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High-performance liquid chromatographic determination of 2-aminoethylphosphonic acid and 2-amino-3-phosphonopropionic acid in seawater matrix using precolumn fluorescence derivatization with *o*-phthalaldehyde-ethanethiol

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ABSTRACT

2-Aminoethylphosphonic acid (2-AEP) and 2-amino-3-phosphonopropionic acid (2-AP3) are two types of abundant and ubiquitous naturally occurring phosphonates used as sources of phosphorus by many prokaryotic lineages. The potential utilization mechanism of 2-AEP and 2-AP3 in eukaryotic phytoplankton is currently under investigation. However, the lack of suitable analytical methods in saline samples are the limitation of such researches. Herein, a high-performance liquid chromatography (HPLC) method for monitoring 2-AEP and 2-AP3 using precolumn fluorescence derivatization with *o*-phthalaldehyde-ethanethiol (OPA-ET) in seawater matrix was developed. The derivatization procedure and HPLC conditions were carefully examined, which included optimization of the fluorescence excitation and emission wavelengths, the ammonium acetate concentration and pH of the mobile phase, the OPA-ET reagent content and composition and derivatization time. Because increasing salinity was observed to lower the derivatization efficiency, working standards were freshly prepared in artificial seawater with the same salinity as that of the samples for the quantification of 2-AEP and 2-AP3. The developed HPLC method showed a wide linear response with high linearity ($R^2 > 0.999$) and high repeatability at three concentration levels. The relative standard deviation was less than 4.1% for 2-AEP and less than 1.7% for 2-AP3 (n = 7). The limits of detection for 2-AEP and 2-AP3 in artificial seawater matrix were both 12.0 μg/L. The recoveries were 83.0–104% for 2-AEP and 72.6–98.6% for 2-AP3 in different aqueous samples, including algal culture medium prepared with filtered seawater. These results indicated the matrix effect of this method was insignificant.

1. Introduction

Phosphorus (P) is a critical nutrient for all living life, and P availability has been identified as a key determinant of marine productivity [1–4]. However, its most bioavailable form, dissolved inorganic phosphorus (DIP), is often depleted in the euphotic zone of the ocean [4]. Many microorganisms have developed the ability to utilize dissolved organic phosphorus (DOP) from various sources, mainly including phosphoesters (which contain C—O—P bond) and phosphonates (which contain C—P bond) [5–7]. Studies have been conducted to understand the utilization of phosphoesters in both eukaryotic and prokaryotic lineages [8,9]. Our current knowledge of the environmental fate of phosphonates, which consist of naturally occurring forms and synthetic commercial products, remains limited [10–12]. In marine systems, phosphonates account for approximately 25% of the high-molecular-weight DOP pool [6] and can be utilized by most bacteria and *Trichodesmium* as a P source [13–15]. However, only a small amount of data exists on eukaryotic phytoplankton potentially using phosphonates as a P source. Thus, this research area requires further biological study using representative phosphonates [16,17].

2-Aminoethylphosphonic acid (2-AEP) and 2-amino-3-phosphonopropionic acid (2-AP3) are two of the most widely distributed biogenic phosphonates in the ocean [18,19]. The existence of C—P compounds as biogenic molecules was not discovered until 1959, when 2-AEP was isolated from rumen protozoa [20]. The first report of 2-AP3 occurred in 1964 in the sea anemone *Zoanthus sociatus* and the protozoon *Tetrahymena pyriformis* [21].

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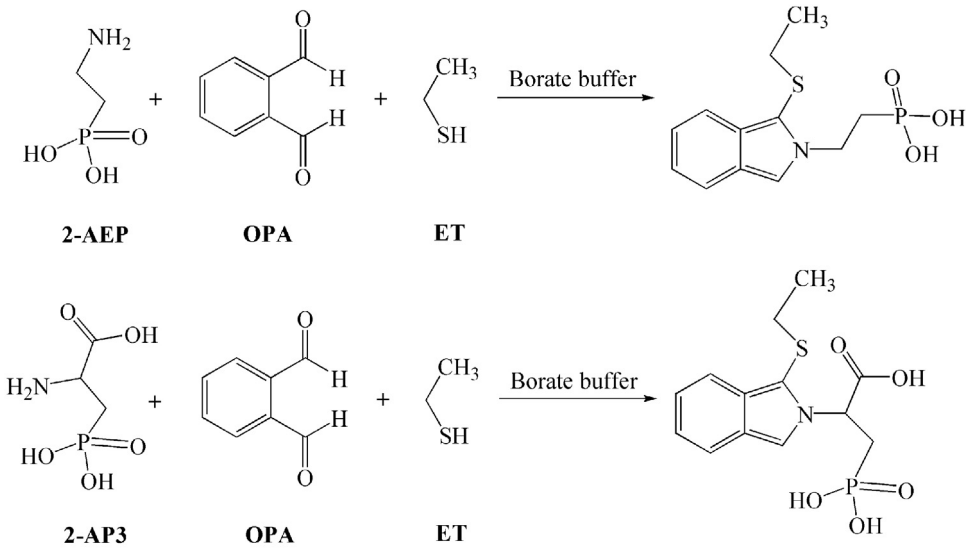

Fig. 1. Derivatization reaction equation of 2-AEP and 2-AP3 with OPA-ET.

