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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Quantitative determination of AI-2 quorum-sensing signal of bacteria using high performance liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 14 February 2016
Revised 21 April 2016
Accepted 25 April 2016
Available online 27 May 2016

Keywords:
Autoinducer-2
Quorum sensing
Signal transduction
HPLC–MS/MS
Quantitative analysis
Escherichia coli wild type

ABSTRACT

Autoinducer 2 (AI-2), an important bioactive by-product of the *LuxS*-catalyzed S-ribosylhomocysteine cleavage reaction in the activated-methyl-cycle, has been suggested to serve as a universal intra- and inter-species signaling molecule. The development of reliable and sensitive methods for quantitative determination of AI-2 is highly desired. However, the chemical properties of AI-2 cause difficulty in its quantitative analysis. Herein, we report a high performance liquid chromatography-tandem mass spectrometric method that enables reproducible and sensitive measurement of AI-2 concentrations in complex matrixes. 4,5-Dimethylbenzene-1,2-diamine (DMBDM), an easy-to-obtain commercial reagent, was used for the derivatization treatment. The assay was linear in the concentration range of 1.0–1000 ng/mL (R^2 = 0.999) and had a lower limit of quantification of 0.58 ng/mL. The method exhibited several advantages, *e.g.*, high selectivity, wide linear response range, and good sensitivity. Furthermore, the effectiveness of the method was further validated through measuring AI-2 concentrations in the cell-free culture supernatant from *Escherichia coli* wild type.

Introduction

Quorum sensing is a term used to describe cell-to-cell communication that allows cell-density-dependent gene expression (Chen et al., 2002; Schaefer et al., 2008). These bacterial activities are not useful when expressed by just one bacterium, but they can become beneficial to the bacteria when expressed in a group-based manner. These signaling systems are recognized to be crucial for bacteria to enact group beneficial behaviors only when sufficient members of the population are present to successfully carry out the desired task (Diggle et al., 2007; Yeon et al., 2009; Song et al., 2014). With proper signaling systems, bacterial communities can behave as pseudomulticellular organisms, such as by biofilm formation, virulence, and production of antibiotics. In the past two decades, this phenomenon has emerged as a research hotspot due to its involvement in various biochemical processes. However, our understanding of intra- and inter-species signaling in bacteria is still in its infancy (Diggle et al., 2007).

Bacteria secrete small molecules called autoinducers for the purpose of cell-to-cell communication. When the autoinducer concentration reaches the threshold level in

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http://dx.doi.org/10.1016/j.jes.2016.04.018
1001-0742/

proportion to the cell density, it will combine with the corresponding receptor protein and activate the transcription of specific genes to induce group behaviors ((Diggle et al., 2007). Gram-positive bacteria use oligopeptides, while Gram-negative bacteria employ acylhomoserine lactones as autoinducers for species-specific signaling (Vendeville et al., 2005). Among these, autoinducer-2 (AI-2) is the only non-species specific, quorum-sensing molecule known that mediates intra- and inter-species communication among bacteria. This quorum sensing system, which operates in both Gram- and Gram-negative bacteria, uses derivatives of the metabolic product 4,5-dihydroxy-2,3-pentanedione (DPD) (Chen et al., 2002; Song et al., 2014). DPD undergoes further rearrangements to yield molecules generically termed the "AI-2" family, which are active in signaling. Biosynthesis of DPD requires the enzyme *LuxS*, which is present in over 60 species of bacteria (Schauder et al., 2001; Semmelhack et al., 2004). The widespread nature of *LuxS* and DPD production has led to the idea that AI-2 functions in inter-species communication. DPD is a highly bioactive molecule that rearranges and undergoes additional spontaneous reactions in solution. Distinct but related molecules are derived from DPD, and different bacterial species recognize various forms of DPD as AI-2 signals (Bassler et al., 2007). The complex chemical properties of DPD and its derivatives cause difficulty in the quantitative analysis of AI-2.

