

## RESEARCH ARTICLE

# Method optimization of the simultaneous detection of B<sub>12</sub> congeners leading to the detection of a novel isomer of hydroxycobalamin in seawater

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**Rationale:** More than half of surveyed microalgae and over 90% of harmful algae have an obligate requirement for vitamin B<sub>12</sub>, but methods for directly measuring dissolved B<sub>12</sub> in seawater are scarce due to low concentrations and rapid light-induced hydrolysis.

**Methods:** We present a method to detect and measure the four main congeners of vitamin B<sub>12</sub> dissolved in seawater. The method includes solid-phase extraction, separation by ultrahigh-performance liquid chromatography and detection by triple-quadrupole tandem mass spectrometry utilizing an electrospray ion source. This method was applied to coastal field samples collected in the German Bay, Baltic Sea and the Danish Limfjord system.

**Results:** The total dissolved B<sub>12</sub> pool ranged between 0.5 and 2.1 pM. Under ambient conditions methyl-B<sub>12</sub> and adenosyl-B<sub>12</sub> were nearly fully hydrolyzed to hydroxy-B<sub>12</sub> in less than 1 h. Hydroxy-B<sub>12</sub> and a novel, corresponding isomer were the main forms of B<sub>12</sub> found at all field sites. This isomer eluted well after the OH-B<sub>12</sub> peak and was also detected in commercially available OH-B<sub>12</sub>. Both compounds showed very high similarity in their collision-induced dissociation spectra.

**Conclusions:** The high instability of the biologically active forms of Me-B<sub>12</sub> and Ado-B<sub>12</sub> towards hydrolysis was shown, highlighting the importance of reducing the duration of the extraction protocol. In addition, the vitamin B<sub>12</sub> pool in the study area was mostly comprised of a previously undescribed isomer of OH-B<sub>12</sub>. Further studies into the structure of this isomer and its bioavailability are needed.

## 1 | INTRODUCTION

Phytoplankton, including microalgae and cyanobacteria, and bacterioplankton are the base of the oceanic food web and are responsible for roughly 50% of global primary production.<sup>1,2</sup> Thus, they play a major role in the biogeochemical cycles of most major elements in the ocean, including carbon, nitrogen, phosphorus and silicon.<sup>3</sup> Even though animals have an obligate requirement

(are auxotrophic) for vitamin B<sub>12</sub> and thus need to acquire it through their diet, it is only produced by few bacteria and archaea.<sup>4</sup> In contrast, higher plants and fungi neither synthesize nor require vitamin B<sub>12</sub>, but instead possess B<sub>12</sub>-independent pathways (e.g. for methionine synthesis).<sup>5,6</sup> Microalgae on the other hand are photosynthetic organisms with a high occurrence (>50%) of vitamin B<sub>12</sub> auxotrophy.<sup>4,5,7,8</sup> Interestingly, there seems to be no apparent evolutionary pattern to this requirement with groups losing or

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gaining B<sub>12</sub>-dependent pathways throughout history.<sup>4</sup> In the last decades many phytoplankton species have been identified as auxotrophic for one or more B-vitamins (thiamin (B<sub>1</sub>), biotin (B<sub>7</sub>), B<sub>12</sub>) confirming that B-vitamin requirement in algae is the norm rather than the exception.<sup>7</sup> More recently, vitamin enrichment studies have illustrated that vitamin B<sub>12</sub> concentrations can significantly influence growth rates of phytoplankton communities in coastal and open ocean environments by co-limiting phytoplankton biomass with other nutrients (e.g. iron, nitrogen).<sup>9–13</sup>

Vitamin B<sub>12</sub> itself is an organometallic tetrapyrrolic cofactor, which is structurally and biosynthetically related to heme and chlorophyll.<sup>14</sup> Many essential metabolic processes are catalyzed by B<sub>12</sub>-dependent enzymes, such as intramolecular rearrangements, methylations and reduction of ribonucleotides to deoxyribonucleotides.<sup>6,15,16</sup> B<sub>12</sub> consists of a central cobalt ion that is coordinated to an  $\alpha$ -ligand of 5,6-dimethylbenzimidazole (DMB) known as the nucleotide loop and a  $\beta$ -ligand, such as methyl, adenosyl, cyano or hydroxy group (Me, Ado, CN, OH, respectively).<sup>15</sup> While the biologically active forms are Me-B<sub>12</sub> and Ado-B<sub>12</sub>, CN-B<sub>12</sub> and OH-B<sub>12</sub> have also been isolated from natural sources such as dairy products.<sup>17,18</sup> It is assumed that even though CN-B<sub>12</sub> and OH-B<sub>12</sub> cannot be utilized for intracellular functions, they are actively transformed into the active cofactors Me-B<sub>12</sub> and Ado-B<sub>12</sub> after transportation into the cell.<sup>19</sup> This transportation via a complex capturing mechanism has been reported to not distinguish between the various B<sub>12</sub> derivatives in mammals and bacteria.<sup>20–22</sup> It is not yet clear however if this is also the case for phytoplankton and if the  $\beta$ -ligand influences the respective bioavailability. Consequently, it is beneficial to distinguish the four B<sub>12</sub> congeners in biological studies.

Historically, vitamin B<sub>12</sub> concentrations were quantified with a laborious and error-prone bioassay method using B<sub>12</sub>-requiring microalgae.<sup>23,24</sup> Today's methods directly measure vitamin B<sub>12</sub> concentrations in seawater through a series of steps including