Subsequent studies have shown 2-AEP and 2-AP3 are components of membrane phospholipids, protecting cells from enzymatic degradation and conferring additional rigidity due to their stable C—P bonds [6,19,22,23]. The adsorption behaviors of 2-AEP and three other phosphonates found in three typical Baltic Sea sediment samples were reported in a previous study [24]. As such, different culture studies in natural environments have verified that 2-AEP and 2-AP3 could serve as sole P sources for microbial growth [22,23,25,26]. Recently, Cui et al. [17] reported that although dinoflagellates possess an enzyme system capable of breaking C—P bonds, dinoflagellates are unable to utilize 2-AEP when exposed to antibiotics but could utilized P derived from bacterial degradation of 2-AEP.. However, in these physiological studies, the 2-AEP and 2-AP3 concentration data were not determined due to the lack of the analytical techniques.

The ability to determine the concentrations of 2-AEP and 2-AP3 in environmental and biological samples is essential. However, only limited information is available concerning the analytical methods needed. Gas-liquid chromatography combined with mass spectrometry (MS) [27] and a colorimetric method based on a reaction with ninhydrin [26] was previously used for 2-AEP quantification. In addition, Takeshi et al. [28] used 2-AP3 as an internal standard in a gas chromatography-MS simultaneous assay of organophosphorus compounds in human serum and urine. Recently, Skeff et al. [29] developed analytical methods for quantifying several phosphonates, including glyphosate, glufosinate, aminomethylphosphonic acid (AMPA) and 2-AEP, in salt water. These methods were based on studies examining the effects of salt matrices on liquid chromatography-tandem MS (LC—MS/MS) after derivatization of the target compounds with 9-fluorenylmethylchloroformate. The results indicated the presence of glyphosate and AMPA in the estuaries of the Baltic Sea [30]. However, the existing methods for 2-AEP and 2-AP3 quantification have several shortcomings, including their tedious operation, relatively high detection limit, serious interference and high cost. Comparatively, high-performance liquid chromatography (HPLC) methods employ a simple sample preparation procedure and exhibit satisfactory separation efficiency and accurate quantification. The lack of a chromophore or fluorophore in the 2-AEP and 2-AP3 molecules prevents their direct detection. Fortunately, 2-AEP and 2-AP3 are active aminoalkanephosphonic acids with primary amine groups, which can react with *o*-phthalaldehyde (OPA)-thiol (SH) to produce strongly fluorescent derivatives [31]. Therefore, the use of HPLC separation and fluorescence detection (FLD) after a derivatization step might be preferred to existing methods.

Reacting OPA with 2-mercaptoethanol to determine amino acid content by monitoring fluorescence was first proposed by Roth [32]. Subsequently, precolumn fluorescence derivatization with OPA-SH and reverse phase- (RP-) HPLC separation were used to determine the amino acid and amine content of seawater [33–35]. Comparison of the different derivatives obtained from OPA reagents containing various (SH)-additives was comprehensively reviewed by Hanczkó et al. [31] and Molnár-Perl [36]. The stability and molar response data confirm the most excellent performance of OPA–ET derivatization [31,36]. Thus, this method could be modified to determine the 2-AEP and 2-AP3 content as both compounds contain primary amino groups. Fig. 1 shows the derivatization reaction equation of 2-AEP and 2-AP3 with OPA-ET. To the best of our knowledge, no reports on the simple and accurate quantification of 2-AEP and 2-AP3 using OPA-ET derivatization and HPLC-FLD detection exist. The effects of different chromatographic and derivatization parameters as well as the use of high-salt-concentration seawater samples (e.g., salinity = 35) on the method were also explored.

The purpose of this research study was to develop a simple HPLC-FLD method based on a one-step OPA-ET derivatization for the determination of the 2-AEP and 2-AP3 content in seawater matrices. The analytical parameters of the chromatographic and derivatization process and salinity effect on the effectiveness of this method were carefully evaluated. This established method was successfully applied to determine the concentration of 2-AEP in seawater algal culture media and in different matrices during the degradation process. The simultaneous determination of the 2-AEP and 2-AP3 content in various spiked water samples was indicative of minimal matrix interference.