The AI-2-related quorum sensing system has been widely recognized to play important roles in many biochemical processes and has been of great interest in the fields of energy recovery, waste treatment, and environmental remediation (Rickard et al., 2006). Many analytical methods have been developed for the detection of AI-2 in bacterial samples. In general, four classes of methods for the detection of AI-2 have been utilized, *i.e.*, the *Vibrio harveyi* luminescence bioassay (Surette et al., 1999; Bodor et al., 2008; Learman et al., 2009), biosensors derived from AI-2 receptor proteins (Zhu and Pei, 2008), gas chromatography–mass spectrometry (Thiel et al., 2009) and high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) (Campagna et al., 2009) analysis of a DPD derivative. The *V. harveyi* luminescence bioassay is sensitive, and is the most commonly used method for the analysis of AI-2. However, the reproducibility of this method is affected by the complex culture medium required, which hinders the quantitative analysis of AI-2 (Vilchez et al., 2007). The biosensors based on AI-2 receptor proteins are sensitive only to the fraction of DPD that has converted to the form of borate esters. Gas chromatography–mass spectrometry analysis requires a series of complex sample pretreatments, including two-step derivatization, extraction, and a sample concentration step (Thiel et al., 2009). HPLC–MS/MS has been proposed as the most feasible solution in the quantitative analysis of AI-2 (Campagna et al., 2009). However, a wide variety of complicated derivatization reagents are needed and most of them are not easy to obtain, which significantly limits the wide application of this method for the analysis of AI-2. Therefore, a sensitive, selective and simple method for detecting and quantifying AI-2 in various biochemical processes is highly desired.

In this study, a simple, rapid, and sensitive HPLC-MS/MS method was developed for the quantitative detection of AI-2. After cells and other possible particulates had been removed by centrifugation, all samples needed only a simple one-step derivatization procedure. In this method, a simple, easy-to-obtain commercial reagent, 4,5-dimethylbenzene-1,2-diamine (DMBDM), was used for the derivatization of DPD. Furthermore, this method has been validated through measuring AI-2 concentrations in the cell-free culture supernatant from *Escherichia coli* wild type.

1. Materials and methods

1.1. Materials and reagents

DPD solution (0.3 mg/mL, dissolved in ultrapure water) was purchased from Omm Scientific Inc. (USA). DMBDM and HPLC-grade formic acid were purchased from Sigma-Aldrich Inc. (USA). HPLC-grade acetonitrile was purchased from Merck Inc. (Germany). Ultrapure (Milli-Q) water (resistivity of 18.2 MΩ/cm) was used in the experiments. Other chemicals or solvents used were of analytical grade.

1.2. Sample preparation

The working standard solutions with a range of 1.0–1000 ng/mL were obtained by diluting the stock solution of DPD (0.3 mg/mL). The DMBDM solution was prepared by dissolving 20 mg DMBDM into 100 mL HCl solution (0.1 mol/L). 500 μL of the standard solution or supernatants after pretreatment was transferred to 2-mL autosampler vials (Agilent Inc., USA) containing an equal volume of DMBDM solution. The two solutions were thoroughly mixed for 1.0 min. Then, these samples were incubated in a temperature-controlled shaker at different temperatures (25, 30, 40, 50 and 60°C). After the samples cooled down, they were analyzed by HPLC–MS/MS directly.

To further evaluate the effectiveness of this method for real biological sample analysis, the concentration of DPD was measured in cell-free supernatants for *E. coli* strain K-12 substrain MG1655 (wide type, ACCC11202, bought from the Microbial Preservation Centre in China). This strain was grown at 37°C in Luria–Bertani (LB) medium supplemented with 0.25% glucose. During the incubation period, aliquots were withdrawn at regular intervals of 2 hr in the initial 12 hr and collected every 12 hr in the subsequent 48 hr, and OD_{600} was recorded to determine the cell density. Then, the culture was centrifuged at −10°C for 10 min (8000 × *g*) and filtered through 0.22 μm membranes to remove the cells. The cell-free supernatants were quickly frozen and stored at −80°C until use or underwent derivatization immediately.

1.3. HPLC-MS/MS determination

A TSQ Quantum Ultra AM triple quadrupole mass spectrometer (Thermo Finnigan, USA), coupled with an electrospray ionization (ESI) source, a Surveyor LC pump and Surveyor autosampler (Thermo Finnigan, USA), was used for HPLC–MS/MS analysis. Data acquisition was performed with Xcalibur 1.4 software (Thermo Finnigan, USA). Chromatographic analysis was performed using a Phenomenex Luna C_{18} column

(150 × 2.0 mm i.d., 5 μm) operating at 20°C. A mixture of HPLC-grade water (0.1% formic acid) and HPLC-grade acetonitrile (65:35, V/V) was used as the isocratic mobile phase at a flow rate of 0.2 mL/min. The signal intensities obtained in positive ion mode were much higher than those in negative ion mode. A source collision-induced dissociation collision energy of 20 eV further improved the sensitivity. The temperature of the heated capillary was set at 270°C, and the electrospray voltage was set at 5.0 kV. Argon was used as collision gas at a pressure of 1.0 mTorr. Nitrogen was used as sheath gas and auxiliary gas, and the pressures were set at 30 and 6, respectively (arbitrary units). The scan width for selective reaction monitoring was set at *m*/z 0.20, and the scan time was set at 0.50 sec. The peak widths of Q_1 and Q_3 were both set at 0.70 full width at half maximum (FWHM).

