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Determination of four forms of vitamin B_{12} and other B vitamins in seawater by liquid chromatography/tandem mass spectrometry

Katherine R. Heal[1], Laura Truxal Carlson[1], Allan H. Devol[1], E. Virginia Armbrust[1], James W. Moffett[3], David A. Stahl[2] and Anitra E. Ingalls[1]*

[1]School of Oceanography, University of Washington, Seattle, WA, USA
[2]Department of Civil and Environmental Engineering, University of Washington, Seattle, WA, USA
[3]Departments of Biological Sciences and Earth Sciences and Civil and Environmental Engineering, University of Southern California, Los Angeles, CA, USA

RATIONALE: Vitamin B_{12} is an essential nutrient for more than half of surveyed marine algae species, but methods for directly measuring this important cofactor in seawater are limited. Current mass spectrometry methods do not quantify all forms of B_{12}, potentially missing a significant portion of the B_{12} pool.
METHODS: We present a method to measure vitamins B_1, B_2, B_6, B_7 and four forms of B_{12} dissolved in seawater. The method entails solid-phase extraction, separation by ultra-performance liquid chromatography, and detection by triple-quadrupole tandem mass spectrometry using stable-isotope-labeled internal standards. We demonstrated the use of this method in the environment by analyzing B_{12} concentrations at different depths in the Hood Canal, part of the Puget Sound estuarine system in Washington State.
RESULTS: Recovery of vitamin B_{12} forms during the preconcentration steps was >71% and the limits of detection were <0.275 pM in seawater. Standard addition calibration curves in three different seawater matrices were used to determine analytical response and to quantify samples from the environment. Hydroxocobalamin was the main form of B_{12} in seawater at our field site.
CONCLUSIONS: We developed a method for quantifying four forms of B_{12} in seawater by liquid chromatography/mass spectrometry with the option of simultaneous analysis of vitamins B_1, B_2, B_6, and B_7. We validated the method and demonstrated its application in the field.

Phytoplankton play a pivotal role in global carbon and nitrogen cycles as the base of the marine food web and as a major vehicle for the uptake of carbon dioxide from the atmosphere. Recent work suggests that auxotrophy for specific B vitamins can control primary productivity and phytoplankton diversity in certain ecosystems.[1,2] Croft and coworkers[3] found that of 300 surveyed eukaryotic phytoplankton cultures, over 50% were auxotrophic for thiamine (B_1), biotin (B_7), or cobalamin (B_{12}). Community composition and activity of vitamin producers could influence marine eukaryotic primary productivity or diversity through these important biomolecules. For instance, B_{12} is produced only by select bacteria and archaea but is also required by many marine eukaryotes.[3–5] While these B vitamins appear to be important and potentially limiting nutrients for some phytoplankton, our understanding of B vitamin cycling in the ocean is largely limited by unvetted and still evolving methods.

Vitamin B_{12} is a suite of compounds that contain a cobalamin with different chemical groups attached to the cobalt atom (Fig. 1). Four main types have been well studied in the medical field but have been largely ignored in environmental settings: cyanocobalamin (CN-Cbl), methylcobalamin (Me-Cbl), hydroxocobalamin (OH-Cbl), and deoxyadenosylcobalamine (Ado-Cbl). Aquacobalamin is another potentially important form, but under acidic conditions is converted into its conjugated base, OH-Cbl.[6] The cobalamins have different bio- and photo-chemistries, making it important to differentiate them in studies of marine nutrient cycling. Of the four forms of cobalamin, Me-Cbl and Ado-Cbl are actively used as cofactors, while OH-Cbl and CN-Cbl are not used directly and are first converted into an active form of B_{12}.[7] All forms of cobalamins are light sensitive, with Me-Cbl and Ado-Cbl identified as extremely labile, photodegrading into OH-Cbl in a matter of seconds after light exposure.[8]

