A NOVEL APPROACH TO THE ANALYSIS OF DISSOLVED FREE AMINO ACIDS IN SEAWATER BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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A Novel Approach to the Analysis of Dissolved Free Amino Acids in Seawater by High-Performance Liquid Chromatography

by

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ABSTRACT

Accurate determination of the dissolved free amino acids (DFAA) in seawater is critical in understanding the nitrogen cycle and marine food web in the ocean.

Traditional ion-exchange chromatographic analysis (IC) is very tedious requiring pre-concentration and desalting steps before analysis. Using conventional *o*-phthaldialdehyde (OPA) pre-column derivatization high-performance liquid chromatography (HPLC), measurements of DFAA concentrations "at time of sampling" is not possible unless an HPLC is on board. An alternative approach to DFAA analysis is HPLC using 9-fluorenylmethyl chloroformate (FMOC) as a fluorescence labelling reagent whereby the amino acids are derivatized (and thus fixed) in the field and brought back for analysis.

The effects of the ionic medium pH, chemical nature and concentrations of the mobile phases on the separation of the FMOC derivatives were investigated. Under optimized HPLC conditions, the twelve of the sixteen amino acid derivative standards were well-resolved using a reversed-phase column. Interference from the FMOC hydrolysis product, FMOH, was significantly minimized. The newly-proposed FMOC derivatization approach was compared with that of the well-established OPA derivatization in terms of stability, precision, sensitivity and recovery. FMOC

derivatives were found to be very stable and were more sensitive (lower detection limit) than OPA derivatives.

Comparisons were also made of DFAA measurements in seawater, obtained by FMOC derivatization at time of sampling, and, as the result of sample storage followed by OPA derivatization. Losses of DFAA in stored samples were found to be as high as 200%.

During 1992, various field studies were conducted using the FMOC method for the analysis of DFAA in real seawater. The variable levels of DFAA measured during an annual spring plankton bloom in a coastal region is studied.

DEDICATION

This thesis is dedicated to my parents, Junxian Liu and Yiju Duan.

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GLOSSARY

DAA	Dissolved Amino Acids
DCAA	Dissolved Combined Amino Acids
DOM	Dissolved Organic Matter
DFAA	Dissolved Free Amino Acids
Dns-Cl	Dansyl Chloride
FAA	Free Amino Acids
FMOC	9-Fluorenylmethyl Chloroformate
FMOH	9-Fluorenylmethanol
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
IC	Ion-Exchange Chromatography
ISTD	Internal Standard
K'	Capacity Factor ($K' = \frac{t_R - t_m}{t_m}$)
OPA	*o*-Phthaldialdehyde
t_m	Retention Time of Unretained Components
t_R	Retention Time of Retained Components
Tris	Tris(hydroxymethyl)aminomethane Hydrochloride

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CHAPTER 1

INTRODUCTION

1.1. Introduction to Dissolved Free Amino Acids in Seawater

In recent years, there has been increasing attention to dissolved free amino acids (DFAA) in seawater due to the rapid improvement in analytical methodology[1-5] and their significant role in the nitrogen cycles, in the marine food web, and in the chemical and biochemical processes in the ocean[6-10]. Although DFAA represent only 0.1 - 0.5% of the total dissolved organic carbon in seawater[11-13], DFAA take part in an important number of chemical, biochemical and physicochemical reactions in the ocean. Accurate determination of DFAA will help us to better understand the nitrogen cycle and transformations in the marine ecosystem.

1.1.1. Dissolved Organic Matter

In a commonly accepted definition, dissolved organic matter (DOM) includes the sum of the organic compounds passing through filters with a 0.45 - 1 μm pore size[12-13]. In actual seawater samples the particle size distribution is continuous[14]. Thus, DOM includes truly dissolved organic matter (particle size below 0.001 μm) and colloidal organic matter (0.001- 0.1 μm), as well as a small proportion of larger

particles (>0.1μm). DOM consists of many classes of naturally occurring compounds such as amino acids, carbohydrates and lipids as well as uncharacterized organics which have been chemically altered through various reactions.

In general, there are several sources of DOM in the ocean. These include phytoplankton[15-16], detrital materials[17], zooplankton[18], as well as river water and land runoff along the coastal and estuarine regions [19]. But the major source of DOM is from phytoplankton which can account for as much as 90% of total DOM in seawater during their metabolism [13]. As a major source of nitrogen and carbon, DOM can be readily taken up by heterotrophic organisms and bacteria[20-21].

From a marine chemistry point of view, DOM is in the intermediate position between living organisms and biogenic inorganic salts. DOM in the ocean is the product of bacterial decomposition and autolysis of plants and animals and their metabolic excretions. Some reports have shown that primary productivity can be influenced by the presence of DOM[22-23]. DOM also participates in the processes occuring at the sediment-water and air-sea interface because of its surface activity. It has been suggested that DOM also influences the speciation of trace metals in seawater[24] as well as metal toxicity to phytoplankton and bioavailability to plankton growth[25].

Although DOM actively influences a number of biochemical and chemical reactions and plays a significant role in marine food web and organism activity in the

oceans, unfortunately, a large portion of DOM is still uncharacterized. The major obstacle for proper chromatography of DOM is it's very low concentration in seawater where total organic carbon is about 0.5 - 1.5 mg $C \cdot L^{-1}$. Hence, the concentration of any single organic compound is likely to be less than 10^{-7} M[126]. The proper determination of one of the DOM subgroups, i.e. dissolved free amino acids (DFAA), is the subject of this thesis.

1.1.2. Nitrogen Cycle and Amino Acids in the Ocean

Amino acids are the building blocks of proteins which serve as components of enzymes to catalyze biochemical reactions and as structural components of organisms. Their importance in living systems make amino acids play significant roles in marine chemistry and marine ecology.

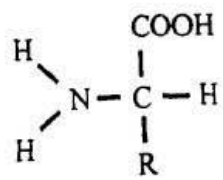


Figure 1.1. Typical Structure of Protein Amino Acids

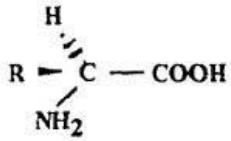
In general, amino acids are simple molecules having a minimum of one carboxylic acid and one amino functional group. Most of the amino acids existing in

seawater are classified as protein amino acids. Protein amino acids are α-amino derivatives of carboxylic acids and all but glycine contain at least one asymmetric carbon atom (see Figure 1.1).

Amino acids with one asymmetric carbon atom exist in two configurations which are mirror images called optical isomers or enantiomers, i.e., D- and L-amino acids. The enantiomers of the amino acids have the same physical properties (except for optical properties) and cannot be resolved by chromatography without the aid of optically active reagents or chromatographic stationary phases. In living organisms, protein amino acids exist as L-enantiomers only. In non-living and dissolved states, their optical activity changes by undergoing interconversions leading eventually to equilibrium mixtures of D- and L-amino acids. Table 1.1 lists the amino acids (and their structures) commonly found in seawater.

For several decades, it has been known that marine animals release dissolved free amino acids into the seawater[27-28]. But in recent years, it has been well understood that there are many kinds of producers of amino acids. The major source is phytoplankton, especially during phytoplankton blooms where concentrations of DFAA will double or triple the regular concentration levels due to an increased release rate from phytoplankton. This coincides with increasing concentrations of total DOM during phytoplankton blooms. Another important source of amino acids is zooplankton[29] via cell lysis and grazing[30] or direct release from zooplankton[18]. Amino

Table 1.1. Structures and Abbreviations for L-Amino Acids Commonly Found in Seawater



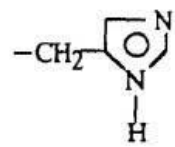
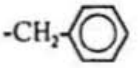
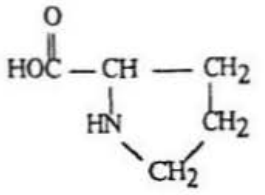
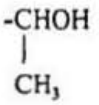
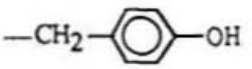
AMINO ACIDS	ABBREVIATION	STRUCTURE OF **R**
Alanine	Ala	$-CH_3$
Arginine	Arg	$-CH_2CH_2CH_2NH-\overset{\overset{NH}{\|\|}}{C}-NH_2$
Aspartic Acid	Asp	$-CH_2COOH$
Cystine	Cys	$-CH_2SH$
Glutamic Acid	Glu	$-CH_2CH_2COOH$
Glycine	Gly	$-H$
Histidine	His	
Isoleucine	Ile	$-\underset{\|}{\overset{CH_3}{\overset{\|}{C}}}HCH_2CH_3$
Leucine	Leu	$-CH_2CH(CH_3)_2$

Table 1.1. (continued)

AMINO ACIDS	ABBREVIATION	STRUCTURE OF **R**
Lysine	Lys	$-CH_2CH_2CH_2CH_2NH_2$
Methionine	Met	$-CH_2CH_2SCH_3$
Phenylalanine	Phe	
Proline	Pro	 *
Serine	Ser	$-CH_2OH$
Threonine	Thr	
Tyrosine	Tyr	
Valine	Val	$-CH(CH_3)_2$

* Complete structure

acids can also be released from detrital material and bacteria[17]. DFAA account for an average of 12% of the dissolved nitrogenous release products of phytoplankton[29-31].

