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A Derivatization Method for Tagging Low-Molecular Weight Organic Acids in Seawater with a Fluorescent Coumarin

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B.S., University of Florida, 2012

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

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Master of Science Thesis

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Major Advisor _____
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Presented is a new method for quantifying organic acids at sub-micro molar concentrations by derivatizing the acids with a fluorescent coumarin. The method has lower detection limits than previous methods and also identifies a larger number of acids. The derivatization method is based on an acid-amine coupling strategy that is widely used and potentially applicable for derivatizing a wide range of LMW organic acids. The fluorescent coumarin amine is commercially available or easily derived from commercially available compounds. In addition, pre-derivatized HPLC standards can be synthesized for most organic acids by a simple and effective acid-chloride coupling strategy that is carried out at high organic acid concentrations. The method has been successfully applied to marine water column and sediment pore water samples. Thus, this new method supplies a new tool to determine the role of many organic acids in carbon cycling in marine water column and sediment pore water samples.

1 Introduction

1.1 Low-molecular weight organic acids in the marine environment

The amount of carbon in marine DOM (~670 Pg) (Ogawa and Tanoue 2003; Hansell 2013) is approximately equal to the atmospheric carbon pool (~700 Pg). DOM is the second largest (second to inorganic carbon only) and least understood marine carbon pool. The molecular size of oceanic DOM decreases with increasing depth (Benner et al. 1992; Ogawa and Ogura 1992), consistent with biological degradation of organic compounds into more refractory molecules. Most DOM resides in the low-molecular weight (LMW; <1000 Dalton) fraction of organic molecules (Burdige and Gardner 1998) comprising about 70-80% of total DOM in the open ocean and as much as 70% in coastal systems (Amon and Benner 1996; Skoog and Benner 1997), while dominating pore water DOC (Burdige and Gardner 1998).

The bulk of LMW DOC represents highly degraded material and is the least reactive fraction of DOM and, as such, accumulates in the deep ocean (Benner et al. 1992). It is estimated that 80% of DOC in the deep ocean is of LMW with an average residence time of 4,000-6,000 yrs (Benner et al. 1992). Therefore, the global ocean reservoir has a distribution that is heavily skewed toward the nanometer size range (Burdige and Gardner 1998).

Within the pool of LMW compounds, organic acids (such as formate, acetate, pyruvate, and glycolate) are ubiquitous in marine environments (Yang, Lee, Scranton 1993; Albert and Martens 1997). Sources for LMW organic acids to marine and coastal environments include: living and decomposing biological cells (Yang, Lee, Scranton 1993; Wu, Green, Scranton 1997), photochemical degradation of organic material (Dahlén, Bertilsson, Pettersson 1996), river water, atmospheric deposition (Galloway et al. 1982; Keene, Galloway, Holden 1983; Legrand

and De Angelis 1996), and chemoautotrophic formation of OM through acetogenesis at redox interfaces in the sediment (Hoehler et al. 1999) and the water column (Taylor et al. 2001).

Organic acid concentrations vary with the environment. Organic acids have concentrations ranging from 100s of nM to μM in sediments and water overlying sediments (Yang, Lee, Scranton 1993; Albert and Martens 1997; Wu, Green, Scranton 1997). In anoxic sediments, the high LMW organic acid concentrations are mainly caused by degradation of OM through anaerobic fermentation, forming acetate. In oxic water columns, organic acid concentrations are much lower than in sediments and range from ~85-600 nM (Wu, Green, Scranton 1997). In water columns with oxic surface water but deep water anoxia, acetogenesis at the anoxic interface results in concentrations in the range 100 nM to μM (Taylor et al. 2001).

Organic acids are highly biologically labile and are rapidly assimilated into bacterial cells (Wright and Hobbie 1966; Wu, Green, Scranton 1997). Due to the small size of organic acids, heterotrophic organisms can directly transport LMW compounds across their cellular membrane. In some marine environments, the relatively high concentrations of LMW organic acids coupled with high biological lability make LMW organic acids the dominant carbon source for heterotrophic organisms (Wu, Green, Scranton 1997; Taylor et al. 2001).

Due to the large number of processes that produce LMW organic acids it is likely that LMW organic acids are important carbon sources in the water oxic column where the majority of oceanic organic carbon cycling takes place. For example, Wright and Hobbie (1966) showed that acetate has uptake rates similar to those of glucose in the water column. However, to date, we have not been able to determine the role of LMW organic acids in carbon cycling in most of the water column. This is due to the difficulty in detecting LMW organic acids at biologically relevant low concentrations. Existing methods for concentration determinations of LMW organic

acids have detection limits on the order of few nM with the exception of formate and acetate which are generally ~ 1 µM (Yang, Lee, Scranton 1993; Albert and Martens 1997). For comparison, most compounds that have been shown to be important as heterotrophic carbon sources have concentrations much lower than 100 nM. Examples are dissolved free amino acids and glucose, which have typical concentration ranges on the order of 10-40 nM (Jørgensen and Jensen 1997; Skoog, Biddanda, Benner 1999). Hence, it is crucial to be able to determine the low concentrations of LMW organic acids in the water column for our understanding of the marine carbon cycle.

The concentration and turn-over determinations carried out to date have centered on anoxic environments or oxic/anoxic interfaces, where high LMW organic acid concentrations can be expected, and indeed have been found (Hoehler et al. 1999; Taylor et al. 2001). However, the importance of LMW organic acids as carbon sources in the marine environment in general is unknown and this compound group is likely to be more important in the marine carbon cycle than we presently realize.

1.2 Existing methods for determining organic acid concentrations in seawater

The most sensitive methods for determination of LMW organic acids in seawater are those reported by Yang et al. (1993) and Albert and Martens (1997). Yang et al. (1993) reported nM-level instrument detection limits for a method that quantifies organic acids using GC separation with FID detection after an innovative pre-concentration step. However, the method has some drawbacks: a pre-concentration step is necessary, large volumes are needed (500 to 1000 mL), and there are contamination problems for ubiquitous and volatile formate and acetate. The method's low detection limit is the result of a 1000-fold pre-concentration step, where the LMW organic acids are diffused in protonated form across an organic membrane. This step

necessitates minimum volumes on the order of 50-100 mL, making the method difficult to use for pore water samples. In addition, the pre-concentration step increases the amount of time necessary to process one sample. Further, Yang et al. (1993) report contamination problems for acetate originating in the membrane used for concentration. In addition, they report no concentration results for formate, presumably a result of contamination problems as well. Studies that have used this method (Wu and Scranton 1994; Wu, Green, Scranton 1997; Taylor et al. 2001) have decreased the blank somewhat (and thereby the detection limit) by using double-distilled milli-Q water for the blank. However, the reported detection limits for acetate are still on the order 60-150 nM in seawater and ~ 2 μM in pore water (Wu, Green, Scranton 1997).

The method developed by Albert and Martens (1997) quantifies organic acids using HPLC with absorbance detection after derivatization of the organic acids with a chromophore. This method requires smaller sample volumes than the method developed by Yang et al. (1993) and there is no pre-concentration step prior to the HPLC analysis. However, this method has detection limits in the range several 100 to 1000 nM, which is much higher than concentrations of other important heterotrophic carbon sources (Jørgensen and Jensen 1997; Skoog, Biddanda, Benner 1999). These high detection limits are mainly due to contamination problems. Albert and Martens (1996) report contamination problems for acetate and formate, and to a minor degree, lactate. The authors identified the chromophore 2-nitrophenyl hydrazine (NPH) and pyridine (used to buffer pH during the derivatization) as two major sources for the contaminants. This highlights the need for the use of only the highest purity chemicals, and the minimization of the number and quantities of reagents needed for the derivatization.

In summary, major obstacles in determining the low natural concentrations of LMW organic acids in seawater include the isolation of small LMW compounds at low concentrations

from a solution with high concentrations of inorganic salts, the high water solubility of organic acids increasing the potential for contamination, and the ubiquity of formate and acetate in chemicals and materials used in the analysis, as well as in the atmosphere of most indoor environments.

Desirable target characteristics of a method for quantifying organic acids in the low nM range include:

- Quantifies organic acids over a wide range of concentrations (nM to mM)
- Low detection limits achieved through the combination of a low blank and use of a sensitive fluorescence detector (detection limits in the low nM range)
- High specificity for a large number of organic acids, including formate and acetate
- Derivatization carried out under neutral or acidic conditions
- Small required sample volumes (mL range or below)
- Few sample preparation and handling steps to minimize the chance of contamination
- Minimal sample exposure to air to avoid contamination from volatile compounds
- Reaction times (seconds to minutes at ambient conditions)
- Small required reagent and solvent volumes for economy

1.3 Proposed method for determining organic acid concentrations in seawater

The proposed method for increasing the detectability of LMW organic acids in seawater is to 1. use a carboxylic acid-amine coupling strategy to derive fluorescent carboxylic acid amides, and 2. use HPLC analysis with fluorescence detection to identify and quantify the amide derivatives.

1.4 Fluorescent coumarin amine tag

Fluorescent amides are formed from the dehydrating reaction of a carboxylic ester (derived from the carboxylic acid) and a fluorescent primary amine (Figure 1) (Montalbetti and Falque 2005; Bode 2006). The fluorescent primary amine used in this study is a coumarin. Coumarins are oxygen-rich compounds that are partially water-soluble, yet cell membrane permeable; they can be used as reagents in an aqueous system and extracted into organic solvents or bound to columns with high efficiency (Gismervik 2012). The parent coumarin 4-bromomethyl-6,7dimethoxycoumarin (Figure 2) was chosen based on its hydrophilicity, excitation/emission wavelengths, and intense brightness. However, the parent coumarin requires an amine group to be used as a precursor in an amide forming coupling strategy. A derivative coumarin amine, 4-aminomethyl-6,7-dimethoxycoumarin hydrochloride (Figure 2) has the potential to form amides with acids, and be modified with respect to lipo/hydrophilicity. Further, the underivatized coumarin is only weakly fluorescent compared to the coumarin –carboxylic-acid derivatives due to a photo-electron (PET) quenching process by the lone electron pair on the amine (Figure 3) (Sasamoto et al. 1996). The method for deriving 4-aminomethyl-6,7-dimethoxycoumarin has been described in the literature (Sasamoto et al. 1996; Lim, Pavlova, Brückner 2008) and was carried out as described in the methods and procedures section.

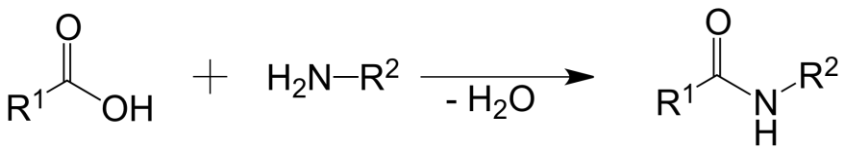


Figure 1. Carboxylic acid coupling strategy via amide bond formation.

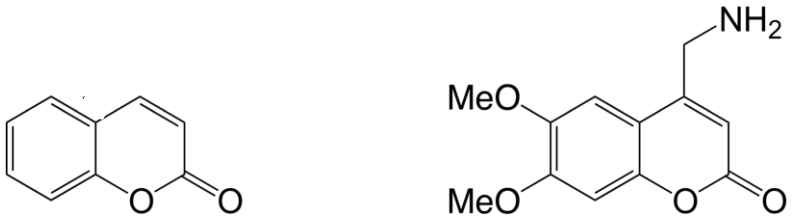
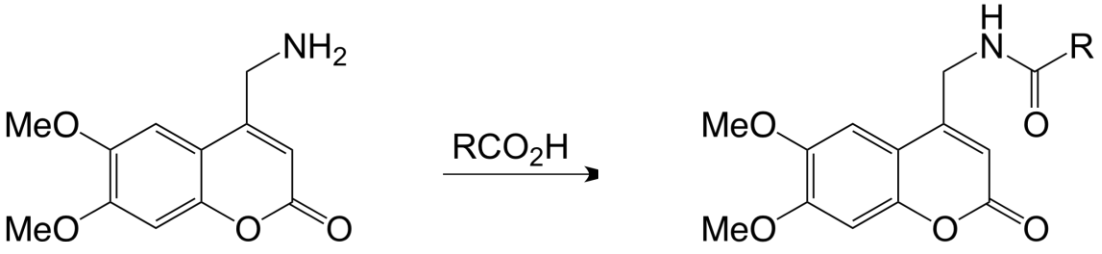


Figure 2. Parent coumarin (4-bromomethyl-6,7-dimethoxycoumarin) and coumarin derivative (4-aminomethyl-6,7-dimethoxycoumarin hydrochloride).



2
weakly fluorescent,
moderately hydrophilic

3
highly fluorescent,
more hydrophilic

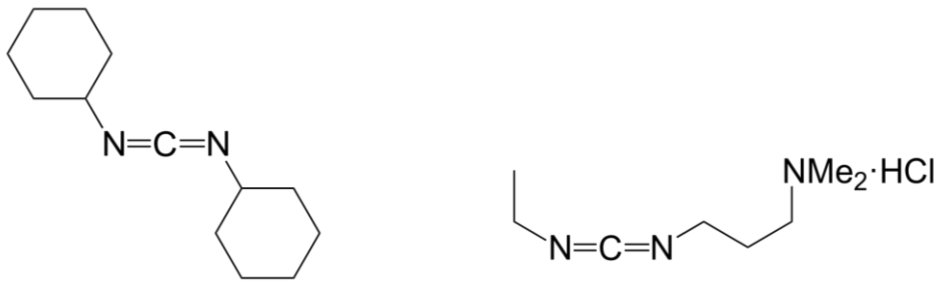
Figure 3. PET quenching and amide formation with high fluorescence.

1.5 Carboxylic acid-amine coupling in aqueous solution

The problem of coupling organic acids at low concentrations with a fluorescent amine is the efficiency of the amide bond formation (Gismervik 2012). Amide bond formation between an acid and an amine are formally condensation reactions (Montalbetti and Falque 2005) and are not spontaneous at ambient temperatures (Yang, Lee, Scranton 1993). It is usually required to first activate the carboxylic acid by converting the –OH of the acid into a good leaving group before reacting with the amine (Valeur and Bradley 2009). In general, organic acids are reacted either in a one- or two-step, one-pot reaction with the amine in the presence of a coupling reagent, whereby the coupling reagent may activate the acid or amine (Gismervik 2012).

Choosing the right combination of coupling reagent and catalyst is critical. Efficiency is key; including high conversion efficiency, minimal epimerization, and limited amounts of by-product (Valeur and Bradley 2009). Hundreds of coupling reagents exist, although many have not been compared and most are not efficient for a broad range of amide bond formation (Valeur and Bradley 2009). The vast majority of amide couplings traditionally require non-aqueous conditions because of the solubility properties of protected amino acids, the most common substrate for amide formations. However, we aim to achieve the coupling reaction in aqueous solution – seawater.

Gismervik (2012) evaluated amide bonding strategies for carboxylic acid-amine coupling in aqueous solution. Among the coupling methods tested, only the carbodiimide mediated reactions led to completion (Gismervik 2012). The carbodiimides tested have been most extensively used in the past and are also most common; including N,N'-dicyclohexylcarbodiimide (DCC) and 1-ethyl-3-(3’-dimethylamino)carbodiimide HCL (EDC) (Figure 4). Carbodiimides were the first acid-amine coupling reagents to be synthesized (Valeur and Bradley 2009), and are very efficient (Mikoz. xl et al. 1981; Nakajima and Ikada 1995; Sheehan and Hess 1955).



A B

Figure 4. Carbodiimide coupling reagents DCC (A) and EDC (B).

On the carbodiimide molecule, it is the carboxylate anion bonded to the central carbon that reacts with the carboxylic acid, theoretically without the presence of additional amine, to form an activated *O*-acylisourea mixed anhydride (Figure 5) (Montalbetti and Falque 2005). The O-acylisourea mixed anhydride then directly reacts with the deprotonated aminomethylcoumarin or another carboxylic acid to form the desired amide product and a urea by-product (Figure 5). The formation of the urea by-product is the driving force for this reaction (Montalbetti and Falque 2005).

During the reaction, undesired racemization and acyl transfer forming the unreactive *N*-acylurea occurs (Montalbetti and Falque 2005; Valeur and Bradley 2009). Addition of a catalyst that reacts faster than the competing acyl transfer and generates an active intermediate can increase yield by inhibiting side reactions and reducing racemization (Carpino 1993; Gismervik 2012; Montalbetti and Falque 2005). This intermediate can then react with the amine to yield the desired amide and the urea by-product.

The most common coupling catalysts used in combination with carbodiimide coupling reagents include N-hydroxysuccinimide (NHS), 1-hydroxy-benzotriazole (HOBt) and Hydroxy-7-azabenzotriazole (HOAt) (Figure 6) (Carpino 1993). These catalysts react with *O*-acylurea to produce an activated ester, which has higher reactivity than an unactivated ester. The increased reactivity of the activated ester is a result of stabilizing the approach of the amine via hydrogen bonding (Valeur and Bradley 2009). HOAt is more efficient than HOBt in terms of yield, kinetics, and reduced racemization (Lim, Pavlova, Brückner 2008). The increased efficiency of HOAt may be due to the additional chelation or to the neighboring effect provided by the pyridine nitrogen during the aminolysis step (Figure 7) (Carpino 1993; Gismervik 2012). The nitrogen on the pyridine moiety of HOAt may act to direct the amine on 4-aminomethylcoumarin

to attack the activated ester through chelation with the amino protein and thereby speed up the overall coupling (Gismervik 2012).

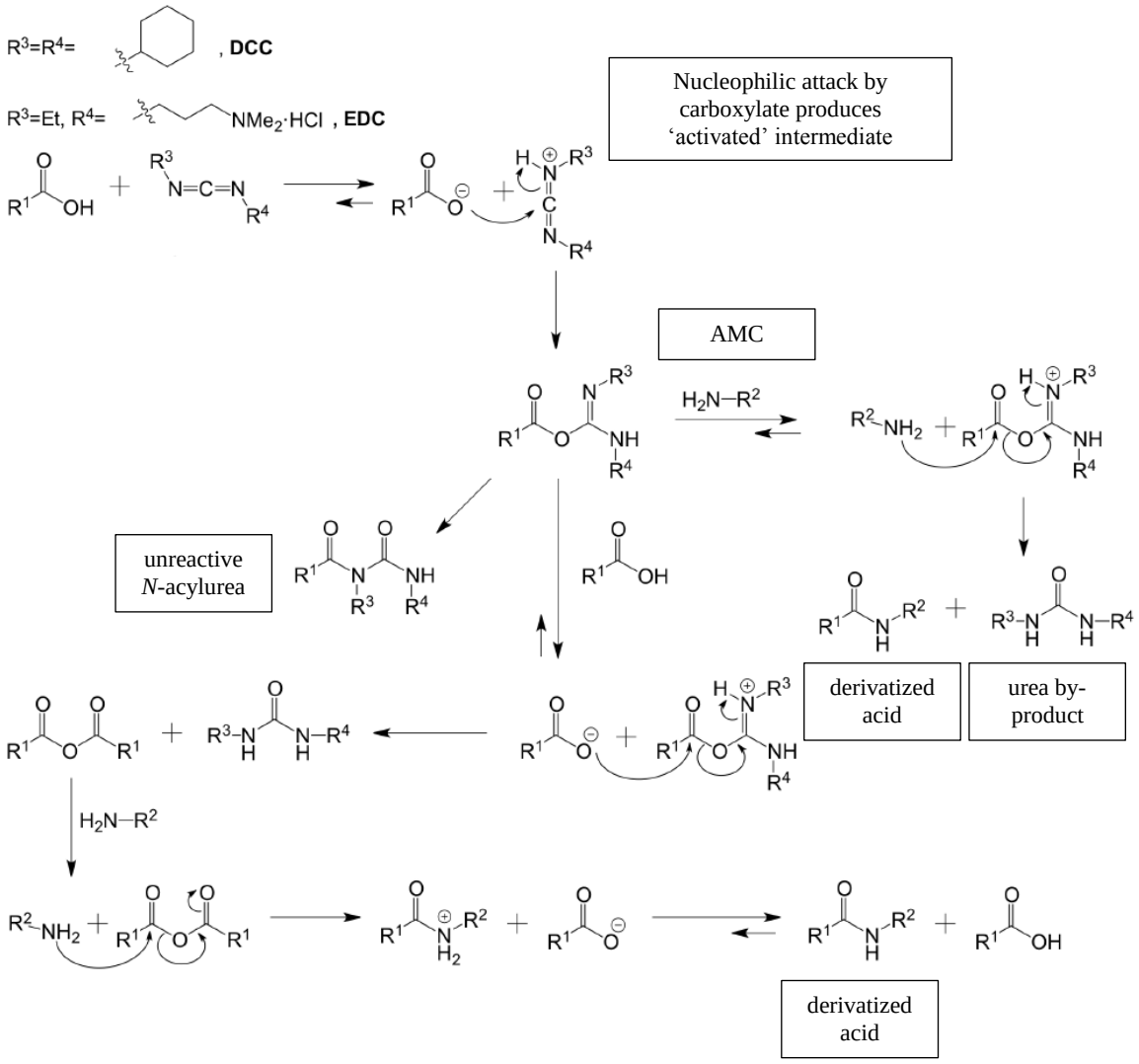


Figure 5. Carbodiimide coupling strategy.

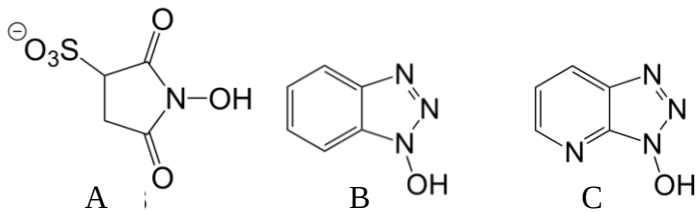


Figure 6. Carbodiimide coupling cataylsts NHS (A), DCC (B), EDC (C)

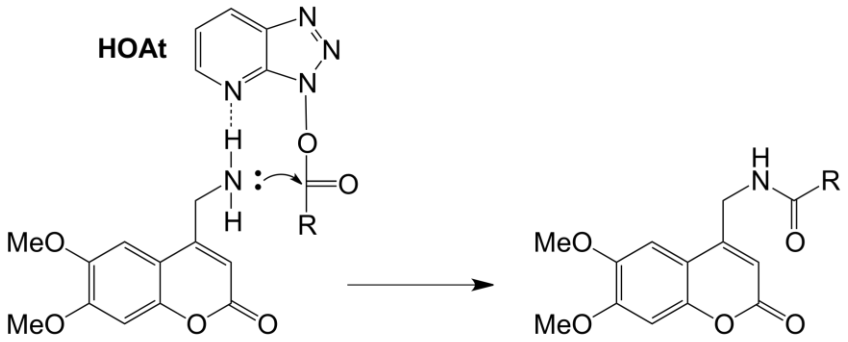


Figure 7. HOAt directs AMC to attack the activated ester.

Gismervik (2012) tested DCC and EDC in combination with NHS, HOBt, HOAt, (Appendix 7.1.1). The organic solvents methylene chloride (DCM) and toluene were also tested for efficiency of extracting the amide products from the aqueous solution. The purpose for including organic solvent in the reaction was the potential to concentrate the amide products and for HPLC compatibility. Triethylamine (TEA) was included in the reaction mixture to deprotonate the 4-aminomethylcoumarin to free aminomethylcoumarin, providing a thermodynamic driving force for the reaction. Propionic acid was used in all screenings of the method.

In the final screening of the method (Appendix 7.1.2), the concentrations of derivatizing reagent, catalyst and TEA were reduced to determine the most efficient combination of derivatizing reagent and catalyst. The reaction with EDC/HOAt was fastest with comparable yield to EDC/HOBt (Gismervik 2012). The efficiency and water-solubility of EDC/HOAt led to the conclusion that this was the best method for derivatization of LMW organic acids at low concentration in aqueous solution (Figure 8).

