Limnology and Oceanography - Methods
June 2013, Volume 11, Pages 327-336
http://dx.doi.org/10.4319/lom.2013.11.327

Selective liquid chromatographic determination of trace domoic acid in seawater and phytoplankton: improvement using the ophthaldialdehyde/9-fluorenylmethylchloroformate derivatization

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Abstract:

ABSTRACT: Domoic acid (DA), a toxin produced worldwide by some species of the genus Pseudo-nitzschia, is responsible for contamination of marine molluscs, mammals, birds, and for human intoxication, and when detected in high levels results in closures of shellfish farms, thus causing severe economic losses to aquaculture. Studies on algal production of DA in cultures and field samples require sensitive methods capable of measuring trace concentrations of domoic acid. Measuring domoic acid concentrations at trace levels is still a significant challenge. A sensitive and reliable double derivatization using o-phthaldialdehyde-mercaptoethanol/9-fluorenylmethylchloroformate (OPA-MeSH/FMOC-Cl) followed by high-performance liquid chromatography with fluorescence detection has been adapted for the determination of trace levels of dissolved (dDA) and particulate domoic acid (pDA). The selectivity toward domoic acid is greatly improved by eliminating the reaction between FMOC-Cl and primary amines. Compared with the original Pocklington method, the sensitivity obtained by the double derivatization is largely enhanced (×6) and the reproducibility is significantly increased (<4% relative standard deviation [RSD] for nanomolar domoic acid concentrations). A better precision and an improved interoperator reproducibility were obtained with this two-step derivatization optimized protocol. This methodology will be useful for monitoring domoic acid production both in Pseudo-nitzschia cultures and in natural environmental marine samples.

Introduction

Domoic acid (DA), the amnesic shellfish poisoning toxin (ASP), is an excitatory amino acid which can accumulate, under certain environmental conditions, in shellfish, finfish, birds and mammals, by direct filtration or by feeding on contaminated organisms (Mos 2001). This potent neurotoxin with three carboxylic groups, responsible for its high polarity and hydrophilicity, is a cyclic amino acid and a secondary amine with a molecular weight of 311 Daltons. DA belongs to a neurotransmitter class of compounds with a structure very similar to an important neurotransmitter excitatory, acid glutamate, and indeed mimics glutamate in its interaction with some of its receptor subtypes. DA can damage the neurons by activating R-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, causing an influx of calcium, and then be the cause of several symptoms such as nausea, disorientation, temporary amnesia and, in more serious cases, persistent memory loss and / or coma, and ultimately even death (Costa et al. 2010). This toxin was originally isolated from a red microalga *Chondria armata* by Japanese researchers (Takemoto and Daigo 1958) and is now reported to be produced in several species of marine diatoms from the genus *Pseudo-nitzschia*. DA was identified as the causative agent of the *"famous"* tragic intoxication event observed in 1987 (Prince Edward Island from Cardigan Bay in Eastern Canada). More than one hundred people became ill and three victims died, by septic shock or pneumonia, 11–24 days after intoxication. While most victims recovered within 10 days, after a period of confusion and nausea, the others continued to show signs of selective short-term memory loss (Bates et al. 1989). Additional consequences of the DA presence included the temporary closure of shellfish aquaculture industries and a strong impact on tourism activities (Pistocchi et al. 2012; Trainer et al. 2012).

The detection limit of the method generally considered as the reference for particulate DA quantification in marine organisms (high-performance liquid chromatography with ultra-

violet detection at λ = 242 nm, HPLC-UV) ranges between 13 and 250 nM depending on the detector (Quilliam 2003). This sensitivity threshold is often insufficient to follow the dynamics of both dissolved and particulate domoic acid production in *Pseudo-nitzschia* cultures and phytoplankton field samples (Trainer et al. 2007). Although an improvement in sensitivity for domoic acid trace level analysis by HPLC-UV has been recently described (Mafra et al. 2009), more sensitive methods based on high-performance liquid chromatography with pre- or post-column derivatization have been developed in order to decrease the detection limit of domoic acid (He et al. 2010). Enzyme linked immunosorbent assays, such as the Biosense ELISA kit, provide a very high sensitivity (10 pg mL^{-1} ~ 0.03 nM) for pure domoic acid solutions and a high sample analysis throughput. However, for both algal extracts and seawater, samples must be diluted at least 1/30 to eliminate any unspecific assay response and matrix effects (Kleivdal et al 2007), thus the "effective" sensitivity on these natural samples (300 ng mL^{-1} ~ 0.96 nM) falls within the range of the most sensitive pre-column derivatization followed by fluorescence detection.

Reagents used for amino acid (AA) derivatization like: 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, Maroulis et al. 2008), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F, James et al. 2000), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, Sun and Wong 1999) and 9-fluorenylmethylchloroformate (FMOC-Cl, Pocklington et al. 1990) have been proposed for the measurement of DA. In fact, the methodology developed by Pocklington and co-workers (1990) is most commonly used for DA determination in seawater and particulate marine material (for example, some harmful *Pseudo-nitzschia spp*. – Quilliam 2003; Armstrong-Howard et al. 2007; Besiktepe et al. 2008; Amato et al. 2010; Lundholm et al. 2012; Trainer et al. 2012).

Within phytoplankton cells, the pool of internal free amino acid concentrations ranges between 50 and 300 mM (Haberstroh and Ahmed 1986; Martin-Jézéquel et al. 1988; Flynn

1990; Péter et al. 1999) whereas internal DA concentrations are much lower. With an average value of 5 mM (Amato et al. 2010), DA only represents a small percentage of the internal free amino acids (Smith et al. 2001). Moreover, except during large blooms, *Pseudo-nitzschia spp.* may constitute only a few percent of the natural phytoplankton assemblage (Trainer et al. 2012) and then, the dissolved and particulate DA concentrations should be comparatively low relative to those of amino acids. As a consequence, the presence of a large numbers of amino acid peaks observed after derivatization may interfere with DA detection (Mafra et al. 2009).