Some researchers have found that amino acids can serve as both nitrogen and energy sources, and, as growth factors for bacteria and other marine saprotrophs[32-34]. Recent laboratory studies have shown that phytoplankton may also use free amino acids as a source of nitrogen and that certain phytoplankton can grow on specific amino acids. It is believed that amino acids are transported into the phytoplankton cell as the initial step in its utilization[35-37].

The concentration, uptake rate, assimilation and metabolism of the inorganic forms of nitrogen such as nitrate and ammonia have been extensively studied to understand the nitrogen nutrition source of phytoplankton and marine organisms, but the relation between organic nitrogen and the marine food web system has received less attention. As with nitrate and ammonia, amino acids take part in a number of biochemical and chemical processes. Figure 1.2 illustrates this by showing the nitrogen cycle in the ocean.

It is obvious that amino acids play an important role in the nitrogen cycle of the oceans and in marine food webs. Amino acids may serve as an important intermediates between living organisms and biogenic inorganic nitrogen forms. Amino acids can be released from both living and dead marine organisms. Amino acids can be taken up by

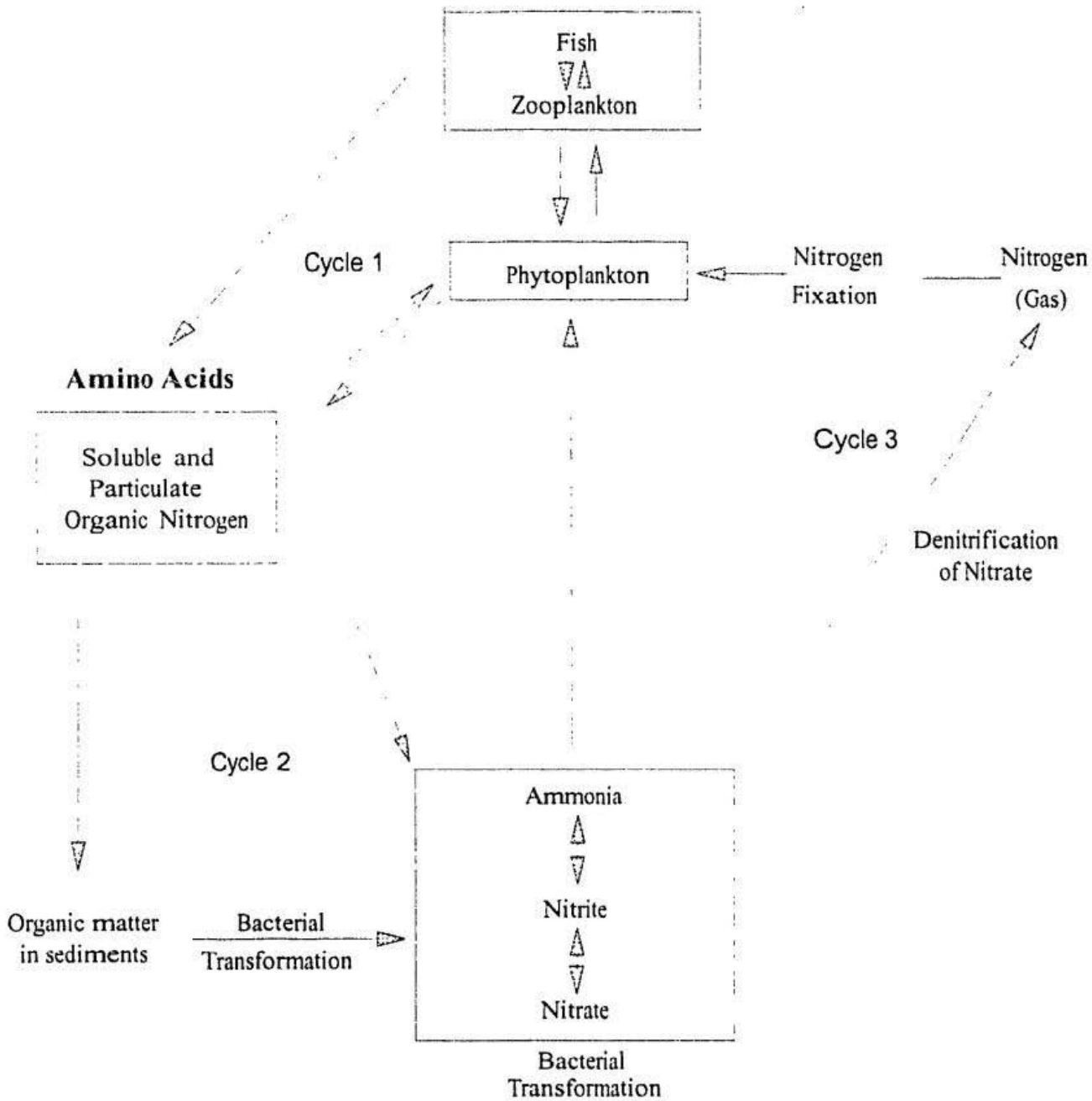


Figure 1.2. Nitrogen Cycle and Amino Acids in the Ocean

zooplankton and phytoplankton (nitrogen cycle 1, Figure 1.2). Amino acids can be transformed to inorganic nitrogenous forms such as nitrate, ammonia and ammonium via mineralization. After mineralization, nitrate and ammonia can be utilized by phytoplankton and bacteria as a major nitrogen and energy source. Mineralization of organic nitrogen is important in maintaining a balance in the nitrogen cycle in the ocean. Finally some amino acids will be lost through sinking of particulate matter and burial in the sediment (nitrogen cycle 2, Figure 1.2)[38-39].

Through a different cycle, nitrate can be reduced to N_2, N_2O and NO by the process called nitrate denitrification[38,40-41](nitrogen cycle 3, Figure 1.2). This is the major pathway for oceanic nitrogen back to the atmosphere. Although molecular nitrogen is the most abundant nitrogen form on earth, it is inert and stable, and only a limited number of organisms can utilize it as a source of nitrogen for growth. Nitrogen fixation plays an important role as it converts inert N_2 to other nitrogenous compounds such as amino acids.

In summary, as one of the organic nitrogen forms, amino acids are intimately involved a number of biochemical and chemical reactions and will influence the health of marine organisms. Proper determination of amino acids in seawater combined with other classes of DOM such as lipids, carbohydrates, can enrich our knowledge of the complex dynamics of marine ecosystems.

1.1.3. Dissolved Free Amino Acids in Seawater

There are two major forms of amino acids in the ocean, particulate amino acids (PAA) and dissolved amino acids (DAA), respectively. Here, as with the definition of DOM, dissolved amino acids are those which pass through filters having 0.45 - 1 μm pore size. Total dissolved amino acid concentrations in seawater are commonly about 50- 300 $\mu g \cdot L^{-1}$ [42-45] and is the major pool of amino acids in seawater, the total concentration of particulate amino acids usually being lower than that of dissolved amino acids.

Dissolved amino acids can be further classified as dissolved free amino acids (DFAA) and dissolved combined amino acids (DCAA). DFAA represent a small proportion of total dissolved amino acids with the concentration range at about 2 - 50 $\mu g \cdot L^{-1}$ [46-48]. DCAA consist of amino acids which are covalently bound as oligomers such as peptides and have a molecular weight range of 200 - 1000[49-50]. The concentration of DCAA is usually 4 to 5 times higher than that of DFAA[51]. Free amino acids (FAA) are released from DCAA only upon hydrolysis in strong acids and high temperatures.

Although DFAA represent a small pool of total dissolved amino acids, they actively participate in many biochemical and chemical reactions involved in marine

ecolog' al processes and in nitrogen cycling in the ocean. DFAA are very dynamic components in seawater and can be quickly utilized by microorganism. They thus have high cycling rates.

Laboratory studies have shown that 35 species of marine invertebrates, representing ten different phyla, are capable of removing amino acids from solution. On the other hand, more than 30 species of marine invertebrates are also able to release DFAA into seawater[52]. The same species of Lamellibranch was found to take up amino acids from solution as well as releasing DFAA[53]. Studies by Jorgensen found that short-term variation in bacterial assimilation of amino acids may also change the concentrations of DFAA in seawater[54]. Another report indicated that the variation in the concentrations of DFAA present in seawater could be related to the variation in the animals' state of nutrition[55].

Compared to DCAA, the DFAA are more biologically dynamic and thus unstable in seawater. Recent studies have shown that bacteria prefer to take up DFAA over DCAA and that the efficiency of utilization of DFAA by bacteria is higher than that of DCAA[56]. But the complex roles DFAA play in marine ecological system are still poorly understood. The accurate determination of DFAA concentrations at time of sampling is still a challenge for marine analytical chemists.

1.1.4. Distribution of Dissolved Free Amino Acids in Seawater

DFAA concentrations in seawater are highly variable seasonally and spatially. Owing to their involvement in the metabolism of marine organisms, DFAA concentrations vary with changes in temperature and in biological and ecological environments in the ocean.

As mentioned before, the total DFAA concentration in the ocean ranges from 2 - 50 $\mu g \cdot L^{-1}$ [46–48]. The lowest values are usually found in the open ocean, particularly in the Pacific Ocean. The Northeastern Atlantic has had consistently higher values than the Pacific[13]. Highest concentrations are normally reported in estuaries[9].