Early analyses quantified B_{12} in seawater by monitoring the growth of B_{12}-requiring diatoms.[5,9,10] New approaches allow the simultaneous measurement of several B vitamins (B_1, B_2, B_6, B_7) in seawater using solid-phase extraction (SPE) followed by high-pressure liquid chromatography (HPLC) coupled to triple quadrupole mass spectrometry (MS), but identify only one form of B_{12}, CN-Cbl.[11,12] The most recently published MS method has a detection limit of 0.18 pM for cyanocobalamin concentrated from 2 L of seawater.[12] All four forms of B_{12} have been quantified in seawater using a diode-array detector (DAD) method,[13] but this required 4 L of seawater to achieve detection limits

* *Correspondence to:* A. E. Ingalls, School of Oceanography, University of Washington, Box 355351, Seattle, WA 98107, USA.
E-mail: aingalls@uw.edu

Rapid Communications in Mass Spectrometry

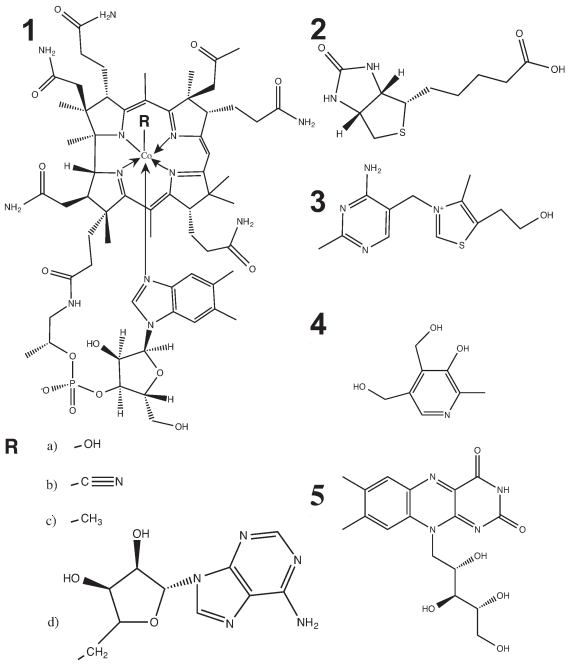

Figure 1. Structures of analytes in this study. (*1*) Cobalamin (B_{12}), with four functional forms: (*a*) hydroxocobalamin (OH-Cbl), (*b*) cyanocobalamin (CN-Cbl), (*c*) methylcobalamin (Me-Cbl), and (*d*) 5′-deoxy-5′-adenosylcobalamin (Ado-Cbl); (*2*) biotin (B_7); (*3*) thiamine (B_1); (*4*) pyridoxine (B_6); (*5*) riboflavin (B_2).

of 0.37 pM for OH- Me-, 0.148 pM for CN-, and 0.044 pM for Ado-Cbl. With processing rates of 1 mL min^{-1}, SPE of this large volume is time consuming, subject to breakthrough and unsuitable for unstable analytes.

Measured B_{12} half-saturation constants for cultured algae range from >0.02 to 13 pM[14] and ambient concentrations of this nutrient are often below existing detection limits,[12] demonstrating the need for a highly sensitive analysis. Published extraction methods in the food and medical fields for measuring different B vitamins do not transfer well to the marine environment due to the low analyte concentrations and high salt content of seawater.[15–24] Here we present an improved method to measure dissolved vitamins in seawater, focusing on the different forms of vitamin B_{12}.

EXPERIMENTAL

Sample collection

Environmental samples were taken near Hoodsport in the Hood Canal (HC), Washington, USA at 47° 25.309 N, 123° 6.755 W on 31 May 2013 at approximately 1 pm. Water used for standard curves was collected from the surface at HC, along historical Line P at station P4 (48° 39.0 N, 126° 40.0 W, collected 21 May 2012) and at station ALOHA (22° 45.62 N, 158° 0.03 W, collected 4 September, 2013). These three stations represent very different oceanic regimes (HC, coastal; P4, transitioning between coastal and North Pacific gyre; ALOHA, oligotrophic subtropical gyre) and provide three distinct matrices. Seawater was collected in Niskin bottles using a conductivity-temperature-depth (CTD) rosette in a single cast. Temperature and chlorophyll fluorescence measurements were obtained with a Seabird CTD sensor package. Samples were collected into opaque, acid-cleaned, high-density polyethylene bottles after rinsing three times with the collection water. Seawater was 0.2 µm filtered immediately with a peristaltic pump. The filtrate was collected and separated into two 500 mL acid-cleaned opaque bottles. These samples were frozen at –20°C until analysis.