2. Experimental

2.1. Chemicals and materials

The 2-AEP (99.0%) and L-2-AP3 (≥98.0%) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). OPA (99.0%) was obtained from J&K Scientific Co., Ltd. (Beijing, China). ET (≥99.5%) was supplied by Macklin Biochemical Technology Co. Ltd. (Shanghai, China). HPLC-grade methanol (MeOH) was obtained

from TEDIA Co., USA. All other chemicals were purchased from Sinopharm Chemical Reagent Co., China, and were reagent grade or better. Ultra-pure water (≥18.2 MΩ cm) used throughout the study was purified using a Millipore water purification system (Millipore Co., MA, USA).

2.2. Solutions

Separate standard stock solutions of 2-AEP (4.00 g/L) and 2-AP3 (500 mg/L) were prepared by dissolving the accurately weighed powders in purified water. The stock solutions were stored in brown glass bottles at 4 °C. These solutions were stable for months. Working standard solutions with a concentration range of 0.100–20.0 mg/L were prepared daily by appropriate stepwise dilutions of the stock solution. Borate buffer stock solution (0.40 mol/L, pH = 10.5) was prepared by mixing 1.6 mol/L boric acid, 1.6 mol/L potassium chloride and 0.8 mol/L sodium hydroxide in a 1:1:2 vol ratio. OPA-ET was freshly prepared by mixing the following reagents together: 200 μL OPA stock solution (0.268 g OPA in 10 mL MeOH), 16.00 mL 0.40 mol/L borate buffer (pH = 10.5), 29 μL ET and 63.771 mL MeOH. A stock solution of ammonium acetate (0.50 mol/L) was prepared in water and stored at 4 °C when not in use. This stock solution was diluted to 5.0 mmol/L with water and adjusted to pH 9.0 with ammonium hydroxide when used as the mobile phase for HPLC. Artificial seawater (salinity = 35) was prepared by dissolving 31 g NaCl and 10 g $MgSO_4 \cdot 7H_2O$ in 1 L of water and was stored in polyethylene bottles when not in use. Different salinity levels were obtained by diluting the artificial seawater solution with pure water.

2.3. Samples

Tap water and lake water were collected at Xiamen University (Xiang'an campus), and reclaimed water was collected from the water outlet of the sprinkling irrigation on campus. River water was collected from Beixi of the Jiulong River, Fujian, China. Seawater (salinity = 35), which was used as the sample matrix, was collected using Niskin bottles during a cruise in the Western Pacific in April 2015 and frozen at −20 °C immediately after collection. Two additional seawater samples (salinity = 30) were collected from Wuyuan Bay and the vicinity of a fish farm in Xiamen City. Before analysis, the water samples were thoroughly mixed and filtered through 0.45-μm syringe-type polyethersulfone filters.

Seawater algal culture medium samples were used in the study of investigating the bioavailability of 2-AEP for different eukaryotic phytoplankton. L1 medium, made with seawater from Taiwan Strait (salinity = 28, filtered through 0.22 μm membranes and autoclaved) spiked with 2-AEP, was used to evaluate whether dinoflagellate was able to use 2-AEP as its sole P source upon antibiotic treatment [17]. These samples were collected on different culture days, filtered through 0.45 μm cellulose membranes in a sterile environment and stored in a refrigerator at 4 °C until analyzed.

2.4. Chromatographic conditions

A modular HPLC chromatography system (Shimadzu, Japan) equipped with a binary pump (LC-20AB), an auto-sampler (SIL-20 A) and an FLD (RF-20 A) was used. System control, data acquisition and processing were provided by the Shimadzu CLASS-VP software. The separation of OPA-ET derivatives was performed on a reversed-phase ZORBAX SB-C18 column (150 × 4.6 mm, 5 μm, Agilent, United States) using a gradient elution at a flow rate of 1.0 mL/min. The FLD excitation and emission wavelengths were set at 330 and 462 nm, respectively. The optimized mobile phase comprised water buffered with 5 mmol/L ammonium acetate at pH 9.0 (solvent A) and MeOH (solvent B). The composition of the mobile phase was changed linearly as follows: 0–3 min, 20% B; 3–6 min, 20%–70% B; 6–13 min, 70% B; 13–15 min, 70%-20% B; and 15–20 min, 20% B. The injection volume used for HPLC analysis was 20 μL.

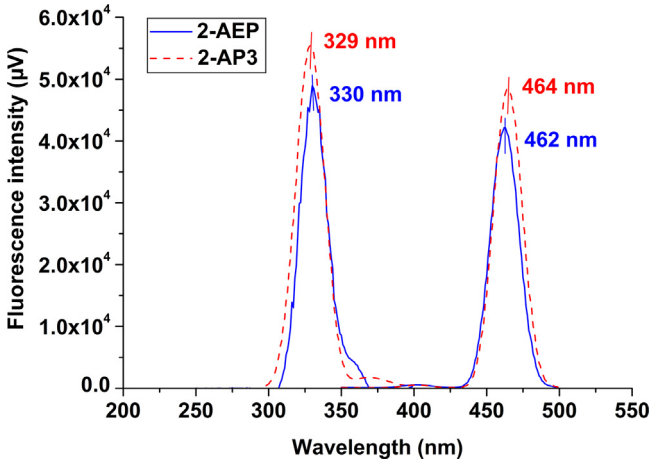

Fig. 2. Excitation and emission spectrum of 2-AP3 and 2-AEP derivatives.