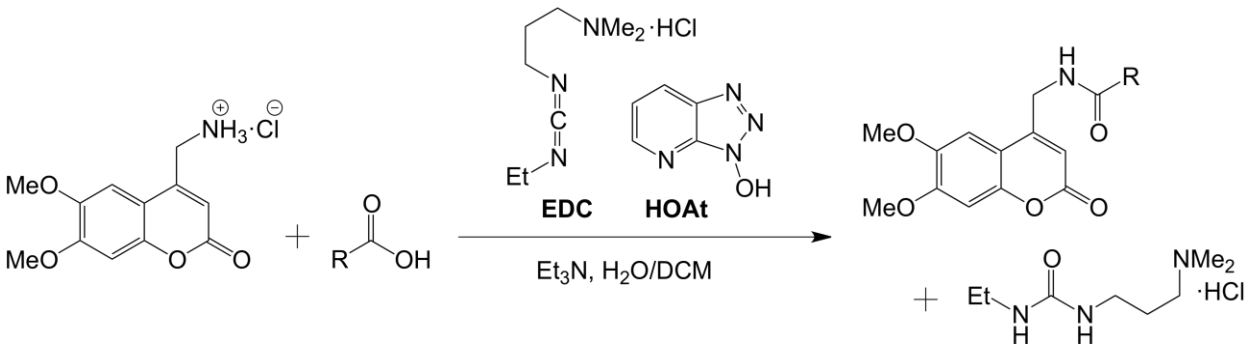


Figure 8. Most efficient coupling strategy for aqueous conditions (Gismervik 2012).

1.6 Derivatization method for aqueous solution

The use of 1 to 1 equivalent concentrations of EDC, HOAt, and Et_3N in the presence of 1 equivalent of 4-aminomethylcoumarin forms the derivatized organic acids when stirred vigorously in minimal volumes of DCM and H_2O (5 mL each) (Table 1). The reaction occurs under mild conditions (ambient temperature) and in quantitative yields (100% completion as determined by TLC and by isolation of the product) (Gismervik 2012).

Table 1. Reagent concentrations in initial derivatization method.

Reagent	Moles	Volume, mL	Concentration, mM
HOAt	1.98e-5	5	1
EDC	1.98e-5	-	1
Coumarin	1.98e-5	-	1
TEA	1.98e-5	-	1
DCM	1.98e-5	5	1

2 Aim of Thesis

This thesis aims to determine the best amide coupling strategy for derivatization of LMW organic acids with a fluorescent coumarin at sub-micromolar concentrations in natural seawater. The research was conducted in two parts. First, an HPLC method was established for separating a suite of pre-derivatized amide standards. Second, the previously determined best derivatization method for LMW organic acids in aqueous solution (Gismervik 2012) was optimized for derivatizing LMW organic acids in seawater at relevant concentrations.

3 Materials and Procedures

3.1 Synthesis of fluorescent coumarin amine

The fluorescent coumarin tag for the organic acid derivatization, 4- aminomethyl-6,7-dimethoxycoumarin hydrochloride was prepared (Appendix 7.2.1) from commercially available 4-bromomethyl-6,7-dimethoxycoumarin by a Delépine reaction (Figure 9) (Lim et al. 2008; Gismervik 2012). Carrying out the Delépine reaction to form 4-aminomethylcoumarin (AMC) is cost-efficient and yields products and intermediates as crystalline solids, allowing their purification through repeated recrystallization. AMC is also commercially available from multiple vendors and varies in price between $250-$300 per 50 mg. The derivatization optimization for aqueous solution carried out in Dr. Bruckner's laboratory required a large amount of AMC. To reduce cost, we chose to synthesize the coumarin in Dr. Bruckner's laboratory.

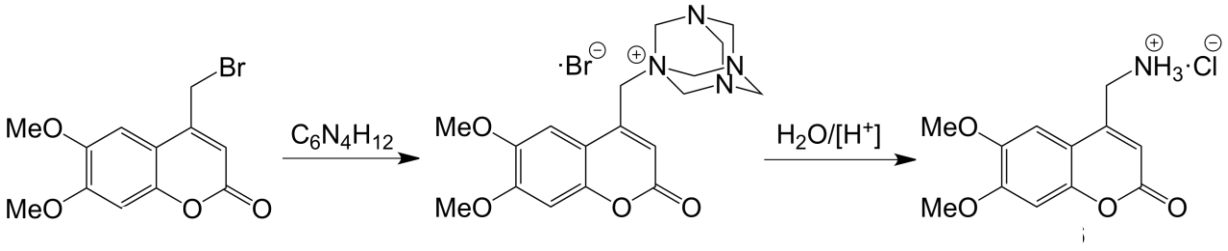


Figure 9. Delépine reaction forming 4-aminomethyl-6,7-dimethoxycoumarin hydrochloride.

3.2 HPLC analysis: standards and methods

3.2.1 Synthesis of coupled amide standards

Brian Gismervik and our collaborators in the laboratory of Christian Brückner at the University of Connecticut carried out the synthesis of a suite of fluorescent amide standards (Appendix 7.2.2). The amide standards were chosen based on their occurrence in marine environments (Yang, Lee, Scranton 1993; Albert and Martens 1997; Wu, Green, Scranton 1997) and include C1-C7 organic acids, branched iso-butyric acid, and aromatic benzoic acid (Figure 10). Derivatized acid standards were prepared (Appendix 7.2.2) using a standard acid chloride-amine coupling strategy (Gismervik 2012). Acid chlorides are a chemically active form of the organic acids. Therefore the acid chlorides form the corresponding amide with an amine without the necessity of coupling reagents. The derivatized acid standards remain stable at room temperature for months in a tightly sealed vial, however they should be stored in a desiccator at 8º C.

To prepare amide standard solutions, determined amounts of individual amide standards were initially dissolved in ACN then diluted with milli-Q water and vigorously vortexed prior to injection. Prepared standard solutions were stored in the freezer at -20ºC. Amide standards are stable in solution for over 120 days when stored in the freezer at -20ºC (Appendix 7.3.1).

3.2.2 HPLC method

The separation and detection of derivatized organic acids was performed with an Agilent Hewlett Packard HPLC 1100 system equipped with an autosampler, fluorescence detector (FLD) and ChemStation software. Derivatized amides were separated using an Agilent Zorbax Eclipse Plus C18 reversed-phase column (4.6 mm x 150 mm, 5 μm) with an Agilent Zorbax Eclipse Plus-C18 guard column guard column (4.6 mm x 12.5 mm, 5 μm).

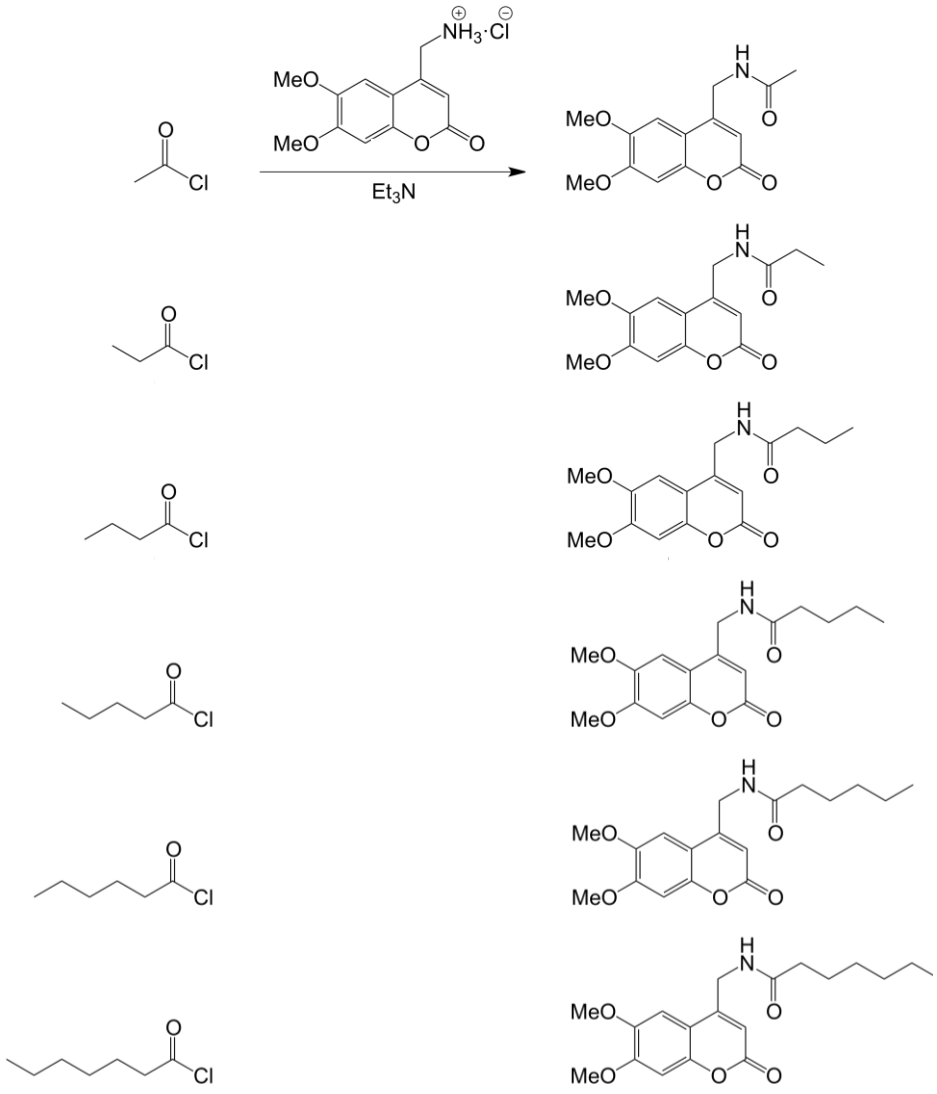


Figure 10. Carboxylic acid standards (right side of figure) formed by reacting acid chlorides with (left side of figure) aminomethylcoumarin.

Gradient elution method

Amide derivatives were separated (Figure 11) with an empirically determined water-acetonitrile gradient (Table 2). Mobile phase A was 10 mM potassium phosphate buffer (pH 3) prepared with milli-Q water. Mobile phase B was 100% acetonitrile. The gradient started with 95% of mobile phase A and decreased to 80% A at 3.0 min, 66% A at 8.0 min, 65% A at 11 min,

41.5% A at 15 min with a 3 min hold, 0% A at 19 min with a 1 min hold, and a return to 95% A at 23 min. The column temperature was maintained at 40°C. The flow rate was 1.0 mL min^{-1}, and the total run time was 23 min. An optimum excitation wavelength (240 nm) and emission wavelength (425 nm) was determined for simultaneous detection of all derivatized acids based on the iso-absorbance chromatogram in the ChemStation software (Table 3). The iso-absorbance plot displays the acquired spectra as a color-contoured map of wavelength against retention time. The critical band pair, i.e. the pair of peaks with the lowest resolution (distance in time) between peaks in a chromatogram, is formate at 9.8 min and acetate at 10.0 min.

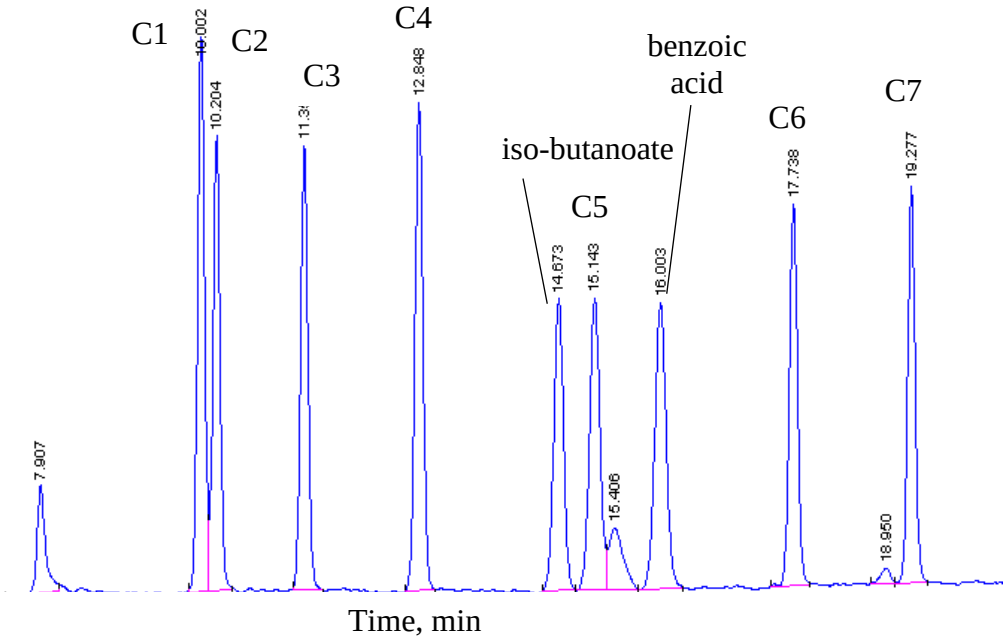


Figure 11. Separation of C1-C7 organic acids, branched iso-butanoate and aromatic benzoic acid at 500 nM each (represented in arbitrary fluorescence units).

Table 2. HPLC gradient elution method.

HPLC Method		
Time, min	Mobile phase A	Mobile phase B
0.0	95.0	5.0
3.0	80.0	20.0
8.0	66.0	34.0
11.0	65.0	35.0
15.0	41.5	58.5
18.0	41.5	58.5
19.0	0.0	100.0
20.0	0.0	100.0
3.0	95.0	5.0

Table 3. Optimum emission and excitation wavelengths.

Reference Peak	Excitation at 240 nm (optimal emission wavelength, nm)	Emission at 425 nm (optimal excitation wavelength, nm)
Formate	415-440	230
Acetate	423-430	228-232
Propionate	415-430	230
Butanoate	415-440	230-232
iso-butanoate	420-430	230-231
Pentanoate	415-435	228-234
Benzoate	420-430	230
Hexanoate	415-427	230-231
Heptanaote	424-425	230
Average	425	230

3.3 Optimization of aqueous derivatization method for seawater samples

3.3.1 Cleaning procedures

Before use, all glassware and plasticware were soaked in acid (15% HCl) for 24 h and rinsed three times with milli-Q water. Glassware was wrapped with aluminum foil and muffled at 500°C for three hours. The glassware remained foil wrapped until use. Plasticware was rinsed in plastic bags, oven dried at 48°C, and kept in closed plastic bags until used.

3.3.2 Chemicals

A single batch of milli-Q water was collected into two Kimex 1-L glass bottles and stored at room temperature. This water was used to make all solutions and was assumed to have constant and very low background concentrations of organic acids.

Seawater collected from the Bermuda Atlantic Time-Series Study (BATS) is available by request and is generally used as a control sample for total and dissolved organic carbon concentration determinations. We intended to use it as a control sample for the organic acid determinations. The BATS seawater had been collected in July 2014. Samples from a depth of 300 m or below where consolidated, filtered with a muffled Whatman GF/F 0.7 μm pore size glass microfiber filter, and stored in a 2.5-L glass bottle at room temperature.

A 24 mM (2.3 g/L) sodium propionate (99%, Aldrich Chemical Company, Inc.) solution was prepared with milli-Q water in a 100-mL volumetric flask. The solution was filtered with a 0.2 μm pore size syringe tip filter into a Kimex 250-mL glass bottle and refrigerated until use. For each experiment, an aliquot of the stock solution was transferred to a 25-mL glass vial with a glass pipette.

A 0.1 M PO_4 buffer was prepared according to Gomori (1955). Buffer solution A, 0.1 M (6.7 g/L) monobasic sodium phosphate (99.2%, Fisher Scientific) was prepared with milli-Q water in a 250-mL volumetric flask. Buffer solution B, 0.1 M (3.5 g/L) dibasic sodium phosphate (99.8%, Fisher Scientific) was prepared with milli-Q water in a 250-mL volumetric flask. The final buffer solution (pH 5.6) was prepared by mixing 237 mL of solution A and 13 mL solution B. This buffer (0.1 M) was used to determine the amount necessary to achieve an optimal reaction pH. Once the necessary amount was determined a more concentrated buffer (0.4 M) was prepared (see below) to avoid diluting the seawater samples and was used in all later studies.

A 0.4 M PO_4 buffer was prepared according to Gomori (1955). Buffer solution A, 0.4 M (107.2 g/L) monobasic sodium phosphate (99.2%, Fisher Scientific) was prepared with milli-Q water in a 250-mL volumetric flask. Buffer solution B, 0.1 M (55.2 g/L) dibasic sodium phosphate (99.8%, Fisher Scientific) was prepared with milli-Q water in a 250-mL volumetric flask. The final buffer solution (pH 5.6) was prepared by mixing 237 mL of solution A and 13 mL of solution B.

A 51 mM (6.9 g/L) solution of HOAt (99%, AK Scientific, HPLC grade) was prepared with milli-Q water in a 100-mL volumetric flask. To completely dissolve the HOAT, the solution was heated to 37°C in an ultrasonic bath for 1 h. Once dissolved, the solution was transferred to a Kimex 250-mL glass bottle and refrigerated at 8°C until use. Before use, the solution was again gently heated to 35-37°C in a water bath until completely dissolved. Once dissolved, an aliquot of the stock solution was transferred to a 25-mL glass vial with a glass pipette. HOAt is considered hazardous to skin, eyes, and respiratory system, and has explosive properties (Fisher Scientific, Inc), therefore special precautions should be taken when handling this compound.

All EDC solutions were prepared immediately before experimentation. EDC solutions at concentrations of 51 mM (9.6 g/L) - 200 mM (38.3 g/L) (98-100%, Thermo Scientific) were prepared with milli-Q water. The compound was weighed directly into a 25-mL glass vial, and tightly capped until dissolved.

A 20 mM (2.3 g/L) solution of AMC was prepared in milli-Q water. The compound was weighed directly into a 2-mL glass vial. The solutions were prepared immediately before experimentation.

A TEA dilution was not carried out due to the low solubility of TEA in water (5.5% at 20°C). Instead, an aliquot of pure TEA (99%, Acros) was transferred to a 25-mL glass vial with a glass pipette and TEA was added directly to the reaction.

3.3.3 Initial assessment of derivatization method for aqueous solution

The initial derivatization method for aqueous solution as described by Gismervik (2012) was applied to milli-Q water samples and natural seawater samples using duplicate samples. However, the concentration of reagents was reduced from 3.4 mM to 1 mM, and after the reaction, the products were analyzed with HPLC as opposed to TLC.

Methods

To carry out the derivatization, chemicals were added to a 25-mL round bottom flask in the following order: seawater (except for milli-Q water only treatments), milli-Q water, propionate acid solution, HOAt, EDC, AMC, and TEA (Table 4). 5 mL of DCM was added to each reaction. A magnetic stir bar was added to each flask and the flask was tightly capped with a glass stopper, sealed with seal view, and stirred for 24 h. After 24 h, an aliquot of DCM (with extracted propionate and underivatized coumarin) was transferred using a glass pipette to a pre-weighed 2-mL amber vial. The vials with DCM were weighed and the volume of DCM was determined by mass and density. The volume of DCM is necessary to calculate the concentration of organic acids in the original sample based the concentration of derivatized amide products in the DCM. The DCM was removed using an Organomation Nitrogen Evaporator (N-Evap111) and the extracted amide products, as well as underivatized coumarin, were recovered as a yellowish crystalline powder. The compounds were re-dissolved in 1 mL acetonitrile for HPLC analysis.

Table 4. Conditions for testing initial coupling method for aqueous solution.

Sample	Sample volume, mL		Concentration of reagent in reaction, mM				
	milli-Q water	BATS seawater	Propionate	HOAt	EDC	AMC	TEA
Milli-Q Water	5.0	-	1.0	1.0	1.0	1.1	1.0
BATS Seawater	-	4.5	1.0	1.0	1.0	1.1	1.0

[a]Reported concentration represents moles per total reaction volume (5 mL).

Results and Discussion

In contrast to the findings of Gismervik (2012) we found that the proposed outlined derivatization method for aqueous solution did not yield 100% of the derivatized carboxylic acid (Table 5). Therefore, the method needed additional improvement to derivatize organic acids in seawater at relevant concentrations.

Table 5. Yield of derivatized propionate (1 mM) as propanamide using initial conditions given in table 4.

Sample	Average amide concentration[a], M	Yield, %
Milli-Q Water	2.30E-04	23.0
BATS seawater	2.26E-05	2.3

3.3.4 Experiments optimizing the derivatization method for seawater samples

Nine hypotheses were tested to improve the method for derivatization of organic acids.

3.3.4.1 Experiment 1

The carbonate buffering system of seawater results in a pH of ~8.1. EDC crosslinking is most efficient under acidic conditions, with optimum pH between 4.7-6.0 (Thermo Fisher

Scientific Inc). However, phosphate buffers and neutral pH (up to 7.2) conditions are compatible with the reaction (Thermo Fisher Scientific Inc). Based on preliminary studies (Table 6), the minimum concentration of PO_4 buffer in the reaction should be about 9 mM to achieve pH (5.5) in the optimal range for efficient coupling with EDC. Further additions of PO_4 buffer do not decrease pH below 5.3.

Table 6. Effect of phosphate buffer concentrations on reaction pH.

[PO_4 buffer] mM	pH
0.0	7.8
9.1	5.5
16.7	5.3
23.1	5.3

Hypothesis

If PO_4 buffer is added to the seawater sample to achieve a pH between 4-6, the recovery of propionate as derivatized propanamide will increase.

The effect of adding PO_4 buffer to the method was tested in triplicate with the following treatments:

A. Derivatization method for aqueous solution

B. Addition of PO_4 buffer (8 mM; 10% 0.1 M v/v of reaction mixture)

Methods

The aqueous derivatization method was applied to milli-Q water and BATS seawater. To accommodate a large number of samples, each reaction was carried out in a 22 mL glass vial instead of a round bottom flask, the reactions were shaken at 182 rpm rather than stirred, and

DCM was not included in the reaction mixture, but was used after the completed reaction to extract the derivatives.

Reagents were added to a 22 mL glass vial in the following order: Seawater (except for milli-Q water only treatments), milli-Q water, propionate solution, PO_4 buffer, HOAt, EDC, AMC, and TEA (Table 7). The seawater volume was 4 mL. The 0.1 M PO_4 buffer volume was 400 µL (10%v/v seawater). The total reaction volume was 5 mL. The concentration of propionate and all other reagents in the reactions were 1 mM. The pH was recorded after the addition of each reagent for each treatment. The vials were tightly capped with PTFE/silicone septa and shaken for 24 h at 182 rpm. After 24 h, 5 mL of DCM was added to each vial and then vigorously vortexed 3 times for 5 seconds intervals, allowing the phases to separate between vortexing. The samples were placed in an ultrasonic bath for 1 minute to remove air bubbles from the aqueous-DCM interface. An aliquot of DCM was transferred to a 2 mL amber vial for each reaction. The volume of DCM was determined by mass and density as previously described. The DCM was evaporated under N_2 gas and the extracted derivatization products, as well as uncoupled AMC were recovered as a yellowish crystalline powder. The compounds were re-dissolved in 1 mL acetonitrile and vigorously vortexed for 5 seconds each. To avoid negative peaks caused by uncoupled coumarin at high concentrations, small sample volumes (2 µL) were injected.

Conclusion

The addition of PO_4 buffer to the derivatization method decreased the pH of the reaction (Table 8) and increased the recovery of propionate as propanamide derivatives (Figure 12). PO_4 buffer will be used to decrease the reaction pH in the derivatization method.

Table 7. Concentration of reagents for treatments in experiment 1.

Treatment	Reaction volume, mL	Sample volume, mL		Concentration of reagent in reaction, mM					
		milli-Q water	BATS seawater	Acid[a]	HOAt	EDC	AMC	TEA	PO_4 buffer
A	5.0	1.0	4.5	1.0	1.0	1.0	1.1	1.0	-
B	5.0	1.0	4.0	1.0	1.0	1.0	1.1	1.0	8.0

[a]Propionate

Table 8. The pH difference for samples treated with buffer compared to samples without buffer.

	Reaction pH	
	Without PO_4 buffer	With PO_4 buffer
Initial	8.0	8.1
Final	9.8	6.4

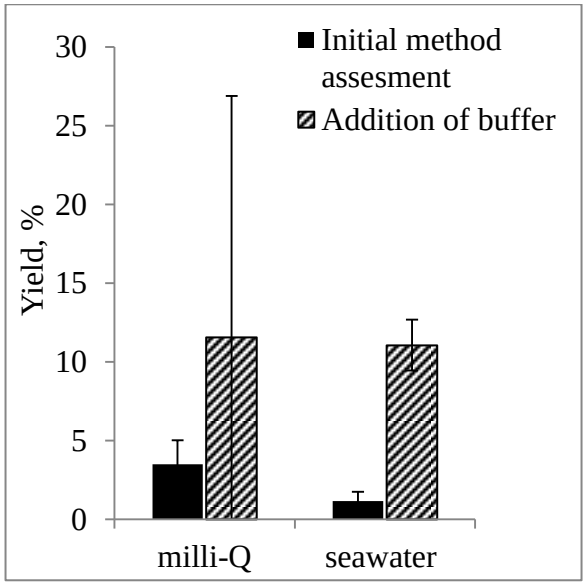


Figure 12. Effect of buffering on propionate (1 mM) derivatization yield.