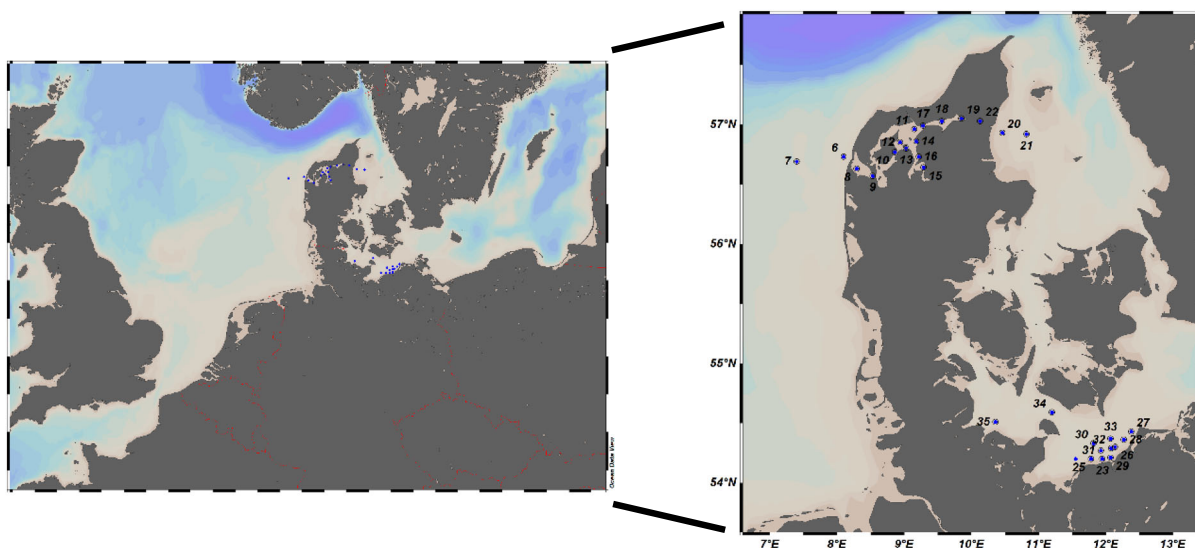
filtration and preconcentration followed by analytical detection. Okbamichael et al developed a C18 solid-phase extraction protocol followed by high-performance liquid chromatography (HPLC) separation and detection of CN-B<sub>12</sub> using ultraviolet-visible spectroscopy.<sup>25,26</sup> More recently advances have been made to establish simultaneous detection of all four B<sub>12</sub> congeners via triple-quadrupole mass spectrometry.<sup>27,28</sup> These new methods reduced sample volume and processing time, thereby making vitamin B<sub>12</sub> data collection more accessible for marine sciences. However, even though these studies have increased knowledge about the importance and detection of B<sub>12</sub> in marine environments, the cycling of B<sub>12</sub> in the oceans remains mostly unexamined as data of particular and dissolved B<sub>12</sub> concentrations are scarce. This lack of data is partly a consequence of the low vitamin concentrations (picomolar range) in seawater and rapid light-induced hydrolysis of Ado-B<sub>12</sub> and Me-B<sub>12</sub> to OH-B<sub>12</sub>.

The work presented here aimed both to set up and improve the previously established liquid chromatography tandem mass spectrometry (LC/MS/MS) methods for the simultaneous detection of four vitamin B<sub>12</sub> congeners and to gather additional information on the stability of B<sub>12</sub> towards light-induced hydrolysis.<sup>27,28</sup> The modified method was then applied to field samples collected from a variety of northern European marine systems.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

Environmental samples were taken in coastal waters in the German Bight, Danish Limfjord and western Baltic Sea (Figure 1). Seawater used for the following experiments was taken with Niskin bottles mounted on a conductivity-temperature-depth



**FIGURE 1** Sampling stations of the expedition with R/V Uthörn in autumn 2020; stations 6–18 were sampled in the middle of August and stations 23–35 in late September [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

rosette and subsequently mixed 1:1 from 10 m (or maximum depth) and surface water. Seawater was filtered immediately over 0.2  $\mu\text{m}$  filters (AcroPak™ 1500, Pall Corporation, Port Washington, USA) and collected in polyethylene bottles. During sample collection and subsequent processing, care was taken to minimize the exposure of samples to light by wrapping all bottles and hoses in tinfoil.

## 2.2 | Preconcentration

A preconcentration system was set up using a peristaltic pump with rotor heads (Multi Channel Cassette Pump 205S, Watson-Marlow Limited, Falmouth, UK). Self-packed 3 mL C18 resin columns (HF-Bondesil C18, Agilent Technologies) and commercially available 1 and 6 mL C18 columns (Supelclean™ LC-18, Supelco, Deisenhofen, Germany) were used for preconcentration. In the case of self-packed columns, about 2–3 mL of the resin was loaded onto the column and conditioned with 6 mL of HPLC-grade methanol (Merck, Darmstadt, Germany), followed by at least 10 mL of deionized water. Conditioning followed by washing with water was also performed for commercially available columns. The pH of filtered seawater samples was adjusted to 6.2–6.6 with HCl (1 M) and loaded onto the solid-phase-extraction (SPE) column at a flow rate of approximately 1 mL min<sup>-1</sup>. SPE columns were kept wet during the entire process and were washed with 10 mL of deionized water before freezing them at  $-20^{\circ}\text{C}$  until further processing in the laboratory. SPE columns were then thawed, additionally flushed with 2% methanol in deionized water, followed by sample elution with 10 mL of 100% methanol. Clogged columns had to be eluted with compressed air (2–5 bar) and additionally spin-filtered (Millipore Ultrafree 0.45  $\mu\text{m}$ , Eschborn, Germany) to remove co-eluted column material. The samples were concentrated using a SpeedVac (SC210A-230, Thermo Fisher Scientific, Schwerte, Germany) at  $25^{\circ}\text{C}$ , re-dissolved in methanol (100  $\mu\text{L}$ ) and stored at  $-20^{\circ}\text{C}$  in the dark until mass spectrometric analysis. An initial subset of field samples was analyzed in order to characterize the background noise. If a high nonpolar background was detected in this subset of samples, the remaining samples were further extracted with *n*-hexane (Merck) in a HPLC vial and stored again at  $-20^{\circ}\text{C}$  until mass spectrometric analysis.

**TABLE 1** MS conditions and retention times for each vitamin B<sub>12</sub> congener; second SRM transition (in italics) used to confirm identity

Analyte	SRM	CE (V)	CV (V)	C <sub>cap</sub> V (kV)	RT (min)
OH-B <sub>12</sub>	665.3 → 147.4 (534.0)	35	10	0.5	2.35
CN-B <sub>12</sub>	678.7 → 147.4 (534.0)	45	10	0.5	2.96
Iso-OH-B <sub>12</sub>	665.3 → 147.4 (534.0)	35	25	0.75	3.35
Ado-B <sub>12</sub>	790.8 → 147.4 (592.0)	50	6	0.25	3.44
Me-B <sub>12</sub>	673.2 → 147.4 (592.0)	45	30	0.25	4.02

SRM, single reaction monitoring; CE, collision energy; CV, cone voltage; C<sub>cap</sub>V, capillary voltage; RT, retention time.

