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Analysis of vitamin B_{12} in seawater and marine sediment porewater using ELISA

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Abstract

Vitamin B_{12} (B_{12}) is a set of closely related organocobalt compounds required by phytoplankton. A highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) was developed for the determination of B_{12} in seawater and marine sediment porewater. An antibody directed against B_{12} was coated on the surface of a 96-well microtiter plate, and horseradish peroxidase (HRP) was used as a labeling enzyme. In the indirect competitive immunoassay format, water samples or standards and a constant amount of HRP-labeled B_{12} were added into the microtiter plate wells, HRP-labeled and free B_{12} compete for binding to the plate-bound antibody. After immunoreaction, the immunochemically adsorbed HRP-B_{12} conjugate was determined by measuring the absorbance produced in a solution containing substrate tetramethylbenzidine (TMB) and hydrogen peroxide. The calibration graph for B_{12} was linear over the range of 0.4–100 ng/mL (0.3–74 nM; higher concentrations were not evaluated) with a detection limit of 0.2 ng/mL (3σ). Coupled with C-18 column extraction–preconcentration, the method is readily applicable to seawater levels (~1–10 pM). No interferences from humic acids, total dissolved organic matter (DOM), and salinity were observed. ELISA determined B_{12} in coastal seawater and surface tidal flat porewater (0–2 cm) ranged from 4.5–38 pM and 12–47 pM, respectively.

Vitamin B_{12} (B_{12}) refers to a set of closely related organometallic compounds, cobalamines, required by most phytoplankton species for marine primary production (Bertrand et al. 2007; Croft et al. 2006, 2005; Andersen 2005; Sañudo-Wilhelmy et al. 2006; Swift 1981; Guillard and Cassie 1963). B_{12} contains carbon-bonded cobalt and is a representative of multiple trace organometallic compounds essential for biological growth and functioning. Despite its importance in marine ecology, research on the biogeochemical cycling of B_{12} is at its initial stages, and its dynamics are just beginning to be understood (e.g., Panzeca et al. 2008; Bonnet et al. 2010). In particular, the distribution, transport, and factors controlling patterns of B_{12} in marine sediments, where heterotrophic microbial communities may be major sources, are essentially undocumented. A large part of this lack of knowledge is due to the trace concentration of B_{12} in seawater (picomolar levels) and the absence of suitable analytical methods.

The common vitamin B_{12} quantification methods include microbiological assays and high performance liquid chromatography (HPLC) with UV detection. Microbiological assays are based on the requirements of B_{12} as growth factors for certain organisms such as *Lactobacillus leichmannii* (Ross 1950; Carlucci and Silbernagel 1966; Kelleher et al. 1990). However, assay organism growth may not always be truly B_{12}-specific, and B_{12} availability or physiological responses to B_{12} may change during the long incubation times required (1 week) (Haines and Guillard 1974). Compared with microbiological assays, HPLC combines high performance separation and identification and provides a relatively rapid method for determination of B_{12} and its related compounds. Many HPLC systems have been developed for B_{12} determination (Frenkel et al. 1979; Binder et al. 1982; Lambert et al. 1992; Vinas et al. 1996; Moreno and Salvado 2000; Wongyai 2000), but they are not sufficiently sensitive to directly detect B_{12} at the low concentrations found in natural waters. More recently, C-18 solid phase extraction (SPE) techniques have been combined with HPLC–UV detection to concentrate and measure trace amounts of B_{12} in seawater (Okbamichael and Sañudo-Wilhelmy 2004) and have improved our understanding of vitamin B_{12} in some biogeochemical and ecological processes (Sañudo-Wilhelmy et al. 2006; Gobler et al. 2007; Taylor and Sullivan 2008; Panzeca et al. 2008, 2009). The detection of vitamin B_{12} can be greatly enhanced by combining SPE technique and using large sample volumes. High loading of the C-18 column, however, may also give rise to the coextraction of

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DOM and produce a high background in HPLC analysis because C-18 sorbent retains analytes primarily by reverse-phase adsorption rather than specific binding. Thus a significant increase in absolute sensitivity may not be obtained in DOM rich samples such as coastal seawater and sediment porewater. Therefore, development of new sensitive and specific methods for measurement of trace B_{12} in seawater and porewater remains desirable.

Immunoassay techniques provide an alternative approach for specifically analyzing organic compounds in complicated biological and environmental matrixes. These techniques take advantage of the molecular recognition of a targeted molecule (antigen) by its antibody based on an antigen-antibody reversible affinity interaction. Thus, both analytical specificity and a measure of biological reactivity can be obtained simultaneously. Up to now, a great number of immunoassay methods have been developed and widely used for biological, clinical, and environmental analyses (Knopp 2006; Hage 1999; Anderson et al. 1997). These methods are often characterized by rapid and easy operation, high selectivity and sensitivity, high reliability and low cost. Immunoassays of B_{12} in biological and food samples have been reported with a picomolar sensitivity level and without important interference from vitamin B_{12} analogues or nonspecific binders (O'Sullivan, et al. 1992). In this work, an Enzyme-Linked ImmuoSorbent Assay (ELISA) method for sensitive and specific determination of vitamin B_{12} in seawater and porewater is described for the first time. Effects of salinity, natural dissolved organic matter, humic acids, and other water soluble vitamins on the method were investigated, and the method was successfully applied to the measurement of B_{12} in coastal seawater and sediment porewater.

