible spectrophotometry was defined as the lowest concentration of DHA that reacted with diphenylamine to form blue solution, which could be detected at 615 nm. For the

concentration that could be accepted as the LOQ, the intra-day accuracy and precession were to be less than 20%. All samples were assayed in triplicate (Table I and II).

Result and Discussion

HPLC method for DHA

Dihydroxyacetone could be well separated from the fermentation broth when 90% acetonitrile was used as mobile phase and packing material with $–NH_2$ as bonded phase (12). The ketone group in DHA has two main UV absorption bands, one is at 195 nm with molar absorbency index (ε) around 1000, another is at 270–285 nm with a relatively small ε around 200. In order to eliminate the interference of the absorption caused by mobile phase (acetonitrile has no UV absorption at wavelengths greater than 210 nm), DHA was detected at 271 nm where it had the maximum absorption in the range of 270–285 nm. The chromatographic condition proposed allowed an efficient and simple analysis. The retention time of DHA was around 5.4 min, which allowed a rapid determination.

The values obtained for method validation are presented in Table I. Good linearity was observed in the 2.00–12.00 mg/mL range with correlation coefficient of 0.9994. The precision was determined by intra-day repeatability and was expressed as relative standard deviation of 6 measurements of the same concentration in the same day. The accuracy was determined by recovery test, presented as the percentage of the found value against the true value (Table II). The specificity was evaluated by comparison between chromatograms of standards in water (Figure 1A) and in fermentation broth without DHA (DHA blank fermentation media), no interfering peaks were observed (Figure 1B).

Table I. Results of Validation Data for the Proposed Methods

Statistical parameters	HPLC for DHA	HPLC for glycerol	VIS for DHA
Calibration range (mg/mL)	2.00–12.00	0.50–20.00	0.04–0.30
Regression equation	$y = 144.61x - 13.487$	$y = 166859x + 30968$	$y = 2.1868x + 0.0197$
Correlation coefficient	$r = 0.9994$	$r = 0.9998$	$r = 0.9997$
Precision (RSD)	0.93%	1.27%	0.92%
LOD (mg/mL)	0.06	0.22	0.0017
LOQ (mg/mL)	1.20	0.50	0.019

Table II. Results Obtained in the Recovery Test of Standard DHA and Glycerol Solutions Added to Blank Fermentation Media

	Sample	Added (mg)	Found	Recovery (%)*
HPLC for glycerol	A	30.0	29.8	99.46 ± 0.21
	B	120.0	119.7	99.74 ± 0.25
	C	180.0	179.3	99.60 ± 0.33
HPLC for DHA	D	24.0	23.8	99.06 ± 0.44
	E	48.0	48.1	100.32 ± 0.24
	F	108.0	108.3	100.27 ± 0.45
Visible Spectrometry for DHA	G	0.60	0.62	103.7 ± 0.19
	H	1.20	1.27	105.8 ± 0.22
	I	2.70	2.92	108.3 ± 0.34

* Mean of three determinations.

HPLC method for glycerol

The determination of glycerol in the fermentation broth was significant since excessive amounts of residual glycerol would interfere with the isolation process of DHA. Consequently, the concentration of residual glycerol in the fermentation broth was an important parameter to determine the completion of the fermentation.

In the proposed HPLC method the retention time of DHA and glycerol were around 5.4 and 9.6 min, respectively, which allowed a good separation of the two peaks. Refractometry was suitable to detect the substance without UV absorption but it also had drawbacks. The baseline was apt to deviate because the detector was very sensitive to temperature, pressure, and any changes in the composition of the mobile phase. These external interfering factors must be conquered before spiking. In order to keep the baseline stable it was necessary to take measurements: the mobile phase must be completely mixed and degassed by ultrasound; the detector should be thermostatted; and the reference cell was purged for approximately 3 h.

The data of method validation is in Table I. The calibration range expands from 0.50 to 20.00 mg/mL wherein the residual glycerol concentration was included. According to Figure 2B, no interfering peak from the matrix was observed, indicating the high specificity of the proposed method.

Visible spectropfotometry for DHA

The visible spectrophotometric method to determine DHA allowed a rapid and low-cost analysis. The acid solution of diphenylamine formed blue color with DHA with maximum absorption at 615 nm. Good linearity was obtained in the range of 0.04–0.30 mg/mL. The accuracy was determined by recovery test, presented as the percentage of the found value against the true value (Table II). Although the recovery of this method in Table II was acceptable, it still had a relatively larger deviation from the true values than HPLC method.

The data of method validation is in Table I. This method was highly specific; no substance in the fermentation broth other than DHA forms a blue solution with diphenylamine.