## 2.3 | UHPLC/MS conditions

An ultrahigh-performance liquid chromatography (UHPLC) electrospray ionization (ESI) MS/MS method was adapted from previously published methods.<sup>23,24</sup> An UHPLC system coupled to a triple quadrupole (ACQUITY/Xevo TQ-XS, Waters, Eschborn, Germany) was used to analyze all samples and standards. Separation was performed on a high strength silica (HSS) C18 column (2.1 × 100 mm, 1.8  $\mu\text{m}$ , ACQUITY, Waters) equipped with a pre-column (HSS C18, 1.8  $\mu\text{m}$ , VanGuard™, Waters) at a column temperature of  $30^{\circ}\text{C}$ . Water was deionized and purified (Milli-Q, Millipore, Eschborn, Germany) to 18 M $\Omega$  cm<sup>-1</sup> or better quality. Formic acid (90%, p.a.), ammonium formate (p.a.) and all organic solvents (methanol and acetonitrile) were purchased from Merck in HPLC and UHPLC grade, respectively. All samples were stored in opaque HPLC vials (Agilent Technologies, Waldbronn, Germany) at  $-20^{\circ}\text{C}$ . Column elution was performed with aqueous solvent (A) containing ammonium formate (10mM) adjusted to pH = 4.0 with formic acid and 80:20 acetonitrile-methanol as an organic solvent (B). A linear gradient was employed for 4.3 min (from 98:2 to 70:30 solvent A:B), followed by organic and aqueous flushing and re-equilibration. The flow rate was 0.6 mL min<sup>-1</sup> and all analyses were performed in positive ion mode using selective reaction monitoring (SRM). The ESI source temperature was  $130^{\circ}\text{C}$  with a desolvation temperature of  $600^{\circ}\text{C}$ . Collision energies, cone and capillary voltages and retention times for each congener are listed in Table 1.

Each vitamin was screened by two transitions in the SRM mode, whereby the first transition was used for quantification and the second transition was used to confirm the identity (Table 1). The mass spectrometric parameters consisting of the collision energy, cone voltage and capillary voltage were optimized for each individual transition.

## 2.4 | Light-induced hydrolysis of B<sub>12</sub> congeners

Triplicates of two concentrations (10 and 100 nM) of each B<sub>12</sub> congener were exposed to ambient natural light (20–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) in both transparent glass and opaque vials. The experiment was conducted at  $20^{\circ}\text{C}$  in deionized water adjusted to pH 6.5 with 1 M HCl. The light radiation was measured with a light data-logger (ULM-500, Heinz Walz GmbH, Effeltrich, Germany) outside of the respective vials. Vitamin B<sub>12</sub> concentrations were quantified at  $t = 0, 1, 13$  h and at  $t = 28$  days.

## 2.5 | SPE recovery rates

Duplicate North Sea seawater samples (2 L) were spiked with each vitamin B<sub>12</sub> congener (ca 100 pM) and concentrated as described in Section 2.2. In addition, untreated seawater in duplicates was concentrated and the obtained values were subtracted from those of each vitamin B<sub>12</sub>-spiked sample.

## 2.6 | OH-B<sub>12</sub> isomer

A standard solution of OH-B<sub>12</sub> in deionized water and in deionized water adjusted to pH 8.2 according to the pH in seawater was prepared and OH-B<sub>12</sub> concentrations were subsequently analyzed via mass spectrometry on a regular basis (Figure A2). In addition, greater amounts of the isomer of OH-B<sub>12</sub> (Iso-OH-B<sub>12</sub>) were prepared by lowering the pH and adding the Lewis acid FeCl<sub>3</sub> to a standard solution of OH-B<sub>12</sub>, followed by incubation for 1 h.

## 2.7 | Quantification

### 2.7.1 | Preparation and storage of standards

Me-B<sub>12</sub>, Ado-B<sub>12</sub> and OH-B<sub>12</sub> (as HCl salt) were obtained from Sigma-Aldrich (Taufkirchen, Germany; CAS 13422-55-4, 13870-90-1, 59461-30-2, respectively) and CN-B<sub>12</sub> was obtained from Fisher BioReagents (CAS 68-19-9). Stock solutions were prepared by dissolving approximately 10 mg of cobalamin in 50 mL of methanol. A subsequent dilution series was prepared to obtain final concentrations (usually 1, 2.5, 5, 10 nM) in the range of measured samples. Stock solutions and all prepared standards were prepared as fresh as possible and stored at -20°C in the dark.

### 2.7.2 | Field application

All vitamin B<sub>12</sub> values represent the mean of duplicate samples and were quantified using a four-point linear regression curve (1, 2.5, 5, 10 nM) of the respective standard in methanol. Me-B<sub>12</sub> concentrations were only calculated through a three-point linear regression (1, 5, 10 nM). Iso-OH-B<sub>12</sub> was expressed as OH-B<sub>12</sub> equivalents. In addition, in order to evaluate the replicability of the SPE preconcentration, four samples were collected at two stations in the Baltic Sea (stations 32 and 33, Figure 1).

### 2.7.3 | Data correction

Since Me-B<sub>12</sub> and Ado-B<sub>12</sub> are very sensitive towards hydrolysis, it was not possible to obtain standard calibration curves without substantial amounts of OH-B<sub>12</sub>. Thus, the obtained peak integral of a standard measurement had to be corrected by the detected amount

of OH-B<sub>12</sub>. This amount of OH-B<sub>12</sub> was quantified using the OH-B<sub>12</sub> standard and Ado-B<sub>12</sub> and Me-B<sub>12</sub> concentrations were corrected accordingly. This data correction was not necessary for CN-B<sub>12</sub> due to its higher stability.

In addition, the transition used for quantification of OH-B<sub>12</sub> consistently had an impurity that could not be removed. This impurity fluctuated between 1 and 2 nM depending on the sampling day; however, it was consistent within each sample run (SD < 5%). Thus, the average blank was calculated from at least 15 blank samples spread over the whole sample run and subtracted from the obtained OH-B<sub>12</sub> values. The duplicate was evaluated as not detected if this resulted in one or two of the duplicates receiving a negative value.