Material and procedures

Reagents and apparatus

All reagents were of analytical grade unless specified otherwise. Vitamin B_{12} standard (Cyanocobalamin) (99%, MP Biomedicals), methylcobalamin (MP Biomedicals), hydroxocobalamin (MP Biomedicals), and HPLC-grade methanol were purchased from Fisher Scientific. Coenzyme B_{12} (>97%), thiamine hydrochloride (vitamin B_1), riboflavin (vitamin B_2), pyridoxin hydrochloride (vitamin B_6), humic acid, tetramethylbenzidine (TMB), and 30% hydrogen peroxide were obtained from Sigma-Aldrich. Vitamin B_{12} ELISA kit and vitamin B_{12} immunoaffinity column were purchased from Immunolab GmbH (Kassel, Germany). HF Bondesil C-18 resin (120 µm) was from Varian Inc.

Natural DOM was extracted from seawater by using a C-18 column at pH 8. Coastal surface seawater (0-1 m) was collected from Stony Brook Harbor (Long Island, NY) on June 2011, and filtered through a 0.2 µm polycarbonate filter (Whatman) after transport to the laboratory (collection and processing completed within 1 h). One-liter filtered seawater was passed through a C-18 column, and the column was then rinsed with 20 mL distilled water. The extracted DOM in the column was eluted with 5 mL methanol. After the eluent was dried with N_2, the residue was redissolved in 1.0 mL distilled water. The vitamin B_{12} enriched in the residue along with the DOM was removed using a vitamin B_{12} immunoaffinity column. The DOM concentration in the final extract (residue) was measured by a DOC analyzer (Total Organic Carbon Analyzer, Shimadzu). This DOM extract was diluted to different concentration for evaluation of DOM interference.

A POLARstar Omega multifunctional plate reader (BMG Labtech GMbH) was used for the ELISA absorbance measurement at 450 nm with reference wavelength at 620 nm. HPLC analysis was performed on an HPLC system (Prominence, Shimadzu) equipped with LC-20AD pumps, DGU-20A degassing unit, SIL-20AC auto-sampler, CBM-20A communications bus model, SPD-20A UV-VIS spectrometer, and CTO-20AC column oven. All pH measurements were made with a digital pH meter (Model 290, thermo Orion).

ELISA procedure

An indirect competitive immunoassay format was applied to the analysis of B_{12} in water samples. The reagents and conjugates including antibody-coated microtiter plate (consisting of 12 strips with 8 breakable wells each coated with anti-vitamin B_{12} antibody), vitamin B_{12}-HRP conjugate, TMB substrate solution, washing solution (phosphate buffered saline (PBS) + Tween 20) and stop solution (0.5 M H_2SO_4) were provided by the ELISA kit. ELISA was run as follows: 50 µL vitamin B_{12} standards or samples were transferred into the appropriate wells of the microtiter plate, and 50 µL vitamin B_{12}-HRP conjugate was immediately added into each well. The microtiter plate was then covered with a plastic foil and incubated for 60 min at room temperature on a plate shaker. After rinsing three times with washing solution (300 µL/well each time), 100 µL substrate solution (TMB/H_2O_2 mixture) was added into each well using a multi-channel pipette. The plate was incubated for 20 min at room temperature in the dark for color development, and 100 µL stop solution (0.5 M H_2SO_4) was added into each well to stop the enzyme reaction. The absorbance at 450 nm (reference wavelength 620 nm) was measured using the plate reader. All determinations were made in triplicate. The calibration graph was constructed in the form of $A/A_0 \times 100\%$ against $\log[B_{12}]$ where A and A_0 are the values of absorbance measured in the presence and absence of vitamin B_{12}, respectively.

Sample collection and treatment

Coastal seawater samples were collected from Flax Pond (19 Jul), Shinnecock Bay (28 Jul), Stony Brook Harbor (2 Aug), and West Meadow Beach (2 Aug 2010) around Long Island, New York. Shinnecock Bay is on the south coast of Long Island, and a bloom of the dinoflagellate *Cochlodinium polykrikoides* was taking place when the sample was collected. Flax Pond, Stony Brook Harbor, and West Meadow Beach are on the north shore of Long Island. Flax Pond is a back barrier, salt marsh lagoon with high organic matter and sulfide concentrations in sediment (Swider and Mackin 1989). West Meadow Beach is open to central Long Island Sound about 2 miles from Flax Pond and

1 mile from Stony Brook Harbor. The water samples were immediately delivered to the laboratory and filtered through 0.2 µm polycarbonate membrane filters. The pH of the samples was adjusted to 6.4 using 1 M HCl prior to solid phase extraction. Surface sediment (0-2 cm) was collected from Flax Pond tidal flats on 19 Jul 2010. Porewater samples were separated by centrifugation of sediment at 9000 rpm and filtered through 0.4 µm polycarbonate membrane filters.

