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# Determination of autoinducer-2 in biological samples by high-performance liquid chromatography with fluorescence detection using pre-column derivatization

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ABSTRACT

Autoinducer-2 (AI-2), as a small-molecular-weight organic molecule secreted and perceived by various bacteria, enables intra- and inter-species communications. Quantitative determination of AI-2 is essential for exploring the bacterial AI-2-related physiological and biochemical processes. However, current strategies for sensitive detection of AI-2 require sophisticated instruments and complicated procedures. In this work, on the basis of the derivatization of AI-2 with 2,3-diaminonaphthalene, a simple, sensitive and cost-effective high-performance liquid chromatography with fluorescence detector (HPLC–FLD) method is developed for the quantitative detection of AI-2. Under the optimized conditions, this method had a broad linear range of 10–14,000 ng/ml ($R^2$ = 0.9999), and a low detection limit of 1.0 ng/ml. Furthermore, the effectiveness of this approach was further validated through measuring the AI-2 concentrations in the cell-free culture supernatants of both *Escherichia coli* and *Vibrio harveyi*.



## 1. Introduction

Bacteria secrete small-molecular-weight molecules, i.e., autoinducers, to communicate with each other, termed as quorum sensing (QS) [1]. QS has been found to regulate biofilm formation, virulence, production of antibiotics, formation of fruiting body, and gene transfer [2,3]. In the past two decades, QS has been extensively studied because of its important roles in the fields of health and environment. Among the autoinducers, autoinducer-2 (AI-2), as one unique autoinducer for enabling both intra- and inter-species communication, is found to be secreted by a variety of bacteria [4]. Many different bacterial species are reported to secrete 4,5-dihydroxy-2,3-pentanedione (DPD), the metabolic precursor of AI-2 [5]. DPD undergoes further rearrangements spontaneously and exists as a mixture of relevant compounds, some of which are recognized by various bacteria [6]. This mechanism enables bacteria to perceive AI-2 derived from DPD secreted by themselves or other bacterial species, and ensures interspecies communication [7].

Because of the crucial roles of AI-2 in QS-related research fields, sensitive and selective detection and quantification of AI-2 in biological samples are greatly desired. However, its various forms and low concentrations found in biological samples make its quantitative analysis difficult. Several methods have been developed to detect and quantify AI-2: e.g., *Vibrio harveyi* luminescence bioassay [8,9], biosensor based on fluorescence yield change when binding AI-2 to receptor proteins [10], high-performance liquid chromatography with UV detector (HPLC–UV), high-performance liquid chromatography with tandem mass spectrometric detector (HPLC–MS/MS) or gas chromatography–mass spectrometry (GC–MS) using 1,2-phenylenediamine or its derivatives to react with DPD to form the corresponding quinoxaline [11–13]. The *V. harveyi* bioassay method using the induction of luminescence of BB170 bioreporters is the most common way to detect AI-2 because of its sensitivity over several orders of magnitude. However, this bioassay is a qualitative method and not suitable for quantitative analysis [8,14]. Signal inhibition caused by high-concentration AI-2 is also observed [15] and some assay conditions such as pH, glucose levels and borate concentration interfere with this assay [8,16]. The biosensors based on AI-2 receptor proteins can response to only the fraction of DPD, which has been converted

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to borate, making this assay sensitive to the borate concentration [10]. Besides, its linear ranges is very narrow (~1–20 μM), and the procedures to purify and label the proteins are complicated, time-consuming and expensive. The derivatization method, which employs 1,2-phenylenediamine as the derivatization reagents and detects the corresponding quinoxaline by HPLC-UV and Nuclear Magnetic Resonance (NMR), fails to give any quantitative results [11,17]. The GC–MS method, which also initially undergoes 1,2-phenylenediamine derivatization and is followed by the treatment with silylation reagents, has a detection limit (LOD) of 0.7 ng/ml and can meet the AI-2 detection requirements in many cases [13]. However, very complicated procedures, such as two-step derivatization, solid phase extraction and concentration, make it difficult to perform. Recently, an HPLC–MS/MS method based on derivatization with derivatives of 1,2-phenylenediamine has been proposed to detect trace AI-2 in human saliva [12]. However, the derivatization reagents with complicated structures are required and usually need painstaking synthetic procedures, which significantly limit its wide application. In addition, expensive facilities, i.e., HPLC–MS/MS, are required for the determination.

In this study, we try to establish a simple, sensitive and cost-effective method for the quantitative analysis of AI-2. To this end, 2,3-diaminonaphthalene (DAN), a simple and easily available reagent, was used to react with DPD to form a new fluorescent substance, which has characteristic excitation and emission wavelengths. Thus, HPLC with fluorescence detector (HPLC–FLD) with an excellent selectivity and sensitivity could be developed to detect the AI-2 concentration in complex matrix like the supernatants of *Escherichia coli* and *V. harveyi*.