Interestingly, the pattern of individual DFAA is quite similar among different oceanic sites. The DFAA pool is dominated by neutral amino acids such as serine and glycine. Lysine, glutamic acid, aspartic acid, alanine, valine, arginine and ornithine are at intermediate levels. The remainder (see Table 1) are at low concentrations and aromatic and sulphur-containing amino acids are almost undetectable[57-59]. Notably, the pattern of the individual DFAA in seawater are similar to those of amino acids found in various algae and their excretion products and suggest that DFAA in seawater may largely originate from phyto- and zooplankton[32]. For example, Webb and Johannes found that the composition of DFAA excreted from various marine and fresh water invertebrates resembled those found in estuarine water[60]. In addition, Brehm reported

that the actual amino acid compositions in diatoms and in algae are similar to that in estuarine water in which the plankton lived[61].

Concentrations of DFAA are higher in summer than in winter due to increasing bacterial activity and phytoplankton metabolism in warmer seasons. The concentrations of DFAA can vary dramatically during a 24 hour period. Mopper and Lindroth reported that DFAA concentrations reached their highest values (about 200 - 400 nM of total DFAA) in the evening and their lowest (30 - 50 nM) in the morning and early afternoon. It is suggested that high zooplankton activity at night may partially be responsible for this change[7].

The concentrations of DFAA are normally higher in surface waters (0 - 20 m), especially in the surface microlayer[62]. The concentrations of DFAA decrease until very close to the bottom where concentrations increase due to input of DFAA from the sediment[63].

1.2. Established Chromatographic Methods for the Analysis of Amino Acids in Seawater

1.2.1. Ion-Exchange Chromatography

Ion-exchange chromatography (IC) remains a standard method for amino acid analysis in biological and clinical fields. Whenever a new method is developed for the

quantitative analysis of amino acids comparison is usually made with the classical ion-exchange chromatographic method[1-2] developed in the early 1950s by Moore and Stein[64], later modified by Spackman and Hamilton[65-66]. Many of the current innovations in liquid chromatography were first developed using ion-exchange chromatography of amino acids.

In IC, post-column fluorescence derivatization is commonly used in amino acid analysis. The eluting amino acids are derivatized to form a fluorescent product, followed by an on-line detection system. Fluorescence detectors are generally more sensitive than absorption detectors, an important feature for trace analysis. Ninhydrin was first introduced by Moore and Stein[64] as a useful amino acid fluorescence labelling reagent. It is a versatile and sensitive reagent and remains in wide use. Another popular fluorescence labelling reagent is *o*-phthaldialdehyde (OPA), especially in trace analysis of amino acids in seawater[67-68].

Amino acid analysis by IC is performed using strongly-acidic sulphonated polystyrene/divinylbenzene copolymer resins. Initially, large particle size resins in traditional ion-exchange chromatographic columns were used[69]. With the availability of high-pressure metering pumps and stainless steel columns, narrower bore columns packed with small particle size resins are now common in modern amino acid analyzers[70]. Improvements in performance and dramatic reduction in analysis time were quickly realized.

In the 1960s and 1970s, IC was widely used in the analysis of amino acids in seawater[71-73]. Dawson and Pertchard developed a standard amino acid analyzer modified to incorporate a fluorimetric detector to measure α-amino acids in seawater[74]. Rosenfeld employed a Beckman automatic amino acid analyzer to determine the concentrations of individual amino acids in nearshore anoxic sediments[75]. There have been several studies on the subject of desalting, preconcentration and analysis of amino acids in seawater since the first publication of the use of ion-exchange chromatography for separation of amino acids in seawater[76-78].

Clearly, the major drawbacks of IC include the large sample volume required, the preconcentration and desalting steps, contamination and the length of time necessary for analysis. The concentration of amino acids in seawater measured by IC were usually overestimated because of probable contamination from the numerous steps in analysis.

1.2.2. Gas Chromatography

The earliest gas chromatographic method (GC) for analysis of amino acids was in 1956 and it involved decarboxylation and deamination with ninhydrin[79]. Since then, advances in the development of GC have provided a sensitive and rapid alternative method to IC[80-82]. A major advantage of GC is that it can be easily connected with a mass spectrometer for amino acid identification and confirmation, especially in the event of co-elution of amino acids[83-84].

The quantitative determination of amino acids is dependent on the initial steps of sample desalting and concentration followed by derivatization. The commonly used derivatization procedure in GC analysis of amino acids includes two steps, esterification and acylation. Successful analysis of amino acids with GC is thus dependent on the synthesis of high yielding derivatives that are volatile and stable[85].

Due to the inherently high sensitivity of GC there have been many attempts to employ GC to measure low concentrations of amino acids in seawater[86-88]. Henrichs and Farrington used GC and GC-MS to identify and detect the amino acids in intersitial water of marine sediments[89]. Whelan compared GC with IC (using fluorescamine) for amino acid analysis in the surface sediment cores of the Atlantic abyssal plain[90]. He suggested that GC may be used as a complement to IC because fluorescamine labelling following analysis using IC does not detect secondary amino acids, (i.e. proline and hydroxyproline). On the other hand, basic amino acids such as lysine and histidine are subject to decomposition during GC analysis.

Although sufficient sensitivity could be achieved with the use of GC, sample handling and derivatization can be a problem. The multiple steps of derivatization are potentially a source of contamination. Another major drawback in GC analysis is the fact that not all DFAA can be chromatographically separated. In addition, for the derivatization procedure, strong acids are used thus hydrolyzing some fraction of the combined amino acids. However, the GC technique remains strong in applications such

as amino acid analysis in protein hydrolysates, in biochemical and clinical fields or total dissolved amino acids in seawater.

In marine chemistry, due to the very low levels of DFAA concentrations in seawater, gas chromatography is limited. With the development of easily synthesized fluorescent derivatives in high-performance liquid chromatography (HPLC), HPLC has largely surpassed GC for amino acid analysis in water samples.

1.2.3. High-Performance Liquid Chromatography

Since the 1970s, HPLC has been widely used as a powerful analytical technique for the analysis of a variety of compounds, particularly compounds which are polar or unstable. Among its various detection modes, fluorimetry has been accepted as a highly sensitive and selective one. Fluorescence derivatization is used for the conversion of nonfluorescent analytes having functional groups such as amino, carbonyl and carboxyl into highly fluorescent compounds. Thus, many of these trace level analytes can be analyzed by HPLC using fluorescence detection[91-97].

The HPLC method combined with on-line fluorescence detection is particularly suitable for the analysis of trace compounds in a complex matrix such as biological fluids and seawater. A major drawback of this technique is the limited number of natural compounds exhibiting sufficiently intense fluorescence. This problem may be

overcome by the use of chemical reactions converting non- or weakly fluorescent compounds into highly fluorescent derivatives[98]. Fluorescence derivatization procedures can be classified into two types: pre-column (derivatization before HPLC separation) and post-column (derivatization after HPLC separation).

The post-column reaction system requires an additional reagent to be continuously metered into the effluent stream from the analytical column. This is usually done with a metering pump but can also be accomplished effectively with a pressure system consisting of a reagent reservoir pressurized with 10 - 20 psi of nitrogen or helium. The reagent flow rate is thus controlled by the applied gas pressure[99].

The trend in recent years has been towards pre-column derivatization in which the sample mixture of amino acids is reacted to form suitable derivatives. The amino acid derivatives are then injected and separated on the HPLC column and detected by a on-line fluorescence detector[100-104].

The pre-column derivatization reaction does not need to proceed rapidly as in post-column techniques, and its conditions can be more easily optimized. It also permits a wider choice of separation modes and mobile phases in HPLC. In certain cases, however, the difficulty of removal of interfering reagent peaks from the chromatogram prevents the application of the method. Although in a few cases the reaction mixture

can be directly injected into the HPLC without clean up, removal of excess reagent and solvent from the reaction mixture is usually required[2]. The stability of the derivatives against hydrolytic cleavage and thermal decomposition is also very important.

Post-column derivatization must proceed rapidly because prolonged reaction time causes peak broadening. As well, the mobile phase should not interfere with the reaction. The choice of suitable conditions for amino acid separation and derivatization is therefore restricted. Occasionally, impure reagent, decomposed products, and sample impurities interfere with detection of the derivatized analytes. However, the post-column derivatization approach can be automated much more easily than pre-column derivatization.

Many fluorescence labelling reagents have been developed and applied to pre- and/or post-column derivatization HPLC. These include dansyl chloride (Dns-Cl)[105-106], fluorescamine[107] and *o*-phthaldialdehyde (OPA)[108-109]. However, Dns-Cl and fluorescamine are not suitable for seawater sample analysis due to the serious interference from both reagent and the reaction by-products, and, in pre-column derivatization, poor chromatographic resolution [110-111].

In 1979, Lindroth and Mopper established a very useful HPLC method using OPA as a pre-column fluorescence reagent followed by separation on reversed phase column with subsequent fluorimetric detection of amino acid derivatives[1]. OPA reacts

with primary amino acids in alkaline media, usually in the presence of a thiol compound (2-mercaptoethanol or ethanediol), to produce highly fluorescent isoindole derivatives (λ_{ex} = 330 nm, λ_{em} = 455 nm)[112]. The reaction proceeds in a borate buffer (pH 9.5 - 10) and is complete within a few minutes at room temperature (see Figure 1.3). The method has been successfully applied for seawater analysis by direct HPLC injection of the amino acid derivatives. Separation of up to 25 amino acids within 25 minutes at sensitivities in the femtomolar (10^{-15}) range can be achieved[11]. Owing to its sensitivity, simplicity and speed of analysis, HPLC using OPA as a fluorescence reagent has almost replaced IC for amino acid analysis in seawater[7,8,113].