Solid-phase extraction of B_{12}

A C-18 solid-phase extraction (SPE) column was prepared using Bondesil C-18 resin and a 6.0 mL polypropylene column. Two grams of C-18 were mixed with 10 mL methanol, and the C-18 slurry was loaded into the column. The column was consecutively conditioned with 10 mL methanol and 10 mL distilled water. The pH of seawater or porewater samples was adjusted to about pH 6.4 using 1 M HCl to enhance the absorption of B_{12} on the sorbent (Okbamichael and Sañudo-Wilhelmy 2004). A known volume (e.g., 0.5–2 L) of water sample was passed through the C-18 column at a flow rate of approximately 1 mL/min, and the column was then rinsed with 10 mL distilled water and dried using N_2. The enriched vitamin B_{12} in the column was eluted with 5 mL methanol. Afterward the eluent was brought to dryness in a gentle stream of N_2 and the residue was redissolved in a small volume of distilled water or 0.01 M phosphate buffered saline (PBS) solution for ELISA.

HPLC analysis

Vitamin B_{12} was detected using a UV-VIS spectrometer at 362 nm. The potential background interferences in the preconcentrated porewater samples were eliminated by passing them through a B_{12} immunoaffinity column, which was filled with anti-vitamin B_{12} antibody immobilized on hydrogel, at a flow rate of 0.2 mL/min, and then eluting with 1 mL methanol into a HPLC sample vial. After gently drying using a N_2 stream, the residue was redissolved into 0.2 mL distilled water for HPLC analysis. The HPLC column was a reverse-phase Premier C-18 column (150 mm × 4.6 mm, 5 µm) from Shimadzu. 40 µL of the extract was injected and gradient eluted with methanol and 5 mM ammonium acetate solution at a flow rate of 1.0 mL/min. Methanol was initially at 2.5% from 0–1.5 min, increased to 16% at 1.6 min, and 50% at 4.6 min, and then decreased to 2.5% at 10 min and maintained for 4 min, respectively.

Assessment and discussion

Solid-phase extraction of vitamin B_{12}

Vitamin B_{12} concentrations in natural seawater reported in the literature vary greatly. Concentrations found in the open ocean are generally in the range of 0.2–5 pM and are often 5–90 pM in coastal seawater (Menzel and Spaeth 1962; Sañudo-Wilhelmy et al. 2006; Panzeca et al. 2008). All reported B_{12} concentrations in seawater samples are far below the direct detection limits of currently available methods including the ELISA method presented here. Therefore, preconcentration of B_{12} is needed before determination. C-18 SPE is one of the most common extraction methods, and it has been applied to enrich and clean-up B_{12} samples. The condition for sample enrichment in this study was similar to that used by Okbamichael and Sañudo-Wilhelmy (2004). Over 90% recovery of standard B_{12} was obtained when the sample pH was in the range of 6.1–6.7 and flow rate at 1 mL per minute. After natural water samples pass through a C-18 column, the cartridge needs to be rinsed with distilled water to remove residual inorganic ions and then dried with N_2 before methanol elution, otherwise extracts can be cloudy. Five milliliters methanol was used to elute the enriched analyte. Because the methanol solution cannot be directly used for immunoassays, the eluent needs to be dried by a gentle stream of N_2 and the residue redissolved in 0.01 M PBS (or distilled water) for ELISA analysis.

C-18 SPE enriches organic or metal-organic analytes primarily by reversed-phase adsorption, and a high DOM background can potentially originate from sample matrix components that are also enriched during extraction. We found that the adsorption of matrix organics on C-18 was so strong that when dissolved organic rich seawater or porewater samples were run through a SPE cartridge, we were not able to effectively clean up interferences using even modified washing procedures. In some cases, the C-18 sorbent became dark brown and the color of the extract was yellow to pink. The accurate measurement of vitamin B_{12} in such complex extracts requires a highly selective or specific method such as an immunoassay method. No interferences from the reagent humic acids and extracted DOM were observed for the determination of vitamin B_{12} using the ELISA method (see below).

ELISA for vitamin B_{12} in natural waters

ELISA has become a predominant immunoassay form in the past several decades because of its specificity, high sensitivity, and reliability. This method utilizes a solid support, for example a 96/384-well microtiter plate, to immobilize an antibody or antigen and to perform immunoassays based on competitive or sandwich format (Fig. 1). In a competitive immunoassay format, the free antigen (analyte) and enzyme-labeled antigen competitively bind to certain antibodies immobilized on the plate based on a single step antibody-antigen immunoreaction. After removing unbound antigen and adding enzyme substrate solution, the resulting signal is inversely proportional to the analyte concentration. The sandwich format is based on two successive immunoreactions. The free antigen binds to the immobilized antibody on the plate first and then the unbound antigen is completely washed away. Second, enzyme-labeled antibody (or secondary antibody) is added to react with the bound antigens in each plate well. After removal of unbound enzyme-labeled antibody by washing, the final signal is directly proportional to analyte concentration. The sampling capacity of a microplate immunoassay technique is very high, and several hundreds of samples can be processed simultaneously (González-Martinez