Finally, vitamin B<sub>12</sub> concentrations were corrected according to the SPE recovery rates.

### 2.7.4 | Limit of detection (LoD) and limit of quantification (LoQ)

LoDs and LoQs were defined as signal-to-noise ratio (S/N) = 3 and 10, respectively. The S/N ratio was determined as the mean of one high- and one low-noise region using MassLynx V4.2 SCN982 software (Waters).

## 3 | RESULTS

### 3.1 | LC/MS/MS conditions

Similar to a previous study, an aqueous eluent consisting of ammonium formate (10 mM) and 0.02% formic acid at pH 4 was found to be optimal. Increasing the buffer and/or the concentration of formic acid did not significantly increase the ionization efficiency, so lower concentrations were used to prolong column lifetime and to prevent contamination of the MS instrument.<sup>27</sup> In contrast to Heal et al and Suffridge et al, using solely methanol or acetonitrile as organic eluents resulted in poor peak separation in this study.<sup>27,28</sup> However, a mixture of acetonitrile and methanol (4:1) reduced peak tailing and additionally resulted in a slightly higher elution strength in comparison to solely acetonitrile, thereby also reducing sampling time.

### 3.2 | Ionization parameters

The ionization parameters were optimized to favor formation of (ideally) one specific molecular ion for each vitamin B<sub>12</sub> congener. This turned out to be the [M + 2H]<sup>2+</sup> ion for all congeners except for OH-B<sub>12</sub>. During the ionization process of OH-B<sub>12</sub>, the Co-OH bond almost completely dissociated resulting in an [M + 2H - 17]<sup>2+</sup> ion which has already been observed in previously published methods.<sup>27,28</sup> This transition is thus not specific to OH-B<sub>12</sub>, but rather pertains to cobalamins in general since removal of the β-ligand results in a molecular framework shared by all cobalamins.<sup>16</sup> This dissociation

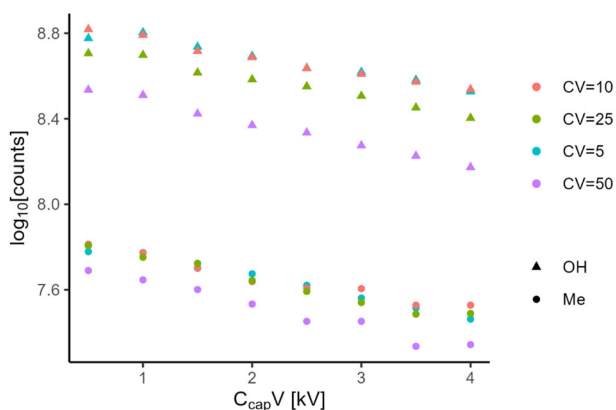
of the  $\beta$ -ligand can also be observed for all other B<sub>12</sub> congeners, however only to a negligible extent.

The highest ionization efficiency was obtained when the desolvation temperature and all interconnected gas flow parameters were set to the highest device-specific settings (Figure A1). These were maintained for the optimization of all other parameters, thereby additionally contributing to a reduction in contamination of the ion source. However, the capillary and cone voltages had major influences on the ionization efficiency of the highest abundant vitamin B<sub>12</sub> ions. A clear trend to higher ionization efficiencies at low capillary voltages was found for all B<sub>12</sub> congeners (Figure 2).

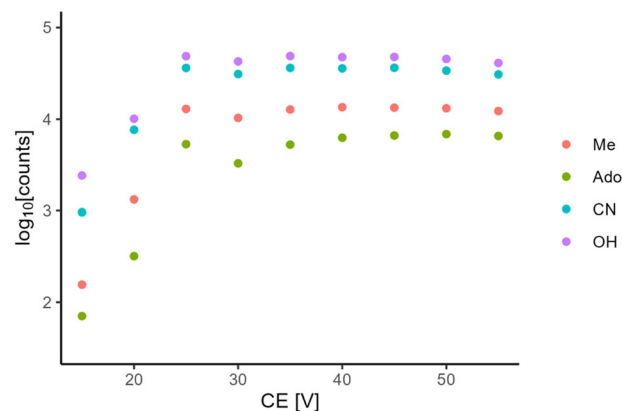
### 3.3 | Fragmentation optimization

After optimization of the ionization parameters, collision-induced dissociation (CID) spectra of each B<sub>12</sub> congener were recorded and the collision energy was adjusted to achieve maximum intensities of the first and second most abundant transitions. In both cases the most abundant fragmentation ion for all B<sub>12</sub> congeners turned out to result in  $m/z$  147 representing cleavage of the DMB moiety. The second most abundant fragmentation ion gave  $m/z$  592 for Me-B<sub>12</sub> and Ado-B<sub>12</sub> and  $m/z$  534 for CN-B<sub>12</sub> and OH-B<sub>12</sub> (Table 1). The fragmentation efficiency increased sharply after 20 V, but only showed minor variations in the range 25–55 V (Figure 3).

Furthermore, it was found that solvent effects of both field samples and standard solutions generally resulted in lower peak integrals when dissolved in water compared to methanol (data not shown). However, the solubility of Ado-B<sub>12</sub> is significantly lower in methanol compared to water (qualitative observation during standard preparation). Thus, strong vortexing and the use of an ultrasonic bath were necessary to prepare higher-concentration (1  $\mu$ M) Ado-B<sub>12</sub> stock solutions.