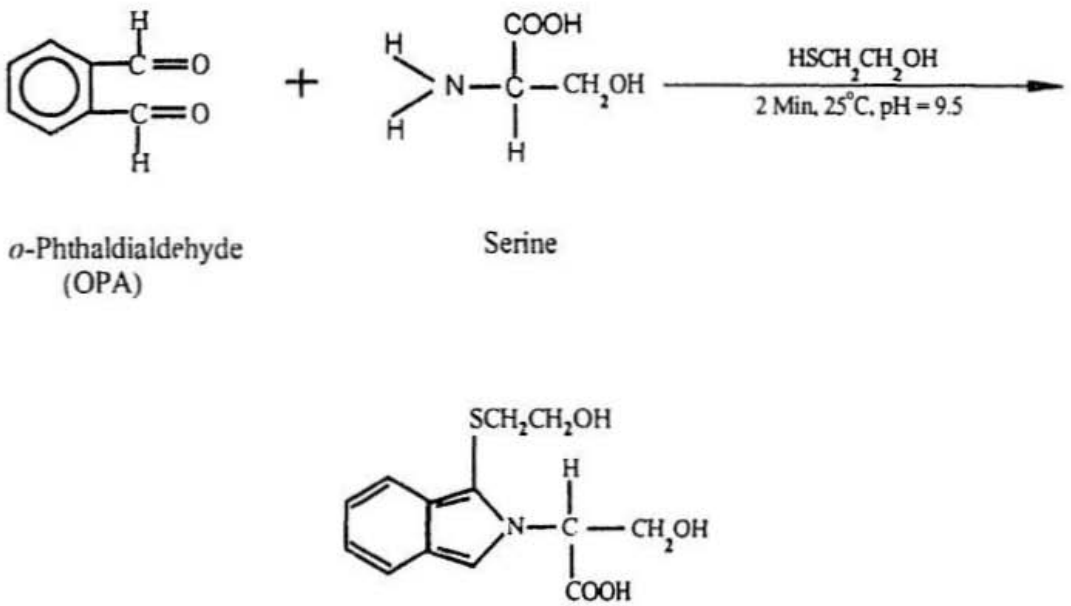


Figure 1.3. Amino Acid Derivatization with *O*-Phthaldialdehyde

In terms of drawbacks, the OPA derivatives are generally unstable and only primary amino acids react. Unless HPLC analysis is done aboard ship, it is not possible to obtained DFAA concentrations at time of sampling. Because various OPA-amino acid derivatives have different stabilities[114-115], derivatized samples must be injected after a precisely determined reaction time, thus limiting the technique in its present form to manual operation.

1.3. A Novel Approach for the Analysis of Dissolved Free Amino Acids in Seawater

In recent years, a new fluorescence labelling reagent, 9-fluorenylmethyl chloroformate (FMOC), has been proposed for the analysis of amino acids[2,3]. Under mild conditions in aqueous solution (pH = 7.5 - 7.8), FMOC reacts rapidly with both primary and secondary amino acids to form highly fluorescent and stable derivatives (Figure 1.4). At room temperature, the FMOC derivatives are stable for several days and if they were stored at 4°C in the dark, for weeks. The separation of a wide range of amino acid derivatives can be easily achieved on the reversed-phase column within 25 min and detected by an on-line fluoremeter (λ_{ex} = 260 nm and λ_{em} = 315 nm).

Various chloroformates are known to undergo very rapid condensation reactions with amino acids in buffered alkaline aqueous media to form carbamates in high yields[116-117]. Carpino and Han used FMOC as a new amino-protecting reagent which is stable towards acids and catalytic hydrogenation[118]. Anson, et al. suggested that

FMOC reagent was suitable for fluorescence labelling of primary and secondary amines[119]. Einarsson et al. were first to use FMOC as a fluorescence reagent to determine primary and secondary amino acids[2,3].

In field studies, FMOC reagent might be used as a means of preserving DFAA in seawater samples owing to the excellent stability of the FMOC derivatives. With no HPLC aboard ship, "at time of sampling" data of DFAA concentrations may be obtained with confidence.

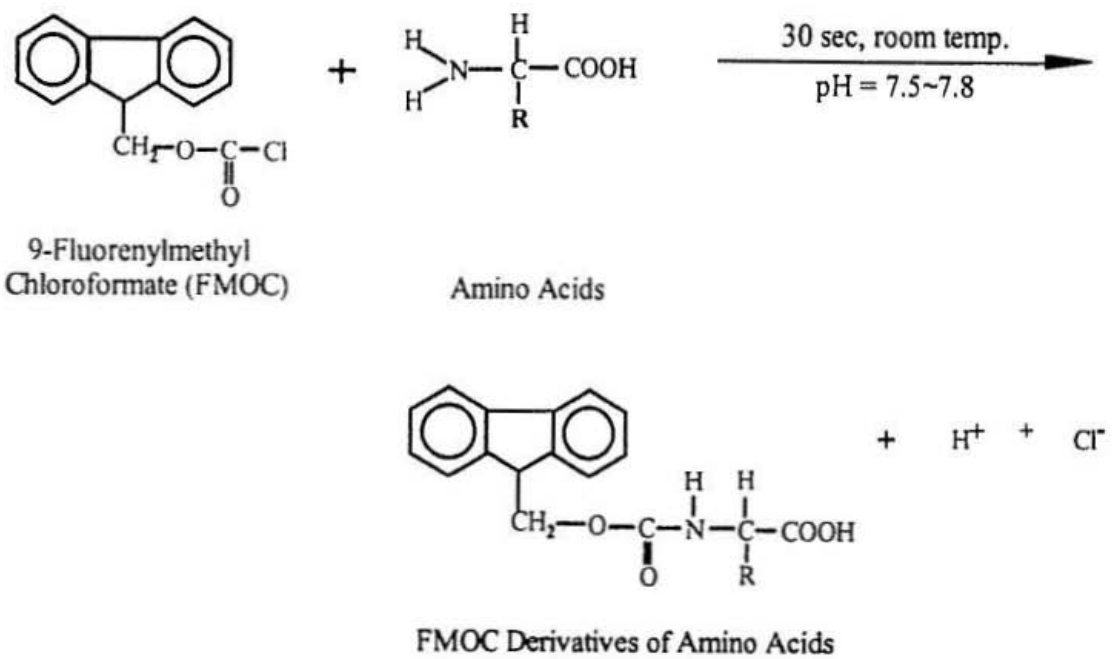


Figure 1.4. Amino Acid Derivatization with 9-Fluorenylmethyl Chloroformate

1.4. Objectives of the Project

The objective of this thesis was to explore and develop an HPLC method using FMOC as a fluorescence labelling reagent for the analysis of DFAA in seawater. Using the conventional OPA-labelling HPLC method, "at time of sampling" information about DFAA concentrations is not possible unless an HPLC is aboard ship. In contrast, FMOC derivatives in seawater are believed to be stable and more sensitive. Thus FMOC may be used as both a preservative of DFAA in seawater samples and as a fluorescence labelling reagent for subsequent HPLC analysis at a later time.

Initial experiments focused on the development of the FMOC method for the analysis of DFAA in seawater. The relation between the retention times of the FMOC derivatives and the pH of the mobile phase was investigated. Results led to an optimized chromatographic system for the analysis of DFAA in seawater. An important chromatographic feature was to lessen the interference of hydrolyzed product of FMOC, FMOH, in amino acid analysis.

The newly-developed FMOC approach was compared with the well-established OPA method. A number of analytical parameters such as stability and sensitivity of the FMOC and the OPA derivatives, precision, recovery and detection limits of the two methods (using the same HPLC system) were investigated and compared.

Finally, the new FMOC method was tested for real seawater sample analysis. During 1992, various field studies were conducted where numerous analytical data were obtained to investigate the application of the method for the analysis of DFAA in seawater. The role of DFAA in the annual spring plankton bloom in the coastal regions of the North Atlantic Ocean is discussed.

CHAPTER 2

EXPERIMENTAL

2.1. Materials

2.1.1. Chemicals

9-Fluorenylmethyl chloroformate (FMOC, 97%), pentane (HPLC grade), petroleum ether (spectro grade) and D-, L-2-aminobutyric acid (ISTD, 99+%) were purchased from Aldrich Chem. Co. (Milwaukee, WI). The FMOC was further purified through recrystallization using petroleum ether and diethyl ether before use (see section 2.1.2). Certified ACS grade diethyl ether (anhydrous) was received from BDH Inc.(Toronto). Acetonitrile and methanol were HPLC grade obtained from Mallinckrodt Canada Inc. (Quebec). Tris(hydroxymethyl)aminomethane hydrochloride ("Tris"), asparagine, and (ISTD) *o*-phthaldialdehyde were obtained from Sigma Chemicals (St. Louis, MO). 2-mercaptoethanol was purchased from Eastman Kodak Co. (Rochester, NY). Sodium hydroxide pellets, concentrated hydrochloric and phosphoric acids and sodium acetate (anhydrous) were certified ACS grade purchased from Fisher Scientific (Ontario). HPLC grade acetone was obtained from Caledon Lab. Ltd. (Ontario). Boric acid (certified ACS grade) was received from Anachemia Ltd. (Toronto). Purified water was obtained using a distillation-Barnstead NANOpure II system (Boston, MA).