Preconcentration

Filtered, frozen sea water samples were thawed prior to vitamin preconcentration using a slight modification of previously published methods.[11,13] Briefly, a C18 SPE column (Waters, 35 mL capacity, 10 g resin) was first conditioned by passing 20 mL of Optima grade (Fisher) methanol followed by at least 20 mL of Milli-Q water through the column. The 500 mL seawater samples were adjusted to pH 5.5–6.5 with HCl and loaded onto the SPE column at 1 mL min^{-1} using a peristaltic pump with tygon tubing. This low flow rate and pH range have been shown[11,25] to be important for high retention of CN-Cbl and B_1 on C18 columns, and our preliminary tests supported this finding. SPE columns were kept wet during the entire process in a closed system to avoid needing to rewet the columns. The column was then washed with 20 mL Milli-Q water (adjusted to pH 6.5 with NaOH). Samples were eluted from the column with 40 mL of methanol, which was dried down under clean N_2 gas at low heat (solution temperature was kept below 35°C to avoid thermal degradation of B_1[26]). Finally, samples were reconstituted in 480 µL of solvent A of the ultra-performance liquid chromatography (UPLC) method (see below) and 20 µL of an internal standard mix. To minimize photodegradation, samples were collected in opaque bottles and preconcentration was performed in a dark, windowless room using opaque tubing and foil-covered vials.

Table 1. MS conditions and retention times (RT) for each analyte and internal standard (IS)

Analyte	SRM	CE (V)	CV (V)	RT (min)
B_1	265.1 → 122.1, *144.1*	12	6	0.63
B_1-IS	269.2 → 122.1, *148.1*	12	2	0.63
B_6	170.1 → 134.1, *79.6*	18	2	0.66
B_7	245.1 → 97.1, *123.0*	28	4	2.14
B_7-IS	247.1 → 99.0, *124.4*	26	2	2.14
B_2	377.2 → 243.2, *172.1*	22	6	2.65
B_2-IS	383.2 → 249.1, *175.1*	22	2	2.65
OH-Cbl	664.7 → 147.1, *359.2*	34	42	3.10
CN-Cbl	678.6 → 147.1, *359.2*	36	22	3.46
Ado-Cbl	790.7 → 147.1, *665.6*	54	26	3.99
Me-Cbl	673.1 → 147.1, *685.6*	38	46	4.52

Selective reaction monitoring (SRM) was used to identify and quantify each precursor and product pair at the indicated collision energy (CE) and cone voltage (CV). First listed product ion was used to quantify with second product ion (italicized) used to confirm identity.