2.5. Derivatization procedure

Standard solutions and samples were analyzed according to the following derivatization procedures. First, 900 μL of the water sample was transferred to a 1.5-mL brown sample vial. Second, 100 μL OPA-ET reagent was added to the vial. After mixing and allowing the samples to rest for 100 min, the samples were injected into the HPLC system with an auto-sampler. For real saline samples, salinities were measured with a handheld salimeter (Master-S/Millα, ATAGO, Japan) before derivatization. Working standards were freshly prepared in artificial seawater with the same salinity levels as the seawater samples and derivatized using the same reagents for each set of samples.

3. Results and discussion

3.1. Parameter optimization

The excitation and emission spectra of the two OPA-ET derivatization products were obtained using the "spectrum" module of the Shimadzu LC instrument. The results, shown in Fig. 2, indicated that the maxima of excitation and emission are located at approximately 330 nm and 462 nm, respectively, and could be used for the simultaneous determination of 2-AEP and 2-AP3.

To optimize the chromatographic and derivatization parameters in this experiment, the effects of the following parameters were investigated using a univariate experimental design: the concentration and pH of the ammonium acetate buffer in the mobile phase, the OPA-ET concentration, the borate buffer pH and MeOH content of the OPA-ET reagent, the volume ratio of the sample and derivatization reagent and the derivatization time. During parameter optimization, the tested parameter was the only variable; the other conditions were held constant. These conditions included the use of a mobile phase comprising water buffered with 5 mmol/L ammonium with a pH value of 9.0, a OPA-ET reagent concentration range of 0.40 mmol/L-4.0 mmol/L in 20% (v/v) borate buffer (pH 10.5) and 80% (v/v) MeOH, a 9:1 (v/v) volume ratio of sample to derivatization reagent and a derivatization time of 100 min. All optimizations were performed using an artificial seawater sample

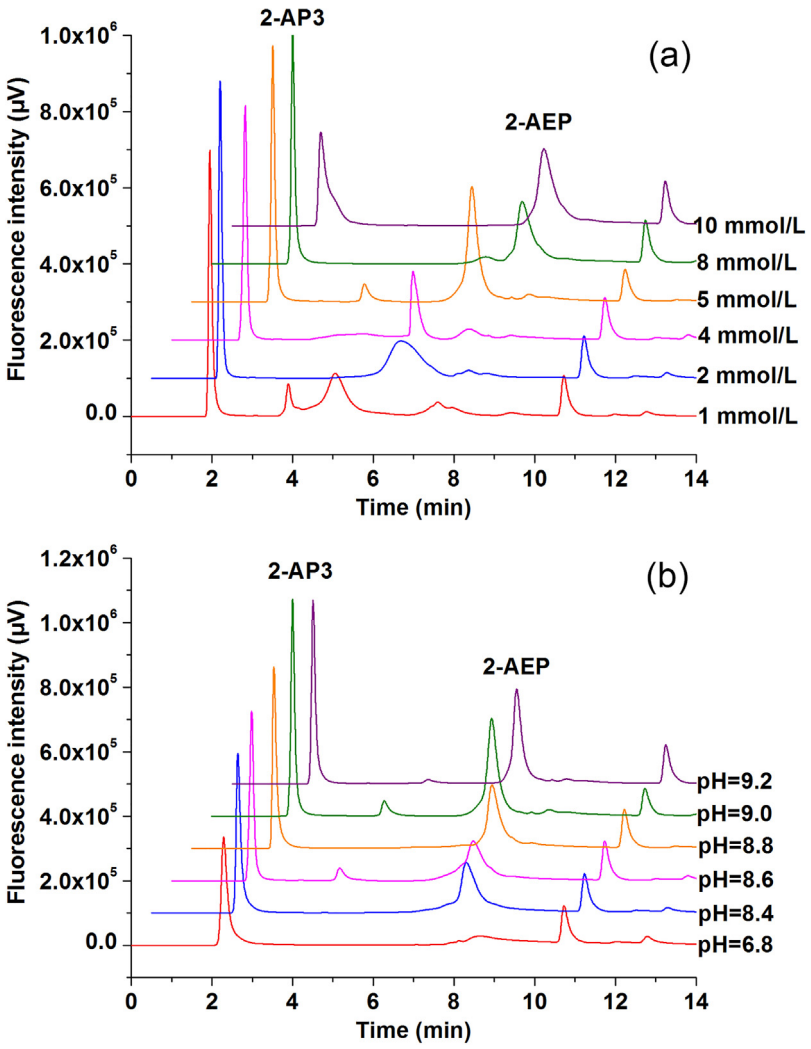

Fig. 3. Chromatograms of 2-AP3 and 2-AEP determination using different (a) concentrations and (b) pH of ammonium acetate buffer as mobile phase.

spiked with 2.00 mg/L 2-AEP and 2.00 mg/L 2-AP3 (salinity = 7) to account for matrix effects.