3.3.4.2 Experiment 2

TEA is included in the method as a basic buffer (pKa 10.8) to deprotonate AMC. However, PO_4 buffer is added to the derivatization method to decrease the reaction pH to 6.4.

Hypothesis

TEA fills no function in the current derivatization method with the use of PO_4 buffer.

The effect of excluding TEA from the method, in addition to adding PO_4 buffer, was tested in triplicate reactions with the following treatments:

A. Derivatization method for aqueous solution (including TEA, excluding PO_4 buffer)
B. Excluding TEA, excluding PO_4 buffer
C. Including TEA, including PO_4 buffer (8 mM; 2.5% 0.4 M v/v seawater)
D. Excluding TEA, including PO_4 buffer (8 mM; 2.5% 0.4 M v/v seawater)
E. Including TEA, including PO_4-buffer (16 mM; 5.0% 0.4 M v/v seawater)
F. Excluding TEA, including PO_4-buffer (16 mM; 5.0% 0.4 M v/v seawater)

Methods

The aqueous derivatization method was applied to milli-Q water and BATS seawater as in section 1.2, with the exception of the designated treatments (Table 9). The concentration of the PO_4 buffer solution was increased from 0.1 M to 0.4 M to avoid diluting the samples. The 0.4 M PO_4 buffer volume added was 100 μL (2.5% v/v seawater) or 200 μL (5.0% v/v seawater). The pH was recorded after the addition of each reagent for all treatments.

Table 9. Concentration of reagents for treatments in experiment 2.

Treatment	Reaction volume, mL	Sample volume, mL		Concentration of reagent in reaction, mM					
		milli-Q water	BATS seawater	Acid[a]	HOAt	EDC	AMC	TEA	PO_4 buffer
A	5.0	1.0	4.0	1.0	1.0	1.0	1.1	1.0	-
B	5.0	1.0	4.0	1.0	1.0	1.0	1.1	-	-
C	5.0	1.0	4.0	1.0	1.0	1.0	1.1	1.0	8.0
D	5.0	1.0	4.0	1.0	1.0	1.0	1.1	-	16.0
E	5.0	1.0	4.0	1.0	1.0	1.0	1.1	1.0	8.0
F	5.0	1.0	4.0	1.0	1.0	1.0	1.1	-	16.0

[a]Propionate

Conclusion

Excluding TEA from the method increased the percent recovery of propionate as propanamide derivatives (Figure 13). The treatments that excluded TEA and included 8 mM PO_4 buffer seawater had the highest recovery of propanamide derivatives (20.3%). Therefore, TEA will be removed from the derivatization method.

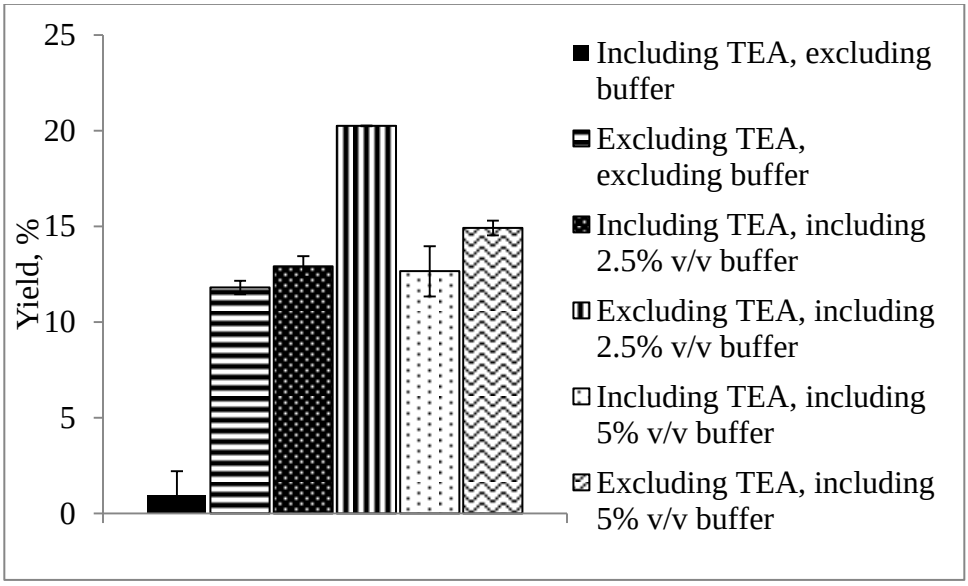


Figure 13. Effect of triethylamine on propionate (1 mM) derivatization yield.

3.3.4.3 Experiment 3

Kinetic studies on the rate of hydrolysis of EDC to its non-reactive urea by-product (EDU) in aqueous solutions demonstrates that, under commonly used conjugation conditions (pH ~5), after 8 min 50% of the starting EDC was converted to EDU, and that after 2 h reaction, less than 0.5% of the EDC will be left (Lei et al. 2002). Previous studies (unpublished research) suggest that adding multiple aliquots of EDC to the reaction over a 12-hour period improves derivatization yield.

Hypothesis

If EDC is added in multiple aliquots, the recovery of propionate as derivatized propanamide will increase.

The effect of adding EDC to the reaction in multiple additions was tested in triplicate reactions with the following treatments:

A. Excluding TEA, including PO_4 buffer

B. Excluding TEA, including PO_4 buffer, 3 aliquots of EDC (0, 1 and 12 h)

Methods

The aqueous derivatization method was applied to natural seawater as in section 2.2, with the treatments specified in 3.1 (Table 10). After 1 h, a second addition of EDC to specified samples was carried out. After the addition, all samples were shaken at 182 rpm. After 10 h, the third addition to specified samples was carried out. After the addition, all samples were shaken at 182 rpm for a total of 24 h. After 24 h, the samples were treated as in section 1.2.

Conclusion

Adding EDC to the reaction in multiple aliquots increased the percent recovery of propionate as propanamide derivatives (Figure 14). Therefore, the derivatization method will be changed such that EDC is added in three aliquots in the first 12 h of the reaction.

Table 10. Concentration of reagents for treatments in experiment 3.

Treatment	Reaction volume, mL	Sample volume, mL		Concentration of reagent in reaction, mM				
		milli-Q water	BATS seawater	Propionate	HOAt	EDC[a]	AMC	PO_4 buffer
A	5.0	1.0	4.0	1.0	1.0	1.0	1.0	8.0
B	5.0	1.0	4.0	1.0	1.0	1.0	1.0	8.0

[a] Initial EDC concentration in the reaction.

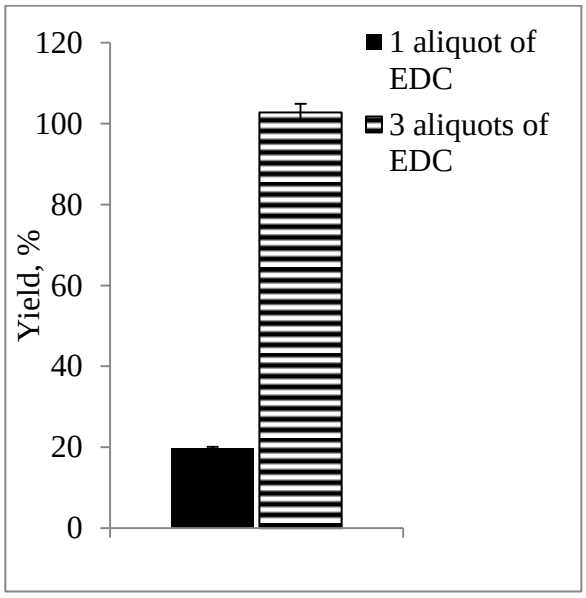


Figure 14. Effect of multiple aliquots of EDC on propionate (1 mM) derivatization yield.

3.3.4.4 Experiment 4

Hypothesis

The aqueous phase of the reaction mixture can be used for the HPLC concentration determinations.

Triplicate samples from experiments 3 and 8 were used to compare the recovery of derivatized propanamide by DCM extraction to direct HPLC injection of the aqueous phase of the reaction.

A. Experiment 3- (A) excluding TEA, including PO_4 buffer
B. Experiment 3- (B) excluding TEA, including PO_4 buffer, 3 aliquots of EDC
C. Experiment 8- (A) excluding TEA, including PO_4 buffer, 200 μM coumarin, 3 aliquots of EDC

Methods

Once the reactions were complete, an aliquot of the aqueous phase of each sample was transferred to a 2 mL amber vial. The volume of each aliquot was recorded. The propanamide derivatives were then extracted and recovered as in section 1.2.

Conclusion

The water-soluble urea by-products of the derivatization reaction, as well as the derivatization reagents, were not detected using the HPLC separation method for isolating amide derivatives. Although recovery of propionate as propanamide derivatives was slightly higher when extracted with DCM from the reaction mixture compared to direct injection of the aqueous phase (Figure 15) the two methods yield nearly equal recovery of propanamide. Therefore, as a step towards HPLC automation, the derivatization method should be changed such that the aqueous phase of the reaction is directly injected into the HPLC for analysis of propanamide

derivatives. However, because DCM extraction can be used to concentrate derivatives this alternative method will have lower method detection limits than direct injection of derivatives in the aqueous phase.

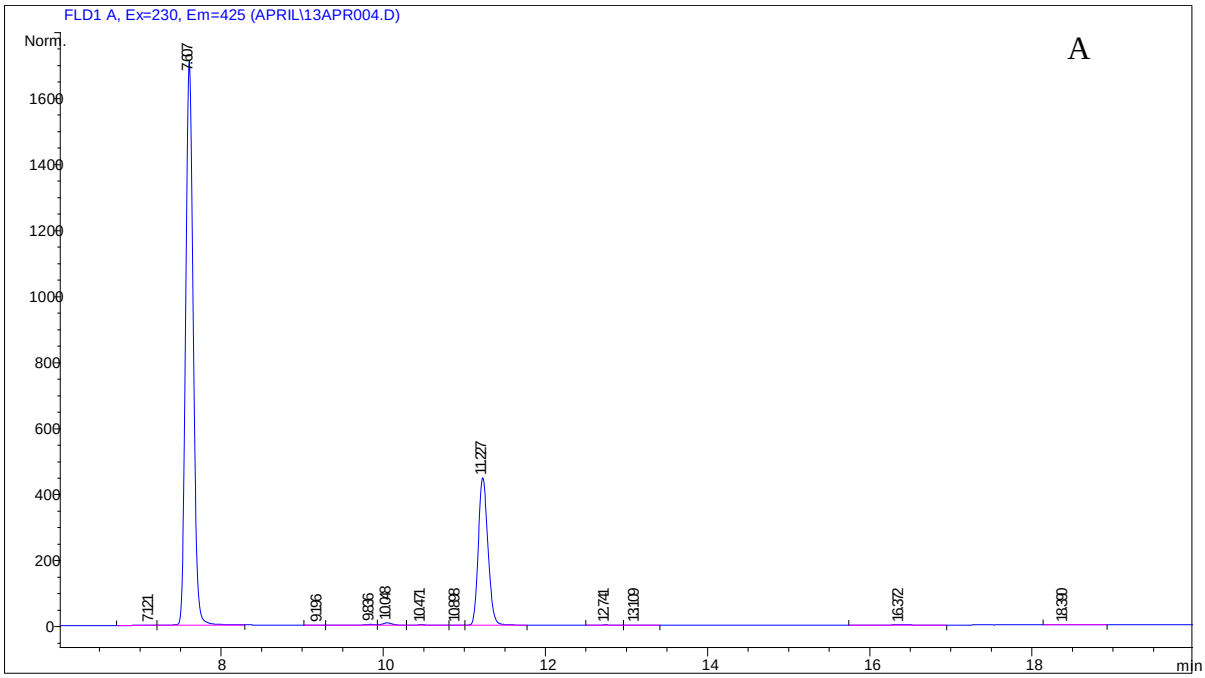


Figure 15. HPLC chromatograms comparing the injection of the aqueous phase of a sample to an injection of acetonitrile with derivatized propionate recovered by DCM extraction. Experiment 3A (excluding TEA, including PO_4 buffer), (A) injection of reaction aqueous phase, and (B) injection of products extracted with DCM. Experiment 3B (excluding TEA, including PO_4 buffer, 3 aliquots of EDC), (C) injection of reaction aqueous phase, and (D) injection of products extracted with DCM.

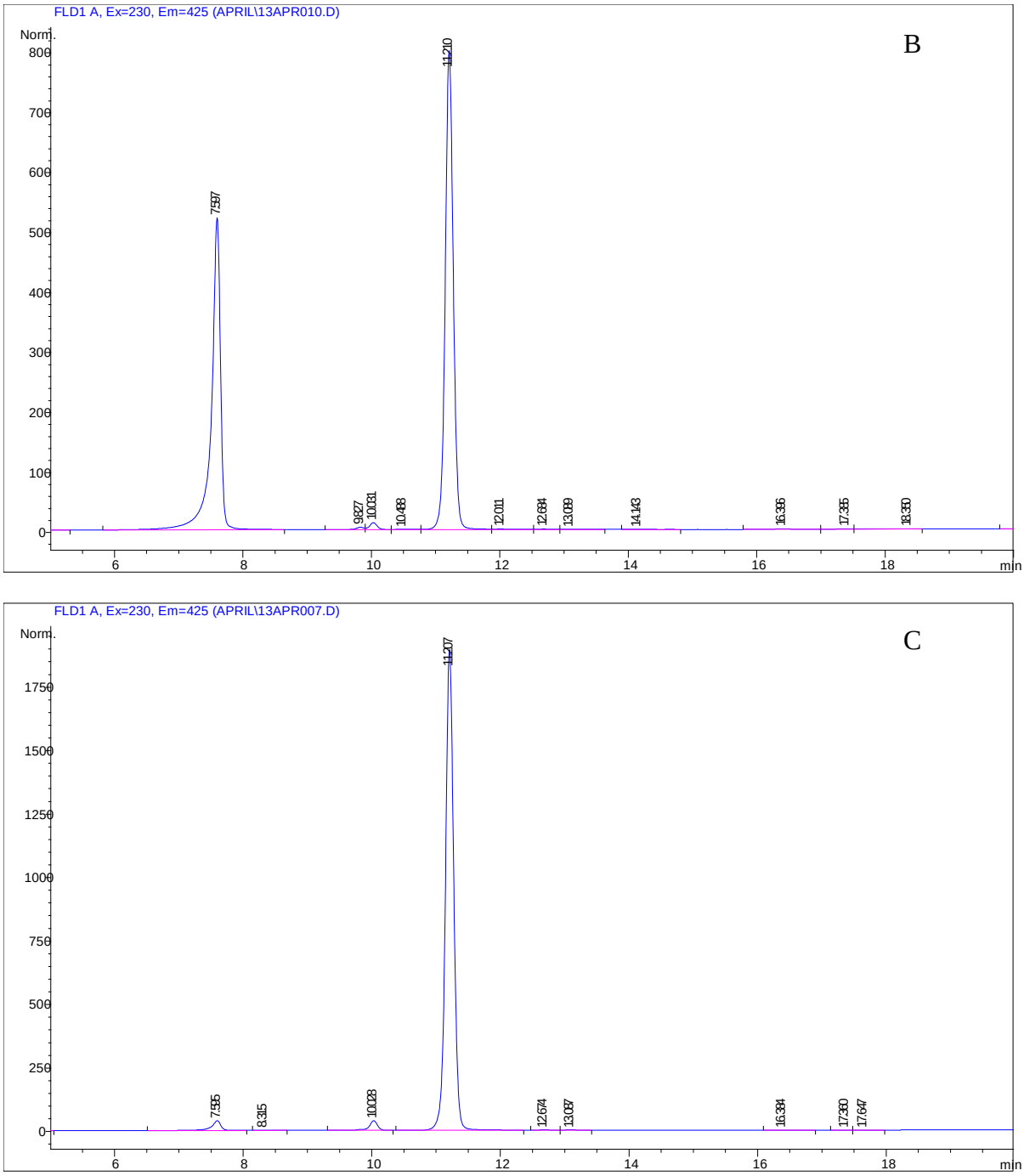


Figure 15. Continued

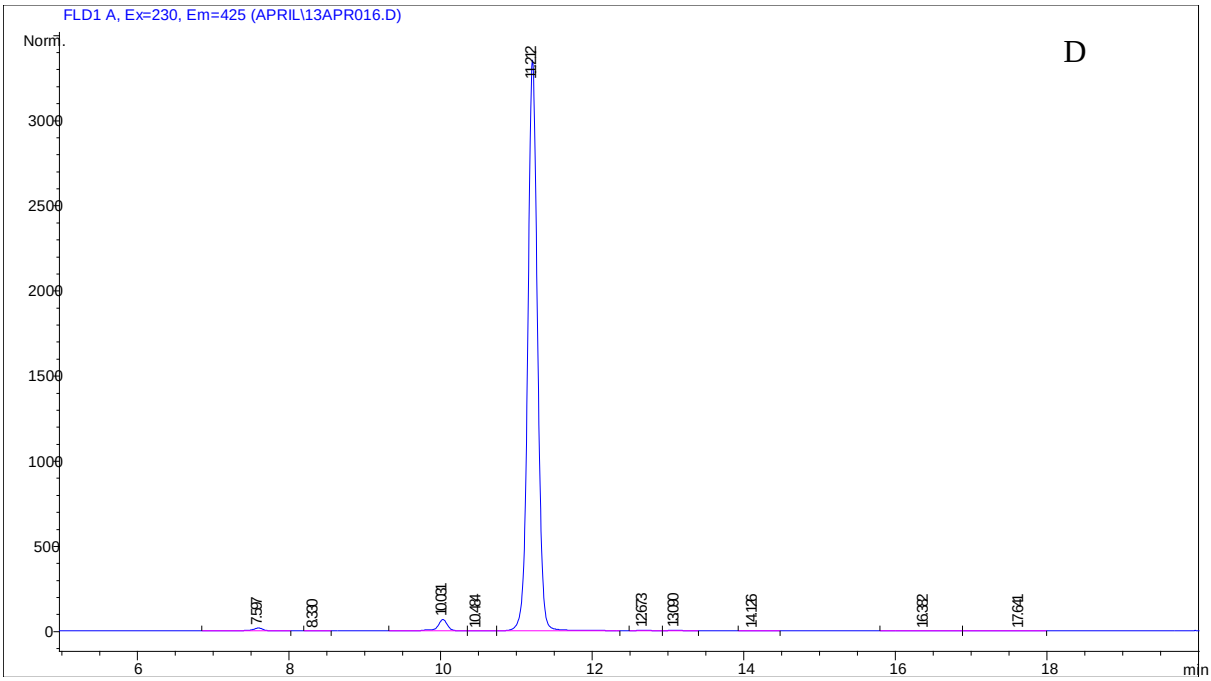


Figure 15. Continued

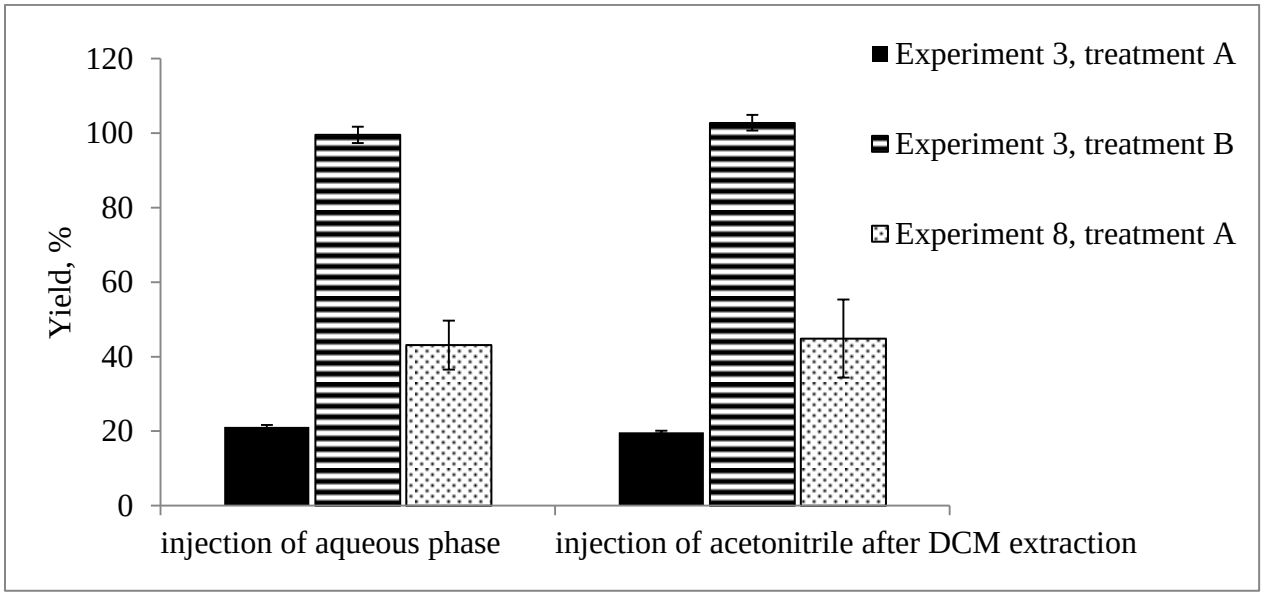


Figure 16. Derivatized propionate yield when injecting either a sample derivatized with aqueous phase only, or injecting derivatized propionate recovered from the aqueous phase by DCM extraction. Propionate is 1 mM in all treatments.

3.3.4.5 Experiment 5

In the previous experiments, the propionate concentration was 1 mM, but should be reduced to simulate relevant environmental concentrations. However, decreasing the concentration may result in lower recoveries, as a result of slower kinetics.

Hypothesis

If the concentration of propionate is reduced to simulate relevant seawater concentrations, the recovery of propionate as propanamide may decrease due to kinetic effects.

Thus, the effect of decreasing the propionate concentration in the reaction was tested in triplicate reactions with the following treatments:

A. 1 mM propionate

B. 10 μM propionate

C. 1 μM propionate

Methods

The aqueous derivatization method determined in 4.3 was applied to natural seawater with the treatments specified in 5.1 (Table 11).

Conclusion

The recovery of propionate as propanamide derivatives decreased as the propionate concentration in the reaction was decreased (Figure 17). The method derivatized propionate at 1 μM concentrations, although the yield was low (11.8%). Therefore, the derivatization method can be applied to samples with low concentrations of organic acids, although with lower yield.

Table 11.Concentration of reagents for treatments in experiment 5.

Treatment	Reaction volume, mL	Sample volume, mL		Concentration of reagent in reaction, mM				
		milli-Q water	BATS seawater	Propionate	HOAt	EDC[a]	AMC	PO_4 buffer
A – 1mM	5.0	1.0	4.0	1.0	1.0	1.1	0.2	8.0
B – 10μM	5.0	1.0	4.0	0.01	1.0	1.1	0.2	8.0
C – 1μM	5.0	1.0	4.0	0.001	1.0	1.1	0.2	8.0

[a] Initial EDC concentration in the reaction.

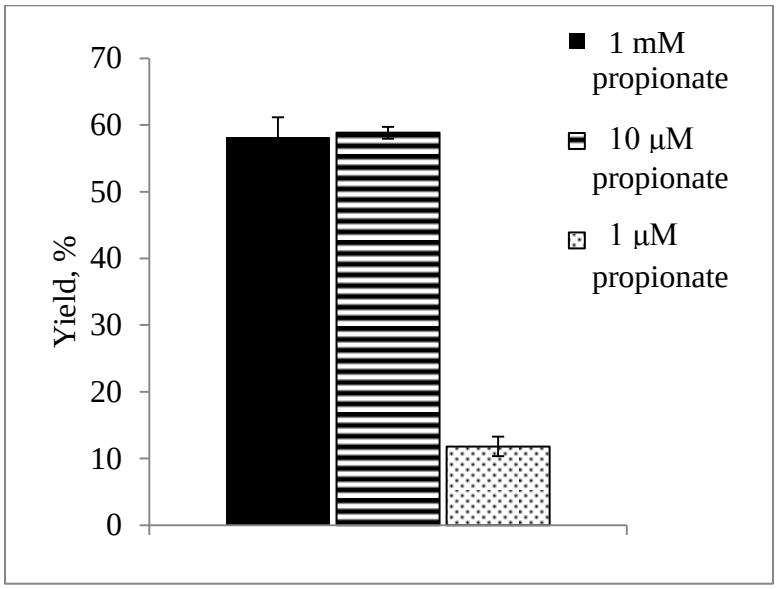


Figure 17. Effect of decreasing the concentration of propionate on propionate derivatization.