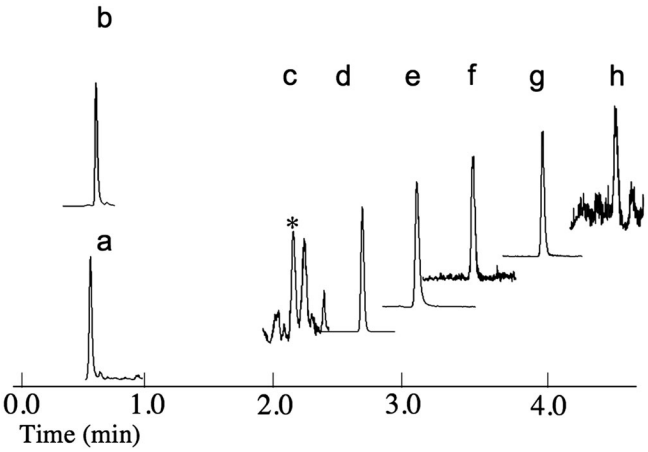

Figure 2. Example chromatograms of extracted ion pairs from a processed sample (surface sample at the Hood Canal): (a) B_1, (b) B_6, *(c) B_7 (* marking peak), (d) B_2, (e) OH-Cbl, (f) CN-Cbl, (g) Ado-Cbl, (h) Me-Cbl. Y axis is intensity of individual transitions, each chromatogram set to 100% relative intensity for visualization since the instrumental response can vary over three orders of magnitude between analytes. For biotin, the peak is identified by both IS and secondary transition. See Table 1 for extracted ion pairs.

Recovery

To determine the percent recovery of each vitamin during SPE, the 0.2 µm filtered seawater samples were spiked with known amounts of the seven analytes in triplicate either before or after preconcentration on SPE. All samples were analyzed in the same fashion. These values of recovery were used in calculations of vitamin concentrations in seawater to correct for SPE recovery.

UPLC/MS conditions

A UPLC/electrospray ionization (ESI)-MS method was adapted from previously published methods.[12,13,27] A Waters Acquity UPLC system coupled to a triple quadrupole (Waters Xevo TQ-S) was used to analyze all samples and standards. A UPLC HSS Cyano column (Waters Acquity, 1.8 µm, 2.1 × 100 mm) was eluted with 20 mM ammonium formate with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). All solvents used for UPLC were Optima LC/MS grade from Fisher. A linear gradient was employed for 5.3 min (from 98:2 to 70:30 solvent A:B), followed by 1 min at 5:95, and re-equilibrated for 1.7 min. The flow rate was 0.6 mL/min and the column temperature was 35°C. All analyses were performed in positive ion mode using selective reaction monitoring (SRM). The ESI source temperature was 130°C, with a desolvation temperature of 600°C. Collision energies, cone voltages, and retention times for each analyte are listed in Table 1. Peak areas of the listed transitions (see Table 1) were used for identification and quantification with internal standards (see below). With the exception of B_7, the SRM method proved very selective, generating a single peak (per transition) within the expected retention window. To identify B_7, we used the corresponding retention time of the internal standard and the ratio of the two transitions. An example chromatogram (with extracted ions) is shown in Fig. 2.

Quantification

Preparation and storage of standards

Standards of B_1 (thiamine hydrochloride), B_2 (riboflavin), B_6 (pyridoxine), B_7 (biotin), and CN-Cbl were obtained from AccStandard (water-soluble Vitamin Kit); Ado-Cbl, Me-Cbl, OH-Cbl, and ^{13}C-labeled B_1, B_2, B_7 (for internal standards) were purchased from Sigma Aldrich. All stock solutions were prepared by dissolving 1 mg of standard in 1 mL of Optima LC/MS grade water (except B_2, which was prepared in 5 mL water). Stock solutions were stored at –20°C in the dark. Working stocks (1 ng/µL) B_1, B_2, B_6, B_7, CN-Cbl and OH-Cbl were prepared fresh for standard curves; no appreciable degradation was noted over a 6-month period when stored at 4°C in the dark. Working stocks of Me-Cbl and Ado-Cbl (1 ng/µL) were prepared daily from frozen stock solutions due to rapid degradation of these compounds. Internal standard stock solutions were kept at –20°C in the dark and did not exhibit degradation over a 6-month period.