3.1.1. Effects of concentration and *pH* of the ammonium acetate buffer in the mobile phase

The concentration and pH of the ammonium acetate buffer in the mobile phase affect the separation efficiency, peak shape, signal intensity and retention time of the analytes. Phosphate and acetate buffers are the most commonly used buffers for the separation of OPA derivatives [33,37–39]. In our study, precipitation occurred when phosphate buffer was used. Thus, ammonium acetate was selected as the buffer for subsequent experiments.

Fig. 3(a) and Fig. 3(b) depict the effects of the concentration and pH, respectively, of the ammonium acetate buffer in the mobile phase when 2-AEP and 2-AP3 OPA-ET derivatives were analyzed. The optimal concentration and pH of the ammonium acetate buffer were selected by considering the fluorescence signal, peak shape and separation problems of the two analytes. The peaks of 2-AEP and 2-AP3 OPA-ET derivatives were easily identified when either 5–10 mmol/L ammonium acetate buffer with a pH value of 9.0 or 5 mmol/L ammonium acetate buffer with a pH value range of 8.8–9.2 was used. However, the fluorescence signal was highest when the 5 mmol/L ammonium acetate buffer with a pH value of 9.0 was used. Therefore, water buffered with 5 mmol/L ammonium acetate to a pH value of 9.0 was used as the mobile phase in this study.

3.1.2. Effects of the OPA-ET concentration, borate buffer *pH* and MeOH content in the OPA-ET reagent

The results of many reviews on OPA-ET derivatization reported that the optimal molar ratio of OPA/ET in the OPA-ET reagent is 1:10 [e.g., 31, 36]. The results of this study also complied with this ratio and investigated the effect of the OPA-ET concentration on the determination of the 2-AEP and 2-AP3 content. The effects of OPA concentration on the signal intensity range of 0.10–4.0 mmol/L are illustrated in Fig. 4(a), and the corresponding ET concentration is 1.0–40 mmol/L. Initially, the fluorescence intensity increased as the OPA concentration increased, but the peak shape of the 2-AEP derivative worsened when the OPA concentration was greater than 0.50 mmol/L. (The corresponding chromatograms are shown in Fig. S1 in Supplementary Materials). Therefore, 0.40 mmol/L and 4.0 mmol/L were selected as the optimal concentrations for OPA and ET, respectively.

In the majority of related studies, borate buffer was used in the OPA derivatization reaction over a wide pH range, 9.4–10.5 [31,33,37–39]. In this study, borate buffer solutions with different pH values were prepared (solution proportioning methods are shown in Table S1). Fig. 4(b) shows the borate buffer pH in the OPA-ET reagent varied from 9.3 to 12.6. The fluorescence intensities of