**FIGURE 2** Influence of capillary voltage on ionization efficiency of the most abundant ions of Me-B<sub>12</sub> (circle) and OH-B<sub>12</sub> (triangle) at four different cone voltages (5, 10, 25, 50 V), representing the least and most polar of the four quantified B<sub>12</sub> congeners, respectively [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



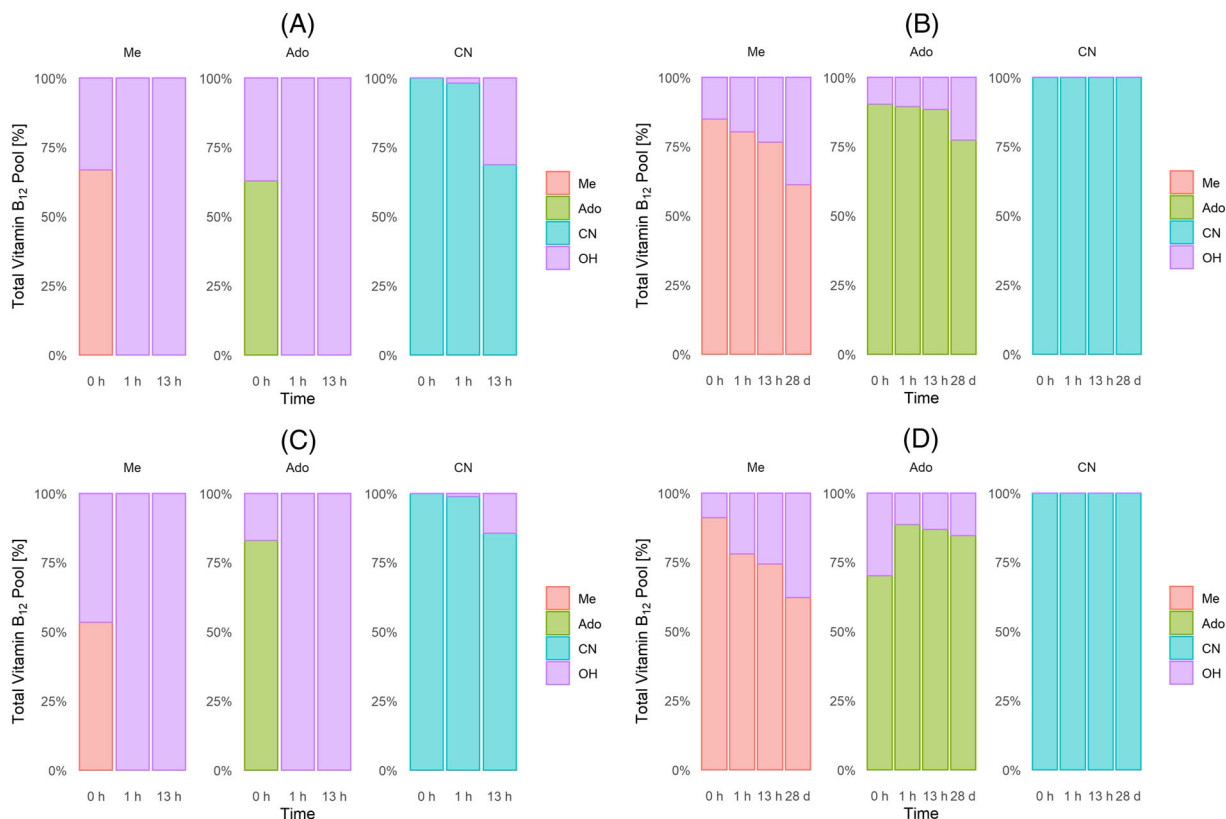
**FIGURE 3** Influence of collision energy on fragmentation efficiency of the quantification trace of four vitamin B<sub>12</sub> congeners [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### 3.4 | Light-induced hydrolysis of B<sub>12</sub> congeners

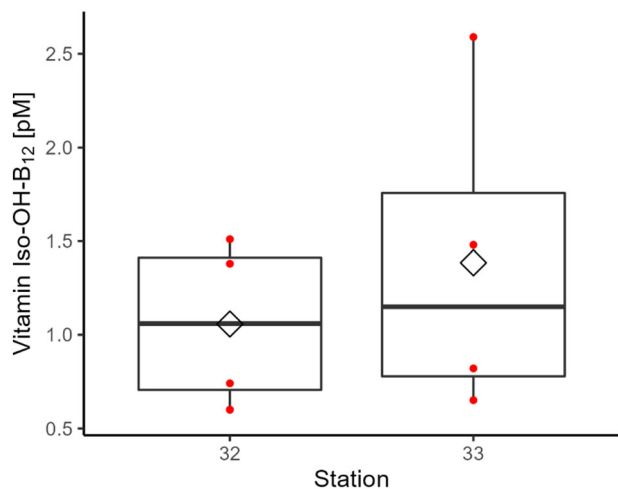
Both Me-B<sub>12</sub> and Ado-B<sub>12</sub>, at concentrations of 10 and 100 nM, were fully hydrolyzed (>99%) to OH-B<sub>12</sub> after 1 h when exposed to the mild acidic conditions (pH 6.2–6.6) of the SPE preconcentration and ambient light irradiation (Figure 4). In contrast, CN-B<sub>12</sub> was only partially hydrolyzed after 1 h (2%; Figure 4). Hydrolysis for all congeners was reduced in opaque compared to transparent vials, resulting in 20%, 10% and 0% conversion for Me-B<sub>12</sub>, Ado-B<sub>12</sub> and CN-B<sub>12</sub> after 1 h, respectively. Nevertheless, hydrolysis of Me-B<sub>12</sub> and Ado-B<sub>12</sub> was also observed even when the samples were mostly protected from light irradiation by utilizing opaque vials.

### 3.5 | Replicability of SPE preconcentration step and recovery rates

The preconcentration step through SPE has previously been described as the highest source of error in the analysis of vitamin B<sub>12</sub>.<sup>27,28</sup> The analysis of four replicate samples, collected at two different stations and preconcentrated using the standard SPE protocol resulted in total vitamin B<sub>12</sub> values of  $1.06 \pm 0.46$  pM at station 32 and  $1.46 \pm 0.97$  pM at station 33 (Figure 5). Furthermore, the SPE preconcentration step is likely also responsible for the high background observed during mass spectrometric analysis in this study. This background could be partly reduced by using a gradient of methanol in water, thereby sequentially eluting the different vitamin B<sub>12</sub> derivatives. However, this is unpractical as water is substantially more difficult to remove than methanol after elution of the column and would result in prolonged sampling times. Nevertheless, flushing the C18 columns with 2% methanol in water prior to vitamin extraction followed by liquid extraction of the samples with *n*-hexane reduced the



**FIGURE 4** Light-induced hydrolysis of vitamin B<sub>12</sub> congeners under ambient light irradiation in transparent (A, C) and opaque (B, D) vials with a starting concentration of  $c_0 = 100$  nM (A, B) and 10 nM (C, D); each bar represents the mean of at least duplicates, mostly triplicates; h, hours, d, days [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 5** Boxplot of Iso-OH-B<sub>12</sub> concentration of four replicates taken at two sampling stations during an expedition with the R/V Uthörn; corresponding sampling area and stations are shown in Figure 1 [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

background and is fast and easy to incorporate into the existing methods.