The FMOC-Cl pre-column derivatization of both primary and secondary amines was first described by Einarsson et al. (1983), their protocol was used by Pocklington and co-authors (1990) for domoic acid derivatization. Later, Einarsson (1985) developed a methodology able to specifically measure the secondary amines in presence of primary amino acids. Primary amino acids were "removed" by the *o*-phthaldialdehyde-mercaptoethanol reagent (OPA which reacts only with primary amines) followed by the labelling of secondary amines with FMOC-Cl. As fluorescence spectra of OPA (340/450 nm) and FMOC (264/313 nm) derivatives do not overlap, highly selective determination of secondary amines can be achieved. This technique is also currently used for the determination of secondary amines such as proline and hydroxyproline in presence of primary amines (Lange and Malyusz 1994; Mazzi et al. 1996; Biondi et al. 1997; Hutson et al. 2003), but has never been tested for selective DA determination.

The aim of this work was to test if the successive double derivatization carried out with OPA and FMOC-Cl improved the selective determination of DA at trace levels in both dissolved and particulate field marine samples without any loss of sensitivity or reproducibility.

Materials and procedures

Chemicals and standards

DA was purchased from the National Research Council (Halifax, Canada) as a certified calibration solution. This CRM (Certified Reference Material - 327.1 ± 6.8 µM) was prepared in acetonitrile/water (1:19, v/v, Hardstaff et al. 1990). FMOC chloride (9-fluorenylmethylchloroformate), *o*-phthaldialdehyde (OPA), mercaptoethanol (MeSH), iodoacetamide, amino acids, dihydrokanaic acid (DHKA), HPLC-grade acetonitrile (MeCN) and ethyl acetate were obtained from Sigma/Aldrich. All reagents were analytical grade, except acetonitrile and ethyl acetate that are HPLC grade.

Cleaning procedure

All glassware was washed, successively rinsed with Milli-Q water (from a Millipore purification system) and alcohol, capped with aluminium foil and heated at 450°C for at least 2 hours. Combustible material (e.g., tips and caps) were rigorously cleaned in 15% hydrogen peroxide to oxidize organic contaminants, then rinsed directly and copiously with alcohol, dried and stored in zip-lock bags.

The Pocklington derivatization: the original method

The borate buffer (1 M) in Milli-Q water was adjusted to pH 6.2 with 2 N sodium hydroxide. The FMOC-Cl was made up daily as a 15 mM solution in acetonitrile. Ethyl acetate was used for clean-up through partitioning during the extraction process.

Borate buffer (250 µL) was added to 1 mL of sample and mixed for 10 s. The FMOC reagent (300 µL) was included and the content mixed. After exactly 45 s, the excess reagent was extracted three times into ethyl acetate (1.7 mL) by manual shaking. In order to avoid the formation of an emulsion and to quickly obtain the separation of the two phases, a four times "up and down" manual mixing seems to be more efficient than the use of a vortex. The organic layers were removed with a disposable tip and discarded. After the final extraction, a brief centrifugation (1 min. at 2000 rpm) allowed a clear phase separation. Subsequently, the

aqueous bottom layer was transferred into a 2 mL glass vial with a glass syringe previously cleaned in an acetone/methanol mix (50/50) and finally rinsed with Milli-Q water.

The extraction procedure is important to eliminate the excess reagent, to accurately control the reaction time and to prevent the formation of a significant level of reagent hydrolysis side-product (FMOC-OH). Immediately prior to injection onto HPLC, 3.5 µL of acetic acid were added to the sample to protonate the amino derivatives (Einarsson 1985; Mafra et al. 2009).

The Einarsson derivatization: the two-step procedure

The borate buffer (0.8 M) dissolved in Milli-Q water was adjusted to pH 9.5 with 10 N sodium hydroxide. The FMOC-Cl was made up daily as a 15 mM solution in MeCN. The OPA-mercaptoethanol reagent was dissolved in acetonitrile and prepared every day (50 mg of OPA and 26 µL of mercaptoethanol per mL). The iodoacetamide reagent (140 mg/mL) was prepared weekly in acetonitrile and protected from the light.

100 µL of borate buffer were mixed with 900 µL of sample. The OPA-mercaptoethanol reagent (100 µL) was added and allowed to react for 30 s after the mixing. Then 100 µL of the iodoacetamide reagent were added and the content mixed**,** after another 30 s, 300 µL of FMOC-Cl was added, mixed and allowed to react another 30 s. Finally, the reagent excess was removed as in the Pocklington derivatization described earlier. Prior to injection onto the HPLC, acetic acid (10 µL) was added.

High-Performance Liquid Chromatography fluorimetric detection

The LC PerkinElmer Flexar system consisted of a solvent manager, a quaternary LC pump, an autosampler, a column oven and a fluorescence LC detector managed by the Chromera software. Chromatographic separations were carried out on a 201TPC18 Vydac column (250 x 4.6 mm, 5 µm) fitted with a 20 mm x 2 mm guard-column (Upchurch) containing 40-µm C_{18} reversed phase packing. The column was maintained at a constant

temperature of 55°C. A 100 µL sample full loop was manually injected. Gradient elution was carried out as in Pocklington et al. (1990) using acetonitrile (MeCN) and 0.1% trifluoroacetic acid in Milli-Q water (TFA) at a flow rate of 1 mL min^{-1}: a linear gradient from 30 to 50% MeCN over 15 min., followed by an increase to 100% MeCN over 2 min. which was maintained for 5 min., before returning to initial conditions over 2 minutes. Initial conditions were maintained for a further 12 minutes before the next injection. The fluorescence detection was performed at the medium sensitivity of the detector (with $\lambda_{excitation}$ = 260 nm and $\lambda_{emission}$ = 313 nm). Blank runs (Milli-Q water and/or seawater) with reagent solutions were performed every day to check the absence of contaminations of both reagents and "aged seawater" (ASW). For each batch of extracts, an additional control was performed on the ASW used for the extraction in order to check for the non-contamination of the samples.

Calibrations and natural-samples processing

Calibrations were carried in the lowest range of DA concentrations expected to be seen in field samples. For this, the primary (100 µM) and secondary (1 µM) domoic acid solutions were prepared after dilutions of the CRM standard; final working solutions (1-40 nM) were made using in ASW and stored at -25°C in glass vials. All dilutions were made at the 0.1 mg level using a balance (calibrated daily).

For dissolved and particulate DA, the limit of detection (LOD) was estimated statistically according to the equation: LOD = [(3 x SD) / b] where "SD" is the standard deviation of repeated runs of the 1 nM DA working standard solution, and "b" the slope of the calibration curve (Mafra et al. 2009).