3.3.4.6 Experiment 6

Based on experiment 5, the method can derivatize organic acids at low micro-molar concentrations. At such low concentrations, most of the AMC is recovered underivatized. A high concentration of AMC injected into the HPLC can oversaturate the fluorescence detector and result in negative peaks. These negative peaks shift the chromatogram baseline and the integrated peak areas for derivatized organic acids are overestimated. To avoid negative peaks

and integration effects, the coumarin concentration should be adjusted relative to the total organic carbon concentration of the sample.

Hypothesis

If the concentration of AMC in the reaction is reduced relative to the total organic carbon of the sample, undesirable fluorescence effects from underivatized AMC will be reduced.

Thus, the effect of decreasing the concentration of AMC in the reaction based on anticipated organic acid concentration was tested in triplicate reactions with the following treatments:

A. 220 μM coumarin, 200 μM propionate
B. 220 μM coumarin, 20 μM propionate
C. 220 μM coumarin, 2 μM propionate
D. 11 μM coumarin, 10 μM propionate
E. 11 μM coumarin, 1 μM propionate
F. 11 μM coumarin, 750 ηM propionate

Methods

The aqueous derivatization method determined in 5.3 was applied to natural seawater with the treatments specified in 6.1 (Table 12).

Conclusion

The recovery of propionate as propanamide derivatives was higher for treatments with 220 AMC than in treatments with 11 μM AMC (Figure 18). Decreasing the concentration of AMC for samples with low TOC is not kinetically favorable, especially for samples with extremely dilute organic acid concentrations. Therefore, the concentration of AMC in the method should be 220 μM.

Table 12. Concentration of reagents for treatments in experiment 6.

Treatment	Reaction volume, mL	Sample volume, mL		Concentration of reagent in reaction, mM				
		milli-Q water	BATS seawater	Propionate	HOAt	EDC[a]	AMC	PO_4 buffer
A	5.0	1.0	4.0	1.0e-2	1.0	1.0	1.1e-2	8.0
B	5.0	1.0	4.0	1.0e-3	1.0	1.0	1.1e-2	8.0
C	5.0	1.0	4.0	7.5e-4	1.0	1.0	1.1e-2	8.0
D	5.0	1.0	4.0	2.0e-1	1.0	1.0	2.2e-1	8.0
E	5.0	1.0	4.0	2.0e-2	1.0	1.0	2.2e-1	8.0
F	5.0	1.0	4.0	2.0e-3	1.0	1.0	2.2e-1	8.0

[a] Initial EDC concentration in the reaction.

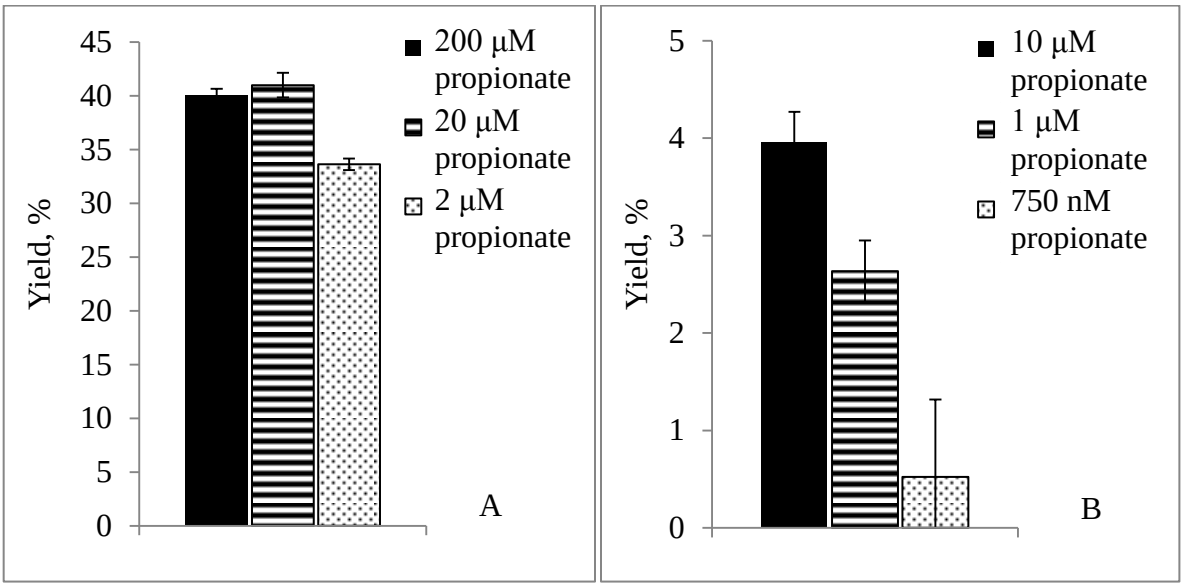


Figure 18. Effect of decreasing the coumarin concentration on yield of derivatized propionate. (A) 220 μM coumarin (B) 11 μM coumarin.

3.3.4.7 Experiment 7

The reaction time determined by Gismervik (2012) is 24 hours. However, with the apparent kinetic effects we suspected a longer reaction time may result in higher yield of amide derivatives. The amide derivatives are stable in solution for days (possibly weeks/months) and should not be affected by increasing the reaction time.

Hypothesis

If the reaction time of the method is increased, then the recovery of propionate as propanamide will increase.

Thus, the effect of reaction time was tested in triplicate reactions with the following treatments:

A. 24 hours reaction time; EDC added at 0, 1, 12 hours

B. 12 hours reaction time; EDC added at 0, 1, 6 hours

C. 8 hours reaction time; EDC added at 0, 1, 4 hours

D. 4 hours reaction time; EDC added at 0, 1, 2 hours

E. 2 hours reaction time; EDC added at 0, 30, 60 minutes

F. 1 hour reaction time; EDC added at 0, 20, 40 minutes

Method

The derivatization method determined in 6.3 was applied to natural seawater with the exception of the treatments specified in 7.1 (Table 13). The time of HPLC injection was considered the end of reaction time.

Conclusion

The recovery of propionate as propanamide derivatives increased with total reaction time (Figure 19). Therefore, the method reaction time should be extended to a minimum of 30 h.

Table 13. Concentration of reagents for treatments in experiment 7.

Treatment	Reaction volume, mL	Sample volume, mL		Concentration of reagent in reaction, mM				
		milli-Q water	BATS seawater	Propionate	HOAt	EDC[a]	AMC	PO_4 buffer
A	5.0	1.0	4.0	1.0	1.0	1.0	1.1	8.0
B	5.0	1.0	4.0	1.0	1.0	1.0	1.1	8.0
C	5.0	1.0	4.0	1.0	1.0	1.0	1.1	8.0
D	5.0	1.0	4.0	1.0	1.0	1.0	1.1	8.0
E	5.0	1.0	4.0	1.0	1.0	1.0	1.1	8.0
F	5.0	1.0	4.0	1.0	1.0	1.0	1.1	8.0

[a] Initial EDC concentration in the reaction.

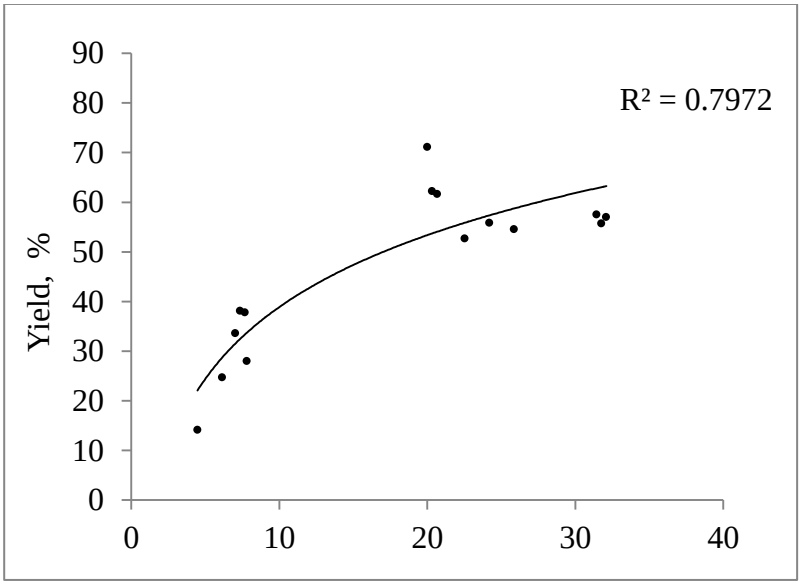


Figure 19. Effect of reaction time (total time until sample injection into the HPLC) on propionate derivatization.

3.3.4.8 Experiment 8

The derivatization reagent EDC is quickly hydrolyzed. Initially, we showed that multiple additions of EDC increased product yield. However, Thermo Fisher Inc. suggests that high concentrations of EDC added only at the start of the reaction also improves yields. To improve

coupling efficiency and avoid multiple additions of EDC over a 12 h period, the initial concentration of EDC could be increased (Thermo Fisher Scientific Inc.).

Hypothesis

If the initial concentration of EDC is increased, then recovery of propionate as propanamide will be similar to adding EDC as 3 aliquots.

Thus, the effect of adding EDC to the reaction at an increased initial concentration was tested in triplicate reactions with the following treatments:

A. 3 aliquots of EDC (6e-5 moles each; ~1 mM)

B. 10 mM initial EDC

Methods

The derivatization method determined in 7.3 was applied to natural seawater with the exception of the treatments specified in 8.1 (Table 14). Samples treated with 3 aliquots of EDC received the second addition of EDC after 1h, and the third after 10 h.

Conclusion

Increasing the initial concentration of EDC to 10 mM resulted in higher recovery of propionate as propanamide derivatives than adding EDC in three aliquots (Figure 20). Therefore, only one EDC addition is necessary and the initial concentration of EDC should be adjusted to 10 mM in the derivatization method.

Table 14. Concentration of reagents for treatments in experiment 8.

Treatment	Reaction volume, mL	Sample volume, mL		Concentration of reagent in reaction, mM				
		milli-Q water	BATS seawater	Propionate	HOAt	EDC[a]	AMC	PO_4 buffer
A	5.0	1.0	4.0	1.0	1.0	1.0	0.22	8.0
B	5.0	1.0	4.0	1,0	1.0	10.0	0.22	8.0

[a] Initial EDC concentration in the reaction.

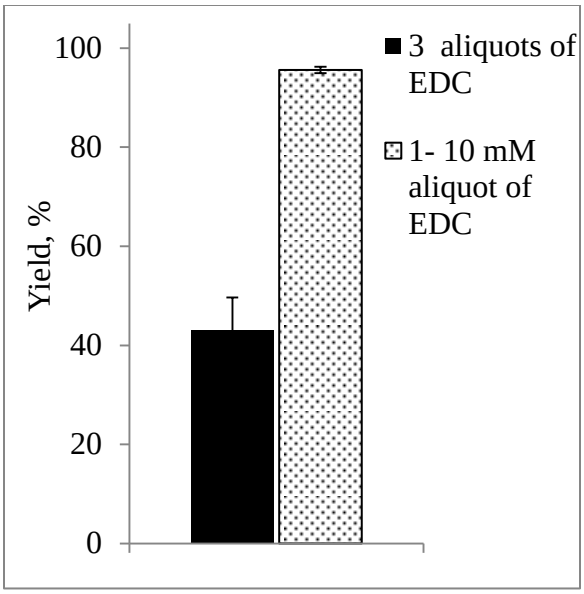


Figure 20. Effect of increased initial EDC concentration on propionate (1 mM) derivatization.

3.3.4.9 Experiment 9

Previous experiments excluded DCM from the reaction due to the volatility and difficulty in pipetting and accurately measuring the volume of the organic solvent. The assumption was that DCM does not have an effect on reaction efficiency. Furthermore, it is desirable to exclude DCM for practical purposes. The properties of DCM make it problematic to carry out the derivatization method in the field, on a research cruise, or as an automated HPLC procedure. However, it is likely that the reaction occurs at the interface of the aqueous-organic phase (Ho et al. 1995).

Hypothesis

If DCM is excluded from the method, the recovery of propionate as propanamide derivatives will not be affected.

To test this, triplicate samples from experiment 8 were used to compare the recovery of derivatized propanamide with the following conditions:

A. DCM included in the reaction

B. DCM used to extract amides after reaction completion

C. No DCM used, only injection of the aqueous phase

Conclusion

Including DCM does increase the yield of the derivatized propionate (Figure 21), likely by influencing the distribution of reacting species, where the polar residues would mainly be distributed on the aqueous side at the interface, while the hydrophobic species remain in the hydrophobic phase (Ho et al. 1995). However, this addition to the method is only practical in the laboratory and is not considered in the final optimal method.

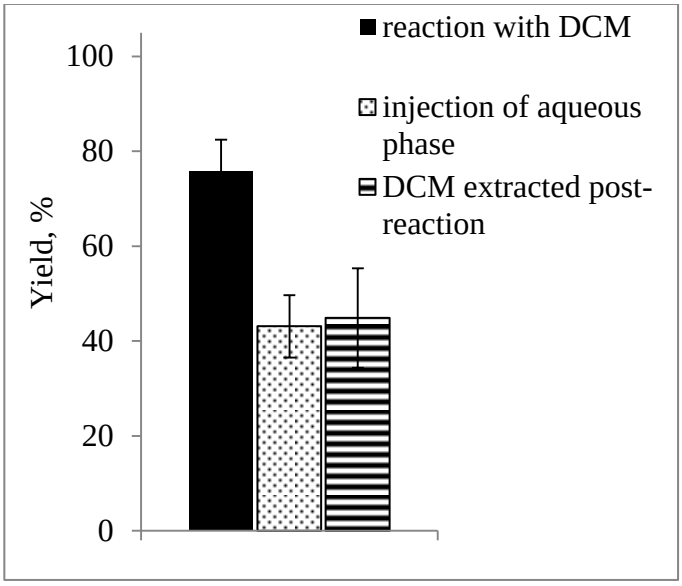


Figure 21. Effect of including DCM during the derivatization on propionate (1 mM) yield.

3.4 Optimal derivatization method for seawater samples

To derivatize LMW organic acids in a 4 mL sample of seawater, add reagents (Table 15) to a 22-mL glass vial in the following order: seawater sample, PO_4 buffer, HOAt, EDC, and AMC. Tightly seal the vials with PTFE/silicone septa and shake vigorously for 30 h. After 30 h, remove an aliquot of the aqueous solution from each sample and transfer with a glass Pasteur pipette to a 2-mL amber autosampler vial. Place the vial in the HPLC for analysis using the previously described HPLC gradient elution method with a 2 μL injection volume.

Table 15. Concentration of reagents in derivatization method reaction.

Total reaction volume, mL	Sample volume, mL		Volume of reagent in reaction, μL			
	milli-Q water	BATS seawater	HOAt (50 mM)	EDC (253 mM)	AMC (11 mM)	PO_4 buffer (8 mM)
5.0	1.0	4.0	99.4	197.8	99.6	100.0

4 Assessment and Application

Cleaning procedures were followed as described in section 3.3.1. Chemical reagents and solutions were prepared as described in section 3.3.2 (Table 16). In addition, a 1 mM stock solution of organic acids containing sodium formate (99.9%, Aldrich Chemical Company, Inc), sodium acetate (99.9%, Aldrich Chemical Company, Inc), sodium propionate (99%, Aldrich Chemical Company, Inc.), pentanoic acid (99%, 99% Acros Organics), and hexanoic acid (99%, Acros Organics) was prepared with milli-Q water in a 500-L volumetric flask. Acid salts were weighed and dissolved with milli-Q water. Acid liquids were dissolved with 2 mM bicarbonate buffer and diluted with milli-Q water. The solution was filtered with a 0.2 µM syringe tip filter and refrigerated at 8°C until used. For experimentation, an aliquot of the stock solution was transferred to a 25-mL glass vial using a glass pipette.

4.1.1 Derivatized propionate recovery

To determine the recovery of derivatized propionate, the optimal derivatization method for seawater was applied to BATS seawater spiked with propionate at a range of concentrations (500 nM- 10 µM), as well as to 3 BATS seawater method blanks and 3 milli-Q method blanks.

Table 16. Chemical reagents for derivatization method assessment and application.

Reagent	final concentration (mM)
organic acid	1
HOAt	50
EDC	253
AMC	1

4.2 Preliminary assessment of derivatized propionate recovery

4.2.1 Recovery determinations and statistical methods

4.2.1.1 HPLC calibration

A calibration curve was generated using the pre-derivatized acid standards. The propanamide standard was weighed (817 µg) with a microbalance, dissolved in 200 µL acetonitrile, and diluted with 1400 µL milli-Q. Standard dilutions were prepared at concentrations of 200 nM, 10 nM, and 5 nM. The standards were injected at varying injection volumes to represent a concentration range of 5 nM to 10 µM using a 2 µL injection volume and were analyzed before and after the 'recovery samples' as a part of the same HPLC sequence.

To determine the derivatized acid concentrations, first the peak area of the acid from the chromatogram was determined and the corresponding average peak area of the method blank (milli-Q water) was subtracted. Then the linear regression model (the standard curve) was used to calculate the number of moles of derivatized acid from the adjusted peak area. Linear regression statistics were generated for the total amount of standard (moles) injected and the resulting peak area using the Excel data analysis toolpak (Appendix 7.3) (Figure 22). Using these statistics, a linear regression model was determined for the pre-derivatized propionate standard.

Instrument detection limits

The limit of detection (LOD) and limit of quantitation (LOQ) was determined for propionate. The LOD is the minimum amount of standard that can be detected reliably and is related to both the signal and the noise of the system. The LOQ is the minimum concentration that can be quantified reliably with a specified level of accuracy or precision and was determined in three ways (Snyder, Kirkland, Glajch 2012). The LOD and LOQ were determined

by using a calibration curve at low concentrations (Figure 23). Linear regression statistics were generated for each calibration curve using the Excel data analysis toolpak (Appendix 7.3), and Equations 1 and 2 were used to calculate LOD and LOQ by substituting the standard deviation of the linear regression line (s_r) for σ and using the slope (m).

Equation 1. $$LOD = \frac{3*\sigma}{m}$$

Equation 2. $$LOQ = \frac{10*\sigma}{m}$$

4.2.1.2 Determination of derivatized propionate recovery

The total amount (moles) of derivatized propionate was determined using the calibration curve of the pre-derivatized propanamide standards. The average peak area of the method blanks was subtracted from individual sample peak areas. The amount (moles) of derivatized propionate was compared to the total amount of underivatized propionate added to the reaction to determine the recovery rate (%) (Figure 24).

4.2.2 Conclusion

The instrument LOD and LOQ for derivatized propionate were 23.6 nM and 78.7 nM, respectively. The limits may be improved if more replicates of the standard are analyzed at lower concentrations. The average recovery of derivatized propionate was 80% (Figure 25). In general, the recovery of derivatized propionate decreased with decreasing acid concentration, however recovery was above 60% at all concentrations (10 μM to 500 nM). In conclusion, this preliminary assessment shows that the derivatization method can be applied to seawater to derivatize propionate at a range of concentrations with relatively high yield.

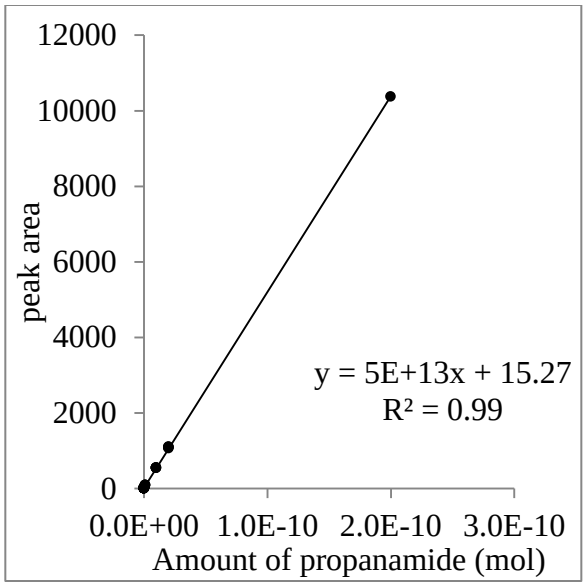


Figure 22. Calibration curve for pre-derivatized propanamide.

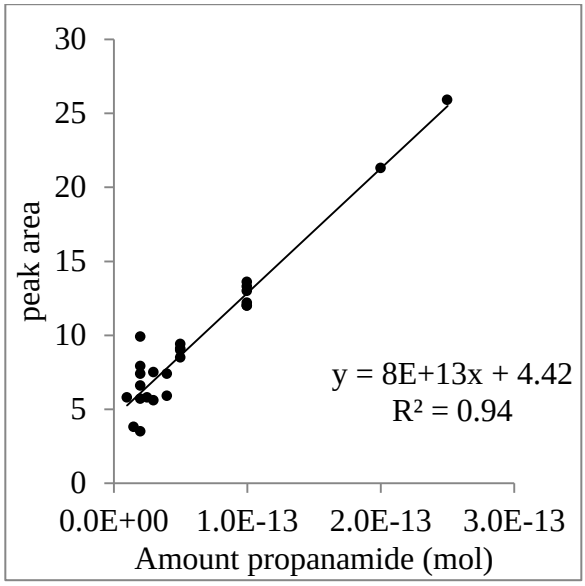


Figure 23. Calibration curve at low concentration (5 nM to 125 nM using a 2 µL injection) used to determine the LOD and LOQ.

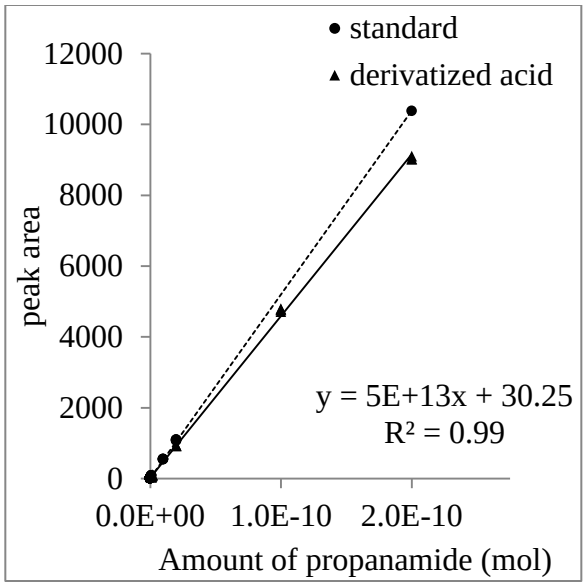


Figure 24. Recovery of propionate taken through the derivatization procedure compared to a standard curve using pre-derivatized propanamide.

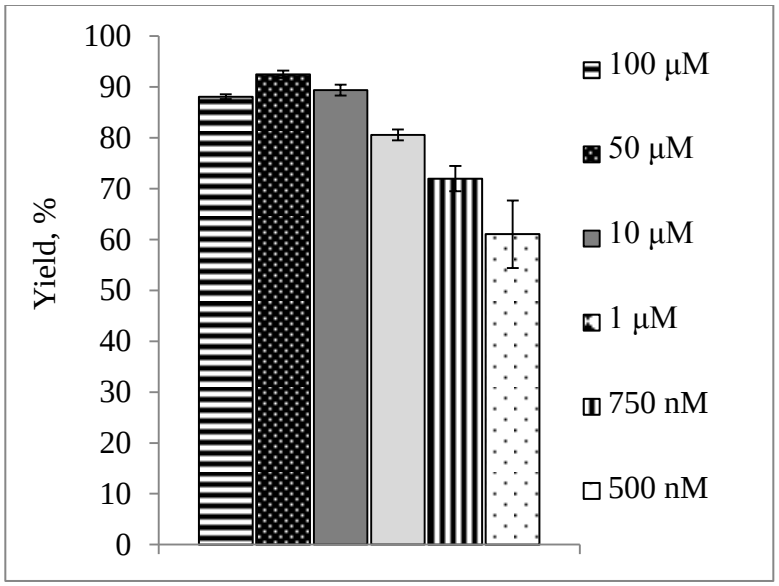


Figure 25. Recovery of propionate added to seawater at a range of concentrations (500 nM to 100 µM) and taken through the derivatization procedure.