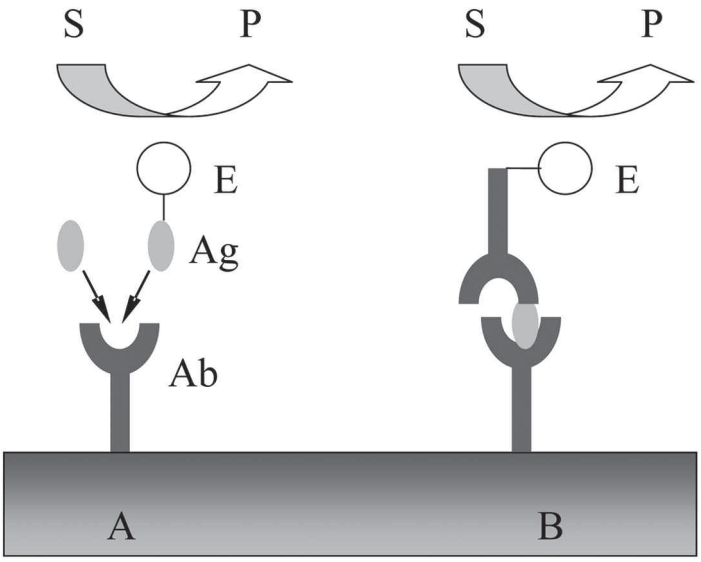

Fig. 1. Schematic representation of ELISA. (A) Competitive assay format between the enzyme-labeled (known concentration) and unlabeled antigen (analyte, unknown concentration) for binding sites on the immobilized antibody. The resulting signal is inversely proportional to the analyte concentration. (B) Sandwich assay format, the unlabeled antigen is determined by an enzyme-labeled antibody. The substrate is transferred to a detectible signal by the enzyme. S: substrate; P: color or fluorescence product; E: enzyme; Ag: antigen; Ab: antibody.

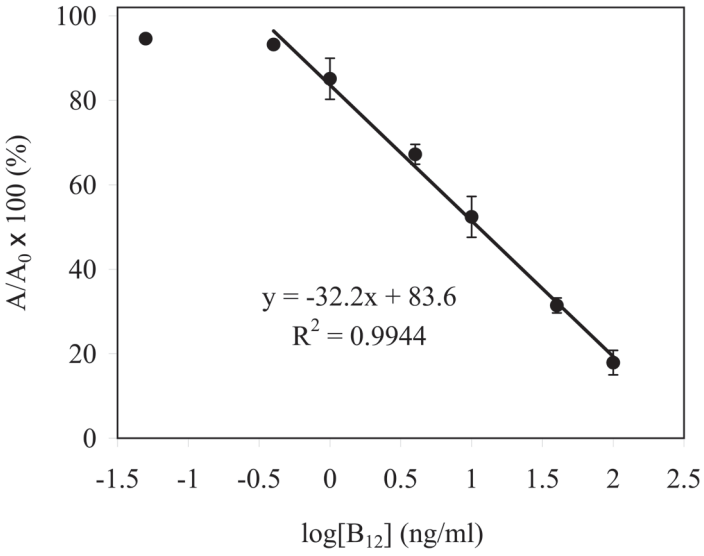

Fig. 2. Calibration graph for the detection of vitamin B_{12} by ELISA. A and A_0 are the values of absorbance measured in the presence and absence of vitamin B_{12}, respectively. The measurement on each concentration was repeated three times, the error bars are standard deviations.

et al. 2007). ELISA has a long tradition of use in the biological, clinical, and pharmaceutical research communities and has been accepted as a standard detection platform, but its application in marine chemistry is not widely reported.

In this work, an antibody directed against vitamin B_{12} was coated on the surface of a 96-well microtiter plate strip, and the indirect competitive immunoassay format was selected because the competitive format is a single step immunoreaction and is simpler and faster compared with sandwich format. Furthermore, the small size of vitamin B_{12} (MW = 1355.4) and relatively few binding sites on the molecule may limit formation of the antibody-B_{12}-antibody sandwich immunocomplex due to the large size of the antibody (MW = 146 kD). In the indirect competitive immunoassay format, horseradish peroxidase (HRP) was used as a labeling enzyme for vitamin B_{12}. Water sample or standard and a constant amount of HRP labeled B_{12} were added into each microtiter plate well, HRP-labeled and free B_{12} compete for binding to the plate-bound antibody. After immunoreaction, the immunochemically adsorbed HRP-B_{12} conjugate was determined by measuring the absorbance produced in a solution containing substrate tetramethylbenzidine (TMB) and hydrogen peroxide. Based on the optimal conditions provided by the ELISA kit, the B_{12} calibration graph was constructed in the form of $A/A_0 \times 100\%$ (where A and A_0 are the values of absorbance measured in the presence and absence of vitamin B_{12}, respectively) against $\log[B_{12}]$ in the range of 0–100 ng/mL vitamin B_{12} (Fig. 2). The analytical working range was about 0.4–100 ng/mL with a detection limit of 0.2 ng/mL (3σ). The relative standard deviation (RSD) of the measured absorbance for three replicates at each standard concentration was lower than 5%.