The *Pseudo-nitzschia australis* strain used in this study was isolated and identified in 2011 by E. Nezan from field samples collected in the Bay of Douarnenez (Brittany coast, France). This strain is well known to be a domoic acid producer (Armstrong-Howard et al. 2007). The culture was grown in f/2 medium (Guillard and Ryther 1962) in sterilized

seawater at 16°C under 70-80 µmol photons m^{-2} s^{-1} with a 12/12 h photoperiod. The culture was sampled at the end of the growth phase for both dissolved and particulate domoic acid measurements.

Field samples were collected during the PSEUTEC cruise (9-16 June 2011) along the coast of Brittany (France) from the plume of the Loire in the south, to the Bay of Brest in the north. At twenty two coastal (15 m depth) or offshore (122 m depth) stations, vertical profiles of temperature, salinity and fluorescence were measured with a SBE25 probe (Sea-Bird Electronic, Washington, USA). Depending on the vertical structure of the water column, discrete water samples were collected using Niskin bottles attached to the CTD-rosette. *Pseudo-nitzschia* cell density was measured by light microscopy counting on buffered formalin preserved samples. During the cruise *Pseudo-nitzschia* abundance range between 10^3 and 50 x 10^3 cells L^{-1}.

For dissolved DA analysis, as filtration cell leakage may happen (Fuhrman and Bell 1985), samples were filtered by gravity through precombusted 0.7 µm glass fiber filters (GF/F) using clean filter-holders and syringes (Delmas et al. 1990). A minimum of 20 mL filtered water was collected in clean polypropylene vials and immediately frozen at - 25°C.

Particulate organic matter, from marine field samples and/or *Pseudo-nitzschia australis* batch cultures, was collected by low vacuum filtration through precombusted GF/F filters and stored frozen until DA extractions. The extractions were performed with ASW by sonication for 1 min, at 30% amplitude by using a 3 mm diameter probe (Vibra-Cell 75115; Fisher Bioblock Scientific, 750 Watts) to disrupt the cells. Then homogenates were filtrated through GF/F precombusted filters to remove any particulate material. Extracted samples were either immediately analysed or stored frozen until HPLC derivatization carried out the next day. For each sample batch extraction procedure, a control of the ASW used for DA extraction was included in order to verify the absence of domoic acid contamination.

Quantification of domoic acid (dissolved: dDA, and particulate: pDA) was achieved by the two derivatization procedures already described; at least triplicate analyses were realized for each sample (e.g., DA standards, batch culture media, and field samples).

Assessment

For both derivatization methods used, the retention times of secondary amines and DA were basically the same (Fig. 1). In our study and for both derivatization procedures, hydroxyproline (HYP) and proline (PRO) appear at 6.95 and 12.2 minutes respectively. Their relevant peaks did not interfere with those of DA (13.4 min) and the internal standard (DHKA, 14.5 min) which appear later (Fig. 1). However for the two-step derivatization, the FMOC-OH peak, which starts at 9.5 min, is much wider. A late eluting peak corresponding to the FMOC-mercaptoethanol derivative (retention time = 15.4 min.), is still present even though iodoacetamide was added before the reaction with FMOC-Cl to block excess mercaptoethanol (Einarsson 1985).

Comparative sensitivity and reproducibility of the two derivatization protocols

Different domoic acid concentrations (1, 2, 4, 8, 16, 24, 32 and 40 nM from certified standard material) were analysed in triplicate by the two methods (Einarsson 1985; Pocklington et al. 1990). Good calibration curves were obtained for both methods (Fig. 2, $r^2 > 0.99$). Looking at these results, acquired with the same domoic acid working standard solutions, two observations can be drawn:

- First, the double derivatization (Einarsson 1985) gives a large increase in fluorescence response (~ 6),
- Secondly, a better reproducibility is achieved (RSD < 4.5%; mean value = 2.8%) with the Einarsson protocol than with the Pocklington method (RSD < 12%; mean value = 5.8%).

A limit of detection of 0.038 nM was achieved using the 1 nM standard solution. For natural samples, triplicate analyses of dissolved domoic concentration (Fig. 3) showed that a very good reproducibility may be achieved even at trace levels (RSD = 1.7%). Using these data, an LOD of 0.045 nM can be estimated for natural samples, similar to that of pure solutions. These LOD, obtained with a 4.6 mm ID column, are comparable to those obtained by Pockington and co-workers (1990) using a 2.1 ID narrow-bore column (0.050 nM) which are known to provide 3 to 5 times greater sensitivity than conventional columns (Vonk et al. 1992).

pH effect on the FMOC-Cl – DA reaction

The higher yield of derivatization obtained with the two-step procedure is quite surprising as the FMOC derivatization is essentially the same for both procedures. One of the main differences between the two protocols is in the pH of the reaction mixture: ~ 6.2 for the direct FMOC-Cl derivatization and ~ 9.5 for the OPA-MeSH / FMOC-Cl. The latter is imposed to allow the reaction between OPA and primary amines. In an extensive literature review on the amino acids / FMOC-Cl reactions, Jambor and Molnar-Perl (2009) found that the reaction pH may vary from 6 to 11.4 and that reaction yield and velocity are controlled by an interplay between buffer pH, FMOC-Cl concentration and reaction time. To our knowledge, exhaustive studies on the pH effect on efficiency of the amino acids / FMOC-Cl reactions are scarce. Only Garside et al. (1988) studied the effect of the pH in the range 6 - 11 and found maximum yield for amino acids at pH above 9. However similar studies have not been carried out for domoic acid.

In order to verify if the pH of the reaction may explain the different yields of the two procedures, we tested the effect of the borate buffer pH on the Pocklington reaction. For this, we used 1 M borate buffer solutions at different pH: 6.2, 7, 7.5, 8, 8.5 and 9 (adjusted with 2 or 10 N NaOH). Triplicate derivatizations were carried out for each pH on a 20 nM domoic

acid working standard solution. There is a clear increase in the derivatization yield of DA with increasing borate buffer pH with a 4.4-fold increase in yield between 6.2 and 9 (Fig. 4A). The relative standard deviation on triplicates decreased sharply as the pH increased from 6.2 to 8.0 (from 7.3% up to 0.8%), then stabilised between 1.3 - 2.2% for higher pH, reflecting an improvement in the reproducibility in parallel to the increase in reaction efficiency.