Determination of DHA and glycerol in the fermentation process

Figure 3 showed the increase of DHA and decrease of glycerol in a typical DHA fermentation process. DHA was determined by HPLC and visible spectrophotometric method, respectively; glycerol was determined by HPLC method. The initial concentration of glycerol was 80.00 mg/mL. At the bioconversion time of 80 h, DHA level reached 72.10 mg/mL while glycerol level decreased to 1.47 mg/mL. The dramatic increase of DHA and decrease of glycerol between 26th h to 76th h

indicated logarithmic phase and the beginning of stationary phase of the bacteria growth. At the early hours of the fermentation when DHA was at low concentrations, there was little deviation between the results obtained from the two methods for determination of DHA. However with the accumulation of DHA, this deviation became more and more significant. The determined concentrations of DHA by visible spectrohpotometric method were usually higher than that of HPLC method, especially at the later stage of the fermentation when DHA had accumulated to a high level. One molecule of glycerol was converted to one molecule dihydroxyacetone by glycerol dehydrogenase and the data suggested that 90% of glycerol was converted into DHA, 8% was consumed by the bacteria and nearly 2% left.

Comparison between HPLC method and visible spectrophotometric method for the determination of DHA

HPLC method and spectrometric method were applied to determine DHA solutions prepared in broth matrix with known concentrations ranging from 2.00 to 70.00 mg/mL. Statistical analysis was used to determine whether there was a significant difference between the two proposed methods. The F-test was applied to determine whether one population was more variable than another in RSD (repeatability). The *t*-test was applied to

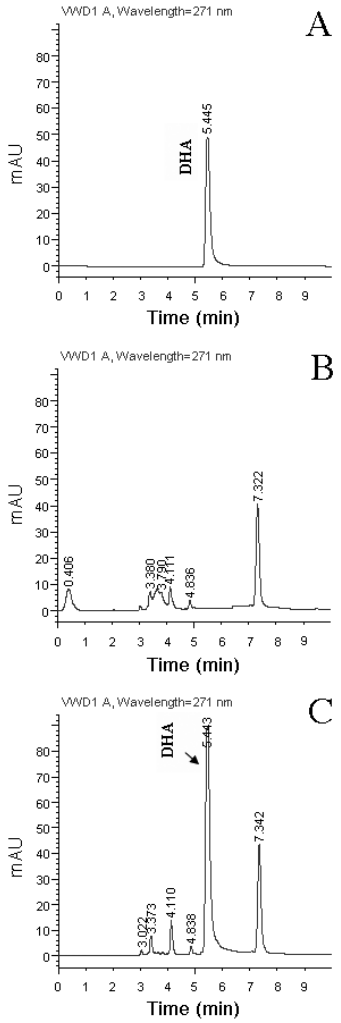

Figure 1. Chromatograph of DHA standard solution 4.00 mg/mL (A), blank fermentation media with glycerol of 5.00 mg/mL (B), and sample solution (C). Conditions: mobile phase acetonitrile–water (90:10, v/v); flow rate: 1.0 mg/mL; injection volume 20 µL; Column: Lichrospher-5NH_2; UV detector at 271 nm at room temperature; sample preparation: centrifuged to obtain the supernatant and diluted 10 times with absolute acetonitrile.

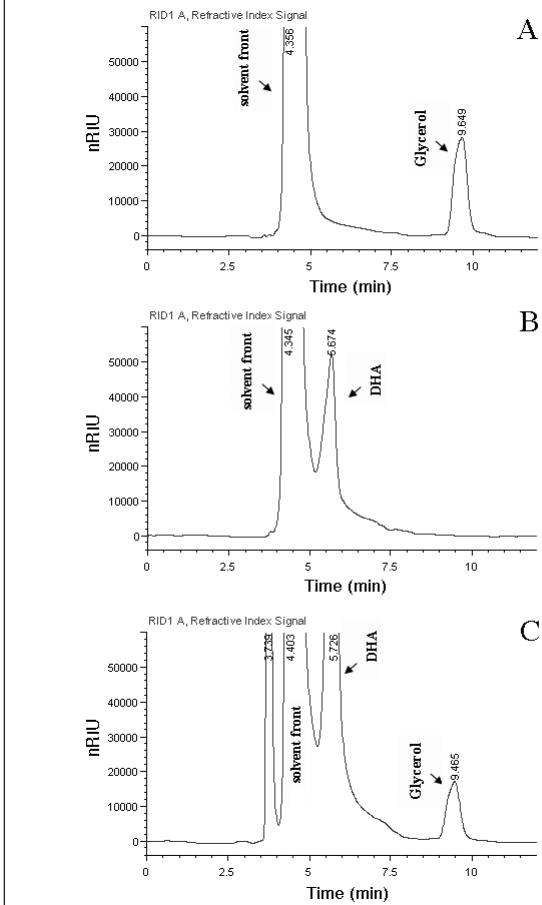

Figure 2. Chromatograph of glycerol standard solution 5 mg/mL (A), blank fermentation media with DHA of 25.00 mg/mL (B), sample solution (C). Conditions: mobile phase, acetonitrile–water (90:10, v/v); flow rate, 1.0 mg/mL; injection volume, 25 µL; Column, Lichrospher-5NH_2; detected with a Aligent refractometer at 35°C; Sample preparation: centrifuged and no dilution was needed.