Effects of DOM, humic acids, and salinity on the ELISA

The separate effects of salinity, natural DOM, and humic acids on the ELISA measurement were studied by detection of 10 ng/mL B_{12} as conditions were varied. 10 ng/mL vitamin B_{12} was chosen here because it was close to the method's IC_{50} values (concentration of B_{12} that produces a 50% decrease from the maximum response) and the concentration of vitamin B_{12} in C-18 SPE extracted samples that were preconcentrated by 100–1000 times. The results summarized in Fig. 3 show that the ELISA signals remain virtually constant when salinity changes from 0 to 35‰, indicating that salinity of the sample does not interfere with the immunoreaction and that immunoassay techniques such as ELISA and immnoaffinity extraction can be directly applied to seawater analysis.

Humic acid is a complex mixture of organic compounds commonly present as a fraction of DOM in environmental samples, including seawater and porewater. Sigma-Aldrich humic acid is used here as a model DOM component, which is easy to obtain and which allows comparison of results among lab groups. It has been reported that nonspecific binding of antigen (analyte) and immobilized antibody to the functional groups of humic acids may result in an ELISA signal change and thus interfere with measurements (Deng et al. 2003). Our results in Fig. 3 indicated that up to at least 30 mg/L added reagent humic acid (higher concentrations were not tested; solution salinity ≈ 0) does not interfere with the present ELISA system for analysis of 10 ng/mL vitamin B_{12}. Because humic acids represent only a portion of DOM in sea-

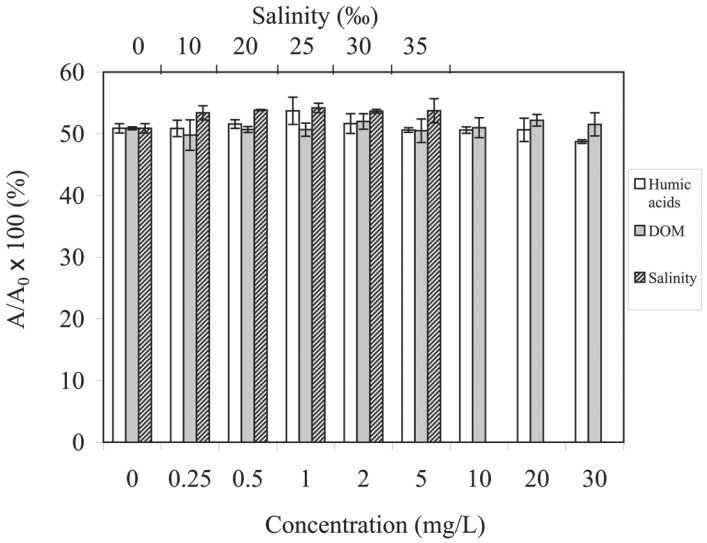

Fig. 3. Effects of salinity, reagent humic acids, and natural DOM on the immunoassay of 10 ng/mL vitamin B_{12} (n = 3, multiple measurement of single sample). Natural DOM was extracted from seawater by using a C-18 column.

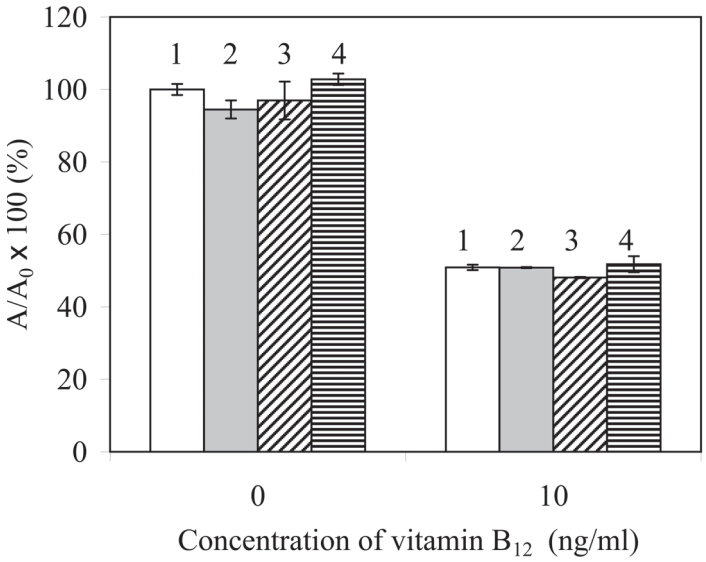

Fig. 4. The specificity of ELISA for detection of vitamin B_{12} (n = 3). (1) Vitamin B_{12} without interference; (2) 100 ng/mL vitamin B_1; (3) 100 ng/mL vitamin B_2; and (4) 100 ng/mL vitamin B_6.

water, to further evaluate the applicability of the present ELISA, natural DOM was extracted from seawater using a C-18 column (as per B_{12} extraction), quantified by a DOC analyzer, and the effects of different amounts of DOM on B_{12} detection also studied. Fig. 3 shows that concentrations as high as 30 mg/L DOM do not interfere with the analysis (higher concentrations were not tested, salinity ≈ 0). These results demonstrate that high levels of natural DOM, separated humic acids, and salinity in marine samples do not affect the vitamin B_{12} immunoreaction, and also imply that an immunoaffinity column, prepared from vitamin B_{12} antibody, could be used as an initial SPE cartridge to enrich the analyte. An immunoaffinity extraction column would significantly lower the nonspecific absorption of matrix components and thus remove potential interferences before HPLC analysis.