## 2. Experimental

### 2.1. Chemicals and preparation of standard solutions

DPD solution (0.3 mg/ml, dissolved in ultrapure water) was purchased from Omm Scientific Inc. (Texas, USA). 2,3-Diaminonaphthalene (DAN) was obtained from Aladdin Inc. (Shanghai, China). 4,5-Methylenedioxy-1,2-phenylenediamine (MDB), HPLC-grade formic acid and acetonitrile were purchased from Sigma-Aldrich Inc. (St. Louis, USA). 18.2 MΩ Milli-Q ultrapure water was supplied by Millipore Co. (Billerica, USA). Other chemicals or solvents used were of analytical grade. The working standard solutions with a range of 10–14,000 ng/ml were obtained through diluting the stock solution of DPD. The DAN solution was prepared by dissolving 10 mg DAN into 50 ml 0.1 M HCl.

### 2.2. Derivatization procedures

400 μl standard solution or supernatants after pretreatment were transferred to 2 ml autosampler vials (Agilent Inc., USA) containing an equal volume of DAN solution. The two liquids were thoroughly mixed for 2 min. Then, these samples were incubated in a water bath at 90 °C for 40 min. After the samples became cooled down, they were analyzed by HPLC–FLD directly.

### 2.3. Chromatographic instrumentation and conditions

20 μl samples was injected for analysis using 1260 HPLC system equipped with a fluorescence detector (Agilent Inc., USA). Separation was achieved on an Agilent ZORBAX SB-C18 reverse-phase column (250 mm × 4.6 mm, 5 μm) set at 30 °C. The mobile phase contained 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml/min. A gradient elution profile was used as follows: $t$ = 0 min, 70% solvent A, 30% solvent B; $t$ = 4 min, 70% solvent A, 30% solvent B; and $t$ = 12 min, 35% solvent A, 65% solvent B; $t$ = 20 min, 35% solvent A, 65% solvent B; $t$ = 24 min, 70% solvent A, 30% solvent B; $t$ = 27 min, 70% solvent A, 30% solvent B. The excitation and emission wavelengths of the fluorescence detector were set at 271 and 503 nm, respectively.

The mass spectral of the derivative products was also recorded by LTQ XL orbitrap high resolution mass spectrometry (Thermo Fisher Sci. Inc., USA) fitted with an electrospray source operated in a positive mode to further confirm its structure. The spray voltage was set to 4 kV, capillary temperature to 275 °C. The sheath and auxiliary gas flow rate (both nitrogen) were optimized at 20 and 5 arbitrary units (a.u.). Full MS scans were acquired in the orbitrap analyzer with the resolution set to a value of 60,000.

### 2.4. Method validation

This HPLC–FLD method was validated in terms of linearity, LOD, LOQ, accuracy and precision following the International Conference on Harmonization (ICH) guidelines [18]. For linearity validation, nine concentration levels (10–14,000 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 concentration that produced a signal-to-noise ratio of 5 ($S/N$ = 5) and a signal-to-noise ratio of 15 ($S/N$ = 15), respectively. The repeatability of this method was evaluated by injecting the standard sample ($n$ = 6, 300 ng/ml) and measuring the relative standard deviations (RSD) of peak area. Precision and accuracy of intra- and inter-day were estimated by analyzing six replicates spiked by three different concentrations (30, 500, and 4000 ng/ml) DPD to LB and Autoinducer Bioassay (AB) media in a single day and six separate days, respectively.

### 2.5. Biological sample preparation

*E. coli* strains MG1655 (wide-type) and DH5α (luxS-null) were grown at 37 °C in LB medium supplemented with 0.25% glucose [19]. *V. harveyi* BB120 (AI-2 positive strain) was cultured in AB medium at 30 °C. During the incubation period, aliquots were withdrawn at regular intervals and $OD_{600}$ was recorded to determine the cell density. Then, the culture was centrifuged at 13,000 rpm for 5 min 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.

### 2.6. AI-2 detection by V. harveyi BB170 bioassay

The bioassay follows the procedures from Surette et al. [19] with a slight modification. The BB170 reported strain was cultured for 13–16 h at 30 °C, and then diluted by 1:5000 into fresh AB medium. 180 μl of diluted reporter strain solution was added to the wells of 96-well plate, which contained 20 μl samples for AI-2 activity. The supernatants of BB120 were used for AI-2 detection and sterile AB medium was employed as the negative control. The 96-well plate was incubated in a rotary shaker at 150 rpm at 30 °C. The bioluminescence was measured using a microplate reader (BioTek, Synergy, USA) at given time intervals and the signal intensity of samples relative to that of the sterile AB medium was calculated as fold induction.

## 3. Results and discussion

### 3.1. Fluorescence spectra, MS confirmation and derivatization

The derivatization scheme is shown in Fig. 1. It was a typical condensation reaction accompanied by the loss of two water molecules. The derivative product, 1-(3-methylbezo[g]quinoxalin-2-yl)-ethane-1,2-diol, a heterocyclic compound with a rigid and planar structure, has its own characteristic