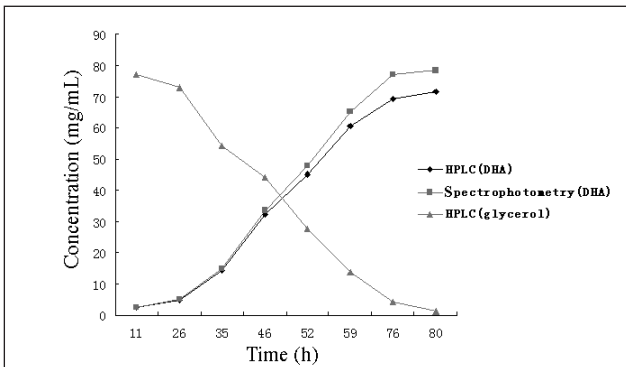

Figure 3. Typical diagram of DHA and glycerol concentration changes in the fermentation time-course.

Table III. Results of Comparison Between HPLC Method and Visible Spectrophotomeric Method*

Sample (mg/mL)	HPLC		Visible Spectrometry			
	$\bar{x} \pm s$	RSD	$\bar{x} \pm s$	RSD	F-test†	T-test‡
2.00	2.02 ± 0.020	0.99%	1.98 ± 0.015	0.75%	1.78	2.771
5.00	4.97 ± 0.033	0.66%	5.02 ± 0.018	0.36%	3.36	2.304
10.00	10.02 ± 0.073	0.73%	10.22 ± 0.096	0.94%	1.73	2.872
15.00	15.08 ± 0.13	0.86%	15.46 ± 0.22	1.43%	2.86	2.576
45.00	45.66 ± 0.36	0.79%	47.52 ± 0.72	1.52%	4.00	4.002
60.00	60.98 ± 0.52	0.85%	65.33 ± 1.09	1.67%	4.39	6.239
70.00	71.80 ± 0.73	1.02%	77.47 ± 1.25	1.61%	2.93	6.784

* Each determination was executed in triplicate.
† Value at 95% confidence level 19.00.
‡ Value at 95% confidence level 2.920.

determine whether there was a statistically significant difference between the means of the two proposed methods. The results are shown in Table III.

The results of F-test indicated that at 95% confidence level there was no significant difference between repeatability of the two proposed methods since no value was above 19.00. t-test suggested that at 95% confidence level there was no significant difference between the means of the two proposed methods for the determination of low DHA concentrations. However at high DHA concentrations, there was significant difference between the means of the two proposed methods. Since the values of HPLC method was closer to the true values, it was reasonable to say that HPLC method was more reliable to determine high DHA concentrations.

The existence of this significant difference at high DHA concentrations could be explained as follows: The linearity range of spectrophotometric method was very narrow (0.04–0.30 mg/mL), samples of high DHA concentrations had to be diluted several hundred times to appropriate concentration within the calibration range before determination. In the recovery test of spectrophotometric method (Table II) the deviation between the true value and the found value was larger than that of HPLC method. In the case of high DHA concentrations, this absolute error was magnified along with the dilution process. The more the dilution times, the larger the absolute error. The linearity range of HPLC method was much broader, ranging from 2.00 mg/mL to 12.00 mg/mL. Even at the end of fermentation when DHA accumulated to 70.00–100.00 mg/mL, ten times dilution was enough, which prevented the absolute error from being greatly magnified.

Both HPLC and spectrophotometric methods can be used to determine DHA at early stage of the fermentation when only small amount of glycerol was converted into DHA. While at later stage when large amount of DHA had accumulated HPLC was more reliable for determination.

Conclusions

The proposed methods for determination of DHA and glycerol can be applied to determine these analytes in the fermentation broth. Also, the methods are highly specific since no interference was observed from the fermentation matrix. The LOD of HPLC methods to determine glycerol is above the residual glycerol concentration, which can serve as a parameter to determine the completion of fermentation. The advantages of HPLC method to determine DHA over existing methods are accurate, specific and time-saving. It is reliable for routine quality control of DHA, especially for determination of high DHA concentrations. The spectrophotometric method for determination of DHA allows rapid and low-cost analysis, which can be used as a substitute of HPLC to determine DHA at the early stage of fermentation since there is no significant difference between these two methods for the determination of low DHA concentrations.

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