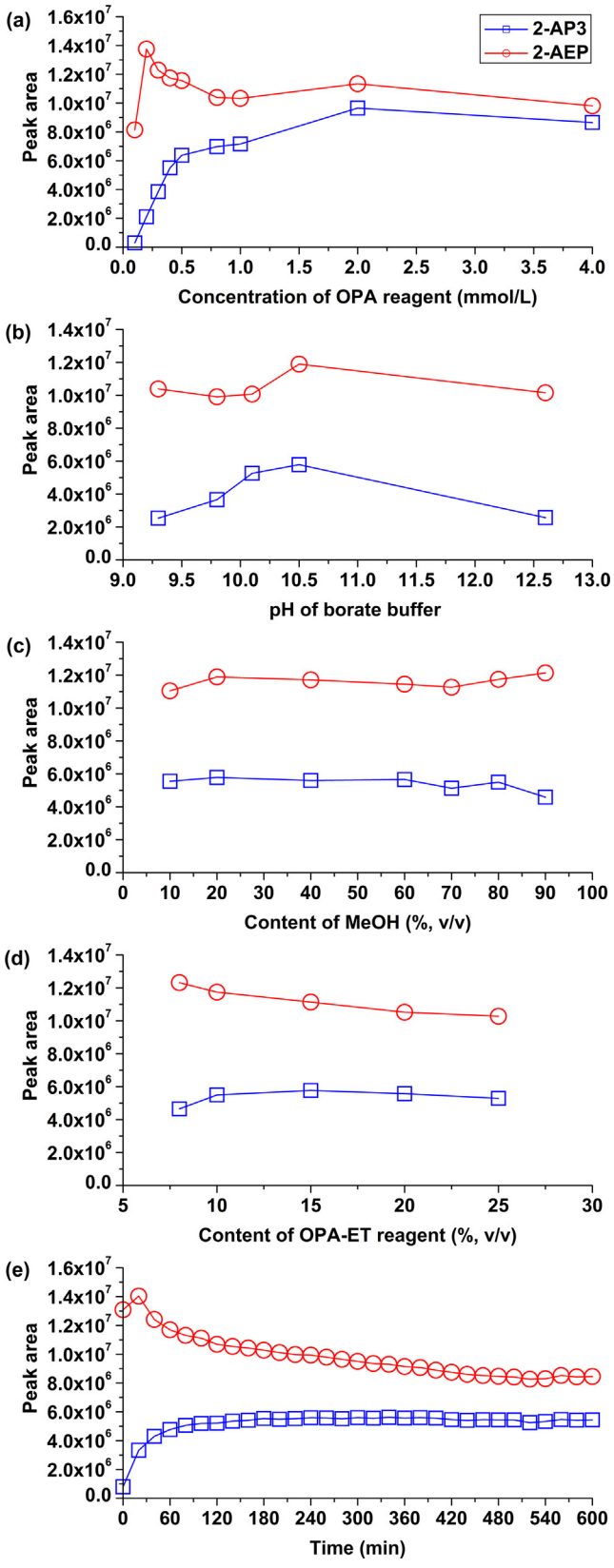

Fig. 4. Effect of (a) OPA-ET concentration, (b) borate buffer pH and (c) MeOH content in OPA-ET reagent, (d) volume ratio of sample and derivatization reagent, and (e) derivatization time.

the 2-AEP and 2-AP3 derivatives decreased when the pH value of the borate buffer was more than 10.5. Thus, borate buffer with a pH value of 10.5 was chosen for subsequent experiments.

The OPA-ET reagent contained OPA, ET, MeOH and borate buffer. Many studies reported the stability and responses of several OPA-ET amino acid and amine derivatives are optimal when ~80% (v/v) MeOH was used in the OPA-ET reagent [31,36]. The MeOH content of the OPA-ET reagent was tested at different levels, and the measured values for 2-AEP and 2-AP3 are shown in Fig. 4(c). However, the MeOH content was not found to be significant in determining the 2-AEP and 2-AP3 content. Thus, OPA-ET reagents with 80% (v/v) MeOH content were used for subsequent experiments.

3.1.3. *Effect of the volume ratio of sample to derivatization reagent*

It is necessary to add a sufficient volume of OPA-ET reagent to achieve maximum derivation efficiency, but excessive derivatization reagent dilutes the sample. To determine the appropriate volume ratio of sample to derivatization reagent, different OPA-ET reagent concentrations, ranging from 8% to 25% (v/v) in the reaction system, were evaluated. Fig. 4(d) depicts the effect of the volume ratio of the sample and OPA-ET reagent and shows that the responses of the 2-AEP and 2-AP3 derivatives decreased when the OPA-ET reagent content exceeded 10% (v/v). Therefore, a 9:1 (v/v) volume ratio of sample to OPA-ET reagent was selected.

3.1.4. *Effect of derivatization time*

The stabilities of different OPA-ET amino acid and amine derivatives were comprehensively reviewed by Hanczkó [31] and Molnár-Perl [36]. These studies showed OPA derivatization products could be classified into two main groups: (a) "=CH—NH_2" moiety-containing compounds provide single OPA-ET derivatives with long-term stability, and (b) "—CH_2—NH_2" moiety-containing isoindoles can react with an additional OPA molecule or SH-compound, resulting in more than one OPA derivative and a rapidly decreasing fluorescence response [31,33,36]. Simons and Johnson [40] explained that the instability of this adduct was due to a slow, spontaneous intermolecular rearrangement in which sulfur is displaced by oxygen from an alcohol group. It was also noted that the presence of an electron donating group, such as '—COOH', α to the isoindole structure has a stabilizing effect on OPA-ET derivatives [33].

To obtain a complete reaction and a high signal, the reaction kinetics of the 2-AEP and 2-AP3 derivatization with OPA-ET were analyzed for up to 10 h. Fig. 4(e) depicts that the fluorescence intensity of the 2-AEP derivative reached a maximum within 20 min and decreased thereafter. Furthermore, the 2-AP3 derivative completely formed after 100 min, and no significant variations were observed in the peak areas. The differences in the reaction kinetics of 2-AEP and 2-AP3 were dependent on the structure of the following two types of phosphonates: 2-AEP with a '—CH_2—NH_2' moiety and 2-AP3 with '=CH—NH_2' and '—COOH' moieties. Thus, 100 min was selected as the derivatization time in this study.