Method specificity

Specificity of a method refers to the capability to discriminate the particular analyte from a complex mixture without interference from other components. Generally the immunoassay method is specific for the corresponding antigen analyte, but cross-reaction can also occur between the immobilized antibody and other components, which have structures or functional groups similar to the analyte. The specificity of the present ELISA method was evaluated by control and cross-reactivity measurements in the presence of other B vitamins including thiamine (vitamin B_1), riboflavin (vitamin B_2), and pyridoxine (vitamin B_6). First, the anti-B_{12} antibody was replaced by bovine serum albumin (BSA) to coat the microtiter plate, otherwise keeping all steps the same as the ELISA. The control results indicated that nonspecific binding of B_{12} on the immobilized protein (BSA) is negative and can be ignored (data not shown), demonstrating that the ELISA absorbance signal was generated from the specific recognition of antibody to vitamin B_{12} in the sample. The cross-reactivity of vitamin B_{12} antibody with other vitamin B species is summarized in Fig. 4. The errors from nonspecific absorption of 100 ng/mL vitamins B_1, B_2, and B_6 on the immobilized B_{12} antibody and protein in the absence of B_{12} are –5.5%, –3.5%, and 3.8%, respectively, and thus can be ignored. The cross-reactivities of these vitamins (100 ng/mL) are within the relative analytical error (5%) for detection of 10 ng B_{12}/mL, indicating that they did not compete with B_{12} during the immnunoreaction because their structures are sufficiently different from vitamin B_{12}.

It is well known that vitamin B_{12} is not a single compound, but consists of a class of chemically related compounds, such as cyanocobalamin, methylcobalamin, hydroxocobalamin, and coenzyme B_{12}, all of which have a similar structure. The cross-reactivities of vitamin B_{12} antibody toward different vitamin B_{12} forms: methylcobalamin, hydroxocobalamin, and coenzyme B_{12} were evaluated. 50 µL of 10 ng/mL cyanocobalamin, methylcobalamin, hydroxocobalamin, and coenzyme B_{12} solution, as well as a mixture of 25 µL cyanocobalamin and 25 µL of other forms of B_{12} (10 ng/mL), was added into plate wells to study their response to the antibody. The results summarized in Table 1 show that the anti-B_{12} antibody used in this ELISA system recognizes all vitamin B_{12} forms with a 100% cross reactivity and is not able to distinguish individual compounds of B_{12}. Therefore, the vitamin B_{12} concentration in samples determined by the proposed ELISA in this work represents a total vitamin B_{12} concentration rather than a single form. It should be pointed out that vitamin B_{12} binding protein, which is important to phytoplankton cultures (Droop 1968), and other similar pseudocobalamin may interfere with the ELISA analysis.

Accuracy and precision

The potential errors in this analytical technique arise from the two components: ELISA detection and C-18 SPE. The accuracy of the ELISA was evaluated by testing the recoveries of standard additions of B_{12} in distilled water and artificial seawater (salinity 35‰). Different known amounts of B_{12} were added to distilled water and artificial seawater, and the spiked samples were directly determined by the ELISA method. The results in Table 2 show that the recoveries of standard addition B_{12} in distilled water and artificial seawater are 99.6–105% and 96.0– 97.0%, respectively. The good agreement of the added and recovered amount of vitamin B_{12} in these samples confirms the ELISA method's accuracy.

The accuracy of ELISA combined with C-18 SPE was evaluated by testing the recovery of standard additions of B_{12} in natural coastal seawater and porewater samples, and by a comparison with the HPLC-based method. 50 ng of vitamin B_{12} was added into 500 mL of 0.4 µm polycarbonate filtered sediment porewater (Flax Pond tidal flat). The spiked and unspiked samples were passed through a C-18 cartridge to enrich the analyte, and the concentrated residue was redissolved in 0.5 mL distilled water. 0.1 mL of this extract solution was diluted by 5 times with 0.01 M PBS solution for ELISA analysis. For HPLC-based vitamin B_{12} analysis, 0.4 mL of the extract solution was further run though a vitamin B_{12} immunoaffinity column, which contained immobilized vitamin B_{12} antibody to eliminate the high DOM or humic acids background. The analyte B_{12} was bound to the anti-B_{12} antibody in the immunoaffinity column and non-target background organics were not retained. The immuno-bound vitamin B_{12} was then eluted by 1 mL 100% methanol. After gently drying by nitrogen stream, the residue was redissolved into 0.2 mL distilled water for HPLC analysis. The results listed in Table 3 indicate that the recoveries of B_{12}, which was added into 500 mL natural porewater and enriched by C-18, determined by either ELISA or by immunoaffinity extraction + HPLC are 101.8% and 97.6%, respectively.

A direct comparison between C-18 extraction/ELISA and C-18 extraction/HPLC (without immunoaffinity column cleanup) for the determination of vitamin B_{12} in coastal seawater was also performed. A seawater sample from Stony Brook Harbor (6 Jun 2011) was filtered through a 0.2 µm polycarbonate filter in the laboratory within 1 h after collection. One liter

Table 1. Cross-reactivity of different vitamin B_{12} forms

Selected antigens	Cross reactivity (%)
Vitamin B_{12} (cyanocobalamin)	100.0
Methylcobalamin	98.3
Hydroxocobalamin	101.7
Coenzyme B_{12}	104.1

Table 2. The accuracy and precision of ELISA for vitamin B_{12} determination.