The HPLC-MS/MS method was validated in terms of linearity, detection limit (LOD), limit of quantification (LOQ), accuracy and precision. For linearity validation, nine concentration levels (1.0–1000 ng/mL) were tested following the method stated above, and calibration curves were constructed by plotting peak area versus concentration. The LOD and LOQ were defined as the concentrations that produced a signal-to-noise ratio of 3 (s/n = 3) and a signal-to-noise ratio of 10 (s/n = 10), respectively. The repeatability of this method was evaluated by injecting standard samples and measuring the relative standard deviations (RSD) of peak areas. The precision and accuracy of intra- and inter-day data were estimated by analyzing ten replicates spiked by three different concentrations (1.0, 10, and 200 ng/mL) of DPD in different matrixes (LB medium, Basal medium (BM), and Pure water, *n* = 10) in a single day and six separate days, respectively.

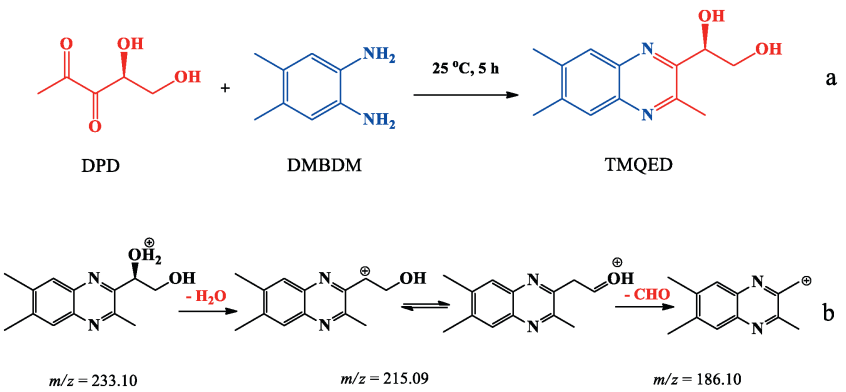
2. Results and discussion

The direct dosing of excess DMBDM to the supernatants of bacterial cultures leads to the formation of the corresponding 1-(3,6,7-trimethylquinoxalin-2-yl)ethane-1,2-diol (TMQED), as shown in Scheme 1a. It is a typical condensation reaction accompanied by the loss of two water molecules. The collision-induced dissociation spectrum of the $[M + H]^+$ precursor ion of TMQED (*m*/z = 233.10) produced two abundant product ions at *m*/z = 215.09 and *m*/z = 186.10 at the optimum collision energy of 21 eV, as shown in Fig. 1a. The product ion at *m*/z = 215.09 derived from the loss of one water molecule from the precursor ion, and the product ion at *m*/z = 186.10 was from a further loss of one molecule of carbon monoxide (Scheme 1b). The product ion at *m*/z = 186.10 was chosen for quantification.

Under optimized conditions, the calibration curves exhibited excellent linearity in the range of 1.0–1000 ng/mL. The calibration coefficient fell between 0.9995 and 0.9990. It was found that the yeast extract of LB medium contained DPD at a low level (3.30 ng/1000 ng yeast extract). Blank LB medium without yeast extract, BM, and pure water were used as matrixes. This method afforded LODs of 0.20, 0.08, and 0.05 ng/mL; and LOQs of 0.58, 0.20, and 0.10 ng/mL, respectively. The stock solutions were prepared by dissolving DPD into different blank matrixes. The highest concentration stock solution was prepared at 1000 ng/mL. DMBDM was dosed at a final concentration of 1000 ng/mL. Fig. 1b and c show the typical chromatograms of a blank LB medium spiked with DPD at 10 ng/mL and a real biological sample.

DPD solutions at 1.0, 10, and 200 ng/mL, which were dissolved in different matrixes (LB medium without yeast extract, BM, and pure water), were used as quality control samples. Accuracy and precision were also assessed by determining the quality control samples using ten replicate (*n* = 10) preparations at three concentration levels (1.0, 10, and 200 ng/mL). The precision of this method in different matrixes was assessed by the values of the relative standard deviation, while the accuracy by relative error, as summarized in Table 1. The RSD (%) for the intra- and inter-assay was less than 9.62% and 11.88%, and the relative error for intra- and inter-assay was 5.20% and −6.00%, respectively.