Evaluation of matrix effect

A representative set of environmental matrices of inorganic and organic molecules consisted of 0.2 µm filtered seawater collected from three locations (Hood Canal, Line P station P4 and station ALOHA) into clear polycarbonate bottles and

Table 2. Results of recovery analysis (with standard deviation of triplicate analysis), limit of detection (LOD), limit of quantification (LOQ), intra- and inter- day % precision, R^2, and internal standards (IS) used

Analyte	% Recovery	LOD (pM)	LOQ (pM)	Intra-day %Precision	Inter-day %Precision	R^2 (w/IS)	IS Used
B_1	55 ± 29	0.059	0.197	4.2	11.1	>0.99	B_1 IS
B_6	101 ± 14	0.149	0.498	6.0	7.5	0.97*	N/A
B_7	67 ± 23	0.898	2.99	5.3	8.2	>0.99	B_7 IS
B_2	93 ± 21	0.124	0.412	5.0	14.0	>0.99	B_2 IS
OH-Cbl	88 ± 13	0.275	0.917	5.7	6.8	0.99	B_2 IS
CN-Cbl	71 ± 4	0.014	0.046	6.9	9.1	0.99	B_2 IS
Ado-Cbl	96 ± 13	0.138	0.459	5.9	15.5	>0.99	B_1 IS
Me-Cbl	90 ± 24	0.024	0.080	2.3	36.9	0.97	B_7 IS

*For B_6, no internal standard was used.

Rapid Communications in Mass Spectrometry

maintained in a full spectrum light incubator for 2 days to degrade the endogenous vitamins. Although this significantly lowered the matrix blanks, some vitamins (B_1, B_6, B_7, CN-Cbl, OH-Cbl) were not completely removed. Therefore, standard addition curves with additions of 0.05 to 5 pg (for B_1, B_2, B_6, and all the forms of B_{12}) or from 0.25 to 25 pg (for B_7, which had a more extreme matrix effect) were made in 500 mL of each matrix. These standards were then preconcentrated and analyzed as described above. Internal standards were added after preconcentration and drying to account for different matrix effects between samples at final concentrations of 2 (B_2) or 5 pg/uL (B_1, B_7).

Limit of detection, precision, carry over

Limits of detection and quantification were defined as three and ten times, respectively, the standard deviation of the most dilute concentration in the calibration curve over several analyses, using the more intense transition (Table 1), though the 2nd transition was always present at LOQ.[28] Intra- and inter-day precision were defined as standard deviation of five injections. MQ blanks were run after the standard curve and between each sample to avoid any carry-over effect, although carry-over was always <1% of the sample peak in these blanks.

RESULTS AND DISCUSION

Here we present a new SPE and LC/MS-based method for the analysis of B_1, B_2, B_6, B_7 and the four major forms of cobalamin (B_{12}) in seawater. This method reduces sample processing time, explicitly accounts for matrix effects by using isotope labeled internal standards, allows chromatographic separation of the four forms of B_{12}, and improves detection limits beyond previous methods.[12,13]

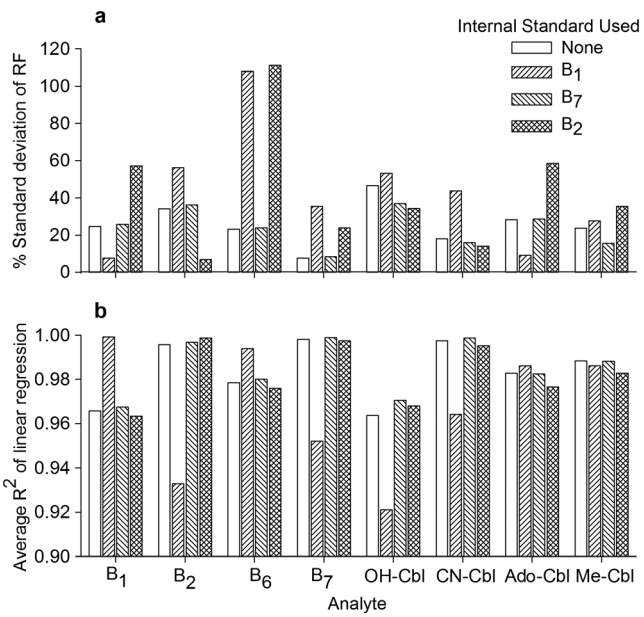

Figure 3. Identification of appropriate standard for analyte quantification. For each analyte, a linear regression was performed on calibration curves prepared in three different matrices using different isotope-labeled internal standards (B_1, B_2, B_7). (a) Standard deviation of the response factor (RF) for each analyte-internal standard pair; (b) average R^2 of the linear regressions in three matrices.