A significant level of hydrolysis side product FMOC-OH occurs during the derivatization process. The shape of this additional peak increases with increasing pH. Such a formation of a huge FMOC-OH peak at basic pH has been previously described (Jambor and Molnar-Perl 2009). However, it does not hinder the accurate integration of the domoic acid peak as the retention times of FMOC-OH (9.5 min) and domoic acid (13.5 min) are clearly different (Fig. 4B).

The use of the two-step derivatization significantly improves the formation of the domoic acid derivative. The better reproducibility obtained may have two explanations. Firstly, at the higher pH used, near the optimum value of the FMOC derivatization reaction, the buffer capacity of borate is close to its maximum. As the pH of the reaction medium is much better controlled than under the conditions used for the simple derivatization, the variability of the formation of the domoic acid fluorescent derivative is greatly reduced. Secondly, as the peaks are larger, their integration becomes easier and there is less uncertainty in the measurement of peak area.

Selectivity of the two-step derivatization on culture media and natural samples

The two-step derivatization may theoretically eliminate reactions between primary amino acids and FMOC-Cl and provide cleaner chromatograms. To investigate if selectivity is really improved by the double derivatization, we carried out, in parallel, analyses on samples with varying complexity of composition. Indeed, more internal free primary amino acids will be present in these types of samples, whereas internal domoic acid concentrations will be much

lower. For this purpose, one natural marine sample collected on the Brittany coasts (47°35'003N, 3°42'070 W, 7 m depth) was chosen in addition to toxic *Pseudo-nitzschia australis* cell extracts and the associated culture medium. Both culture medium and extracts were analysed according to the two derivatization protocols using the same chromatographic conditions (detector sensitivity, injection volume). In agreement with previous observations, DA fluorescence response obtained with the two-step derivatization is more intense than the one acquired from the single step process (Fig. 5). In the culture medium, more additional peaks are present in the chromatogram obtained with the Pocklington derivatization than with the double derivatization. Most of them appear before the FMOC-OH peak (Fig. 5A). The same trend was observed in particulate extracts (Fig. 5B and C). With the Pocklington protocol, a lot of unresolved peaks overlap and produce a large drift of the baseline. This is not the case with the double derivatization method where there are few additional peaks and no drift of the baseline. As in particulate organic matter, domoic acid and intracellular amino acids are extracted simultaneously; the overlapping of a lot of compounds before the FMOC-OH peak is even more important if the sample is rich. Although the majority of peaks appear before FMOC-OH's, as there are tens of amino compounds, we cannot exclude the possibility that some might co-elute with domoic acid.

Both FMOC-Cl and OPA react with primary amino acids to give fluorescent derivatives. Their main difference lies in their reactivity towards secondary amines. However, they both react equally with primary polyamines, biogenic amines and amino sugars (van Eijk et al. 1996; Appuhn et al. 2004; Lozanov et al. 2007; Brückner et al. 2012). All these compounds may be found in marine water and particulate material (Antia et al. 1991; Yang et al. 1993; Nishibori et al. 2001; Müller et al. 2009). Thus, these compounds may give extraneous peaks during chromatographic analyses. During the two-step derivatization procedure, all the primary amines react with OPA-MeSH to give fluorescent iso-indoles derivatives. At that

time, only remaining secondary amines like proline, hydroxyproline and DA may react with FMOC-Cl and will be detected with the excitation and emission wavelength used, improving significantly the selectivity of the method resulting in "clearer" chromatograms, particularly for samples rich in organic matter.

In conclusion, the double derivatization procedure developed during this study allows for the removal of all primary amino acids present in both batch cultures and field samples. This optimization of the Pocklington method by a two-step derivatization process (OPA-MeSH and FMOC-Cl) is more selective, accurate and sensitive and allows quantification of trace levels of dissolved and particulate domoic acid by giving clearer chromatograms without significant co-elutions.

Reliability of the two-step derivatization procedure

In order to compare the two protocols, one natural marine sample and one *Pseudo-nitzschia australis* culture medium were analyzed by both methods. For triplicate analysis, the results obtained for dissolved domoic acid concentrations are quite similar for both protocols (Fig. 6). However, in accordance with the results obtained during the calibration exercise, the two-step derivatization provides more reproducible results. During the sampling cruise carried out within the Southern-Brittany coastal waters, the abundances of *Pseudo-nitzschia spp.* (< 5 x 10^4 cells L^{-1}), as well as dissolved and particulate domoic acid concentrations we measured, were very low (dDA < 1.4 nM and pDA < 0.88 nM, respectively).

Furthermore, comparison between methods was performed on dissolved DA from culture medium and field samples and on particulate material extracts from natural marine samples collected during the PSEUTEC cruise in 2011 (Southern-Brittany coastal waters, France). In the low concentration range tested, both derivatization protocols gave results that were highly correlated ($r = 0.969$, $n = 10$, $p < 0.1\%$) and fully comparable (Fig. 7). These initial results are

encouraging but they need to be confirmed over a wider range of dissolved and particulate domoic acid concentrations.

Finally, in order to test inter-operator variability, some extracted samples were analyzed at 10 d intervals by each of the two authors and a very good agreement has been obtained between analysts (DA_{A1} = 1.008 DA_{A2} - 0.06, r^2 = 0.993, n = 9). Although the numbers of analyzed samples is small these results suggest a low inter-operator variability which should be confirmed by other analysts on a larger sample panel.