Samples	Spike (ng/mL)	Found* (ng/mL) (mean ±SD)	RSD (%)	Recovery (%)
Distilled water	4.0	4.2 ± 0.2	4.76	105.0
	10.0	10.2 ± 0.3	2.94	102.0
	100.0	99.6 ± 3.6	3.61	99.6
Artificial seawater	5.0	4.8 ± 0.2	4.17	96.0
	10.0	9.7 ± 0.2	2.06	97.0
	60.0	58.2 ± 1.7	2.92	96.9

*n = 3, multiple analysis of single sample.

Table 3. Comparison of ELISA and HPLC for determination of vitamin B_{12} in porewater.

			Found ±SD* (ng), (n = 3)		Recovery (%)	
Sample	Volume (mL)	Added (ng)	ELISA	HPLC	ELISA	HPLC
Porewater†	500	0	31.7 ± 2.9	33.9 ± 1.9	—	—
	500	50.0	82.6 ± 8.0	82.7 ± 8.1	100.8	97.6
Seawater‡	1000	0	5.1 ± 0.5	Non-detectable	—	—
	1000	50.0	52.0 ± 0.7	46.6 ± 0.9	93.7	83.2
	1000	150.0	162.1 ± 4.1	146.7 ± 8.5	104.7	94.4

*n = 3, multiple analysis of single sample.

†Porewater sample was obtained from surface marine sediment (0–2 cm) collected on 19 Jul 2010 from Flax Pond, Long Island, NY. The freshly centrifuged anoxic porewater was filtered through a 0.4 µm filter, spiked with a known amount of standard vitamin B_{12}, and preconcentrated using a C-18 column. Portion of the 0.5 mL extract in distilled water was then diluted by 5 times with 0.01 M PBS buffer for ELISA analysis. The extract of the porewater sample from C-18 cartridge was further run through a vitamin B_{12} immunoaffinity column to remove DOM before HPLC analysis.

‡Seawater sample collected on 6 Jun 2011 from Stony Brook Harbor (Long Island, NY) was immediately filtered through 0.2 µm filter, spiked with a known amount of standard vitamin B_{12}, and run through C-18 columns. The extract in 1.0 mL distilled water was directly used for ELISA and HPLC

Table 4. Analysis of seawater and porewater samples using ELISA.

Samples	Added (ng/L)	Found* (ng/L) (mean ±SD)	RSD (%)	Recovery (%)
Seawater samples				
Flax Pond†	0	19.7 ± 0.6	3.05	
	100	106.9 ± 0.8	0.75	87.2
Stony Brook Harbor‡	0	51.6 ± 1.1	2.13	
	40	94.3 ± 5.2	5.51	106.7
West Meadow Beach‡	0	6.1 ± 0.9	14.8	
	20	23.7 ± 0.1	0.42	88.0
Shinnecock Bay‡	0	17.3 ± 2.0	11.6	
	40	57.4 ± 0.9	1.57	100.3
Porewater samples				
Flax Pond§	0	63.5 ± 5.7	8.98	
	100	165.1 ± 15.9	9.63	101.6
Flax Pond#	0	20.7 ± 2.4	11.6	
	100	131.7 ± 7.5	5.69	111.0
Flax Pond¶	0	15.9 ± 1.6	10.1	
	100	111.9 ± 4.9	4.38	96.0

*n = 3, multiple analysis of single sample.
†(2 L) and ‡(500 mL) seawater was filtered through 0.2 µm filter and preconcentrated by C-18 column; the 1.0 mL extract in 0.01 M PBS was then diluted by 5 times with PBS buffer for ELISA analysis.
§500 mL freshly centrifuged anoxic porewater was filtered through 0.4 µm filters and preconcentrated by C-18 column; the 0.5 mL extract in distilled water was then diluted by 5 times with 0.01 M PBS buffer for ELISA analysis.
#100 mL porewater same as that in § was oxygenized and then spiked with vitamin B_{12} and preconcentrated using a C-18 column; the 0.5 mL extract in distilled water was 1:1 diluted with 0.01 M PBS buffer for ELISA analysis.
¶100 mL porewater same as that in # was filtered through a 0.2 µm filter and then spiked with vitamin B_{12} and preconcentrated by C-18 column; the 0.5 mL extract in distilled water was then 1:1 diluted with 0.01 M PBS buffer for ELISA analysis.

samples filtered seawater were spiked with 0, 50, and 150 ng vitamin B_{12}, and then respective samples preconcentrated by a C-18 column. The final preconcentrated residue was redissolved in 1.0 mL distilled water, portions of which were used for the ELISA and HPLC measurements. The results in Table 3 showed that the values obtained from the two independent methods are comparable except the unspiked preconcentrated sample was undetectable by the HPLC method. Further study showed that the HPLC peak of vitamin B_{12} was unrecognizable on the natural DOM background when B_{12} concentration was less than about 40 ng/mL in the preconcentrated seawater sample with a 40 µL sample injection

Precisions of the proposed ELISA method were evaluated by measurement standard deviation (SD) and relative standard deviation (RSD) (n = 3). Results in Table 2 showed that RSD is less than 5% in artificial seawater samples. However, the RSDs are not always less than 5% when real samples were measured. Results in Table 3 and 4 show that although most RSDs are smaller than 5%, RSD from some measurements may reach over 10%. The higher RSDs may result from unknown operation errors or additional matrix effects of porewater samples (for example DOM may not have been totally removed from the microtiter well after immunoreaction). Because the recoveries of standard addition vitamin B_{12} in natural samples are satisfactory (87–111%), the possible small matrix effect should not affect practical application of the ELISA. On the other hand, this variability indicates that sufficient washing of the microtiter plate is a critical step after immunoreaction to completely remove nonspecifically absorbed B_{12}-HRP conjugate and other DOM.