The published stable pH range for all analyzed cobalamins is pH 4–7,[29] with lower pHs resulting in lower retention on a C18 SPE and acid-induced heterolysis of Ado-Cbl.[11,30] Me-Cbl and Ado-Cbl are particularly labile, so we focused on decreasing processing time (from 66 to 8 h for the preconcentration step) and eliminating the 2nd SPE (pH 2) step required in previously published methods.[11] We found poorer recoveries of B_1 (37%) and B_7 (48%) when the pH 6.5 SPE was followed by a pH 2 SPE on a single C18 resin. We therefore chose to eliminate the pH 2 SPE step, which halved the processing time and reduced the potential for methodological artifacts, especially for Me-Cbl and Ado-Cbl.

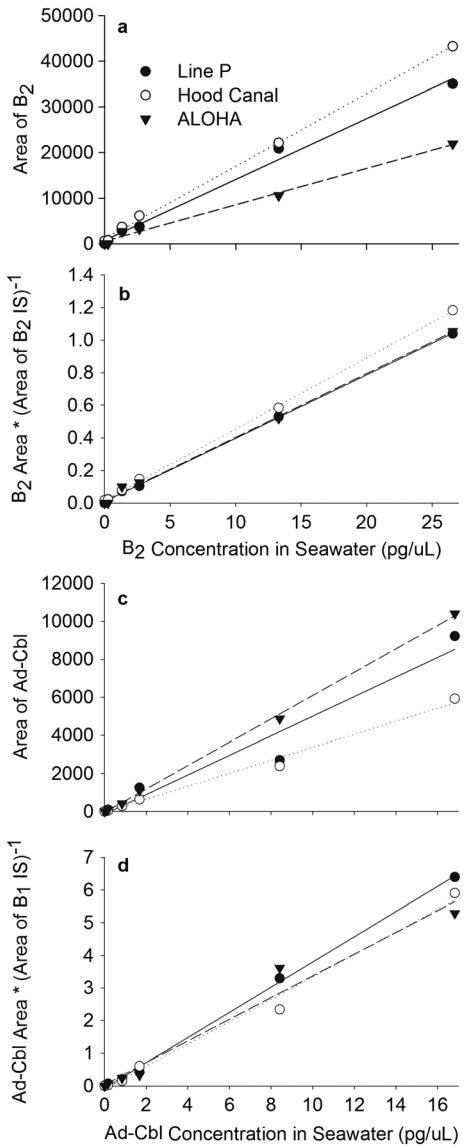

Figure 4. Example standard curves of B_2 (a, b) and Ado-Cbl (c, d) in three different matrices: Line P Station P4, closed circles with solid regression line; Hood Canal, open circles with dotted regression line; and ALOHA, closed triangles with dashed regression line. Plots of raw concentration vs peak areas, for B_2 (a) and Ado-Cbl (c) demonstrate the effect of matrix on instrument response. Plots of concentration vs peak area normalized to internal standard peak area for B_2 (b) and Ado-Cbl (d) demonstrate the uniformity of slopes obtained after normalization.

Our method reduces the required volume 4–8-fold and involves a single SPE step, saving over 58 h of time in the preconcentration step per sample.[12,13]

Method validation

Recoveries for all analytes during the SPE step were high (>70%) at a flow rate of 1 mL min^{-1} and pH 5.5–6.5, except B_1 and B_7, which were never higher than 55% and 67%, respectively (Table 2). The poorer recovery for B_1 is likely due to breakthrough on the C18 SPE column, as the chromatography indicates that this very polar molecule with a quaternary amine elutes with the solvent front at 0.61 min (Fig. 2). The method was specifically optimized for the B_{12} forms.