Discussion

i) The expected results of the two-step derivatization to improve the specificity of the detection were obtained; they provide greater sensitivity and better reproducibility than the original method. While for trace domoic acid analysis (3 nM), the Pocklington method displayed an RSD of the order of 10%, for the two-step procedure the RSD was less than 4% at 1 nM concentration. As the precision is excellent, the use of the internal standard (DHKA) may not be essential, at least for ecological studies on natural marine samples.

ii) The two-step derivatization protocol doesn't cause significant additional costs as the reagents used (OPA and MeSH) are not expensive. Besides, the total reaction time is not much longer than that of the usual method (~1 min) as the ethyl acetate extraction is the longest and critical step for both procedures. Hence, sample throughput is the same.

iii) The inter-calibration of the two protocols showed no difference in outcome; however it was only carried out on a small number of challenging samples and over a low domoic acid concentration range. This inter-calibration exercise should also be performed by other laboratories over a wider range of domoic acid concentrations. Similarly the inter-operator reproducibility must be confirmed by other analysts.

iv) However, if some analysts do not want to use the double derivatization, an improved Pocklington protocol can be obtained from an optimization of reaction pH in order to increase sensitivity and reproducibility.

v) Finally, the performance achieved by the two-step derivatization, such as the detection limit and reproducibility, compare favorably with those of ELISA assay which requires a sample dilution to avoid matrix effects. The main advantage of the latter method lies in their high sample analysis throughput.

Comments and recommendations

The extraction of the excess reagent by ethyl acetate is a critical step, the mixing of phases by vortexing tends to cause the formation of an emulsion, which results in a variable phase separation time. As the derivatization reaction prior to extraction of the excess of FMOC is not 100% complete, the reaction tends to continue in the first extraction. Altering the duration of the extraction step can decrease the reproducibility of the reaction, especially for the Pocklington protocol, where pH conditions are far from their optimal value. A manual "up and down" shaking is preferable because it allows a very fast separation of phases. Finally, during the derivatization processes, operators should carefully control the pH of the borate buffer. The control of the pH is essential for a good reproducibility.

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Acknowledgements

This research project was funded by the DIALTOXE program (ALNIT action n°A090206 — http://wwz.ifremer.fr/dyneco/Thematiques/Biodiversite-du-phytoplancton-des-especes-toxiques-et-eutrophisation). A. Devez was supported by an IFREMER post-doctoral fellowship. A special thanks to J. Quéré and R. Siano (IFREMER, Brest) for the preparation of *Pseudo-nitzschia* batch cultures and to all the crew and scientists present on the RV Côtes de la Manche during the PSEUTEC cruise along the Brittany coasts (France) in 2011. The authors are grateful to Wayne R. Litaker and Phillip Hess and anonymous reviewers for useful comments which improved the manuscript. The Conseil Général of the Finistère and the Brest Métropole Océane are also thanked for their equipment funding help.

Figure legends

Figure 1. Chromatograms of the same 32 nM domoic acid working standard solution containing DHKA and traces of proline and hydroxyproline, obtained with the single (POCK: Pocklington in grey) and the two-step derivatization procedures (EIN: Einarsson in black) respectively.

Figure 2. Calibration curves and relative standard deviation coefficients in % (RSD) obtained with the Einarsson (in black, n = 21) and Pocklington (in grey, n = 24) methodologies respectively. For the two-step derivatization procedure, at medium fluorescence sensitivity, the 40 nM DA working standard saturates the detector.

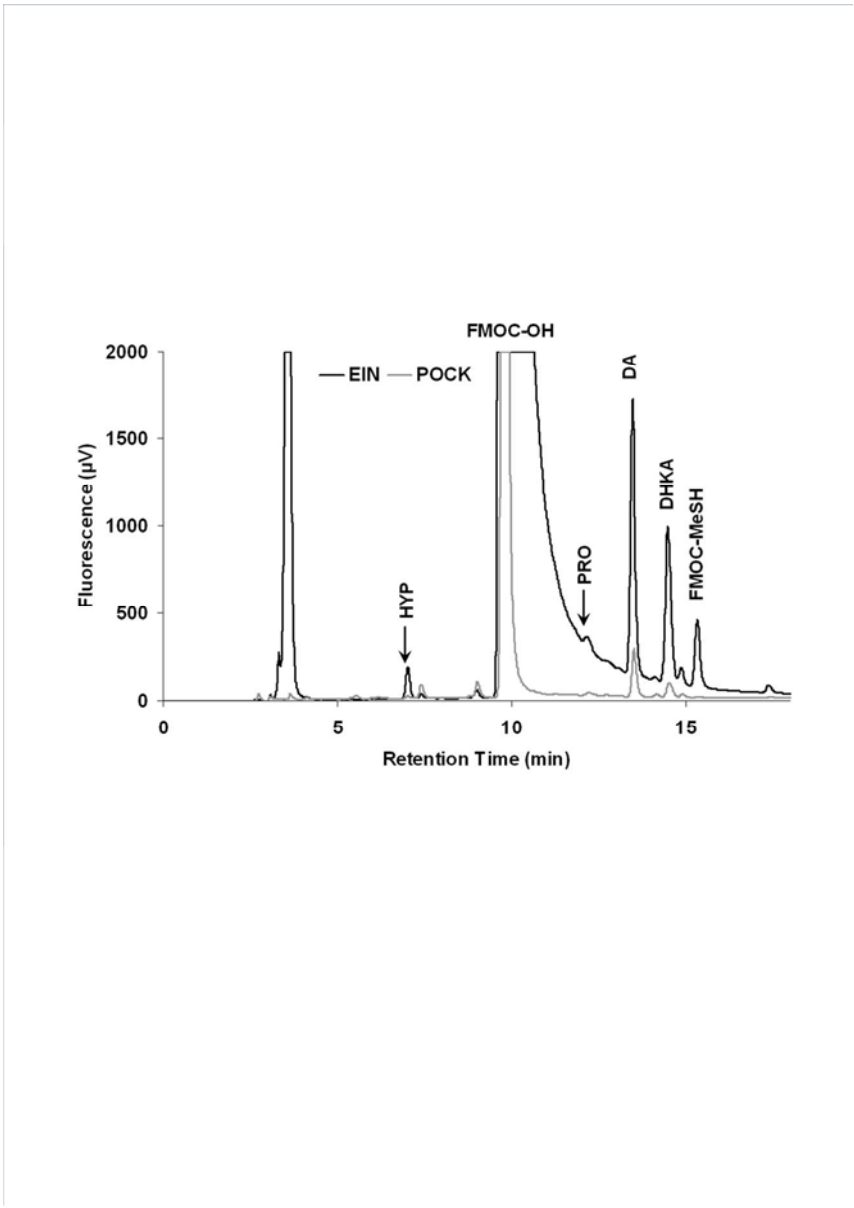
Figure 3. Trace determination of dissolved domoic acid in a natural marine sample carried out according the two-step derivatization procedure; mean value of dissolved domoic acid concentrations : 0.898 ± 0.015 nM with a RSD = 1.7% (n = 3).