4.3 Estimates of carboxylic acid concentrations in seawater

4.3.1 Derivatized carboxylic acid recovery

To determine the recovery of derivatized LMW acids, the optimal derivatization method for seawater was applied to BATS seawater and a known amount of organic acids were added (formate, acetate, propionate, pentanoate and hexanoate) at a range of concentrations (200 nM-10 µM).

4.3.2 Application to seawater samples

The samples were collected from multiple locations and were immediately placed on ice and then frozen at -20°C until analyzed. Seawater salinity and temperature were recorded at the time of sampling (Table 17). Immediately prior to analysis, the samples were thawed at ambient temperature and filtered with a muffled Whatman GF/F 0.7 µm glass microfiber. All samples were kept on ice until analysis. The derivatization method was carried out in triplicate with seawater samples and method blanks of milli-Q water, as well as a method blank of filtered milli-Q water.

4.3.3 Calculations and statistical methods

4.3.3.1 Calibration curves

Calibration curves were obtained using the pre-derivatized acid amide standards; formate, acetate, propionate, pentanoate, and hexanoate. The standards were weighed with a microbalance, dissolved in acetonitrile, and diluted with milli-Q water. Standard dilutions were prepared at concentrations of 5 nM, 50 nM, and 200 nM. The standards were injected at varying injection volumes in triplicate to represent a concentration range of 10 nM to 10 µM using a 2 µL injection volume.

Table 17. Salinity and temperature of samples at collection time.

Sample	Salinity ppt	Temperature ºC
Site 1. Paysen lane salt marsh water	30.2	17.0
Site 1. Paysen lane salt marsh sediment pore water	-	17.0
Site 2. Macaroni beach seawater	35.0	13.0
Site 3. Meadow Head point beach	36.1	14.8
Site 4. Race Point beach seawater	36.5	14.2
Site 5. Sunken Meadow beach seawater	30.8	20.4
Site 6. Thames river water	0.2	20.3
Site 7. Birch Creek Salt marsh sediment pore water	-	-
Site 7. Birch Creek Salt marsh seawater	-	-

Linear regression statistics were generated for the total amount of standard (moles) injected and the resulting peak area using the Excel data analysis toolpak (Appendix 7.3). Using these statistics, a linear regression model (the calibration curve) was determined for each pre-derivatized acid standard; formate, acetate, propionate, pentanoate and hexanoate.

Instrument detection limits

The LOD and LOQ for each acid were determined based on a calibration curve in the concentration range 10 nM - 125 nM using a 2 μL injection volume (Figure 26). Linear regression statistics were generated using the Excel data analysis toolpak (Appendix 7.3) and equations 1 and 2 were used to calculate LOD and LOQ by using the standard deviation (σ) of the linear regression line (s_r) and the slope (m) the linear regression line (Table 18).

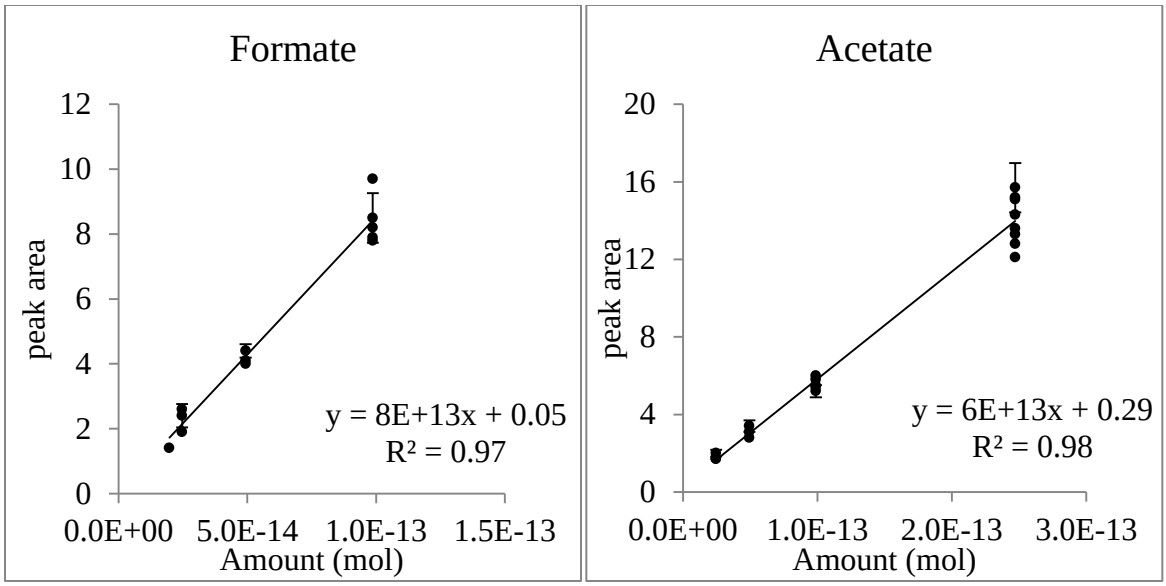


Figure 26. Calibration curves within the range of the LOD and LOQ for each acid standard. Linear regression model statistics for each acid were used to determine the limits.

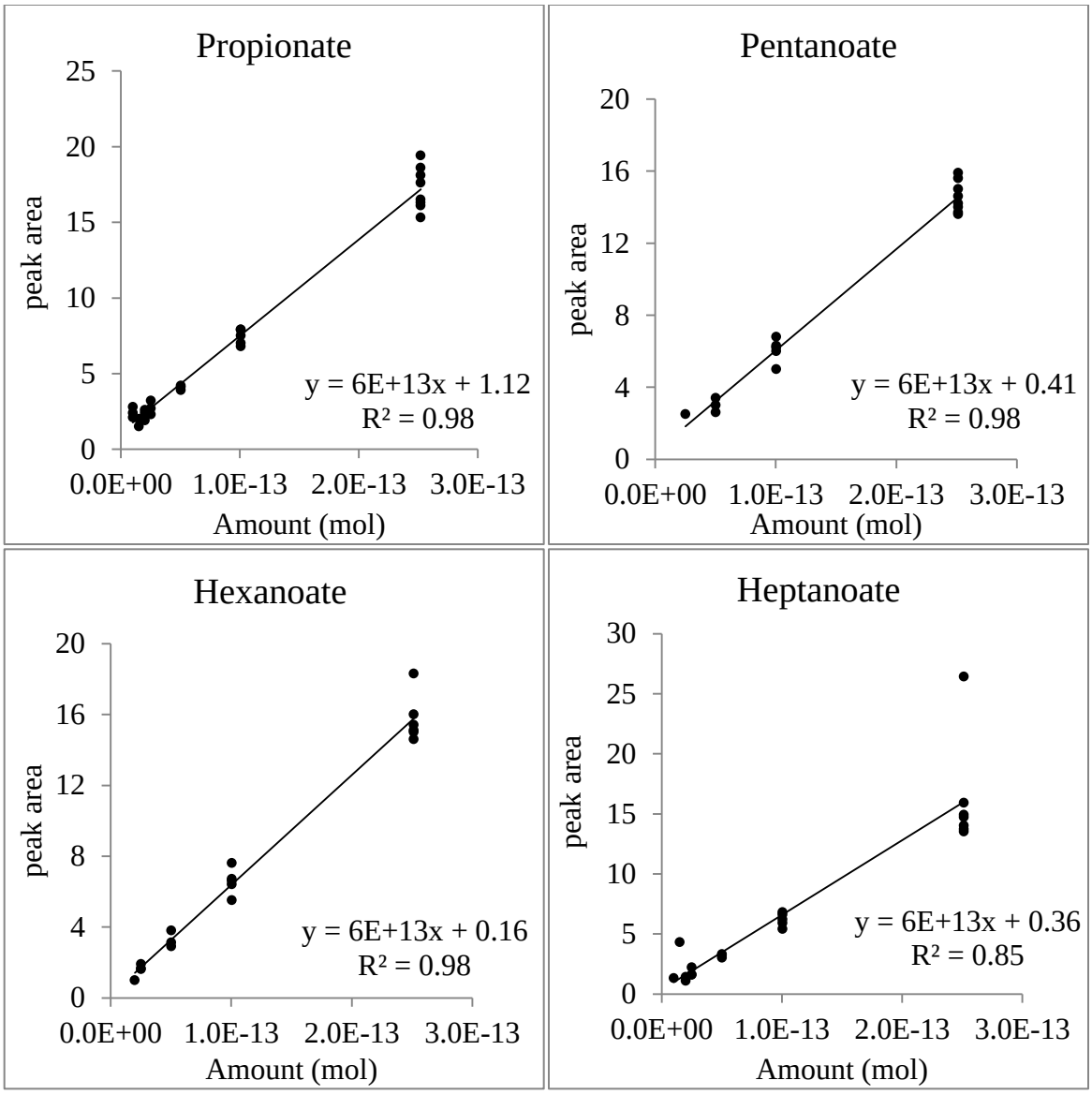


Figure 26. Continued

Table 18. Instrument limit of detection (LOD) and limit of quantitation (LOQ) for pre-derivatized acid standards.

Acid	LOD (nM)	LOQ (nM)
Formate	9.52	31.72
Acetate	22.97	76.58
Propionate	19.93	66.42
Pentanaote	19.30	66.35
Hexanoate	20.95	69.87

4.3.3.2 Determination of recovery for derivatized organic acids in seawater

The linear regression model (4.2.3.1) was used to determine the amount (moles) of derivatized acid in each sample based on the peak area (with the average peak area of the method blank (BATS seawater) subtracted). Triplicate method blanks (milli-Q) were analyzed and the peak areas averaged for individual derivatized acids. The only acid found in method blanks was formate at a concentration of ~ 1 µM (adjusted for recovery rate). The amount (moles) of derivatized acid was compared to the amount (moles) of underivatized acid added to the reaction. The recovery rate (%) and standard deviation s_s (Appendix 7.3) were determined for each set of triplicate samples (Figure 27).

The linear regression statistics were also generated for the amount (moles) of derivatized acid and the corresponding peak area, and a linear regression model (recovery curve) was determined for each acid (Figure 28).

4.3.3.3 Carboxylic acid concentration estimates for natural samples

The recovery linear regression model (recovery curve 4.2.3.2) was used to determine the amount (moles) of derivatized acid in each sample based on the peak area (with the average peak area of the method blank (milli-Q water) subtracted). The average acid concentration of the injection was determined and multiplied by factor 1.2 to account for dilution by the derivatization method. The standard deviations s_c and s_s (Appendix 7.3) were determined for the mean of each set of triplicate samples.

The amount of derivatized acid (moles) was determined based on the peak area (with the average peak area of the method blank (milli-Q water) subtracted) by using the linear regression model. The average acid concentration of each injection was determined and multiplied by factor 1.2 to account for dilution by reagents in the derivatization method and then divided by the

average recovery rate (%) (4.2.3.2) to estimate the actual concentration of carboxylic acid in the sample. The standard deviations s_c and s_s (Appendix 7.3) were determined for the mean of each set of triplicate samples.

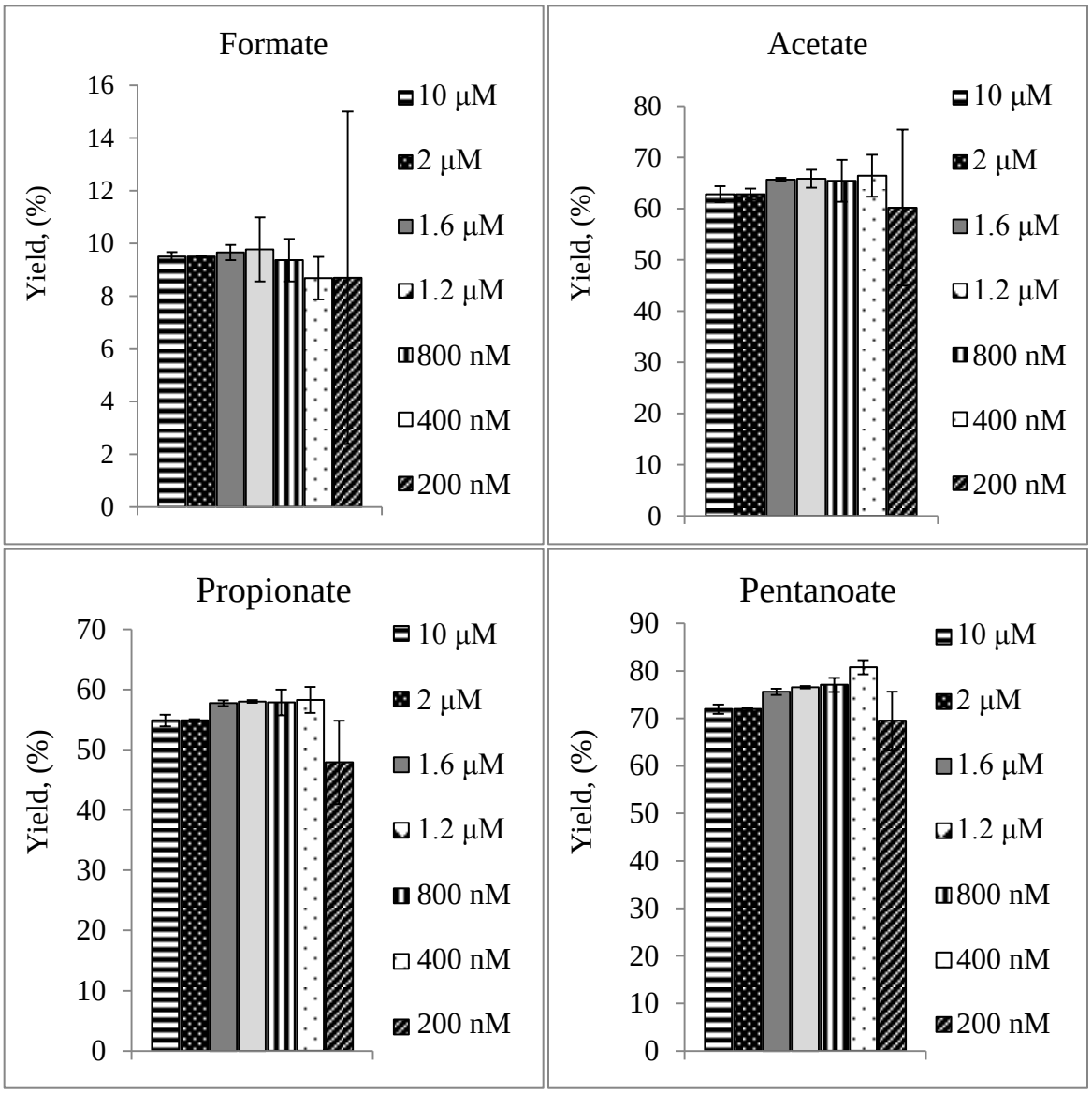


Figure 27. Yield (%) of derivatized acids as a fraction of total underivatized acid added to the reaction. Error bars represent standard deviation (s_s).

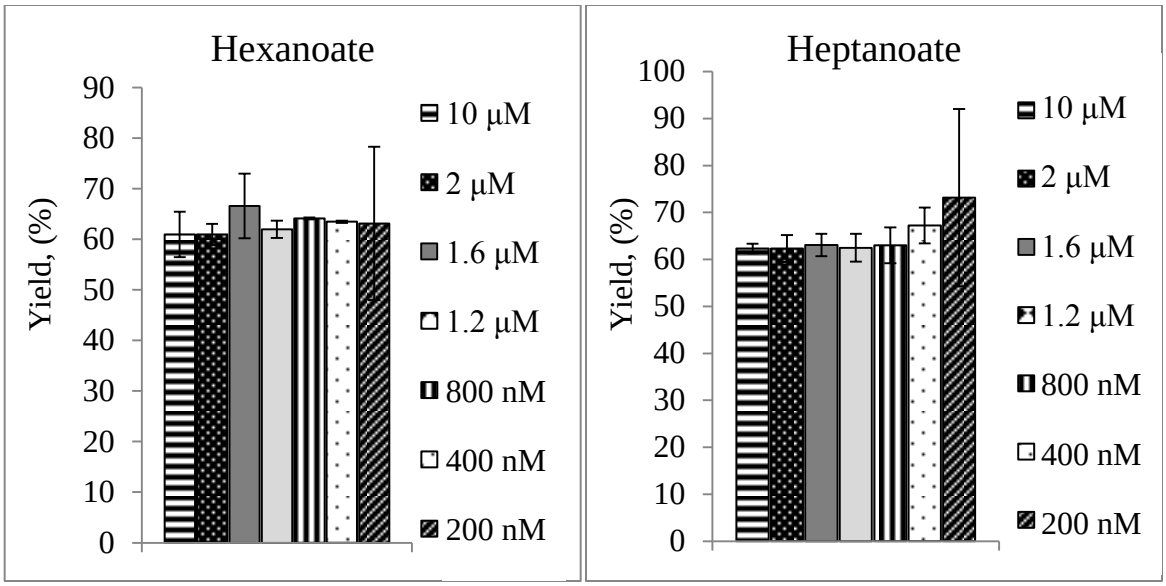


Figure 27. (Con't).

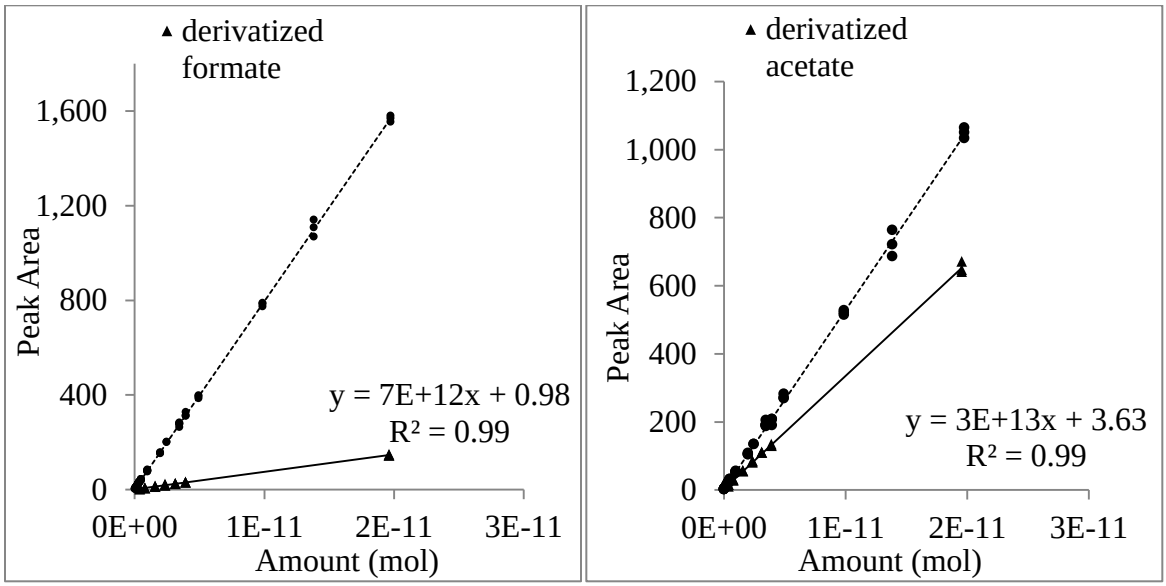


Figure 28. Recovery of derivatized acid linear regression compared to HPLC pre-derivatized standard linear regression (HPLC calibration curve is dashed line).

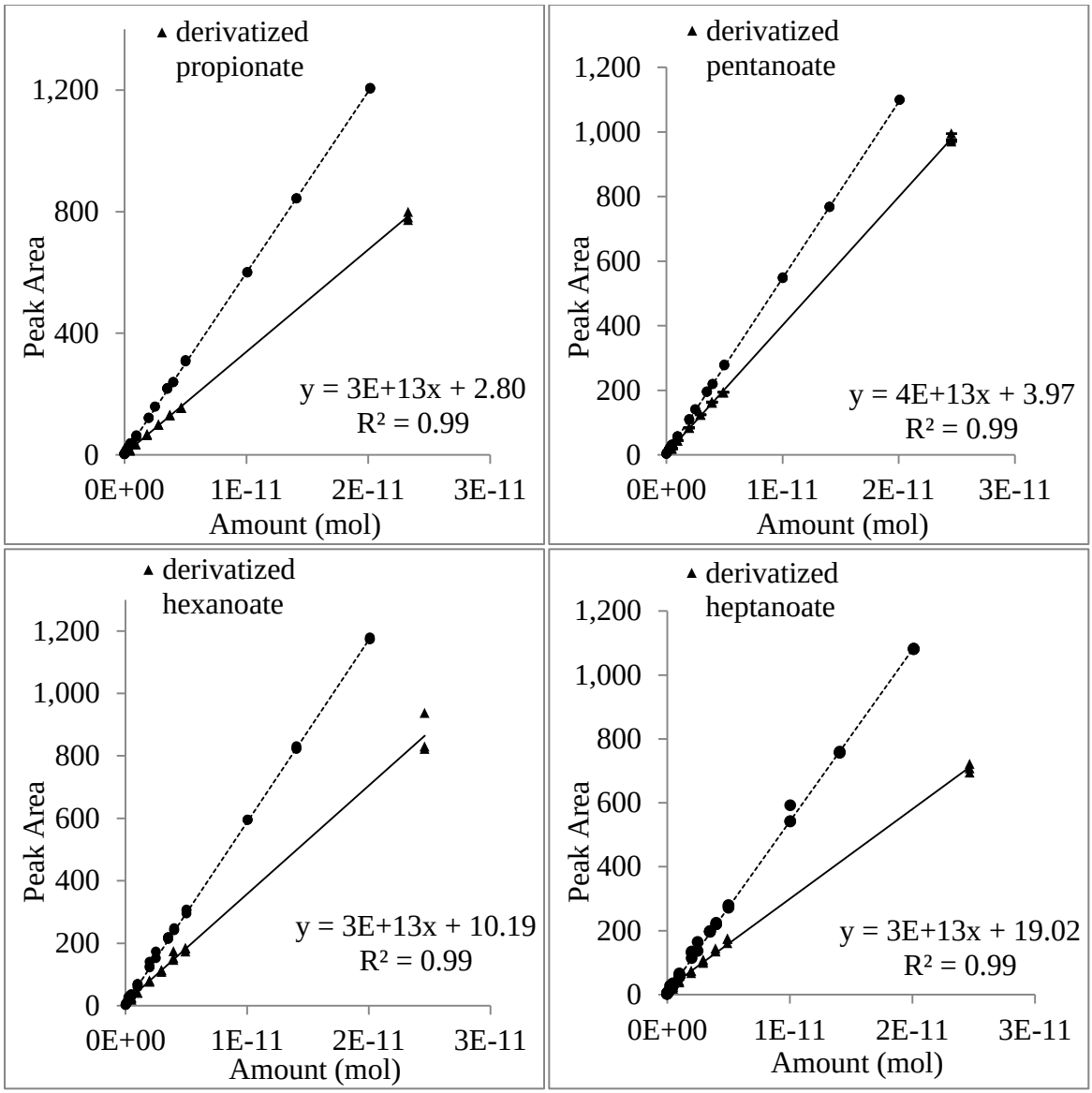


Figure 28. (Con't).

4.2.3 Results

The HPLC limits for simultaneous detection of all acids is 77 nM and the simultaneous quantification for all acid standards is 64 nM, using a 2 µL injection volume. Detection limits ranged from 9.52 nM (formate) to 63.50 nM (hexanoate). Quantification limits ranged 31.72 nM

(formate) to 76.58 nM (acetate). Recovery of derivatized organic acids varied between acids, but in general was more than 50%, with the exception of formate (9%) (Figure 29).

In seawater samples, formate, acetate and propionate were found at nearly all locations (Table 19 and Figure 30). The highest acid concentrations were found in sediment pore water samples, which were dominated by acetate (54.7 mol%; 91.4 mol%).