The recovery rates of the SPE protocol for Me-B<sub>12</sub>, Ado-B<sub>12</sub>, CN-B<sub>12</sub> and OH-B<sub>12</sub> were 41%, 17%, 77% and 62%, respectively.

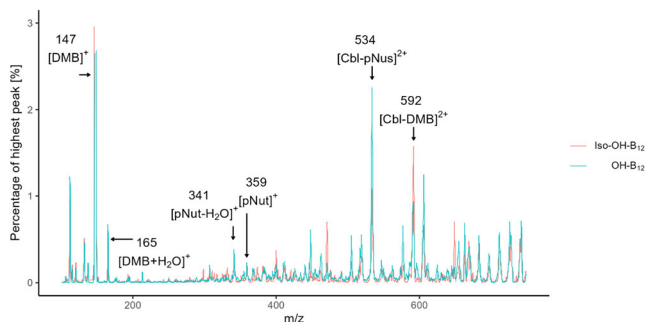
### 3.6 | OH-B<sub>12</sub> isomer

OH-B<sub>12</sub> is the most polar vitamin B<sub>12</sub> congener and thus the first to elute from the hydrophobic C18 column. OH-B<sub>12</sub> could only be quantified with confidence in combination with the retention time and/or qualifier ion trace as the transition selected for the determination of OH-B<sub>12</sub> is not specific for OH-B<sub>12</sub> but shared by all cobalamins. In highly concentrated stock solutions (100  $\mu$ M), OH-B<sub>12</sub> accounted for more than 99% of total isomer abundance and was more stable towards isomerization. Here, we only present data and spectra of OH-B<sub>12</sub> and the main observed isomerization product Iso-OH-B<sub>12</sub>, as these were the only variants detected in field samples. This isomerization was observable even under mild conditions such as using deionized water, where it occurred faster than in deionized water adjusted with NH<sub>3</sub> to pH 8.2 (pH of seawater; Figure A2). After three months, 23% of Iso-OH-B<sub>12</sub> could be observed in deionized water compared to only 15% in NH<sub>3</sub>. However, this conversion was rapidly enhanced by lowering the pH and adding Lewis acids, such as FeCl<sub>3</sub> (Figure A3). These conditions allowed us to obtain CID spectra of Iso-OH-B<sub>12</sub>, which showed a very high similarity with OH-B<sub>12</sub> (Figure 6). Furthermore, the molecular ions  $[M + H]^+$ ,  $[M + 2H]^{2+}$  and  $[M + 2H - 17]^{2+}$  with  $m/z$  of 1347.4, 674.2 and 665.3, respectively, were found in the single ion recording scan of all OH-B<sub>12</sub> variants.



### 3.7 | Method application

We applied our method to coastal field samples collected in the northern and western Baltic Sea and the Danish Limfjord, which connects to the northern and western Baltic Sea (Figure 1). Vitamin B<sub>12</sub> sampling was performed in the frame of a cruise mainly conducted to sample phycotoxins of harmful algae bloom species. For the analysis of phycotoxins, seawater from the surface and 10 m depth was sampled and thus the vitamin B<sub>12</sub> sampling scheme was performed at identical depths to be potentially correlated to any other parameters gathered during the cruise. In general, vitamin B<sub>12</sub> was



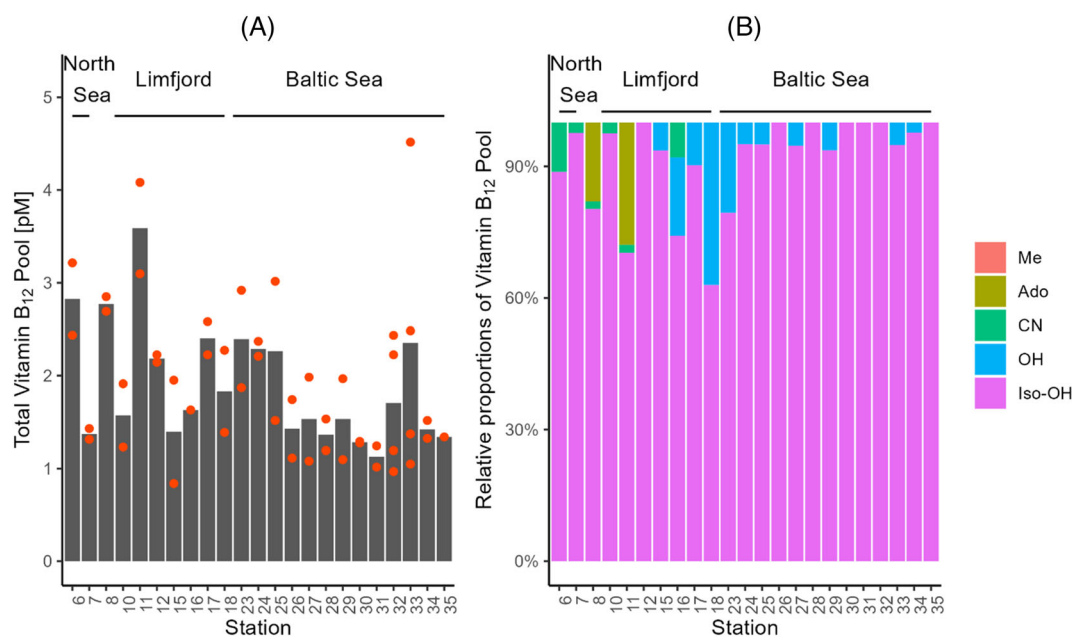
**FIGURE 6** Comparison of CID spectra of OH-B<sub>12</sub> (blue) and its isomer Iso-OH-B<sub>12</sub> (red); peak height is depicted as percentage of the highest peak at  $m/z = 147$ ; molecular structures of highly abundant fragments specific to cobalamins are denoted on top of the peaks. DMB, 5,6-dimethylbenzimidazole; pNut, pseudonucleotide; pNus, pseudonucleosid; Cbl, cobalamin [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