Figure 4. Effects of borate buffer pH during the Pocklington derivatization, on the fluorescence signal and relative standard deviation obtained for triplicate analyses of 20 nM domoic acid working standard solutions **(A)** and changes in the FMOC-OH peak shape at the different pH tested **(B)**.

Figure 5. Comparison of chromatograms obtained after analyses carried out using Einarsson (black) and Pocklington (grey) derivatizations on *Pseudo-nitzschia australis* culture medium (**A**, 100 µL injected), particulate matter collected in coastal waters (**B**, 500 mL filtered, 100 µL injected) and on *Pseudo-nitzschia australis* cell extract (**C**, 40 mL culture, 20 µL injected). Analyses were carried out on same sample for dDA (A) or on the same extracts for pDA (B and C).

Figure 6. Comparison of Einarsson (grey) and Pockington (light grey) derivatization protocols for dissolved domoic acid concentrations found in *Pseudo-nitzschia australis* culture medium (*PN australis*) and in a natural marine sample (PC5). All analyses were carried out in triplicate on the same samples.

Figure 7. Correlation obtained for domoic acid (DA) concentrations present in selected natural samples and culture. Samples (for dDA) or extracts (for pDA) were analysed by both the Einarsson (Ein.) and the Pocklington (Pock.) methodologies.●: seawater, ▲: *Pseudo-nitzschia* culture medium culture medium, ♦: field particulate matter sample extracts.



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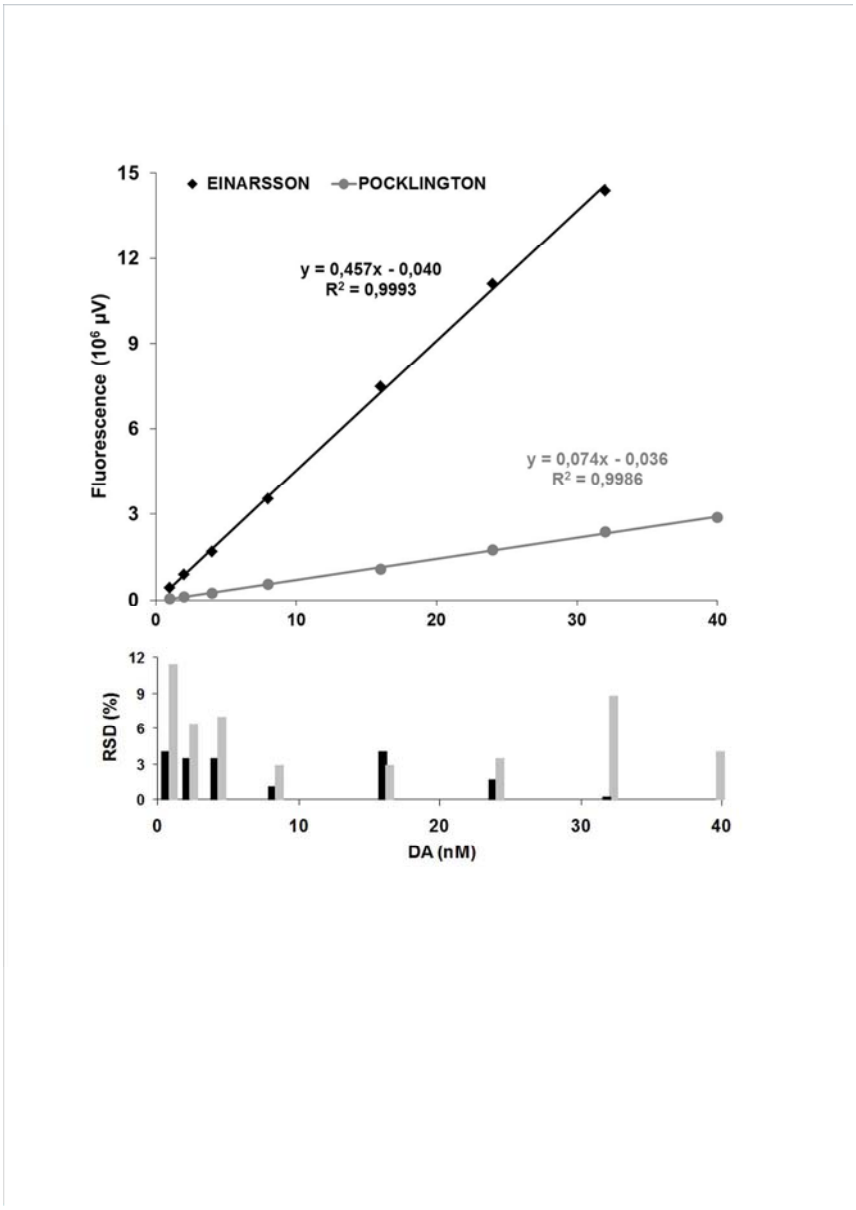