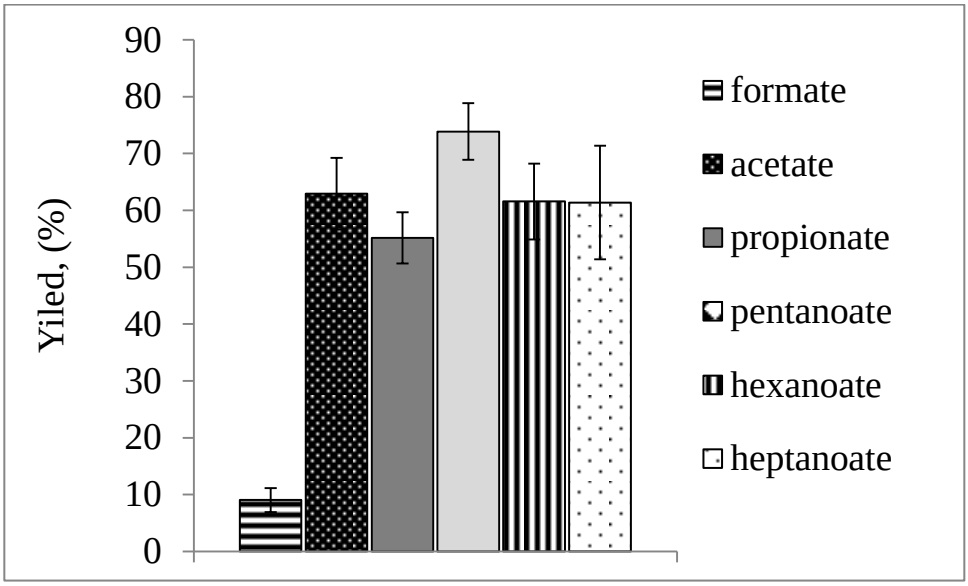


Figure 29. Average recovery (N=3) and standard deviation (s_s) for derivatization of organic acids (200 nM – 10 uM).

Table 19. Carboxylic acid concentration estimates for each sampling site with standard deviation (s_c and s_s).

Site 1. Paysen Lane salt marsh seawater.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	412	66	364
Acetate	219	55	102
Propionate	44	52	41
Pentanaote	-	-	-
Hexanoate	-	-	-

Site 1. Paysen lane sediment pore water.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	59960	358	1010
Acetate	89400	437	562
Propionate	12800	54	154
Pentanaote	596	46	198
Hexanoate	357	216	158

Site 2. Macaroni beach seawater.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	222	66	19
Acetate	342	55	17
Propionate	45	53	19
Pentanaote	-	-	-
Hexanoate	-	-	-

Site 3. Meadow Head Point beach seawater.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	178	66	28
Acetate	220	55	16
Propionate	26	53	5
Pentanaote	-	-	-
Hexanoate	-	-	-

Site 4. Race Point beach seawater.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	455	66	45
Acetate	424	55	13
Propionate	44	53	13
Pentanaote	-	-	-
Hexanoate			

Site 5. Sunken Meadow beach seawater.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	1410	65	304
Acetate	648	55	633
Propionate	393	52	178
Pentanaote	-	-	-
Hexanoate	-	-	-

Site 6. Thames harbor water.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	2380	54	429
Acetate	311	55	91
Propionate	29	53	38
Pentanaote	-	-	-
Hexanoate	-	-	-

Site 7. Birch Creek salt marsh sediment pore water.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	-	-	-
Acetate	3040	53	122
Propionate	157	53	90
Pentanaote	-	-	-
Hexanoate	-	-	-

Site 7. Birch creek salt marsh seawater.

Acid	Concentration (nM)	Standard Deviation (nM)	
		s_c	s_s
Formate	-	-	-
Acetate	5470	54	4530
Propionate	2350	50	832
Pentanaote	64	65	30
Hexanoate	-	-	-

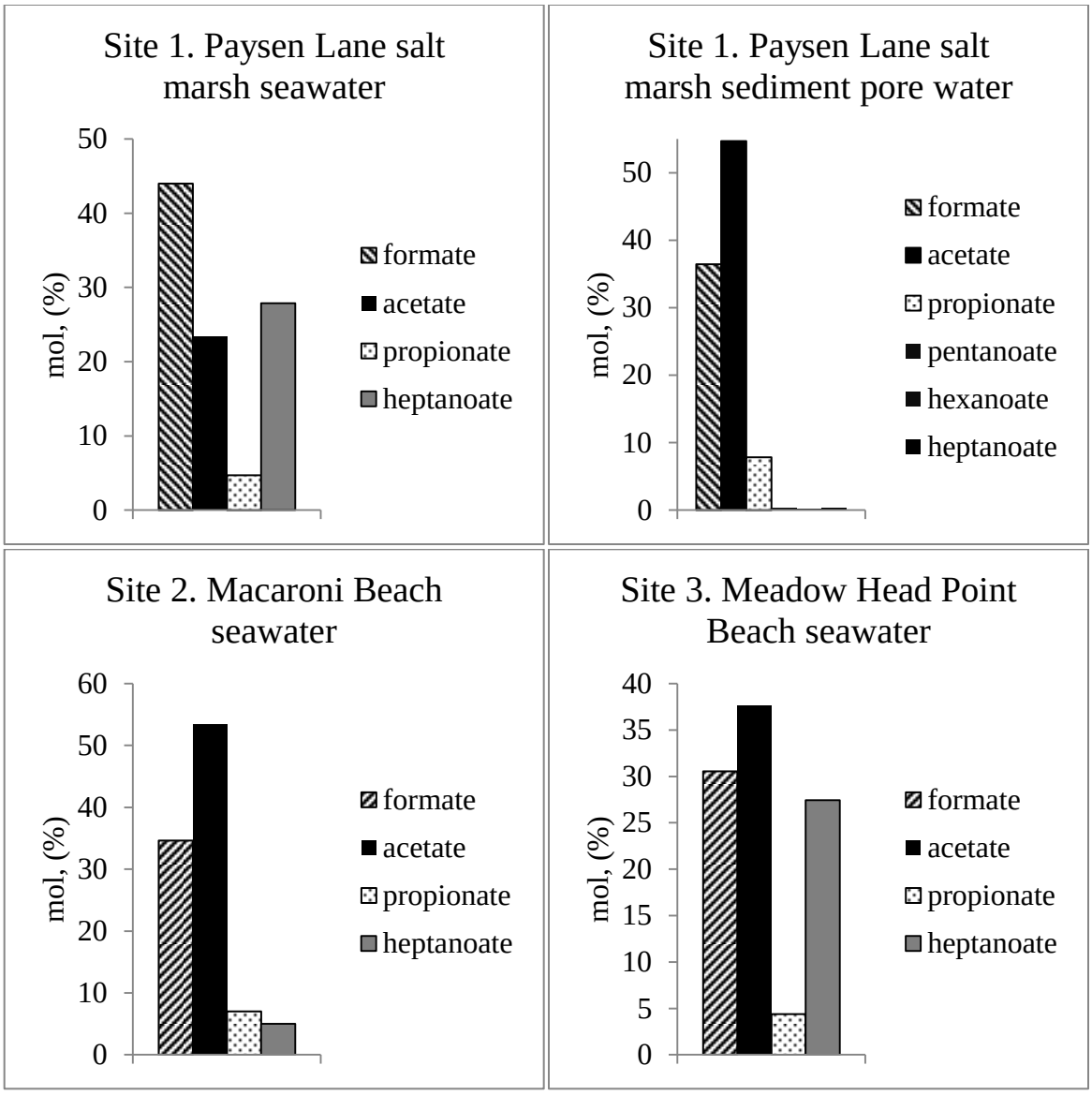


Figure 30. Individual carboxylic acid concentrations as a fraction of total identifiable derivatized organic acids in each sample.

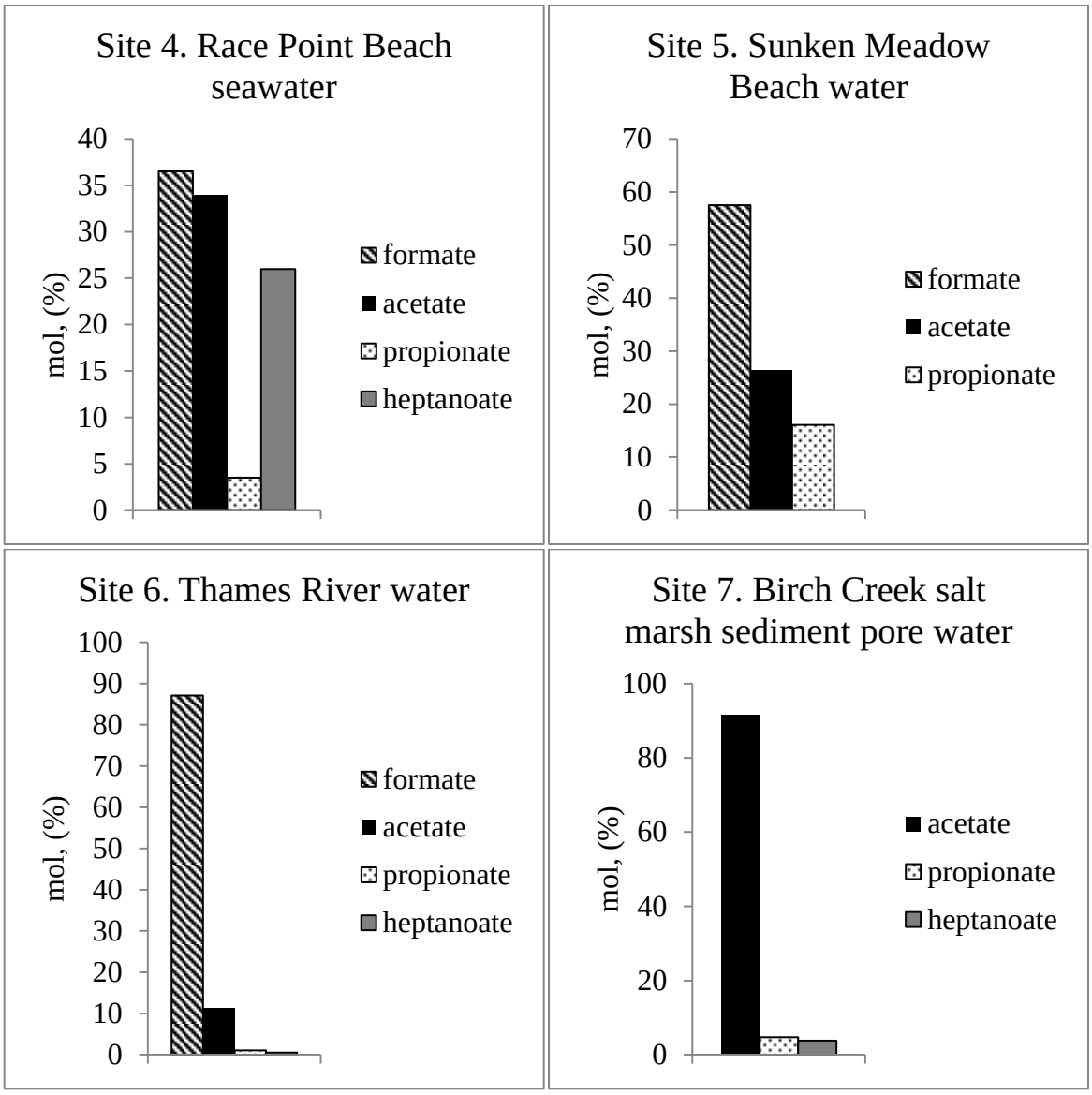


Figure 30. (continued)

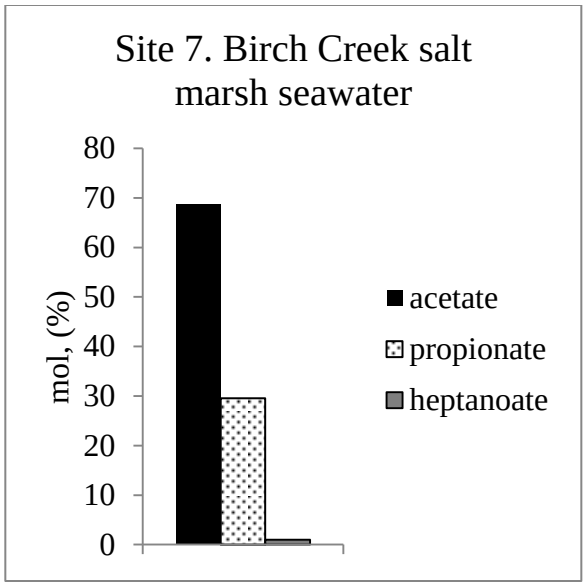


Figure 30. (continued)

5 Discussion

The proposed method has some similarities with and several advantages over previous methods. Similar to previous methods (Yang, Lee, Scranton 1993; Albert and Martens 1997), there are contamination problems that could not be addressed within the scope of this thesis. Formate is the major contamination problem in the two previous methods, and is also a problem in our method. Suggestions for further work to address this issue are discussed in section 5.5. However, in contrast to previous methods, our formate blank allows determination of formate concentrations. A comparison among the detection limits of the three methods is not straightforward since the descriptions of determinations of detection limits are unclear in previous studies (Yang, Lee, Scranton 1993; Albert and Martens 1997). There are essentially three different types of commonly used detection limits to consider. First, the method detection limit, which includes variations in recovery, contamination (as determined by blanks) and uncertainties associated with calibration curves. Secondly, the detection limit set only by the instrument and uncertainties in standard curves. Thirdly, the instrument detection limit, determined only by separation, detector sensitivity and variations in detector output (as seen in baseline noise). Statistical treatments of concentrations derived from standard curves calculated from linear regression are discussed further in section 5.6.

Our calculated method detection limits (Table 18) include uncertainties associated with the calibration curves, while many method detection limits are based only on the method blanks or on signal to noise ratios (Yang, Lee, Scranton 1993; Albert and Martens 1997). At present, the calculation of our method detection limits appear to include uncertainty factors similar to or lower than detection limits reported in previous method evaluations, and the method can

successfully separate and quantify a larger number of acids (formate, acetate, propionate, pentanoate, hexanaote).

The LOD and LOQ of individual derivatized carboxylic acids were determined (Table 18) based on the standard deviation and slope of the calibration curve linear regression model. Note – LOD and LOQ was based on actual standard curves, NOT the recovery curves. The LOD was about 20 nM for all acid standards, except formate (9.5 nM). The high LOD's are most likely a result of the large standard deviation about the regression line, and could possibly be lowered with a better calibration curve. The LOQ was 67 nM to 77 nM for all acid standards, except formate (32 nM).

In contrast to the method detection limit, the instrument detection limit can be pushed much lower. We used a 2 µl injection volume, which is very low. The instrument is capable of directly injecting 100 µl, and can inject any number of 500 µl aliquots on to a concentrator column held in a loop before the actual separation column. At this time, a 10 nM standard concentration is the lowest injected concentration, but this could easily be pushed lower than 0.2 nM.

On average, the recoveries were greater than 50%, with the exception of formate (9%) (Figure 29). Albert and Martens (1997) also found the derivatization rate of formate to be much lower than acetate and propionate.

The number of steps involved in our derivatization method is about the same as in the method outlined by Albert and Martens (1997), and smaller than the number of steps in the method by Yang et al. (1993). Further, Albert and Martens (1997) found that a basification step in their method caused precipitation of carbonate with divalent ions, which incurred losses of organic acids to the formed floc. Our method includes no step that causes precipitation. The

sample volumes are smaller than those required by Yang et al. (1993) and about the same as those reported by Albert and Martens (1997). As discussed above, the fluorescence detection of the derivatized acid is very sensitive, and more sensitive than the UV detection employed by Albert and Martens (1997).

5.1 The importance of pH control for reaction yield

The reaction pH was determined to be a controlling factor in the derivatization of organic acids. The pH affects the efficiency of the EDC coupling reagent and therefore affects the efficiency of the derivatization reaction. The optimal pH for the EDC coupling reagent is 4 to 6. However, in an unbuffered milli-Q solution, pH will be determined by the reagents added and will vary from 4.8 to 6.7 as the different reagents are added to the sample. Seawater solutions are buffered by the carbonate system and pH will vary from 6.9 to 8 as the different reagents are added to the sample. In addition, if the pH is lower than the pKa of any of the organic acids, the volatility of the protonated acid may cause losses. The pKa values for organic acids of interest are 3 to 5 (Table 20). Therefore, it is necessary to carefully buffer seawater samples to a pH between the highest pKa of the organic acids and the lowest pH where EDC is active. We buffered the solution to 5.5. Others using EDC have buffered to pH 3.5 to 5 (Albert and Martens 1997) We found that phosphate buffer worked well, although phosphate-buffered saline (PBS) and 2-ethanesulfonic acid (MES) buffers are also commonly used with EDC.

Table 20. pKa values for organic acids.

Carboxylic acid	pKa
Formic acid	3.75
Acetic acid	4.76
Propanoic acid	4.9
Butanoic acid	4.82
Pentanoic acid	4.82
Hexanoic acid	4.88
Heptanoic acid	4.89
Benzoic acid	4.20

5.2 Optional DCM extraction

Originally, the derivatization was carried out in a two-phase system, including the sample water phase and an added organic phase composed of DCM. The amide compounds formed from the organic acids in the derivatization were extracted with DCM, which was evaporated and the dry amides were then re-dissolved in acetonitrile for HPLC analysis. The initial role of the DCM organic phase was for compatibility with reversed-phase HPLC solvent systems and the ability to concentrate derivatized products. However, the extraction of propanamide derivatives from the aqueous phase into DCM is not necessary for compatibility with the HPLC mobile phase, since it is initially 95% water (mobile phase A). Further, the HPLC method will not detect the water-soluble urea by-product from the reaction, nor the coupling reagents, with the exception of AMC. Finally, the DCM phase will also include the bi-phasic underivatized AMC, which in high concentrations can cause negative peaks in the chromatograms. Therefore, the concentration of derivatization products is only favorable when underivatized coumarin is much less than total derivatized products.

Furthermore, the chemical properties of DCM make it difficult to work with and derivatization without DCM would be ideal. We found that injecting the aqueous phase of the

derivatization reaction resulted in the same recovery of derivatized propionate as injections of derivatized propionate in acetonitrile after extraction with DCM. However, it was also found that including DCM in the derivatization reaction resulted in the highest yields of derivatized propionate, possibly by removing derivatized products from the aqueous phase, thereby driving the reaction forward and potentially towards completion. Thus, the derivatization method is efficient with or without the presence of DCM during the reaction, making this method adaptable for use in the laboratory, in the field, on a research cruise, or even as an automated HPLC method. However, the effect of including DCM on yields of the complete suite of acids should be investigated further and could be included in analyses carried out in non-field environments.

5.3 Additional changes to the aqueous method (Gismervik 2012)

Originally, TEA was included in the aqueous derivatization method to deprotonate AMC by acting as a basic buffer. However, the addition of TEA essentially cancels the effect of the phosphate buffer. We found that excluding TEA and using phosphate buffer further increased derivatization.

The method was also adjusted to account for the hydrolysis of EDC. Initially, we found that adding EDC in multiple aliquots increased derivatization. However, when the initial concentration of EDC was increased from 1 mM to 10 mM, derivatization increased further compared to derivatization with multiple additions of EDC.

The original reaction time of the aqueous derivatization method was 24 h. A shorter reaction time is desired, but we found that 30 h resulted in the highest yields. Since the derivatized acids are stable in aqueous solution in the dark, increasing the reaction time even

longer may result in further increased yields, especially at low carboxylic acid concentrations (<500 nM).

Using low concentrations (10 μM) of AMC resulted in lower derivatization yields. However, at high concentrations (1 mM) underivatized AMC oversaturates the fluorescence detector, resulting in negative peaks in the HPLC chromatograms, ultimately affecting the baseline and peak area integration. The method is easily adaptable to derivatize carboxylic acid concentrations from nanomolar to millimolar without modification to the procedure except for adjusting the concentration of AMC relative to the total carboxylic acid concentration; hundreds of micromolar for seawater and several millimolar for pore water. However, 220 μM AMC was the lowest concentration tested.

5.4 Considerations regarding chemicals used in the method

HOAt is sparingly soluble in water (Carpino 1993). To completely dissolve HOAT, the solution had to be heated to 37°C in an ultrasonic bath for 1 h. HOAt is stable for several months in aqueous solution when stored at 8°C (verbal communication, Prof. Brückner). Before use, the solution was again gently heated to 35-37°C in a water bath until completely dissolved. HOAt is considered hazardous to skin, eyes, and respiratory system, and has explosive properties; therefore special precautions should be taken when handling this compound.

EDC is a solid and highly soluble in hot or cold water (>95 g/L) (Thermo Fisher Scientific, Inc). All EDC solutions were prepared immediately before experimentation, as EDC is easily and quickly hydrolyzed (Thermo Fisher Scientific, Inc.), which destroys its function as a coupling reagent. The quick hydrolysis of EDC when exposed to humidity means that special precautions must be taken. When not in use, EDC should be stored in a tightly sealed container where the air space has been filled with dry, heavier than-air-gas (we used N_2), in a desiccator at

-20°C. To prevent condensation of air humidity into a cold container, EDC should remain in the desiccator while warming to room temperature before opening the container. EDC is also considered hazardous and a skin and respiratory irritant (Thermo Fisher Scientific, Inc.), therefore special precautions should be taken for safe handling.

AMC is highly soluble in water (>15 g/L). The solutions were prepared immediately before experimentation; however AMC remains stable in aqueous solution for months in the dark (verbal communication, Prof Brückner). AMC remains stable as a crystalline powder for months to years at room temperature (verbal communication, Prof Brückner), in a tightly sealed container.

5.5 Sample contamination

Sample contamination issues are still unresolved and need to be addressed. The volatility and ubiquity of formate and acetate in most indoor environments suggests the laboratory air is a potential contaminant. In addition, potential contamination from reagents used in the derivatization procedure needs to be evaluated.

Only formate (~ 1µM) was found in the method blanks (milli-Q). The concentration of formate did not increase when the concentration of EDC was increased by a factor 10, or with the use of phosphate buffer, therefore if the source of formate is the reagents, it must originate from the HOAt and/or AMC. If AMC is found to be the major contributor of formate, it can be simply purified with DCM.

Future work on the potential contamination problem should include: comparing derivatized acid concentrations in samples derivatized in ambient laboratory air with organic acid concentration in samples derivatized under N_2 atmosphere in a glove box, and comparing

derivatized acid concentrations between samples where the concentrations of AMC and HOAt are varied individually.

5.6 Statistical considerations

Two variations of standard deviation for concentration estimates were reported; s_c and s_s. Standard deviation s_c is used to calculate the standard deviation of a mean of replicate samples determined using a calibration curve, and is based on the standard deviation (s_r) of the calibration curve regression line and the average *y* value of the data used in making the calibration curve. Since s_c depends on the average *y* value of the data used to make the calibration curve, calibration curves with narrow concentration ranges yield lower and more accurate standard deviations.

Also, s_c is dependent on the number of sample replicates, and the standard deviation increases with fewer samples. One consequence of using s_c, is that means with similar values and number of replicates will have similar standard deviations. Therefore, it is suggested that the derivatization be carried out with as many replicate samples as possible, and that the calibration curve used to determine the amount (moles) of derivatized acid only include the necessary range, such that the average *y* of the data used to make the calibration curve be relevant to the mean sample concentration estimates. Therefore, multiple calibration curves may be necessary for samples varying in acid concentrations, such as pore water and seawater samples.

Standard deviation s_s is more commonly used to determine the standard deviation of a small set of data (i.e. triplicate samples) and was reported for the mean concentration of replicate samples. Unlike s_c, s_s depends only on the deviation from the mean of the samples and degrees of freedom.

The LOD and LOQ were also determined based on the standard deviation of the calibration curve linear regression model. It is possible that our detection limits were high due to the wide concentration range of the calibration curves, and that the LOD and LOQ can be reduced by using calibration curves with narrower ranges. Also, the detection limits may be improved if more replicates of the standard are analyzed at lower concentrations.

5.7 Organic acid concentrations in natural samples

The derivatization method was applied to samples consisting of seawater, sediment pore water, and low-salinity river water, as well as triplicate method blanks (milli-Q). Formate was found in the method blank at ~ 1 µM. Individual organic acid concentrations were estimated based on the recovery linear regression models.