detected at all stations with total B<sub>12</sub> values ranging between 0.5 and 2.1 pM (Figure 7). However, the biologically active Me-B<sub>12</sub> was not measured at any station and Ado-B<sub>12</sub> was detected only at stations 8 and 11. CN-B<sub>12</sub> could be detected at a total of nine stations with values ranging between 0.02 and 0.34 pM representing 1% and 15% of the total dissolved B<sub>12</sub> pool. OH-B<sub>12</sub> was detected at nearly half of all sampling stations (11 of 23 total stations) and ranged from 0.01 to 0.49 pM, while the novel isomer Iso-OH-B<sub>12</sub> was detected at all sampling stations ranging from 0.49 to 1.87 pM. OH-B<sub>12</sub> including the detected isomer Iso-OH-B<sub>12</sub> accounted for >90% of the total dissolved B<sub>12</sub> pool with Iso-OH-B<sub>12</sub> representing the larger fraction by consistently contributing 80% or more (except for station 18). Experimental LoD and LoQ values for all B<sub>12</sub> congeners were below 0.01 and 0.1 pM, respectively. However, the qualification trace was no longer visible at LoQ values.

## 4 | DISCUSSION

### 4.1 | LC/MS/MS conditions

The LC/MS/MS conditions for the detection of cobalamins that resulted from tuning the individual MS parameters on the TQ-XS instrument were similar to those reported by previous studies.<sup>27–29</sup> However, the instrumental LoD value in this study was 10- to 100-fold lower compared to previous studies. Even though the optimization of the capillary voltage was instrumental in increasing the ionization efficiency here, this parameter had not been reported in previous studies. Additionally, this contrasts with many other LC/MS/



**FIGURE 7** (A) Total dissolved vitamin B<sub>12</sub> pool of field samples taken during an expedition with the R/V Uthörn with red points corresponding to single values. (B) Relative proportions of vitamin B<sub>12</sub> congeners. B<sub>12</sub> concentrations were corrected according to the SPE recovery rates. Corresponding sampling area and stations are shown in Figure 1 [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

MS methods, which show highest ionization efficiency at higher capillary voltages (*ca* 3 kV). This effect might be explained by the low stability of cobalamins because the  $[M + 2H]^+$  ions were most abundant at lower capillary voltages, which in turn indicates a reduced dissociation of the  $\beta$ -ligand. In summary, the relatively low capillary voltage of 0.25–0.75 kV may partly explain the lower LoD determined in this study compared to previous studies.<sup>27,28</sup>

Another factor reducing the LoD is the use of methanol as solvent of field samples and standard solutions, which increased the ionization efficiency and thus sensitivity of cobalamin detection. Furthermore, the use of methanol as sample solvent is more convenient in sample handling as it does not freeze when stored at  $-20^\circ\text{C}$  and additionally reduces the hydrolysis rate of Me-B<sub>12</sub> and Ado-B<sub>12</sub>. Additional drying of the methanol via a molecular sieve or with chemical drying agents may be useful to further reduce the hydrolysis rate. However, additional care must be taken to reduce solvent evaporation and to fully dissolve standards while preparing high-concentration stock solutions as solubility of Ado-B<sub>12</sub> is lower in methanol compared to water.

## 4.2 | Light-induced hydrolysis of B<sub>12</sub> congeners

Previously, photosensitivity of vitamin B<sub>12</sub> congeners was only quantified at high concentrations (10  $\mu\text{M}$ ) and under physiological conditions typically present in humans (pH 7.4).<sup>30</sup> This study extends the available data on the light-induced hydrolysis of B<sub>12</sub> congeners for application in marine science. Hydrolysis was more rapid in lower-concentration solutions exposed to light, emphasizing the need to carefully handle seawater samples in which vitamins are present at picomolar concentrations. It is important to note that this experiment was conducted in deionized water with the aim of quantifying the influence of light and mild acidic conditions on B<sub>12</sub>. Seawater components like a high salt content may further impact the stability of cobalamins and should be investigated. Nevertheless, the results of this experiment emphasize the need to conduct the experimental procedure including filtering, acidifying, SPE and subsequent quantification quickly and with a minimum of light exposure. Otherwise, information about the original B<sub>12</sub> congener distribution may be distorted since continued light exposure will lead to overestimation of the presence and concentration of OH-B<sub>12</sub>. Furthermore, these findings support the importance of reducing the sample volume used for SPE, thus reducing sampling time and thereby lowering the potential for B<sub>12</sub> hydrolysis.

## 4.3 | Replicability of SPE preconcentration step

The preconcentration step through SPE has been described as the highest source of error by other studies.<sup>27,28</sup> Consistent with this, the error margin of the SPE preconcentration step in this study was 50% or higher (Figure 5) as can be seen by the standard deviation

of four identical field samples. While these values were in the range of the other vitamin measurements collected during the expedition, the large standard deviation highlights issues with the SPE method. This large standard deviation causes the larger part of the margin of error of dissolved vitamin B<sub>12</sub> concentrations and should thus be primarily optimized to improve the precision of this method. A possible explanation could be that other components of seawater interfere with the adsorption of vitamin B<sub>12</sub> derivatives to the stationary phase and therefore it may be worthwhile to investigate the use of an additional SPE column upstream of the C<sub>18</sub> column which could filter out interfering compounds without affecting vitamin B<sub>12</sub>. In addition, during field work in this study it was qualitatively observed that higher biomass systems, such as the Danish Limfjord, lead to faster clogging of the SPE columns, thereby affecting the flow rate. This could be the result of high abundance of particles smaller than 0.2  $\mu\text{m}$  that were not filtered out. The clogging does not only prolong the preconcentration time significantly but may also reduce the efficiency of vitamin B<sub>12</sub> binding. The two above mentioned potential error sources may generally be reduced by increasing the amount of utilized resin material, something which would also increase the cost per sample analyzed. In summary, cost-effective improvements to the SPE method which are easy to incorporate into field work are urgently needed.