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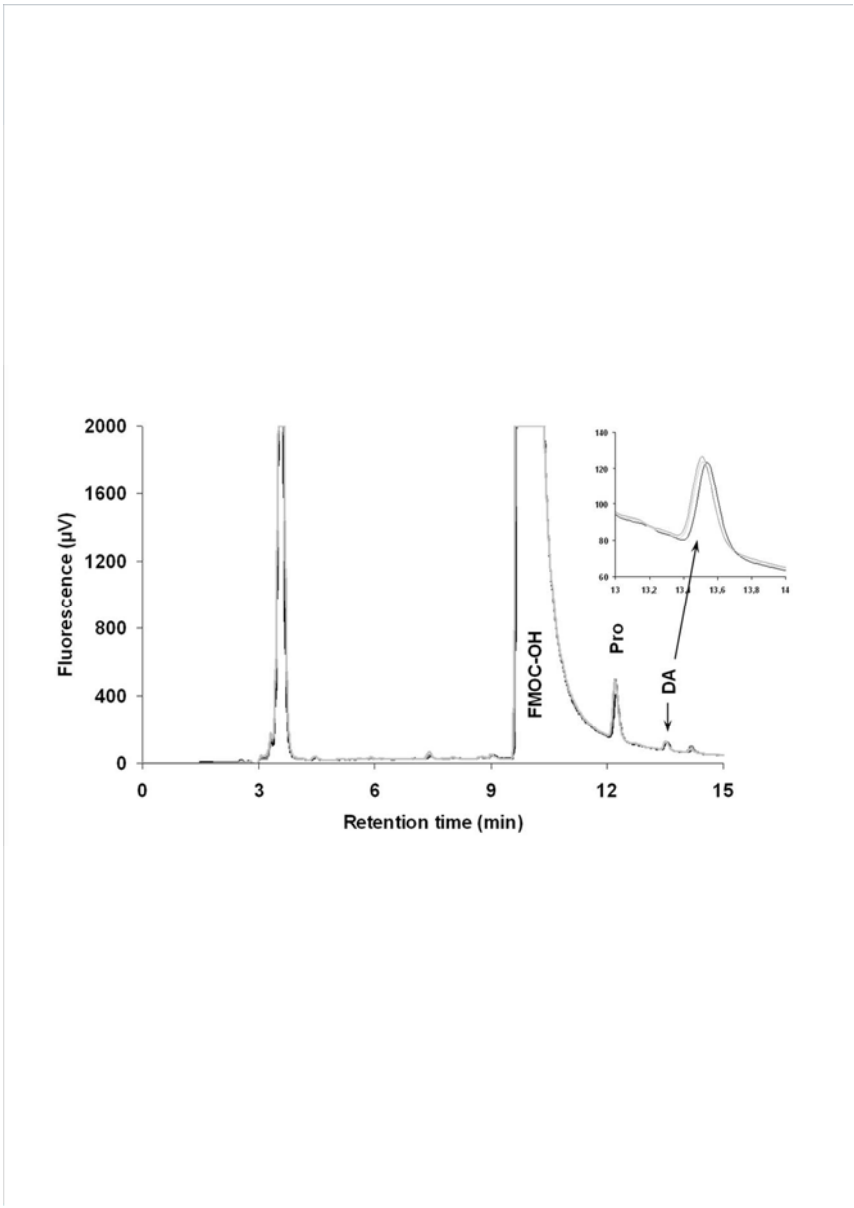

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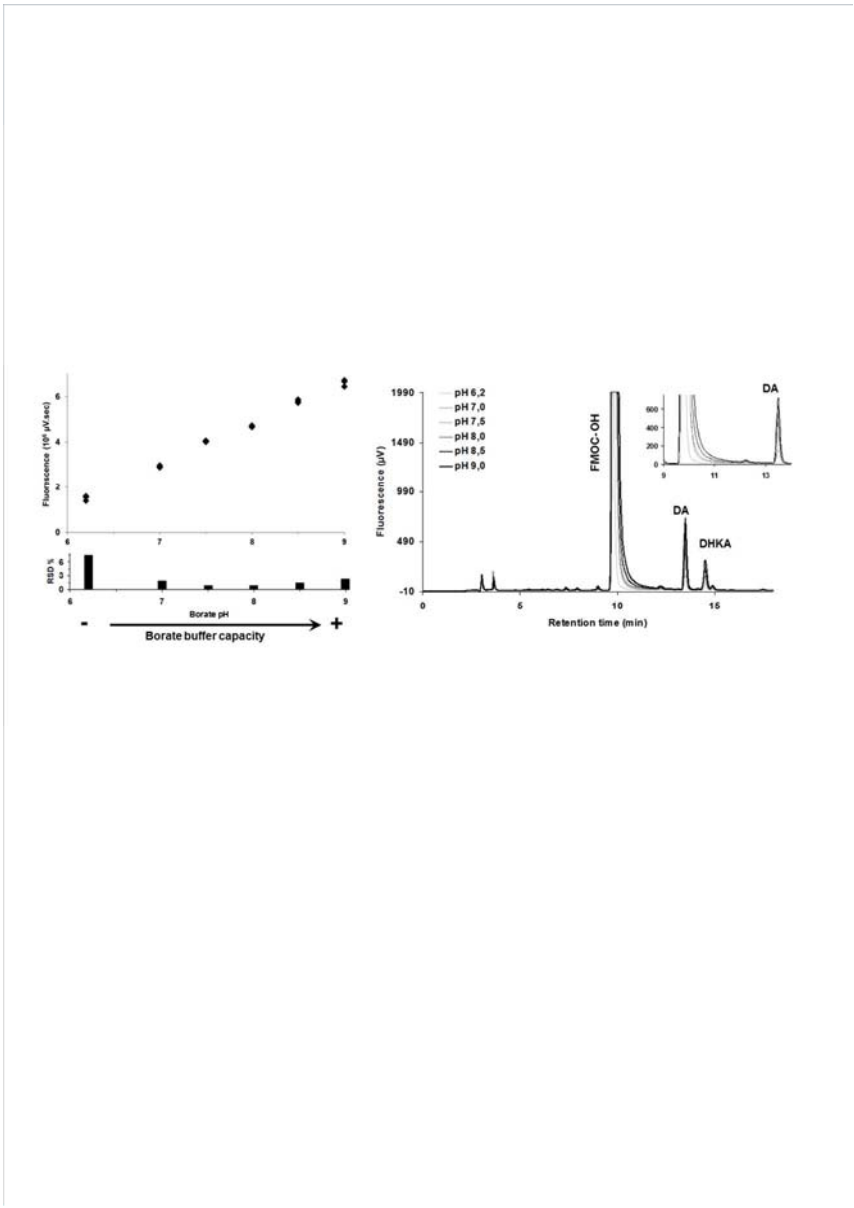