In general, all samples had detectable concentrations of acetate and propionate. In most seawater samples, formate and acetate had the highest mol fractions (Table 21). The highest acid concentrations were found in sediment pore water samples dominated by acetate (54.7 mol%; 91.4 mol%), which is expected as acetate is a product of anaerobic acetogenesis (Albert and Martens 1997). Consistent with our samples collected in early June, Albert and Martens (1997) found that acetate and propionate dominated (80-90%) the carboxylic acid pool in Cape Lookout Bight sediments in June. At site 1 (Paysen Lane salt marsh) sediment pore water acid concentrations ranged from 13 µM (propionate) to 89 µM (acetate) (Table 19), while overlying seawater had much lower acid concentrations; 412 nM (formate) and 219 nM (acetate). Similarly, at site 7 (Birch Creek) sediment pore water had acid concentrations greater than overlying seawater; 5.5 µM acetate and 2.4 µM propionate compared to 3.0 µM acetate and 157 nM propionate (Table 19). The acetate concentrations for sediment pore water at both locations are less than those reported by Albert and Martens (1997) (500 µM to 2 mM) and Hoehler (1999)

(300-600 µM). Previously reported acetate concentrations for seawater range from 160 nM (Wu, Green, Scranton 1997) to 2.6 µM (Yang, Lee, Scranton 1993), and previously reported propionate concentrations for seawater range 60 to 260 nM (Yang, Lee, Scranton 1993).

Table 21. Individual derivatized organic acids as a fraction (%) of the total concentration of derivatized acids.

Sample	total acids[a] (µM)	mol %				
		form	ace	prop	pent	hex
Site 1. Paysen Lane salt marsh water	0.94	44.0	23.4	4.7	-	-
Site 1. Paysen Lane salt marsh sediment pore water	163.00	36.4	54.7	7.8	0.4	0.2
Site 2. Macaroni Beach seawater	0.64	34.6	53.4	7.0	-	-
Site 3. Meadow Head Point Beach	0.58	30.5	37.6	4.4	-	-
Site 4. Race Point Beach seawater	1.25	36.5	34.0	3.5	-	-
Site 5. Sunken Meadow Beach seawater	2.45	57.5	26.5	16.1	-	-
Site 6. Thames River water	2.74	87.1	11.4	1.0	-	-
Site 7. Birch Creek salt marsh sediment pore water	3.32	-	91.4	4.7	-	-
Site 7. Birch Creek salt marsh seawater	7.96	-	68.7	29.5	0.8	-

[a] Total identifiable derivatized acids

Formate was also found in all samples, except for one location (site 7. Birch Creek). pentanoate and hexanoate were found only in pore water samples and only at one location (Site 1) in quantifiable amounts; 596 nM (pentanoate) and 357 nM (hexanoate).

6 References

Albert DB and Martens CS. 1997. Determination of low-molecular-weight organic acid concentrations in seawater and pore-water samples via HPLC. Mar Chem 56(1-2):27-37.

Amon RM and Benner R. 1996. Bacterial utilization of different size classes of dissolved organic matter. Limnol Oceanogr 41(1):41-51.

Benner R, Pakulski JD, McCarthy M, Hedges JI, Hatcher PG. 1992. Bulk chemical characteristics of dissolved organic matter in the ocean. Science 255(5051):1561-4.

Bode JW. 2006. Emerging methods in amide- and peptide-bond formation. Curr Opin Drug Discov Devel 9(6):765-75.

Burdige DJ and Gardner KG. 1998. Molecular weight distribution of dissolved organic carbon in marine sediment pore waters. Mar Chem 62(1):45-64.

Carpino LA. 1993. 1-hydroxy-7-azabenzotriazole. an efficient peptide coupling additive. J Am Chem Soc 115(10):4397-8.

Dahlén J, Bertilsson S, Pettersson C. 1996. Effects of UV-A irradiation on dissolved organic matter in humic surface waters. Environ Int 22(5):501-6.

Galloway JN, Likens GE, Keene WC, Miller JM. 1982. The composition of precipitation in remote areas of the world. Journal of Geophysical Research: Oceans (1978–2012) 87(C11):8771-86.

Gismervik BC. 2012. Fluorescence tagging of marine low molecular weight carboxylic acids.

Gomori G. 1955. [16] preparation of buffers for use in enzyme studies. Meth Enzymol 1(0):138-46.

Hansell DA. 2013. Recalcitrant dissolved organic carbon fractions. Marine Science 5.

Ho G, Emerson KM, Mathre DJ, Shuman RF, Grabowski EJ. 1995. Carbodiimide-mediated amide formation in a two-phase system. A high-yield and low-racemization procedure for peptide synthesis. J Org Chem 60(11):3569-70.

Hoehler TM, Albert DB, Alperin MJ, Martens CS. 1999. Acetogenesis from CO2 in an anoxic marine sediment. Limnol Oceanogr 44(3):662-7.

Jørgensen NO and Jensen RE. 1997. Determination of dissolved combined amino acids using microwave-assisted hydrolysis and HPLC precolumn derivatization for labeling of primary and secondary amines. Mar Chem 57(3):287-97.

Keene WC, Galloway JN, Holden JD. 1983. Measurement of weak organic acidity in precipitation from remote areas of the world. Journal of Geophysical Research: Oceans (1978–2012) 88(C9):5122-30.

Legrand M and De Angelis M. 1996. Light carboxylic acids in greenland ice: A record of past forest fires and vegetation emissions from the boreal zone. Journal of Geophysical Research: Atmospheres (1984–2012) 101(D2):4129-45.

Lim NC, Pavlova SV, Brückner C. 2008. Squaramide hydroxamate-based chemidosimeter responding to iron (III) with a fluorescence intensity increase. Inorg Chem 48(3):1173-82.

Mikoz. xl, lajczyk M, Kiez. xl, lbasiński P. 1981. Recent developments in the carbodiimide chemistry. Tetrahedron 37(2):233-84.

Montalbetti CA and Falque V. 2005. Amide bond formation and peptide coupling. Tetrahedron 61(46):10827-52.

Nakajima N and Ikada Y. 1995. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. Bioconjug Chem 6(1):123-30.

Ogawa H and Tanoue E. 2003. Dissolved organic matter in oceanic waters. J Oceanogr 59(2):129-47.

Ogawa H and Ogura N. 1992. Comparison of two methods for measuring dissolved organic carbon in sea water.

Paula Lei Q, Lamb DH, Heller RK, Shannon AG, Ryall R, Cash P. 2002. Kinetic studies on the rate of hydrolysis of N-ethyl-N′-(dimethylaminopropyl) carbodiimide in aqueous solutions using mass spectrometry and capillary electrophoresis. Anal Biochem 310(1):122-4.

Sasamoto K, Ushijima T, Saito M, Ohkura Y. 1996. Precolumn fluorescence derivatization of carboxylic acids using 4-aminomethyl-6, 7-dimethoxycoumarin in a two-phase medium. Analytical Sciences 12(2):189-93.

Sheehan JC and Hess GP. 1955. A new method of forming peptide bonds. J Am Chem Soc 77(4):1067-8.

Skoog A and Benner R. 1997. Aldoses in various size fractions of marine organic matter: Implications for carbon cycling. Limnol Oceanogr 42(8):1803-13.

Skoog A, Biddanda B, Benner R. 1999. Bacterial utilization of dissolved glucose in the upper water column of the gulf of mexico. Limnol Oceanogr 44(7):1625-33.

Skoog DA, West DM, Holler JF, Crouch SR. 2000. Analytical chemistry: An introduction, thomson, brooks.

Snyder LR, Kirkland JJ, Glajch JL. 2012. Practical HPLC method development. John Wiley & Sons.

Taylor GT, Iabichella M, Ho T, Scranton MI, Thunell RC, Muller-Karger F, Varela R. 2001. Chemoautotrophy in the redox transition zone of the cariaco basin: A significant midwater source of organic carbon production. Limnol Oceanogr 46(1):148-63.

Valeur E and Bradley M. 2009. Amide bond formation: Beyond the myth of coupling reagents. Chem Soc Rev 38(2):606-31.

Wright RR and Hobbie JE. 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology :447-64.

Wu H and Scranton MI. 1994. Cycling of some low molecular weight volatile fatty acids in a permanently anoxic estuarine basin. Mar Chem 47(2):97-113.

Wu H, Green M, Scranton MI. 1997. Acetate cycling in the water column and surface sediment of long island sound following a bloom. Limnol Oceanogr 42(4):705-13.

Yang XH, Lee C, Scranton MI. 1993. Determination of nanomolar concentrations of individual dissolved low molecular weight amines and organic acids in seawater. Anal Chem 65(5):572-6.

7 Appendices

7.1 Screening of coupling reagents for derivatization of organic acids in aqueous solution (Gismervik 2012)

7.1.1 Method for testing Dialkyldiimide-mediated couplings

AMC (5 mg, $1.8x10^{-5}$ mol), TEA (3.08 µL, $2.2x10^{-5}$ mol), DCC (4.54 mg) or EDC (4.22 mg) ($2.2x10^{-5}$ mol), HOBt (3.65 mg) or HOAt (3.67 mg) ($2.7x10^{-5}$ mol), and propionic acid (5.63 µL, $2.2x10^{-5}$ mol) in DCM/H_2O (distilled) and toluene/H_2O (distilled) bi-phasic solutions.

7.1.2 Final screening of dialkyldiimide coupling reagents and catalyst

From the bi-phasic screening, six reactions under four sets of conditions were identified where the fluorescent coumarin amine was completely consumed. To identify the most efficient set of conditions dialkyamide, catalyst, and TEA were lessened (1.2 to 1.1 with respect to AMC).

7.2 Synthesis of compounds

7.2.1 Synthesis of fluorescent coumarin amine (Lim et al 2008, Gismervik 2012)

Halogenated 4-bromomethyl-6,7-dimethoxycoumarin is a convenient and commercially available starting material for the preparation of aminomethyl coumarin. The original literature procedure for 4-aminomethylcoumarin is a Gabriel synthesis. As the reported overall yields for the synthesis of 4-aminomethylcoumarin were very low (under 20%) (Sasamoto et al. 1996). Lim et al. improved the yields to >80% by use of a Delépine reaction (Scheme 11) (Lim, Pavlova, Brückner 2008). This reaction begins with hexamine displacing the halogen to form bromide salt. In the next step, hydrolysis with a 0.5 M HCl/EtOH solution results in ammonium

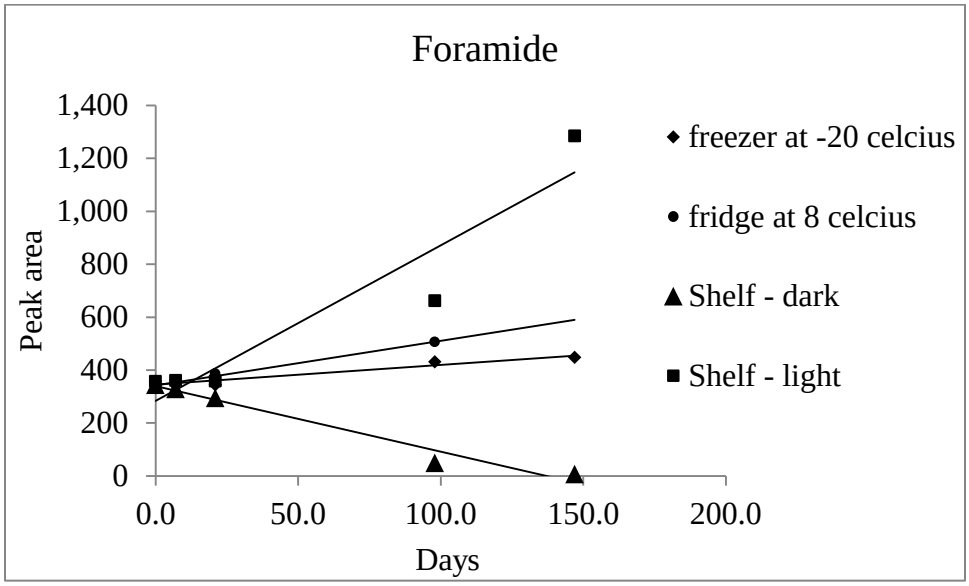
chloride aminomethyl coumarin, which readily is hydrolyzed to form the free amine. The Delépine reaction allows for the facile and cost-efficient preparation of aminomethyl coumarin. Importantly, all the products and intermediates are crystalline solids, allowing their preparation in highest purity through (repeated) recrystallization.

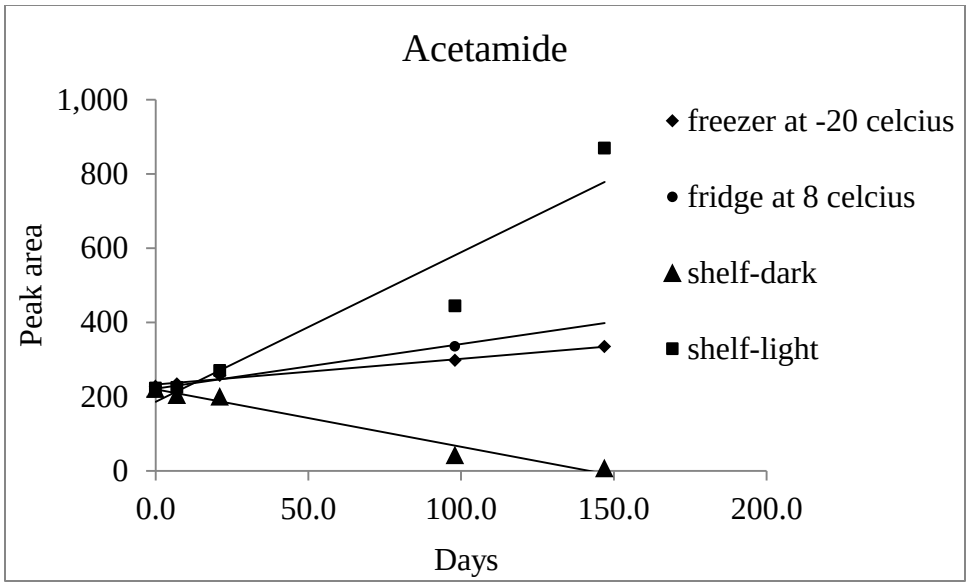
7.2.2 Synthesis of derivatized acid standards (Gismervik 2012)

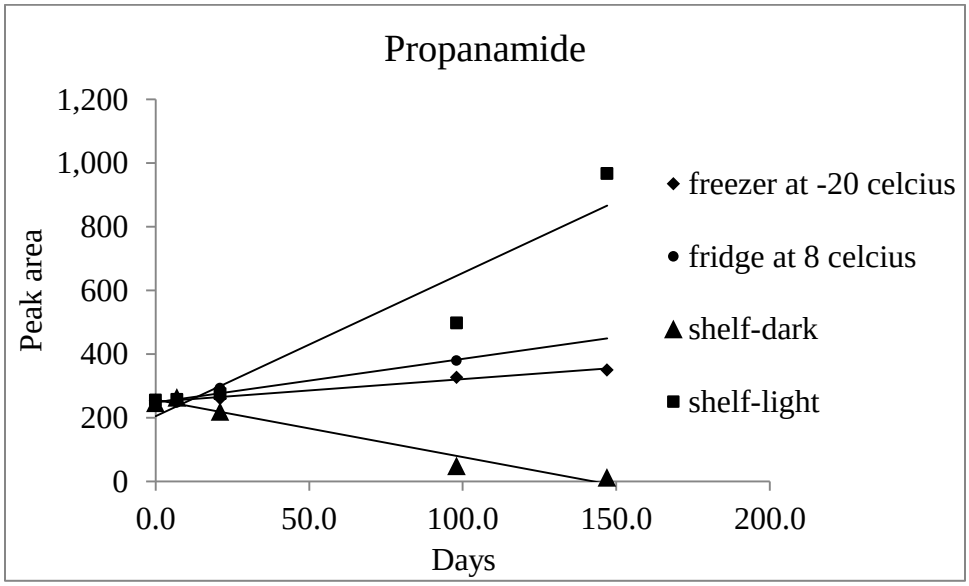
In the synthesis of the pre-derivatized acid standards, the acid chloride was added to the solution of AMC and Et_3N (2.1 to 1 to 1.1) in CH_2Cl_2 and left to stir overnight. Product formation was monitored using TLC and isolated with preparative plates (500 μm silica gel on glass, CH_2Cl_2/MeOH solutions as eluents).

Due to the instability of formyl chloride, a dialkyldiimide coupling strategy was choosen to form foramide from formic acid and AMC. DCC and the coupling catalyst HOBt were added to a solution of AMC and Et_3N in CH_2Cl_2, left to stir overnight, and monitored and isolated as above.

7.2.3 Stability of standard in solution




Acetamide
1,000
800
600
400
200
0
Peak area
0.0
50.0
100.0
150.0
200.0
Days
freezer at -20 celcius
fridge at 8 celcius
shelf-dark
shelf-light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1,000
800
600
400
200
0
Peak area
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150.0
200.0
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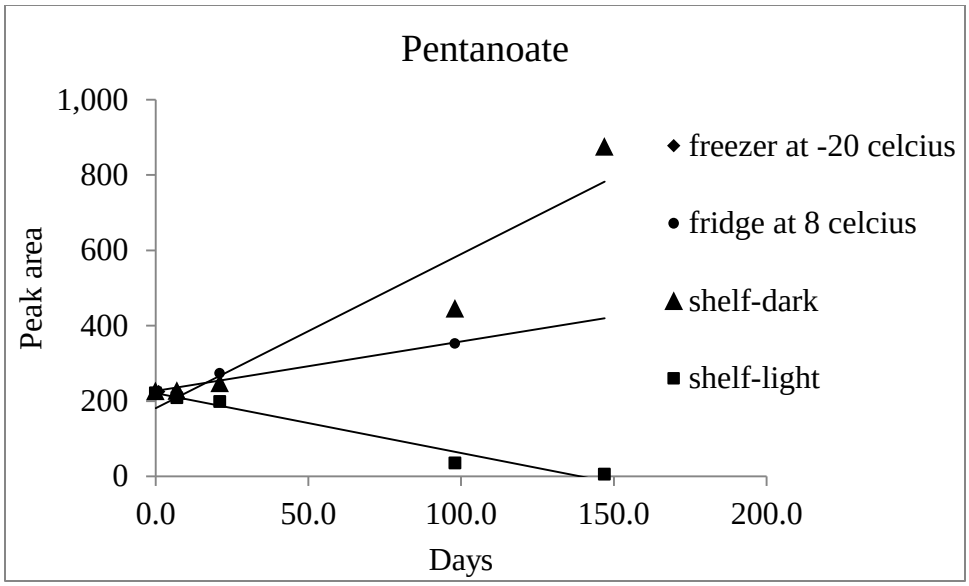
freezer at -20 celcius
fridge at 8 celcius
shelf-dark
shelf-light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600
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0
Peak area
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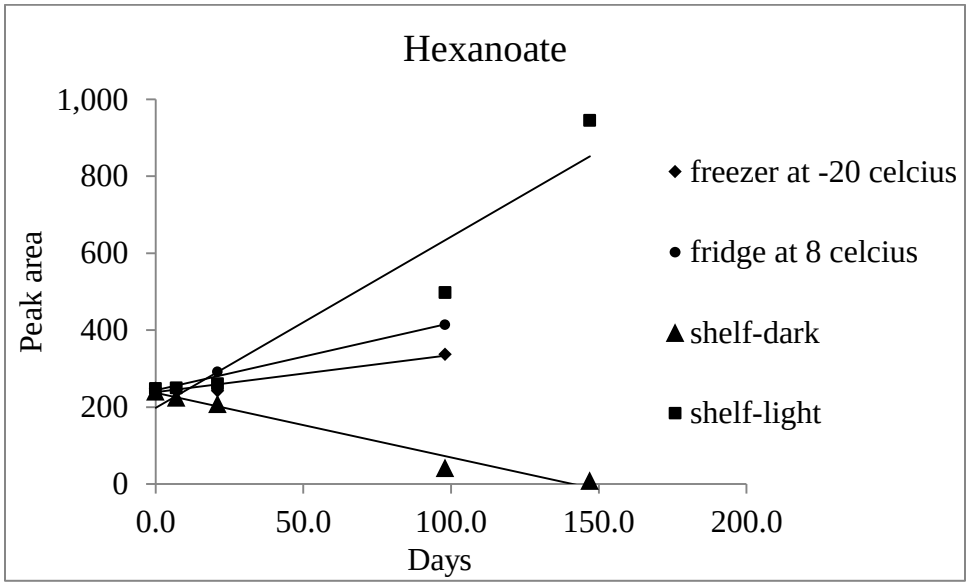
shelf-dark
shelf-light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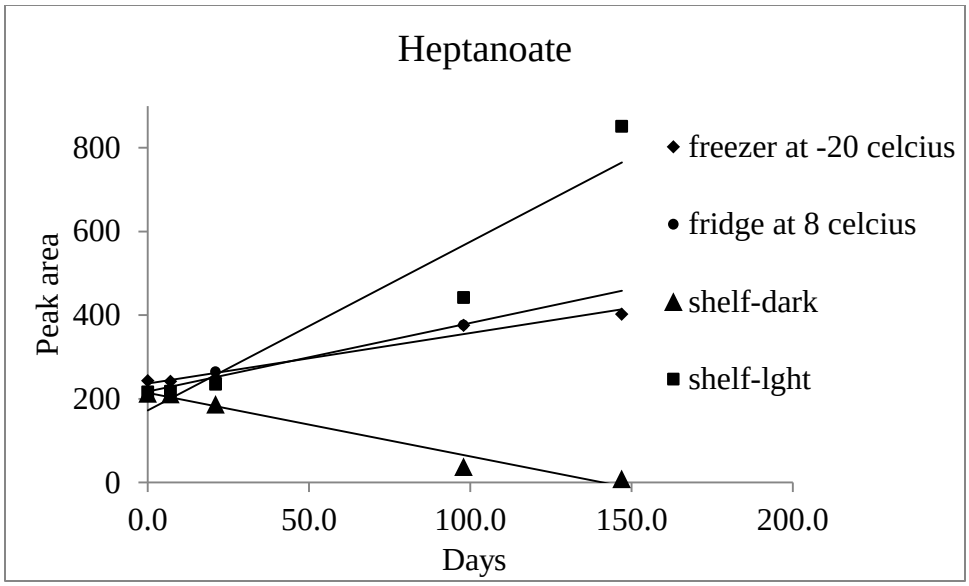


7.3 Determination of calibration curves

7.3.1 Equations for determining calibration curves

Linear regression models based on the method of least squares were used to calibrate peak area data based on equations A-M (Skoog et al. 2000). The equations were solved using the Excel data analysis toolpak.

Equation A. Sxx

$$S_{xx} = \sum {x_i}^2 - \frac{(\sum x_i)^2}{N}$$

Equation B. Syy

$$S_{yy} = \sum {y_i}^2 - \frac{(\sum y_i)^2}{N}$$ Type equation here.