## 4.4 | OH-B<sub>12</sub> isomer

The discovery of a novel OH-B<sub>12</sub> isomer in both the commercially available standard and field samples from all analyzed geographical locations was surprising, as it has not previously been reported in other publications dealing with cobalamins in the marine environment. This is especially noteworthy since the extraction protocol used here was identical to previously published methods.<sup>23,24</sup> In addition, the employed LC/MS/MS conditions were similar to those of previously published methods containing only minor changes of the organic solvent during chromatographic separation and ionization and fragmentation parameters. These changes may influence the sensitivity of cobalamin detection, but it is unlikely that they result in the isomerization of OH-B<sub>12</sub>, thereby artificially creating Iso-OH-B<sub>12</sub>. For this reason, extraction and measurement artefacts are unlikely to be responsible for this discrepancy. Isomerization of hydroxycobalamin has been only reported once as a reversible isomerization process documented by separation of hydroxycobalamin on thin-layer chromatography plates.<sup>31</sup> This isomerization occurred more rapidly at lower temperatures and at high pH producing molecules with a greater retention time than hydroxycobalamin. This published observation in combination with the high similarity in the obtained CID spectra led us to the conclusion that Iso-OH-B<sub>12</sub> indeed represents an isomer of OH-B<sub>12</sub>. It is still unclear as to why this isomer has a much higher retention on a C<sub>18</sub> stationary phase, eluting as much as a minute after OH-B<sub>12</sub> under UHPLC conditions. It may be that the coordination from the DMB



fragment to the central cobalt atom is weakened and thus the DMB moiety may interact more strongly with the hydrophobic stationary phase resulting in longer retention times. This hypothesis could be clarified by converting greater amounts of OH-B<sub>12</sub> to the isomer, isolating it through HPLC separation and elucidating the molecular structure through NMR studies. In addition, studies investigating the bioavailability of the different B<sub>12</sub> congeners are lacking and thus their ecological significance remains unclear. The results from this study highlight the need to include the novel OH-B<sub>12</sub> isomer in future laboratory studies focusing on bioavailability.

## 4.5 | Method application

Mainly a consequence of practical constraints in the available methodologies, to date, B<sub>12</sub> concentrations have only been measured at few locations of the world's oceans even though the ecological significance of vitamins in phytoplankton ecology is well known. This study represents the first dissolved vitamin B<sub>12</sub> concentrations for the North Sea and Baltic Sea. The total dissolved vitamin B<sub>12</sub> pool was between 1.1 and 3.6 pM at all stations and dominated by the newly described Iso-OH-B<sub>12</sub>. With a few exceptions at some stations, all other quantified B<sub>12</sub> congeners contributed little to the total dissolved B<sub>12</sub> pool (Me: 0%; Ado: 0–12%; CN: 0–17%; OH: 0–40%). Thus, excluding Iso-OH-B<sub>12</sub> would result in a dramatic underestimate of the total vitamin B<sub>12</sub> pool and lead to significantly lower total concentrations than previously published.<sup>27–29</sup> A potential explanation could be that the conditions facilitating the conversion of OH-B<sub>12</sub> to Iso-OH-B<sub>12</sub> were not met in the water bodies investigated by other groups or that Iso-OH-B<sub>12</sub> was measured and presented as OH-B<sub>12</sub>. However, since there are only a few publications concerning B<sub>12</sub> concentrations in seawater with no two publications reporting concentrations of the same water body, it is difficult to conclude that absolute B<sub>12</sub> values were unusually low in this study area. Nonetheless, the ratio of vitamin B<sub>12</sub> concentrations (including Iso-OH-B<sub>12</sub> as OH-B<sub>12</sub>) was very similar to the ratios obtained by Heal et al, who also found OH-B<sub>12</sub> to be the major contributor.<sup>27</sup> The biologically active form Me-B<sub>12</sub> could not be detected at any station and Ado-B<sub>12</sub> was only measured at low concentrations (0.5–1.0 pM) at two stations, which may be a result of light-induced hydrolysis in surface waters. Conversely, rapid uptake of vitamins by the plankton community, as has been shown in other estuarine systems during summer, may be responsible for the low pool of biologically active forms of B<sub>12</sub>.<sup>10,32</sup> CN-B<sub>12</sub> concentrations varied between undetectable and 0.44 pM and were thus in the range observed in the Pacific or during a transect covering the Mediterranean Sea and the Atlantic Ocean (0.01–2.5 pM).<sup>33,34</sup> However, obtained CN-B<sub>12</sub> values were two orders of magnitude lower than CN-B<sub>12</sub> values in the Baja California region.<sup>35</sup> These discrepancies once again emphasize the influence of different water bodies on both total B<sub>12</sub> concentrations and relative proportions of B<sub>12</sub> congeners. While the total B<sub>12</sub> values were also within the ranges published by another study, they differ strongly in the ratios of the contributing congeners.<sup>28,34</sup> While the

absolute concentrations of CN-B<sub>12</sub> are similar, there are major differences in the detected Me-B<sub>12</sub> concentrations. While it could not be detected at any station in this study, Suffridge et al found Me-B<sub>12</sub> to be the major contributor of the total dissolved B<sub>12</sub> pool.<sup>28,34</sup> This is surprising since the highest concentrations of Me-B<sub>12</sub> were found in surface waters, which contrasts with the light-sensitivity of Me-B<sub>12</sub> and also because Suffridge et al incorporated a second preconcentration step at pH 2, which prolongs sampling time during which Me-B<sub>12</sub> is prone to hydrolysis.<sup>28,34,36</sup>

## 5 | CONCLUSIONS

In summary, this study has extended the information about the stability of cobalamin congeners under commonly applied SPE conditions. Furthermore, an isomer of OH-B<sub>12</sub> (Iso-OH-B<sub>12</sub>) was found, which was consistently the largest contributor to the total dissolved vitamin B<sub>12</sub> pool at all stations sampled. With respect to the observed distribution of the various congeners, it will be important to include this novel isomer in future studies about vitamin B<sub>12</sub> cycling in the oceans to cover all existing B<sub>12</sub> congeners. Also, further studies about the structural differences and bioavailability of this isomer are needed. In addition, isolation of this isomer to obtain a standard reference material will be necessary in order to account for potential differences of OH-B<sub>12</sub> and Iso-OH-B<sub>12</sub> in the response factor during mass spectrometric analyses.

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## DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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## SUPPORTING INFORMATION

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