We used ^{13}C-isotope-labeled internal standards of vitamins B_1, B_2, and B_7 to account for matrix effects. A standard curve was generated for each analyte in the three different seawater matrices using three different isotope-labeled internal standards (B_1, B_2, and B_7). The standard curves were evaluated based on variation in the response factors in the matrix and the R^2 values of the resulting linear regressions. We normalized the peak areas of each analyte to each isotopically labeled internal standard. The standard deviation of the response factor of each internal standard against each analyte (Fig. 3(a)) represents the degree of variation observed in samples analyzed in each of the matrices. Both the standard deviation of the response factors and the R^2 of the linear regression produced by internal standard normalized curves (Fig. 3(b)) were used to select the best internal standard for each compound (Table 2). We found that in all cases except B_6, using a standard curve normalized to an internal standard corrected the data for variations in the response factor in different matrices (Fig. 4).

Standard addition calibration curves for all analytes gave high R^2 values (>0.97 with internal standards), and low limits of detection (<0.3 pM in seawater), with the exception of B_7, which had a much greater matrix effect (Table 2). The low response factor of B_7 in matrix is likely due to coelution of other organic molecules, which introduced large errors in peak integrations (Fig. 2). Our detection limit was lower than those reported using existing methods for B_1, B_6, B_2, and CN-Cbl, despite using only 25% of the original sample size, but was higher for B_7 due to a large matrix effect. Measurements of vitamins B_1 and B_7 were associated with large errors and poor recoveries, which may explain our inability to reproduce previously reported recoveries.[12] Intra- and inter-day precision emphasized the importance of running a matrix curve and samples on the same day in a single batch (Table 2).

Application

We applied our new method to a depth profile from the Hood Canal (Puget Sound, Washington) field site (Fig. 5). Vitamin concentrations varied with depth and were generally higher in surface waters, lower at mid-depth and then increased in our deepest samples. B_1 concentrations ranged from 0.58 to 1.5, B_2 from 45 to 128, B_6 from 1.3 to 5.7, and B_7 from <0.9 to 16.9 pM. Throughout the water column, B_{12} concentrations were dominated by OH-Cbl (1.56 to 5.8 pM) with Ado-Cbl contributing a significant percentage of the B_{12} pool, especially at the surface (<0.14 to 1.2 pM). By contrast, Me-Cbl and traditionally measured CN-Cbl were much lower, never contributing more than 2% (Me-Cbl) or 21% (CN-Cbl) to total B_{12} inventories at any given depth.