2.1.2. Purification of 9-Fluorenylmethyl Chloroformate

The purity of FMOC from the supplier is unsatisfactory and a number of small interfering contaminants were observed in the chromatograms of derivatized amino acids. When FMOC was re-crystallized following the procedure outlined below the contaminants were minimized.

A 350 mg sample of FMOC was added to 2 mL of petroleum ether and 1 mL of diethyl ether, and the reagent dissolved using a hot water bath (60°). The solution was placed in a fridge (4°C) for 16 hours to allow for slow crystallization. The solvent was decanted off and recrystallization was repeated again. The crystals were dried under a stream of clean nitrogen gas. The purity of FMOC (99+%) was checked by HPLC. The yield of the recrystallization was about 60%.

2.1.3. Amino Acid Standard Solutions

Two stock solutions of amino acid standard were used. Stock solution 1 was purchased directly from Sigma Chem. Co. (St. Louis, MO), consisting of the amino acids L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine as well as ammonium chloride; 25 μM for each of individual amino acid (except L-cystine 12.5 μM) in 0.1 N HCl. The stock

solution was stored in a refrigerator and was stable for months. Standard solutions of amino acids were prepared by the dilution of this stock solution using purified water. Standard solutions were prepared fresh daily.

Stock solution 2 was prepared from individual amino acids purchased from Sigma Chem. Co. Calculated amounts of amino a ls were accurately weighted out using a Perkin-Elmer auto-micrObalance (model AD-2Z, Perkin-Elmer Corp., Norwalk, CO), dissolved in 20% (V/V) methanol/purified water and made up to 100.0 mL. This stock solution contained 500 μM of 16 individual amino acids; L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine. This stock solution was stored in a refrigerator and was stable for 3 - 4 weeks.

2.1.4. Glassware, Storage Containers and Filters

All glassware used was cleaned thoroughly, rinsed with methanol (HPLC grade) and purified water, and heated in a muffle furnace at 450°C overnight.

Two types of storage bottles were used to store seawater samples, scintillation vials (c.a. 20 mL) for the storage of small amounts of seawater, and polyethylene bottles (c.a. 250 mL) for the storage of larger volumes. Special precautions were taken in cleaning storage containers. The glass vials were first soaked for 24 hours in 0.1 N

NaOH solution, then copiously washed with purified water, rinsed with methanol followed again by purified water, and finally combusted at 450°C overnight. The vials were equipped with Teflon lined screw caps cleaned by methanol and purified water. Washed polyethylene bottles and caps were soaked in 0.6 N HCl for one week, soaked with purified water, rinsed with methanol and then purified water, and dried in ambient temperature. The bottles were capped during storage.

Whatman GF/C glass fibre filters (25 mm diam) were combusted at 450°C for 10 hours before use. The 0.2 μm and 0.02μm Anopore Membrane Discs filters (Scientific Products & Equipment Ltd., Concord, Ontario) were used as is.

2.2. Derivatization Procedures

2.2.1. Derivatization with 9-Fluorenylmethyl Chloroformate

Reagent A 233 mg sample of purified 9-fluorenylmethyl chloroformate (FMOC) was dissolved in 40 mL acetone (HPLC grade) to give a concentration of approximately 22.5 mM. The reagent was allowed to stand for 5 min at ambient temperature before use. A 2.5 mL of reagents were added to the sample by means of a 5 mL hypodermic plastic syringe. The reagent was stored in the refrigerator (4°C) and was stable for a month.

Borate Buffer Two borate buffer solutions were prepared and stored in

polyethylene bottles. Boric acid (6.13 g) was dissolved in 90 mL of purified water, the pH adjusted to 6.5 with 6 N NaOH solution and the solution made up to 100.0 mL (borate buffer 1). The same boric acid solution was adjusted so that its pH was 7.0 (borate buffer 2).

Procedure A 2.00 mL sample was placed in a pre-cleaned reaction vial (ca. 15 mL) and 0.5 mL borate buffer and 2.5 mL of the FMOC reagent were added. Borate buffer 1 (pH = 6.5) was used for standard amino acid solutions while borate buffer 2 (pH = 7.0) was used for seawater. The resulting pH of both water and seawater samples after the addition of borate buffer was 7.5 - 7.8. The solution was mixed thoroughly and the mixed solution was allowed to react for 1 min. If the internal standard, asparagine, ISTD (Sigma Chemical Co.) was used, it should be added prior to the FMOC reagent addition (for the analysis of DFAA in seawater samples, 10 μL of ISTD (5μM) was added to the 2.00 mL sample). The excess FMOC was extracted with pentane. Pentane 10 mL (HPLC grade) was added and the mixture shaken for 15 sec. The pentane layer was carefully removed with a pasteur pipette. The extraction was repeated again with 10 mL of pentane. It was noted, for seawater analysis, that after the first extraction the aqueous layer was cloudy, but became clear after second extraction. About 2 mL of aqueous solution remained which contained the FMOC derivatives. This volume was suitable enough for two injections (400 μL each) for HPLC analysis. If HPLC analysis was not performed immediately the derivatized seawater sample was stored at 4°C in the dark.

2.2.2. Derivatization with *o*-Phthaldialdehyde

Reagent *o*-phthaldialdehyde (OPA, 50 mg) was dissolved in 1 mL of methanol (HPLC grade), and 50 µL 2-mercaptoethanol was added . The reagent mixture was stored at 4°C in refrigerator to "age" for at least 24 hours prior to use. The reagent was stable for 4 days at 4°C in the dark.

Borate Buffer Boric acid (6.13g) was dissolved in 90 mL of purified water, the pH adjusted to 12.5 with 12 N NaOH, and the solution made up to 100 mL. The buffer was stored in a polyethylene bottle.

Procedure To a 2.00 mL of sample in a pre-cleaned reaction vial (ca. 20 mL) was added 20 µL borate buffer (pH = 12.5) and 10 µL of 5 µM internal standard (STD) α-aminobutyric acid (Aldrich Chemical Co.). The solution was mixed well and the resulting solution pH was about 9.5. A 20 µL aliquot of OPA reagent was then added and allowed to react for exactly 2 min. The HPLC injection syringe was rinsed with a small amount of derivatized solution and the sample immediately loaded into the HPLC sample loop. It was very important that the time between the addition of OPA reagent and sample injection be constant (within 2 min $\pm$ 10 sec).

2.3. Sampling, Filtration and Storage

2.3.1. Sampling

Most samples were collected with a 5 L Niskin sampling bottle (model 1010, General Oceanics Inc., Miami, Florida). The sampler closure was triggered from the ship through a single cable and a metal messenger.

2.3.2. "Aged" Seawater

Amino acid-free seawater was required in experiments which involved preparation of seawater samples spiked with solutions of amino acid standards. Seawater which has been left to sit at room temperature for 2 months ("aged" seawater) was found be almost free of DFAA and therefore suitable for this purpose.

2.3.3. Filtration

Seawater from the Niskin sampler was immediately filtered through a pre-combusted Whatman GF/C glass fibre filter under slight vacuum. In studies on the effect of different filter sizes, 0.2 and 0.02 μm Anopore filters were used instead of the GF/C filters. All filters were rinsed prior to use with 50 mL of distilled water and 10 mL of seawater sample. The filtering apparatus were rinsed with distilled water and a small amount of filtered seawater between samples. Blanks were determined by

measuring concentrations of DFAA in distilled water which had been filtered the same way as a seawater sample. GF/C filtration was carried out either by gravity or by a 50 mL glass syringe with slight pressure depending on the volume of the seawater filtered. Whenever 0.2 μm and 0.02 μm Anopore filters were used the syringe filtration apparatus was used and the flow rate was kept at about one drop per sec. All samples were filtered into pre-cleaned bottles.

2.3.4. Storage

If storage was required, seawater samples were frozen immediately after filtering in the polyethylene bottles or glass sample vials using dry ice. In the laboratory they were stored at -20°C in the dark. Storage containers were filled up to within 1.5 cm of the top of the container cap.

2.4. Chromatographic Analysis of Amino acids

2.4.1. High-Performance Liquid Chromatographic System

The HPLC system consisted of a Hewlett Packard 1050 series gradient pumping system used for delivering mobile phases, a Rheodyne model 7125 injector equipped with a 400 μL sample loop. The injector valve was immediately turned to the injection position after filling. The valve was turned back to the load position after 1 min and rinsed with 5 mL of purified water and 2 mL of acetonitrile.

Several types of reversed phase column were tested to achieve satisfactory separation of the FMOC derivatives. A Spherisorb-ODS-1 column (250 x 4.6 mm, particle size 10 μm) and a Spherisorb-ODS-2 column (250 x 4.6 mm, 5 μm) were purchased from Chromatographic Science Co. (Montreal). A specialty reversed-phase column for amino acid analysis, a "Pico.Tag" column (300 x 3.9 mm, 10 μm), was received from Millipore Corp (Milford, MA). A Waters precolumn system was mounted between the pumping outlet and the column inlet to guard the analytical column. The C_{18} cartridge of the precolumn was changed every month.