3.2. *Salinity effect and qualification method*

In previous studies, OPA-SH derivatization has been carefully optimized for the determination of amino acids in seawater [41]. However, no detailed data could be found on the effect of salinity on this typical derivatization. Because the potential application of this method is seawater culture medium analysis, the salinity effect on the identification and quantification of 2-AEP and 2-AP3 OPA-ET derivatives was investigated.

Typical HPLC chromatograms of the blank sample and seawater sample as well as 500 μg/L 2-AP3 and 2-AEP in pure water, seawater and artificial seawater are shown in Fig. 5. The chromatograms

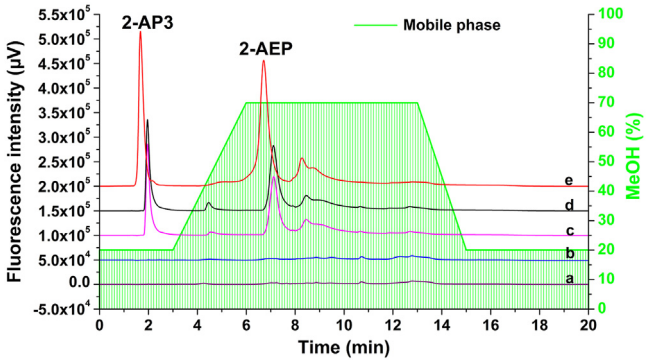

Fig. 5. Typical chromatograms of 2-AP3 and 2-AEP determination: (a) blank sample, (b) seawater sample, (c) 500 μg/L 2-AP3 and 2-AEP in seawater, (d) 500 μg/L 2-AP3 and 2-AEP in artificial seawater, (e) 500 μg/L 2-AP3 and 2-AEP in pure water.

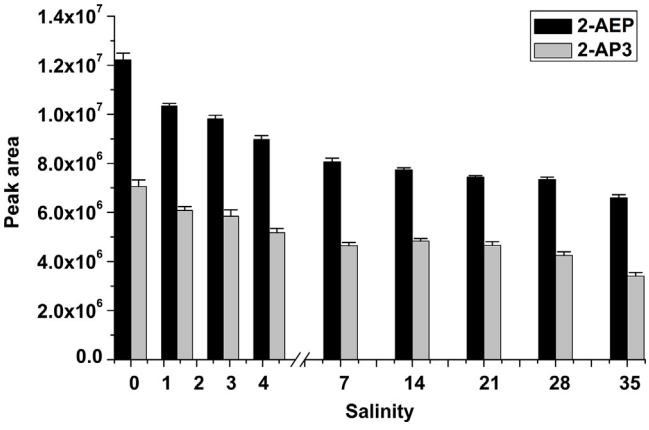

Fig. 6. Effect of salinity (0–35).

of the blank and seawater samples illustrate the blank value of the OPA-ET reagent is negligible. Amino acids and amines in seawater produce little interference when measuring 2-AP3 and 2-AEP. The additional chromatograms show that weaker signals for 2-AP3 and 2-AEP were obtained when seawater and artificial seawater were used as matrices. The retention times of 2-AEP and 2-AP3 derivatives increased slightly when the samples were in seawater matrix because of the salting-out effect. Moreover, no interference was observed when the signals of samples of the same concentration in natural seawater and artificial seawater at the same salinity level were compared.

To determine how salinity variation affects derivatization efficiency, a solution containing 2.00 mg/L 2-AEP and 2.00 mg/L 2-AP3 was derivatized and analyzed in artificial seawater with different salinity values. The relationship of the signal and salinity value is presented in Fig. 6. Increasing salinity was shown to dramatically weaken the derivatization efficiency of 2-AEP and 2-AP3 when the samples had a low salt concentration. The trend eased when the seawater matrix salinity value was greater than 7.

Matrix effects were investigated by comparing the calibration curves of 2-AEP and 2-AP3 prepared in pure water, seawater and artificial seawater. The calibration curves of 2-AEP and 2-AP3 in diluted seawater and artificial seawater (final salinity = 7) produced lower signals than those of pure water (Fig. S2), demonstrating pure water cannot be used for preparing calibration curves to quantify 2-AEP and 2-AP3 in seawater matrix. However, no significant difference was observed between the calibration curves obtained in artificial seawater and natural seawater (P = 0.95), indicating that there was no interference from other natural seawater properties, such as buffering ability. Therefore, working standards were freshly prepared in artificial seawater of the same salinity as the samples for the quantification of 2-AEP and 2-AP3.

3.3. Method validation

To apply the proposed method to different aqueous samples and seawater culture medium samples, the method used to determine the 2-AEP and 2-AP3 content by HPLC-FLD was validated for its performance parameters in both pure water and artificial seawater matrix at the optimized working conditions.