Equation C. Sxy

$$S_{xy} = \sum x_i y_i - \frac{\sum x_i \sum y_i}{N}$$

Equation D. Average values $\bar{x}$ and $\bar{y}$ for the variables x and y, where x_i and y_i are individual pairs of data for x and y, *N* is the total number of pairs of data used in preparing the calibration curve.

$$\bar{x} = \frac{\sum x_i}{N} \quad \text{and} \quad \bar{y} = \frac{\sum y_i}{N}$$

Equation E. Slope of the line, *m*.

$$m = \frac{S_{xy}}{S_{xx}}$$

Equation F. The intercept of the line, *b*.

$$b = \bar{y} - m\bar{x}$$

Equation G . Linear regression model.

$$y = mx + b$$

Equation H. Standard deviation about the regression, s_r (also referred to as standard error in y).

$$s_r = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N - 2}}$$

Equation I. The standard deviation of the slope, s_m.

$$Sm = \sqrt{\frac{{s_r}^2}{S_{xx}}}$$

Equation J. The standard deviation of the intercept, s_b.

$$s_b = s_r \sqrt{\frac{\sum {x_i}^2}{N \sum x_i^2 - (\sum x_i^2)}}$$

Equation K. The standard deviation for results obtained from the calibration curve, s_c. This equation calculates the standard deviation from the mean $\overline{y_c}$ of a set of M replicate analyses of unknowns when a calibration curve that contains *N* points is used; $\overline{y}$ is the mean value of *y* for the *N* calibration data.

$$s_c = \frac{Sr}{m} \sqrt{\left(\frac{1}{M}\right) + \left(\frac{1}{N}\right) + \frac{(\overline{y} - \overline{y_c})^2}{m^2 Sxx}}$$

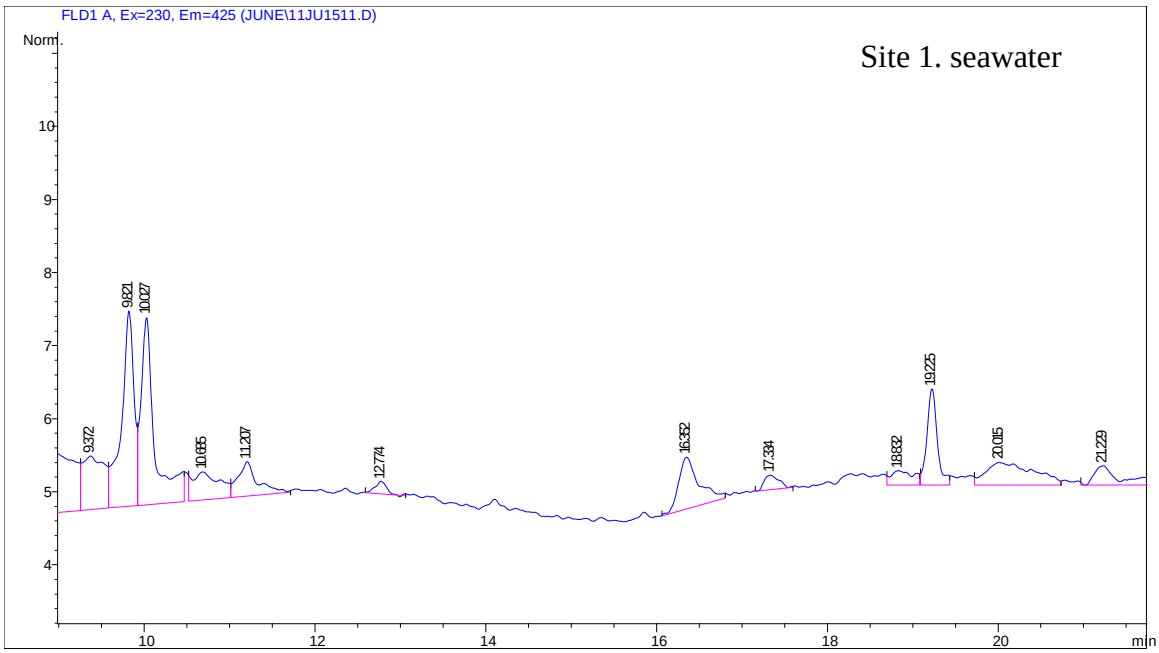
Equation L. The standard deviation for a small set of data (s_s).

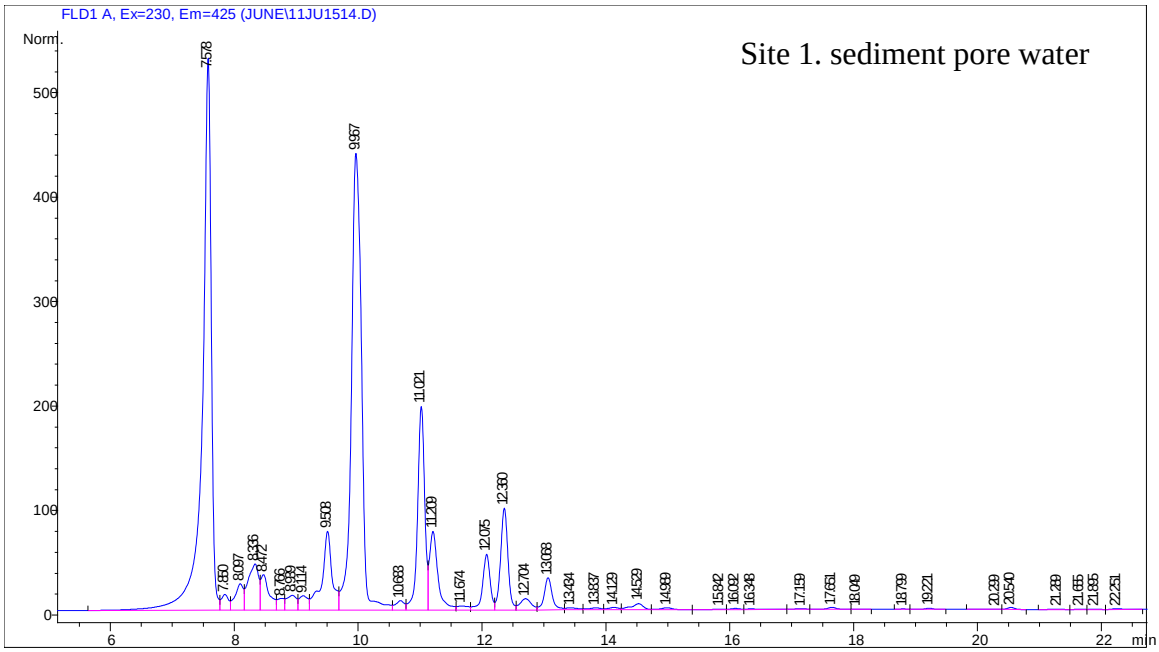
$$s_S = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N-1}}$$

Equation M. Standard error of the mean for each set of triplicates injected.

$$s_m = \frac{s}{\sqrt{N}}$$

7.4 Chromatograms from seawater derivatization

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13088
9503
8472
8336
8097
7880
10668
11674
12704
13464
13837
14123
14539
14988
15842
16032
16348
17159
17651
18009
18739
19221
20239
20540
21239
21665
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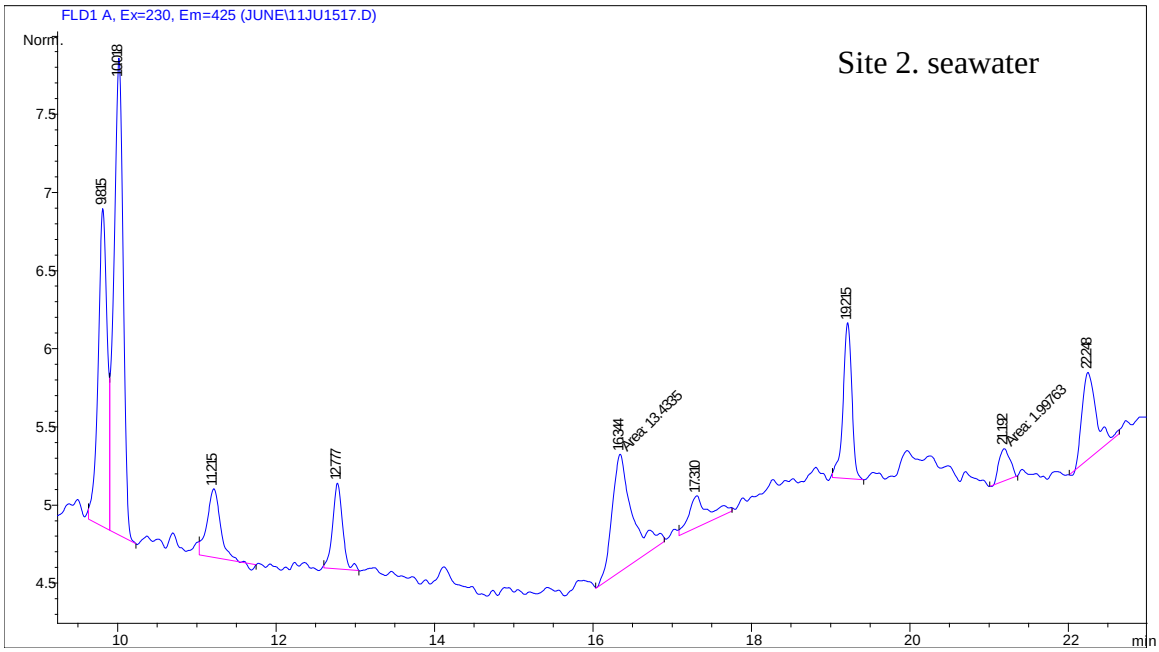
300
400
500
6
8
10
12
14
16
18
20
22
min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Norm.
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Area: 13.4335
21192
Area: 1.99763
11215
12777
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10
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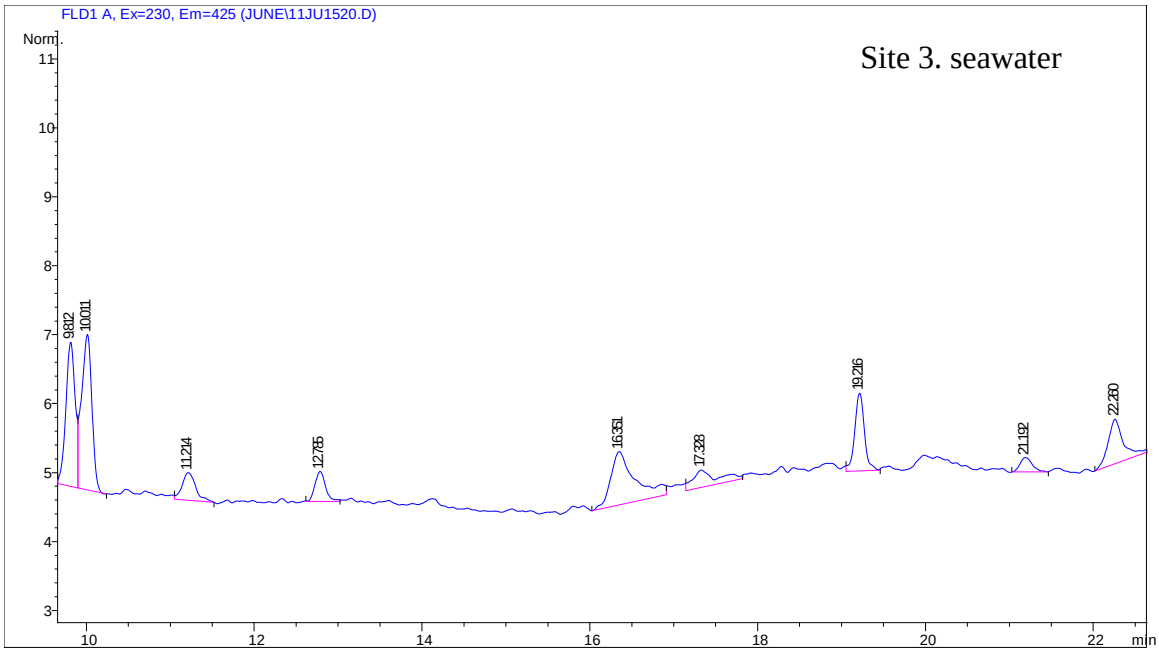
16
18
20
22
min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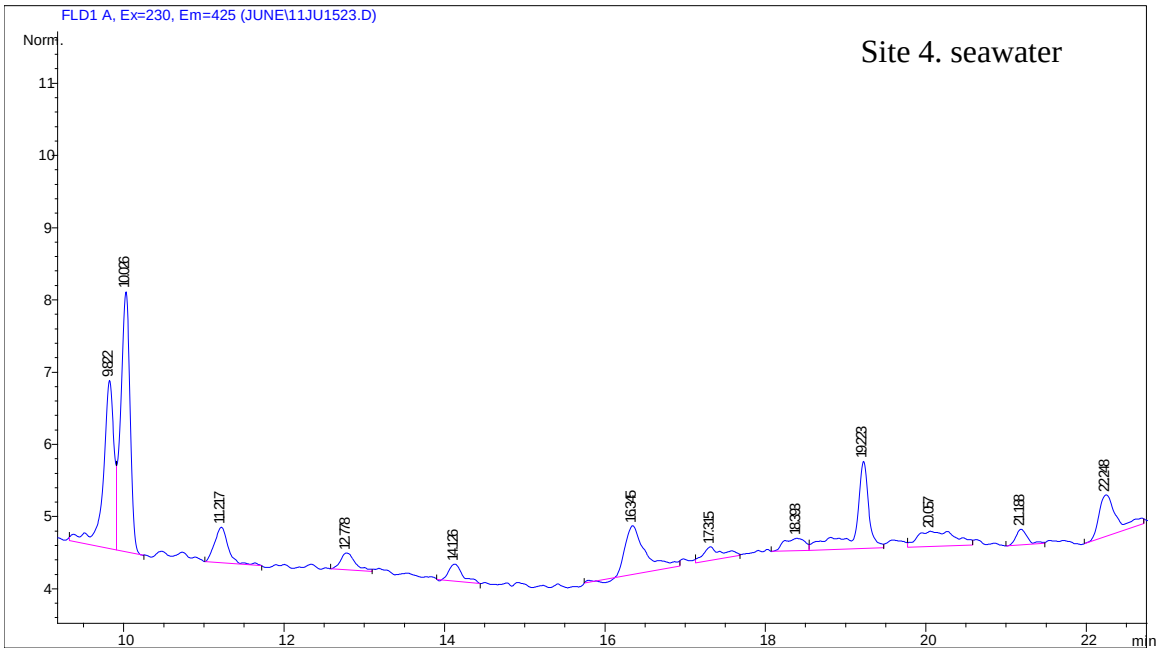
17.328
19.226
21.192
22.280
min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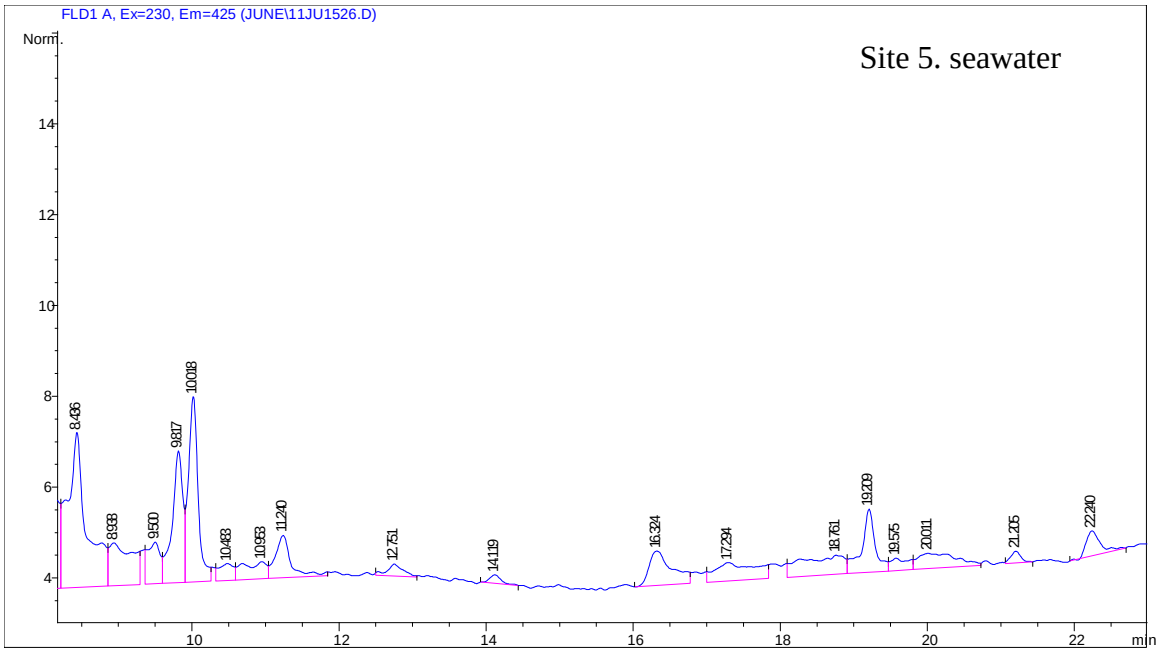
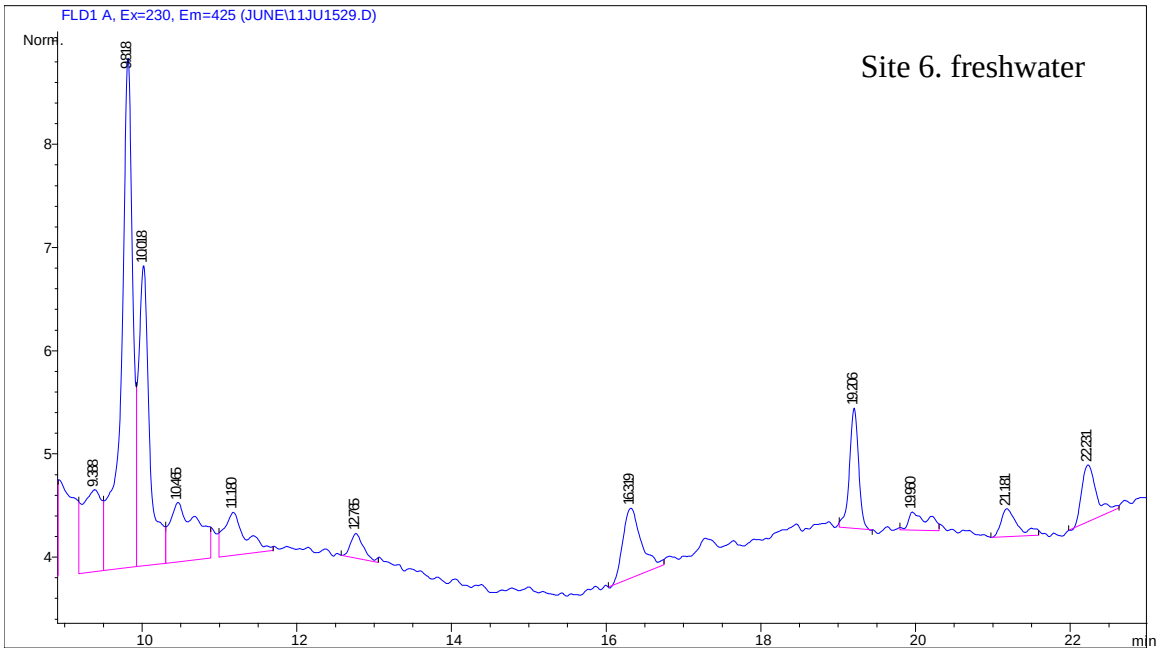
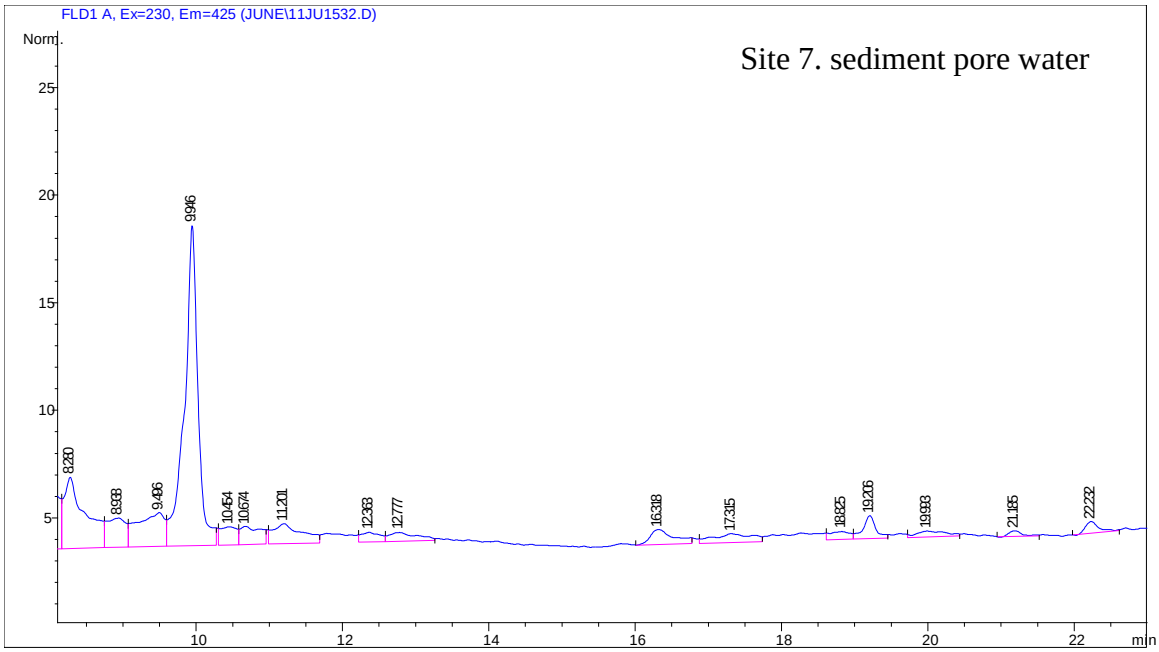
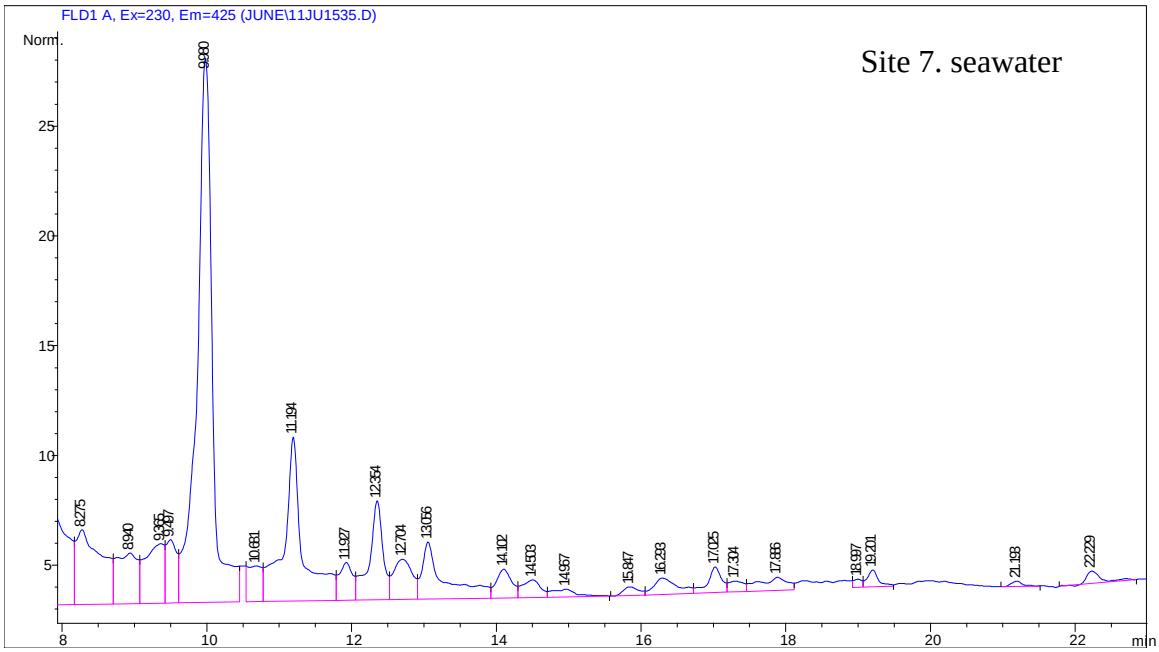
14.126
16.345
17.315
18.333
19.223
20.057
21.188
22.248
min


FLD1 A, Ex=230, Em=425 (JUNE\11JU1526.D)
Norm.
Site 5. seawater
8.406
8.933
9.500
9.817
10.018
10.483
10.933
11.240
12.751
14.119
16.324
17.294
18.761
19.209
19.575
20.011
21.205
22.240
min


FLD1 A, Ex=230, Em=425 (JUNE\11JU1529.D)
Norm.
Site 6. freshwater
9.318
9.938
10.018
10.465
11.160
12.765
16.339
19.206
19.930
21.181
22.231
min


FLD1 A, Ex=230, Em=425 (JUNE\11JU1532.D)
Norm.
Site 7. sediment pore water
8.220
8.933
9.406
9.996
10.454
10.674
11.201
12.393
12.777
16.318
17.315
18.825
19.206
19.993
21.185
22.222
min


FLD1 A, Ex=230, Em=425 (JUNE\11JU1535.D)
Norm.
Site 7. seawater
8.275
8.940
9.365
9.407
9.980
10.681
11.194
11.927
12.354
12.704
13.066
14.102
14.533
14.957
15.847
16.233
17.025
17.304
17.886
18.997
19.201
21.163
22.229
min