For the separation of OPA derivatives of amino acid a reversed-phase Spherisorb-ODS-2 column (250 x 4.6 mm, 5 μm) was used.

Column eluent was monitored with an on-line SF-330 spectrofluorometer equipped with a 150 watt xenon lamp. Excitation and emission wavelength were set at λ_{ex} = 260 nm and λ_{em} = 315 nm for FMOC derivatives, and λ_{ex} = 330 nm and λ_{em} = 458 nm for OPA derivatives. The cell volume was 5 μL.

The chromatograms were recorded on a two-channel Spectra-Physics SP 4290 integrator on a 1-mV scale and all of the data were simultaneously stored on a Winner Labnet software program.

2.4.2. Mobile Phases and Gradient Elution System

Mobile Phases For OPA derivatives, mobile phases were: (A) 98% of 50 mM sodium acetate adjusted to pH 5.8 with acetic acid, 2% tetrahydrofuran; (B) methanol (HPLC grade).

For FMOC derivatives, optimized mobile phase were: (A) 20 mM sodium acetate adjusted to pH 7.5 with 6 N HCl, (B) 20% of 50 mM sodium acetate (pH 7.5), 80% of acetonitrile (HPLC grade). All mobile phases were filtered through Millipore filter (pore size 0.45 μm, Millipore Corp., Bedford, MA). The mobile phases were further degassed daily using an ultrasonic bath and water aspirator vacuum.

Gradient Elution System For OPA derivatives, the gradient elution was as follows: flow rate 1 $mL \cdot min^{-1}$; initial solvent, 25% solvent B; linearly increased to 50% B in 6 min, isocratic step at 50% B for 10 min, linearly increased from 50% to 80% B in 4 min, isocratic step at 80% B for 15 min. The mobile phase was returned to initial conditions in 10 min.

For FMOC derivatives, the gradient elution and flow rate were varied to optimize resolution, but, typically were as follows: solvent B linearly increased from

1% to 50% in 20 min, isocratic step at 50% B for 5 min, linearly increased from 50% to 100% B in 2 min, isocratic step at 100% B for 13 min. The mobile phase was returned to initial conditions in 10 min. A constant flow rate of 1 $mL \cdot min^{-1}$ was employed.

CHAPTER 3

OPTIMIZATION OF CHROMATOGRAPHIC ANALYSIS OF 9-FLUORENYLMETHYL CHLOROFORMATE DERIVATIVES

3.1. Derivatization Procedure for 9-Fluorenylmethyl Chloroformate Derivatives

The reaction of FMOC with amino acids is represented in Figure 1.4. Under weak alkaline conditions in aqueous solution, FMOC reacts rapidly with amino acids to form highly fluorescent carbamate derivatives. The chloroformate can also react with water to form the alcohol 9-fluorenylmethanol (FMOH) as a hydrolysis degradation product. Yields of 88-97% for the reaction of FMOC with amino compounds and yields of 88-89% for the preparative derivatization of amino acids with FMOC have been reported[2, 5, 118].

3.1.1. Reaction time

The reaction rate of FMOC with amino acids is very fast. Some reports have indicated that the reaction is complete within 30 sec[2-3]. In order to reduce the yield of the hydrolyzed side-product FMOH, whose chromatographic peak interferes with the chromatographic separation of the amino acids (see Figure 3.2), the time between the addition of FMOC reagent and pentane extraction is normally limited to 40 sec. For the standard amino acid solution, a 40 sec reaction time was found satisfactory for

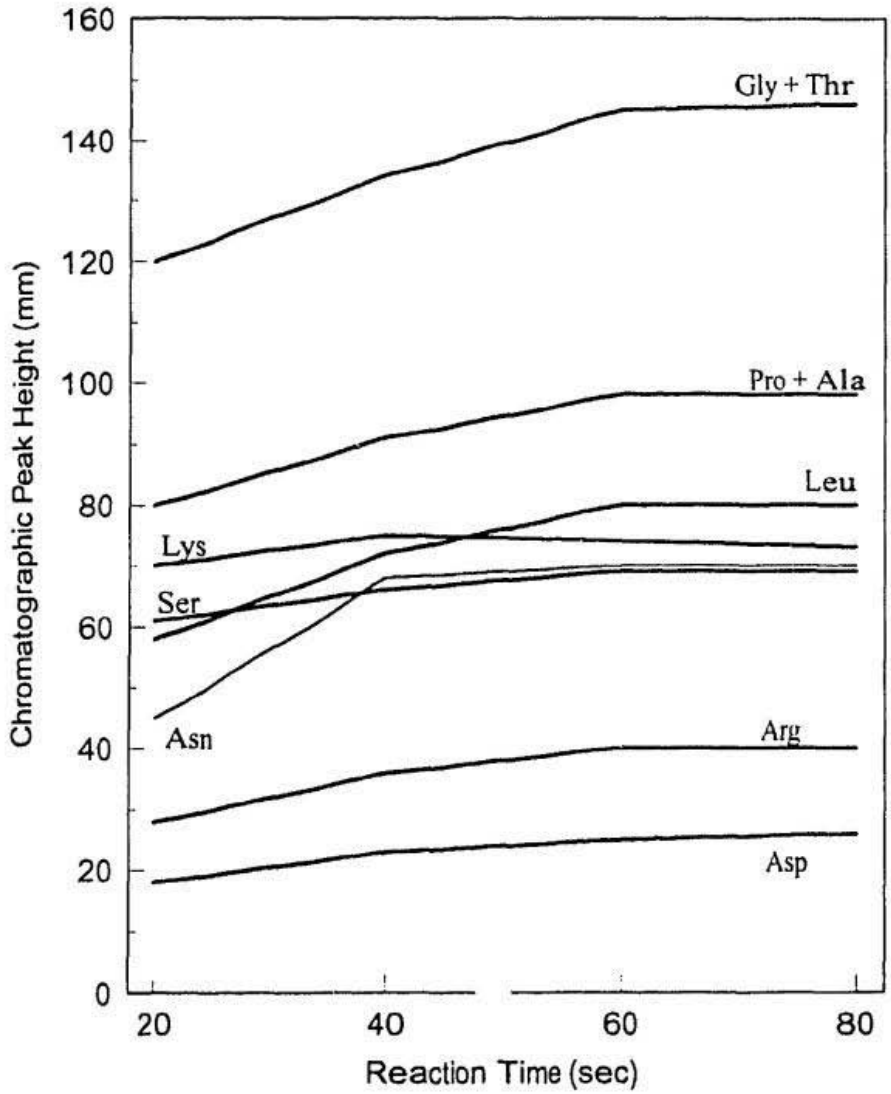


Figure 3.1. Effect of Reaction Time on the Production of Amino Acid Derivative Spiked in seawater

maximum yield. But for seawater samples, a reaction time of 60 sec was necessary to ensure more reproducible results and higher yields (see Figure 3.1).

As the result of high salinity in seawater samples, the reaction mixture turned cloudy after the addition of FMOC reagent indicating limited solubility of FMOC. This would explain the need for larger reaction times for amino acids in seawater. The sample mixture remained cloudy after the first pentane extraction, but became clear after the second extraction. The increase of reaction time from 40 sec to 60 sec for seawater analysis had little effect on the FMOH production.

3.1.2. 9-Fluorenylmethyl Chloroformate Concentration

It was found that for aqueous solutions of amino acids a FMOC reagent concentration of 15 mM was satisfactory. Because of the limited solubility (or lower rate of solubilization) of FMOC in seawater a study was done to measure the effect of FMOC reagent concentration on reaction yields. When the reagent concentration was increased 50% to 22.5 mM yields increased between 5 - 25% for individual amino acids (Table 3.1). Further increasing the reagent concentration showed very little improvement in yields but produced a much larger amount of FMOH. A FMOC reagent concentration of 22.5 mM was thus chosen for all derivatization procedures.

Table 3.1. The Effect of FMOC Reagent Concentration on Derivatization Yields of Amino Acids Spiked in Seawater

DFAA 50 pmol Each Spiked in Seawater	Yields {as Peak Heights (mm)} versus FMOC Concentrations		
	15 mM FMOC	22.5 mM FMOC	30 mM FMOC
Asp	29	36	36
Glu	56	61	62
Ser	93.5	101	101
Gly+Thr	167	178	180
Pro+Ala	114	131	132
Val	86	92	92
Met	62	64	64
Leu	82	82	82
Ile	83	84	84
Arg	45	48	49
Lys	93	91	90

