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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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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; 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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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



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