Linearity was established over several calibration ranges, and each standard curve consisted of five different concentrations. The limit of detection (LOD) and limit of quantification (LOQ) values of this method were estimated on the basis of signal/noise ratios (S/N) of 3 and 10, respectively. This method showed good linear relationships over different concentration ranges for 2-AEP and 2-AP3 with correlation coefficients (r^2) higher than 0.999 from tens of μg/L to 2000 μg/L. Thus, this method enables determination of the 2-AEP and 2-AP3 content at low levels. A series of validation data for 2-AEP and 2-AP3 in pure water and artificial seawater matrix (salinity = 7) is shown in Table 1.

The precision of this method was determined by calculating the relative standard deviation (RSD) for the replicated derivatization followed by HPLC-FLD analysis (n = 7). Three selected concentrations (40.0, 200 and 2000 μg/L) of 2-AEP and 2-AP3 in artificial seawater (salinity = 7), which covered the analytical range of the calibration curves, were analyzed by the same person using the same reagents. The RSD varied between 0.75% and 4.1% for 2-AEP and between 0.75% and 1.7% for 2-AP3 (Table S2), indicating our optimized method demonstrates good repeatability. The results are indicative of the stability of the derivatization reagent within one day, the repeatability of this derivatization reaction and the potentially wide applicability of this method.

3.4. Application and recovery

The HPLC-FLD method developed herein was applied to real seawater culture medium samples collected on different days in triplicate for each day. Then, the 2-AEP content in these seawater samples was determined before and after spiking them with 2-AEP

Table 1
Linear relationships, LOD (S/N = 3) and LOQ (S/N = 10) of 2-AEP and 2-AP3.

Matrix	Analyte	Linear range (μg/L)	Regression equation	Correlation coefficient	LOD (μg/L)	LOQ (μg/L)
Pure water	2-AEP	24–200	y = 6.53E + 03x + 1.02E + 04	0.9997	7.20	24.0
		200–2000	y = 7.13E + 03x – 2.723E + 05	0.9994		
	2-AP3	16–200	y = 4.43E + 03x + 3.34E+03	0.9996	4.80	16.0
		200–2000	y = 4.40E + 03x + 8.54E + 04	0.9997		
Artificial seawater	2-AEP	40–200	y = 4.41E + 03x + 1.06E + 04	0.9998	12.0	40.0
		200–2000	y = 4.49E + 03x – 1.29 E + 04	0.9998		
	2-AP3	40–200	y = 2.84E + 03x + 6.29E + 03	0.9998	12.0	40.0
		200–2000	y = 3.00E + 03x – 4.47 E + 04	0.9999		

standard solutions. There was no obvious change in the 2-AEP concentration during culture, RSD ≤ 2.8% (Fig. S3), and the recoveries were in the range of 79.3–99.3% for all samples. These two results indicated this method was not affected by the matrix or salinity effect during the determination of the 2-AEP content in seawater culture medium (Table S3). Additionally, these results showed good agreement with biological growth and other physiological parameters with antibiotic treatment in the algal culture experiment [17]. Furthermore, 2-AEP degradation experiments in different matrices (pure water, lake water and seawater) and different storage conditions (room temperature and 4 °C) were performed in the laboratory. The results showed 2-AEP degrades to the same molar mass of phosphate most easily at room temperature without salt (Fig. S4).

Biological experiments on 2-AP3 in seawater have not yet been performed; thus, the feasibility of the proposed method for the simultaneous determination of the 2-AEP and 2-AP3 content was validated by calculating the recoveries for the spiked samples. Recovery tests were conducted using several different water samples spiked with the standard solution of 2-AEP and 2-AP3 at three concentration levels. Spiked 2-AP3 was recovered at 72.6–98.6% and 2-AEP was recovered at 83.0–104% in different water samples, including seawater culture medium samples, indicating minimal matrix interference (Table S4).

4. Conclusions

To meet the necessity for new analytical methods in seawater prepared biological culture experiments, a simple HPLC-FLD method based on OPA-ET derivatization for the determination of the 2-AEP and 2-AP3 content in seawater matrix was developed herein. A minimal one-step derivatization procedure and HPLC-FLD were utilized in this method, comparatively reducing analyte loss, the cost of materials and the inconvenience of operating complicated instruments. Therefore, this new method can easily be used by researchers in other related fields. The applicability of this method was demonstrated by the accurate determination of the 2-AEP concentration in seawater algal culture medium and in different matrices during the degradation of 2-AEP. Satisfactory recoveries of 2-AEP and 2-AP3 were observed in spiked samples of different matrices, indicating this method can be a promising tool in marine research. Furthermore, this study functions as a reference for the simultaneous analysis of additional phosphonates in the future.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2018.08.016.

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