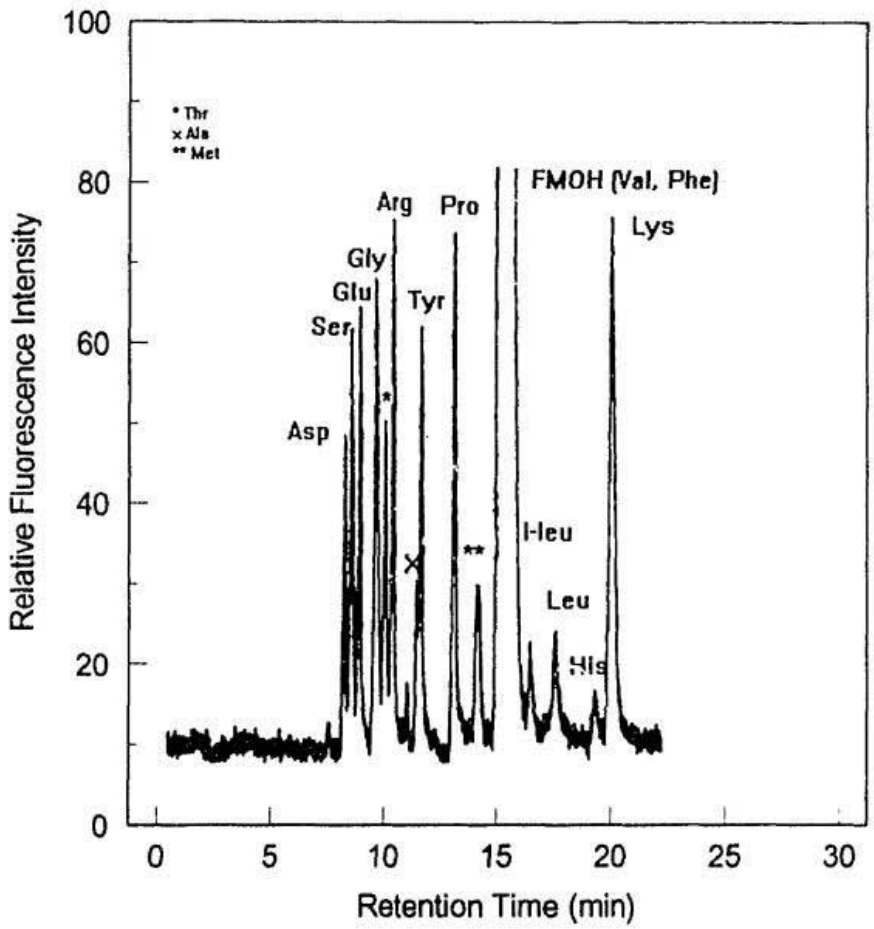
3.2. Chromatographic Separation of FMOC Derivatives of Amino Acids

3.2.1. Conventional Chromatographic Separation

The conventional chromatographic separation method reported in the literature adopted the use of the following mobile phases[2-3]; solvent A, 50 mM of sodium acetate buffer, pH=4.31, and, solvent B, 100% acetonitrile. Initial use of these mobile phases and gradient elution program for the chromatographic separation of trace amounts of amino acid derivatives proved unsatisfactory as revealed in Figure 3.2. The major problem is the elution of FMOH side-product among the analyte peaks thus interfering with observation of a number of amino acid derivatives and co-eluting with valine and phenylalanine. The conventional separation system is more suitable for much higher concentrations of amino acids such as those of plant and animal protein hydrolysates where the FMOH peak is much smaller (1/100 the size of peak observed in Figure 3.2) relative to FMOC-derivatized amino acids. A concerted effort was made to alter the chromatographic separation of the derivatized amino acids in order to lessen the interference of FMOH while retaining satisfactory resolution. The pH of the mobile phase was discovered to be an important parameter (see Section 3.2.2).

3.2.2. The Effect of the Ionic Medium pH

The effect of the pH of the buffered mobile phase on the separation of the amino acid derivatives was investigated over the interval of pH 3.0 to 9.0. In general, the



HPLC conditions: Flow rate: 1mL·min. Mobile phase: (A) Sodium acetate buffer, 50 mM, pH 4.31; (B)acetonitrile. Gradient elution program: 22 - 60 % solvent B from 0.7 to 12 min. Each peak represents 12.5 pmol of individual amino acid.

Figure 3.2. A Typical Chromatogram of FMOC-Derivatives of Trace Amino Acids Using Conventional Chromatographic Separation.

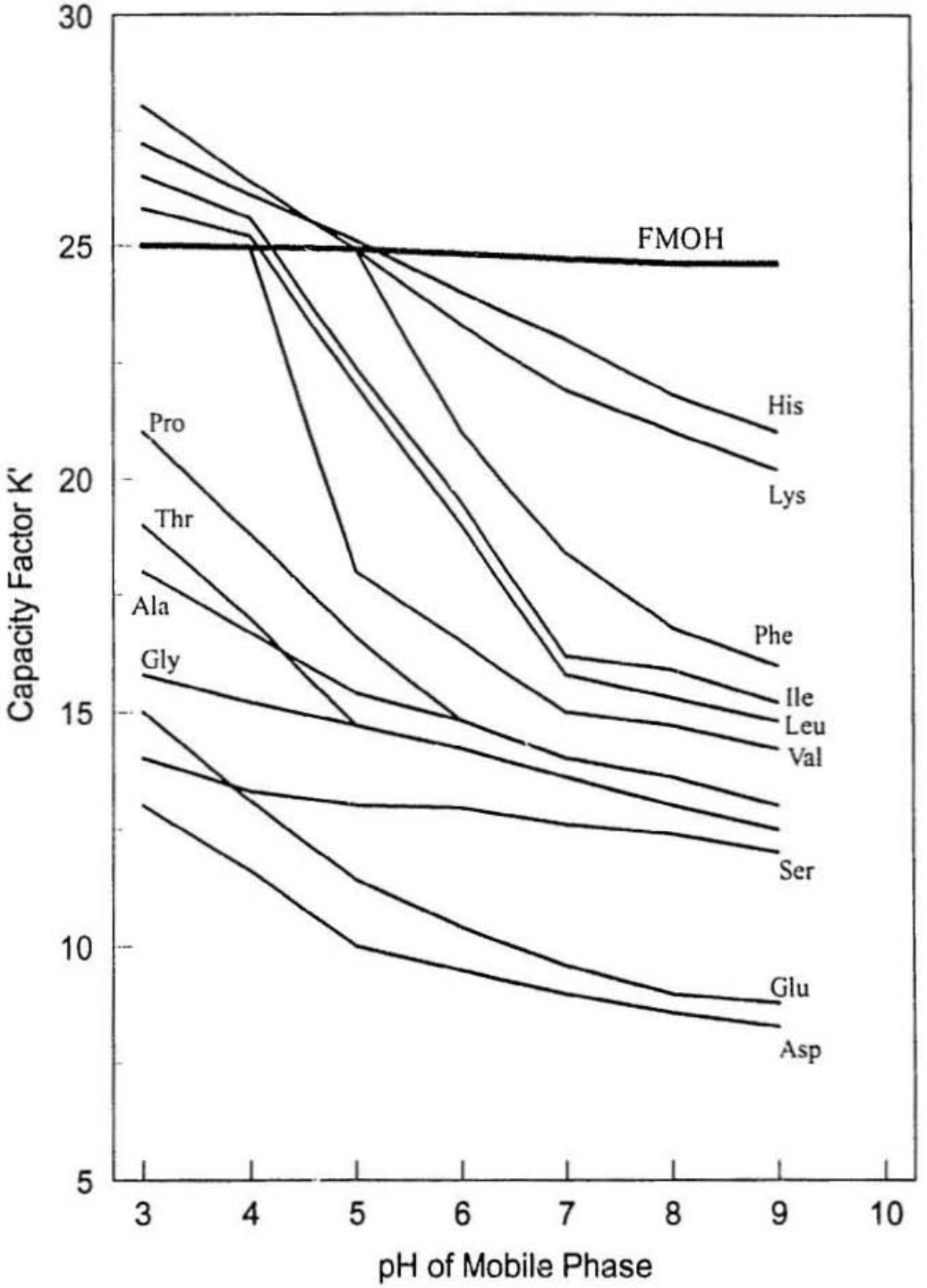


Figure 3.3. Effect of the pH of the NaAc Mobile Phase A on Capacity Factors

capacity factors K' ($K' = \frac{t_R - t_m}{t_m}$) of most amino acid derivatives decreased as pH of mobile phase increased, while k' of FMOH remained almost unchanged (Figure 3.3). By increasing the pH higher than 6.5 all amino acids eluted before FMOH and its chromatographic interference was significantly reduced. An ionic medium pH of 7.5 was selected for optimum resolution of the amino acid derivatives. A higher mobile phase pH would be detrimental to the silica-based HPLC column.

3.2.3. The Effect of the Chemical Nature of the Ionic Medium

Realizing that the pH buffering capacity of HOAc/NaAc is limited to the acidic pH region two different base buffers which would function at pH 7.5 were tested as ionic components of mobile phase A. These were NaH_2PO_4/Na_2HPO_4 (pH 6.1 - 8.1) and tris(hydroxymethyl)aminomethane hydrochloride ,"Tris", (pH 7.0 - 9.0). Buffer concentrations between 20 and 50 mM were tested. Both buffer systems behaved similar to that of NaAc mobile phase (pH 7.5) for elution order of the amino acid derivatives but were proven to be in unsuitable ionic medium because they produced very broad chromatographic peaks and poorly resolved chromatograms.