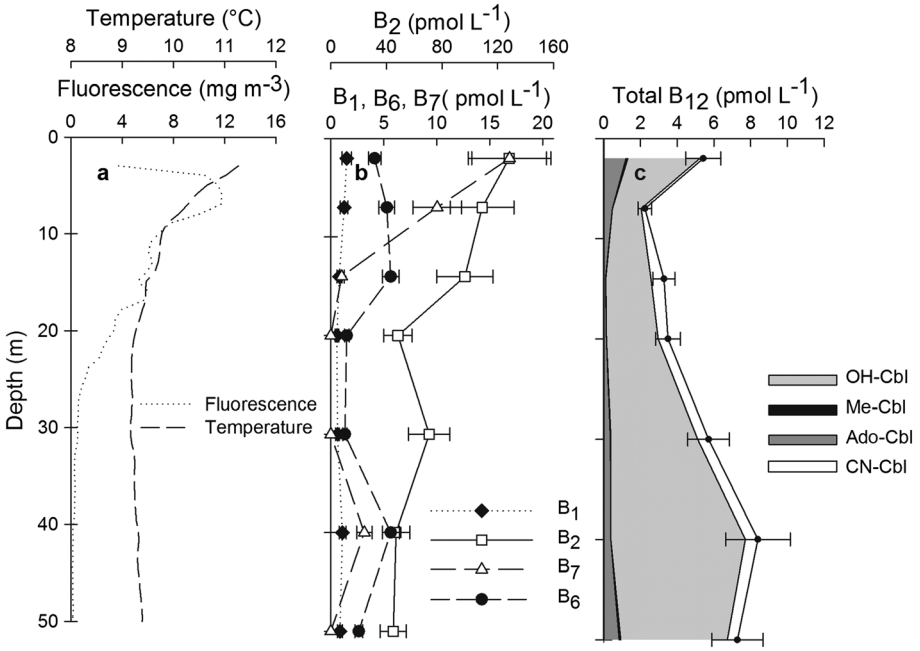

Figure 5. Depth profile of vitamin concentrations in the Hood Canal. (a) Temperature and fluorescence; (b) B_1, B_2, B_6, B_7; (c) Total B_{12} with individual contributions (of CN-Cbl, OH-Cbl, Me-Cbl, and Ado-Cbl) to the pool shown by shaded regions. Data are the means of duplicate measurements for all vitamins. Error bars represent the standard deviation mainly associated with the preconcentration step, which introduces the largest source of error in the method.

Rapid Communications in Mass Spectrometry

The most extensive published datasets of direct measurements of B_{12} in the ocean measure the CN-Cbl form. In the Hood Canal system, the dominant cobalamin was OH-Cbl, a degraded but still bioavailable product of other B_{12} forms, with CN-Cbl contributing little to the pool of B_{12} at most depths (<0.7 pM, Fig. 5).[5,12] Previously published MS methods[12] would suggest a B_{12} concentration an order of magnitude lower than the entire cobalamin pool in the Hood Canal by not including OH-Cbl in the quantification of dissolved B_{12}. Suárez-Suárez *et al*. also found the B_{12} pool distributed between the four forms in surface waters of Palma de Mallorca Bay, Spain,[13] corroborating that measurement of the different forms of B_{12} is paramount when attempting to evaluate the availability of dissolved B_{12} to marine communities.

Our data and those of Sañudo-Wilhelmy and coworkers[12] are the only published B_2, B_6, and B_7 concentrations in seawater. Our B_6 and B_7 values are of the same order of magnitude as those previously reported, while our values for vitamin B_2 are consistently two orders of magnitude higher than those previously reported in seawater (<0.7 pM to 7 pM).[12] Our B_1 values are consistently lower than previously reported,[12] which may reflect a systematic underestimation due to poor recovery, despite the values being corrected for our estimated SPE recovery efficiencies. Concentrations of B vitamins can vary dramatically over space and depth and often vary independently of one another.[5,12] The use of consensus reference materials would bolster the ability to compare datasets between labs. We are just beginning to understand the spatial and temporal variability of these metabolites, but accurate measurements of labile B_{12} forms in the smaller sample volumes processed here should allow us to better constrain the variability through time series and rate experiments. There are no published methods for direct measurement of vitamins in marine particulates, which may be a large pool of vitamins in the ocean.

It is unclear whether measurements of the thiamine or pyridoxine (the B_1 and B_6 forms measured in this study respectively) are representative of the suite of molecules that make up the total pool of those vitamins. Some forms of B_1 and B_6 are extremely polar, which would not be efficiently retained on the SPE column (like the form of B_1 analyzed in this study). The ecological importance of the varied forms and moieties of B_1 is only beginning to be understood,[5,31] and a second methodology utilizing a concentrating mechanism that incorporates these polar molecules would further improve our ability to study these compounds.

CONCLUSIONS

In summary, we have improved upon existing methods to simultaneously quantify four forms of vitamin B_{12} as well as B_1, B_2, B_6, and B_7. The method greatly decreases sample size, processing time, and pH manipulations. We quantify our analytes using an isotope-labeled internal standard, giving us high confidence that in a complex mixture of organic molecules a change in the analytical response is due to a change in concentration rather than a difference in matrix effects. We applied this technique by quantifying the eight analytes throughout a water column in the Hood Canal, Washington. This work demonstrates the importance of measuring all forms of B_{12} in order to understand the cycling of this important nutrient in the marine environment.

Acknowledgements

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