Example applications

The proposed C-18 SPE-ELISA method was applied to the determination of vitamin B_{12} in a series of coastal seawater and porewater samples. The samples, 0.1, 0.5, or 2 L in volume, were extracted on C-18 and B_{12} was measured by ELISA. Results from Shinnecock Bay, Flax Pond, Stony Brook Harbor, and West Meadow Beach (Long Island, NY) are summarized in Table 4. As indicated by previous studies (e.g., Sañudo-Wilhelmy et al. 2006), these data show that B_{12} concentrations can vary substantially between different locations separated by small distances. For example, B_{12} concentration in Stony Brook Harbor, enclosed and bordered by salt marsh, is about 9 times higher than nearby West Meadow Beach, which is open to central Long Island Sound.

Another interesting result shown in Table 4 is that B_{12} concentrations in filtered porewater can be altered by handling and redox conditions. Dissolved vitamin B_{12} in filtered (0.4 µm) anoxic porewater from Flax Pond was 63.5 ng/L, however, it decreased to ~20 ng/L after porewater was exposed to air, allowed to oxidize, and passed through a C-18 column.

Although scavenging of B_{12} by the precipitation of Fe, Mn oxides may contribute to the decrease, we think that, in this case, the decrease was likely caused by the scavenging of B_{12} by colloidal sulfur particles formed under oxic conditions and removed during passage through the C-18 column. Ambient hydrogen sulfide was found to be as high as 8 mM in Flax Pond during summer (Swider and Mackin 1989; unpubl. data), and oxygenation of such porewater can produce colloidal elemental sulfur. Light scattering (Tyndall Effect) in oxidized samples, regardless of acidification with HCl, is ~ 3× stronger than anoxic porewater (data not shown), consistent with the formation of colloidal sulfur during oxidation and polysulfide decomposition.

Two experiments were performed to investigate the nature of particle scavenging during oxidation. In one case, B_{12} standard was added after colloidal precipitates were formed so that any B_{12} standard scavenging was largely by surface adsorption rather than co-precipitation. 10 ng standard vitamin B_{12} was added to 100 mL (100 ng/L) oxygenized porewater and then passed through a C-18 column. Any colloidal precipitates were presumably absorbed or filtered by the C-18 resin. A 111% recovery of standard was measured (Table 4d), indicating that the irreversible loss of dissolved vitamin B_{12} occurred during the precipitation stage and that any subsequent adsorption of B_{12} onto colloids could be desorbed (recovered) during elution with methanol, or that colloidal particles, once formed, were not reactive to B_{12}. In the second experiment, oxygenized porewater was refiltered using a 0.2 µm filter to remove most particles initially formed during oxidation. A lower B_{12} concentration of 15.9 ng/L was measured in subsequently processed and eluted sample, confirming that B_{12} was removed by co-precipitation with particles. B_{12} standard was added to a sample of the refiltered porewater (10 ng B_{12} to 100 mL) and the sample extracted, eluted with methanol, and analyzed. A 96% recovery of standard was found (Table 4, e), demonstrating that any residual particles do not irreversibly remove B_{12} and again that B_{12} was lost during the initial colloid precipitation process rather than subsequently.

The reliability of ELISA is demonstrated by the standard additions of vitamin B_{12} to the natural seawater and porewater samples. The recoveries of added vitamin B_{12} in the various samples ranged from 87–111%, and we consider these satisfactory (Table 4).

Comments and recommendations

The proposed ELISA method has a wide working dynamic range and a low direct detection limit (0.2 ng/mL, 150 pM), and when combined with SPE, has been successfully applied to the quantification of trace dissolved B_{12} in seawater and marine sediment porewater. In practice, ~10–500 mL sample is needed for preconcentration depending on the range of B_{12} concentration. Salinity, DOM, humic acids, and other vitamin B substances do not interference with the ELISA measurement. The method has the advantages of high specificity, easy operation, rapid measurement, and relatively low cost. By using 96- or 384-well microtiter plates and multifunctional plate readers, the proposed ELISA method can readily measure hundreds of sample extractions within 2 h. Although we have used commercially available analytical components in this study, the fabrication of antibody-coated microtiter plates is straightforward for research applications. Because biologically active elements are used, the calibration and sample measurement should be performed at the same time. Additionally, the antibody-coated microtiter plate and reagents should be stored at 4°C (up to a year). For longer term storage, small aliquots of antibody solutions should be kept at –20°C or below.

In summary, we propose the SPE-ELISA method as an excellent and powerful approach for quantifying dissolved vitamin B_{12} in a range of environmental samples.

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