Effects of temperature and reaction time on the AI-2 signal intensity were also investigated. DPD solution (200 ng/mL) was reacted with DMBDM solution at different temperatures (25, 30, 40, 50 and 60°C) over a 7 hr period. Then, the peak area



Scheme 1 – (a) Derivatization of 4,5-dihydroxy-2,3-pentanedione (DPD) with 4,5-dimethylbenzene-1,2-diamine (DMBDM) for electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis; TMQED: 1-(3,6,7-trimethylquinoxalin-2-yl)ethane-1,2-diol; and (b) ESI-MS/MS fragmentation schemes of the derivatization product (TMQED).

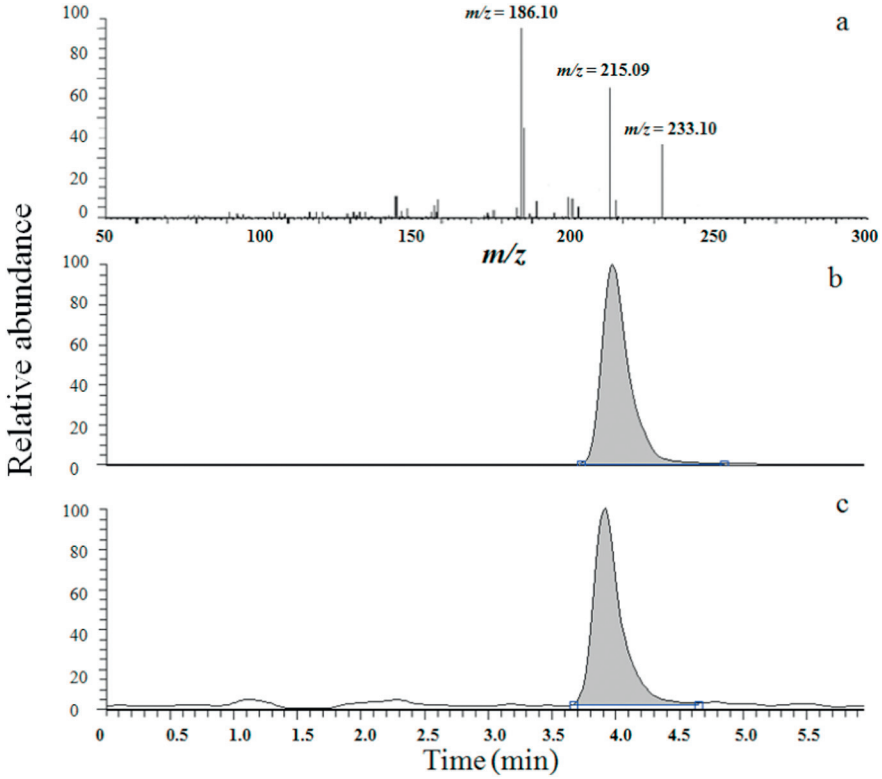


Fig. 1 – (a) ESI-MS/MS analysis of the derivatization product of 4,5-dihydroxy-2,3-pentanedione (DPD); (b) typical chromatogram of blank Luria-Bertani (LB) medium spiked with DPD at 10 ng/mL; and (c) typical chromatogram of a real biological sample. The product ion *m/z* = 186.10 was used for quantification.

determined by the HPLC–MS/MS method was plotted against time at different temperatures (Fig. 2). A high incubation temperature might lead to the decomposition of the derivative product (TMQED), while reaction at a low temperature required a very long time to complete the derivatization reaction. In order to avoid the decomposition of TMQED, the temperature and reaction time in this work were selected as 25°C and 5.0 hr, respectively.

To further evaluate the effectiveness of this AI-2 analysis method for real biological sample analysis, the concentration of DPD was measured in cell-free supernatants for *E. coli* wide-type. The strain was cultured in LB medium at 37°C with aeration. Samples were collected at regular intervals of 2 hr in the initial 12 hr and every 12 hr in the subsequent 48 hr. After OD_{600} analysis and centrifugation to remove cells, a final concentration of 3000 ng/mL DMBDM was dosed to each

Table 1 – Intra- and inter-day precision and accuracy data for the analysis of DPD in different matrixes (Luria–Bertani (LB) medium without yeast extract, basal medium (BM), and pure water, *n* = 10).