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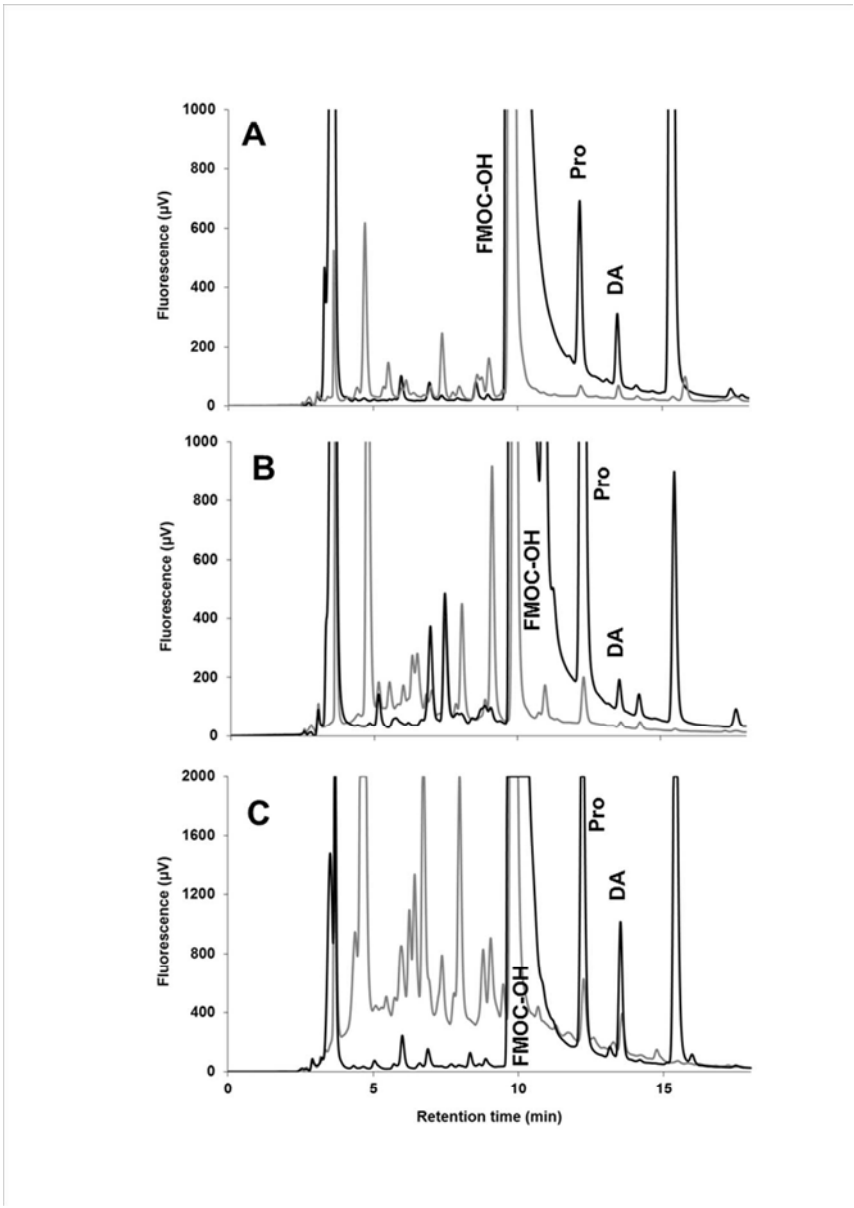

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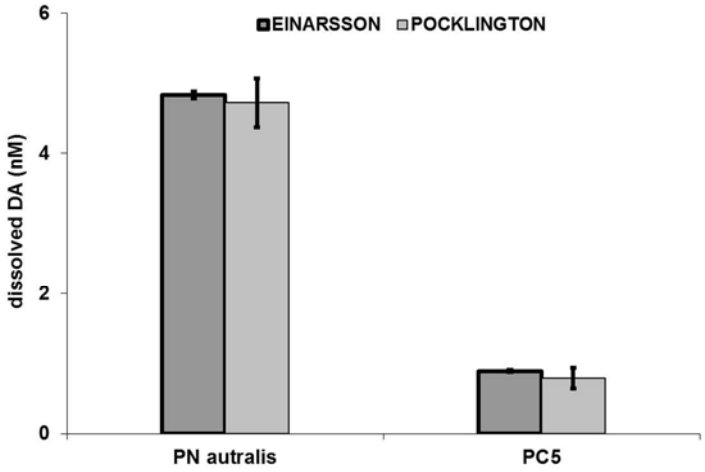

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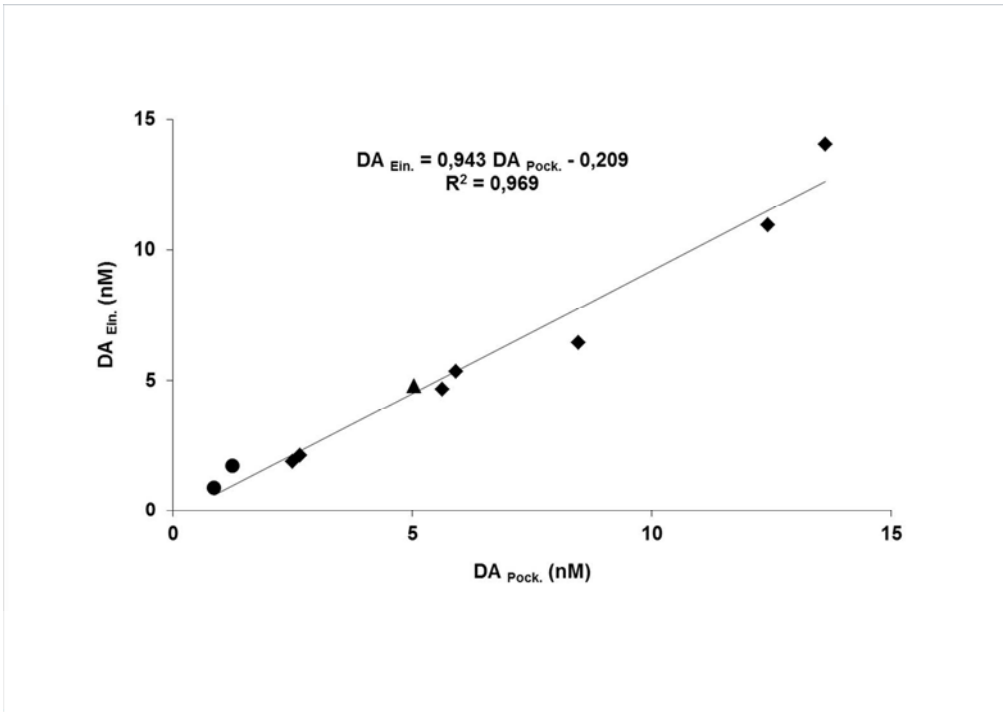

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