	Matrix	LB			BM			Pure water		
	Concentration (ng/mL)	1.0	10	200	1.0	10	200	1.0	10	200
Intra-day (*n* = 10)	Mean ± S.D. (ng/mL)	1.04 ± 0.10	10.52 ± 0.47	208.59 ± 5.50	0.97 ± 0.07	9.82 ± 0.63	195.61 ± 6.50	1.01 ± 0.05	10.44 ± 0.38	203.82 ± 3.23
	RSD (%)	9.62	4.47	2.64	7.22	6.42	3.32	4.95	3.64	1.58
	R.E. (%)	4.00	5.20	4.30	−3.00	−1.80	−2.20	1.00	4.40	1.91
Inter-day (*n* = 10)	Mean ± S.D. (ng/mL)	1.01 ± 0.12	10.35 ± 0.72	202.59 ± 8.42	0.94 ± 0.08	9.67 ± 0.81	193.22 ± 8.21	0.98 ± 0.09	10.12 ± 0.74	201.59 ± 8.50
	RSD (%)	11.88	6.96	4.16	8.51	8.38	4.25	9.18	7.31	4.22
	R.E. (%)	1.00	3.50	1.30	−6.00	−3.30	−3.39	−2.00	1.20	0.80
	Mean ± S.D. (ng/mL)	1.01 ± 0.12	10.35 ± 0.72	202.59 ± 8.42	0.94 ± 0.08	9.67 ± 0.81	193.22 ± 8.21	0.98 ± 0.09	10.12 ± 0.74	201.59 ± 8.50

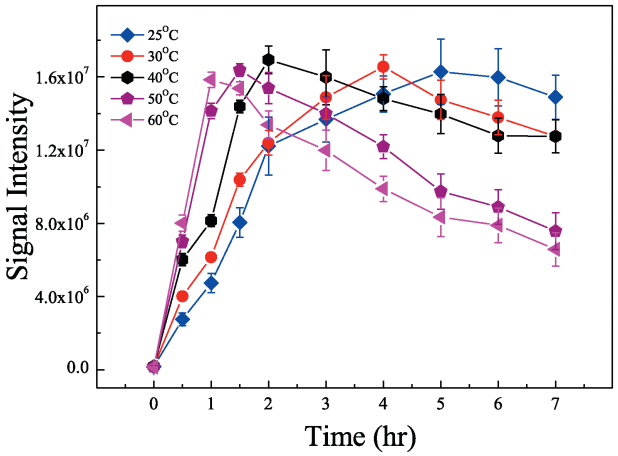


Fig. 2 – Effects of temperature and incubation time on the AI-2 signal intensity in the derivatization treatment.

sample. After 5-hr incubation, the samples were subjected to HPLC-MS/MS analysis. Fig. 3 shows the AI-2 concentration profiles during the cultivation period of *E. coli* wide-type. To determine the reproducibility of these results, three parallel experiments were conducted. The results show that the concentration of DPD peaked at 921.3 ± 40.0 ng/mL at 10 hr, but was then consumed.

3. Conclusions

In summary, the method described in this paper is a relatively simple, sensitive HPLC-MS/MS method for the quantitative analysis of AI-2 quorum sensing signal molecules. The method shows good sensitivity, precision and linearity, and can be widely used for analysis of biological and environmental samples. An advantage of this method is that DMBDM, an easy-to-obtain commercial reagent, was used for derivatization treatment. Thus, this method provides a good choice for AI-2 quorum sensing signal quantitative analysis.

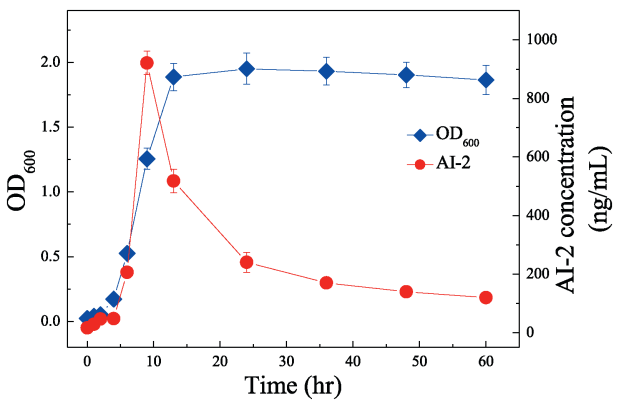


Fig. 3 – DPD concentration profiles during the cultivation period of *Escherichia coli* wild type.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 21261160489, 21207031 and 51538011) and the Program for Changjiang Scholars and Innovative Research Team in University, China.

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