3.2.4. The Effect of Acetonitrile Concentration in Mobile Phase B

In the conventional chromatographic system, mobile phase B was 100% acetonitrile while A was 50 mM sodium acetate pH 4.31[2-3]. When the pH of mobile

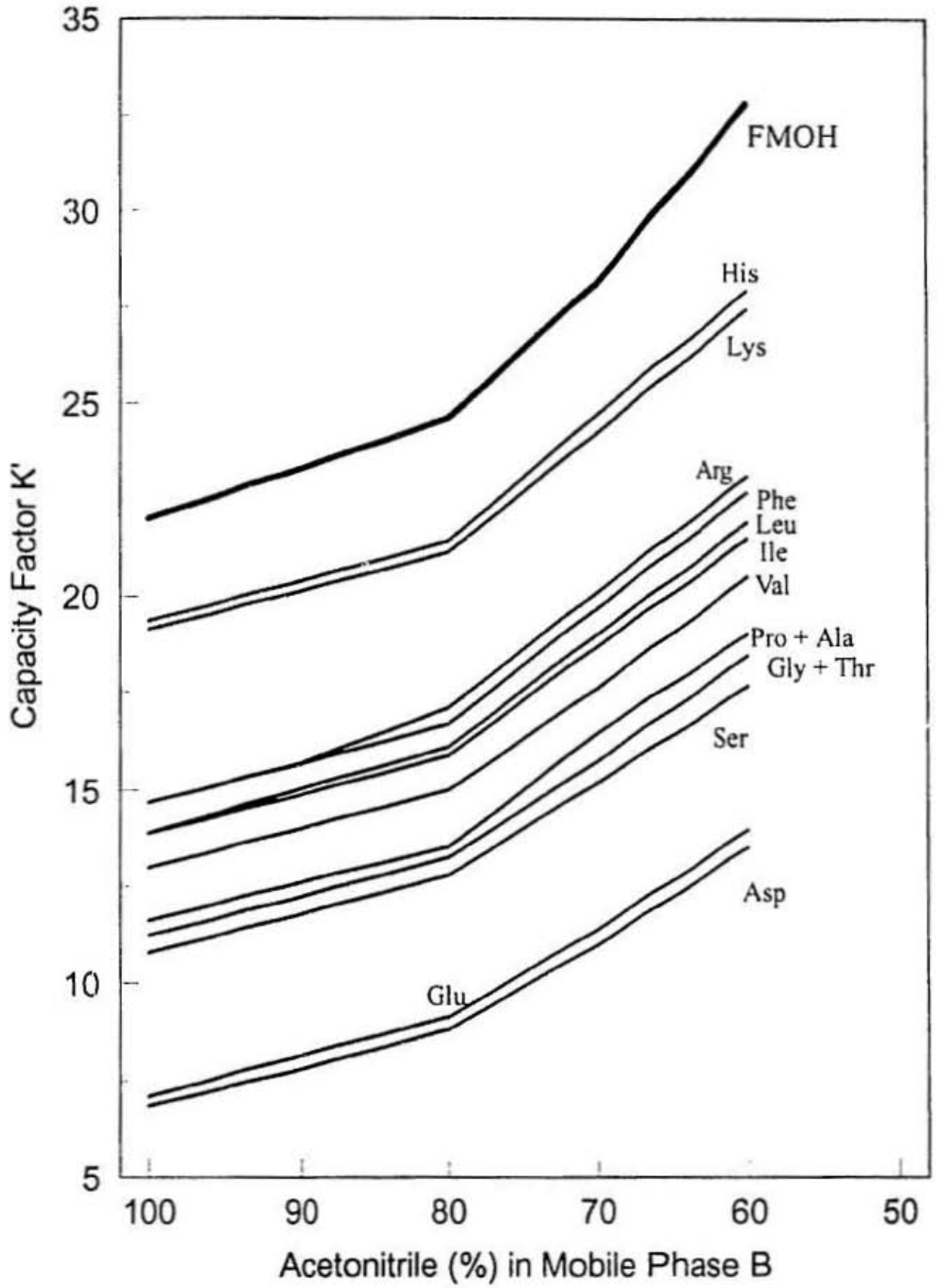


Figure 3.4. Effect of Acetonitrile Concentration in Mobile Phase B on Capacity Factors

phase A was increased above 6.5 (as described in Section 3.2.2) the use of 100% acetonitrile resulted in poor resolution of the amino acid derivatives as illustrated in Figure 3.4. After decreasing acetonitrile concentration to 80% in B, better resolution of amino acid derivatives from both standards and seawater was observed. When the acetonitrile concentration was further decreased to 60%, the selectivity factor α ($\alpha = k'_2/k'_1$) for many of the derivatives were better, but, the total analysis time became too long and peaks for Asp and Glu broadened. Thus, the optimum conditions which were selected for mobile phase composition B were 80% acetonitrile and 20% sodium acetate buffer (pH 7.5).

3.2.5. The Effect of Ionic Medium Concentration

The resolution of some amino acid derivatives, particularly the co-elution of Arg and Phe, was still unsatisfactory. A series of experiments were conducted in order to observe the effect of ionic medium concentration on the individual capacity factors of the derivatives. It was observed that when the NaAc concentration in mobile phase A was decreased from 50 mM to 20 mM better separation was obtained and Arg and Phe were well resolved as illustrated in Figure 3.5.

When 20 mM concentration of NaAc was incorporated into mobile phase B (i.e. 20% of 20 mM NaAc/80% acetonitrile) very poor peak shapes were observed for Asp

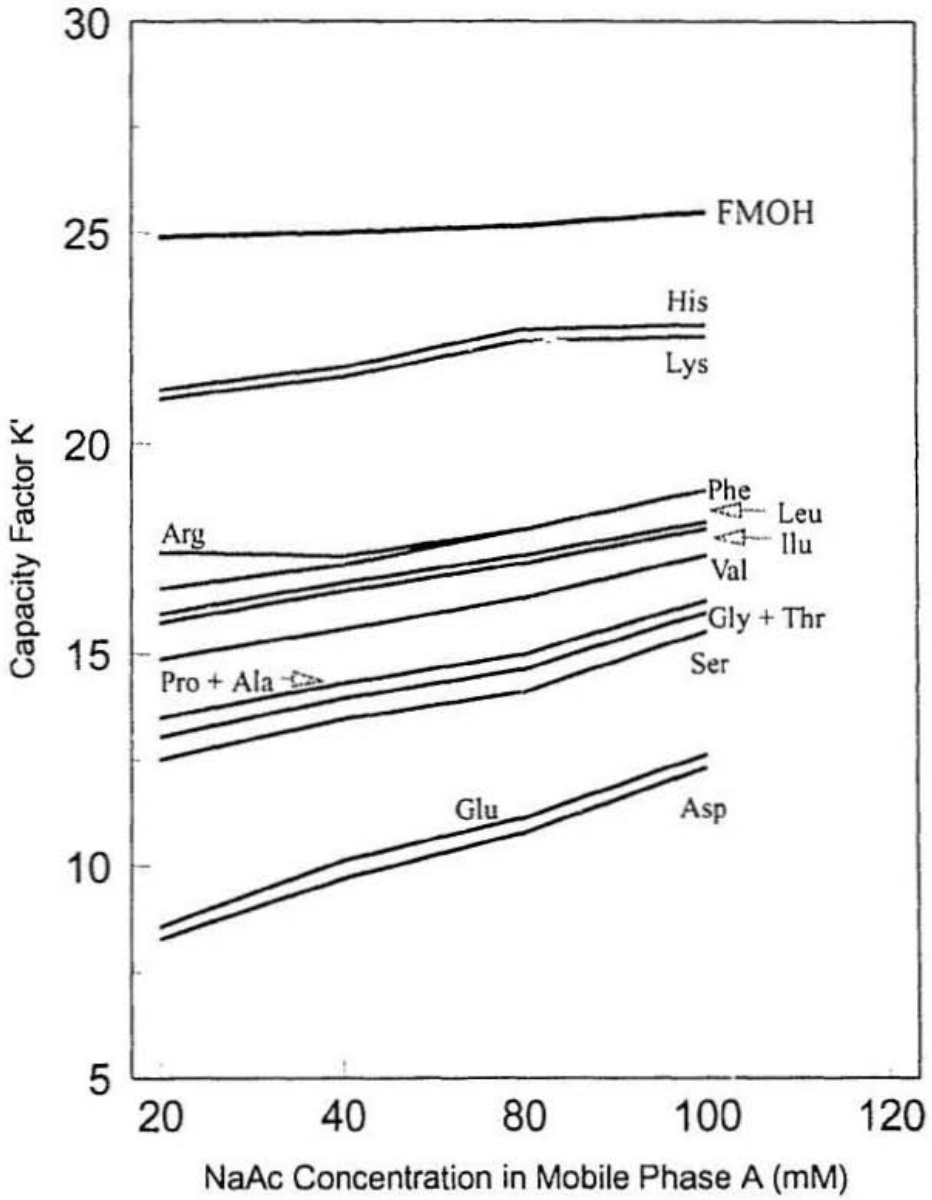


Figure 3.5. Effect of Ionic Medium Concentration in Mobile Phase A

and Glu in the chromatogram. This was improved when 50 mM NaAc was used instead of 20 mM.

3.2.6. Gradient Elution System

As a strong organic eluting solvent in HPLC, acetonitrile had a very significant influence on the retention time and resolution of the amino acid derivatives. When a isocratic run with 40% of acetonitrile was performed, the total analysis time was longer than 2 hours. When a isocratic run with 80% of acetonitrile, the analysis time was less than 15 min (including FMOH), but, most amino acids co-eluted. Due to the wide range of polarities of the amino acid derivatives, gradient elution was preferred over isocratic elution. Figure 3.6 shows the optimized gradient elution program used for the chromatographic resolution of 16 amino acids.

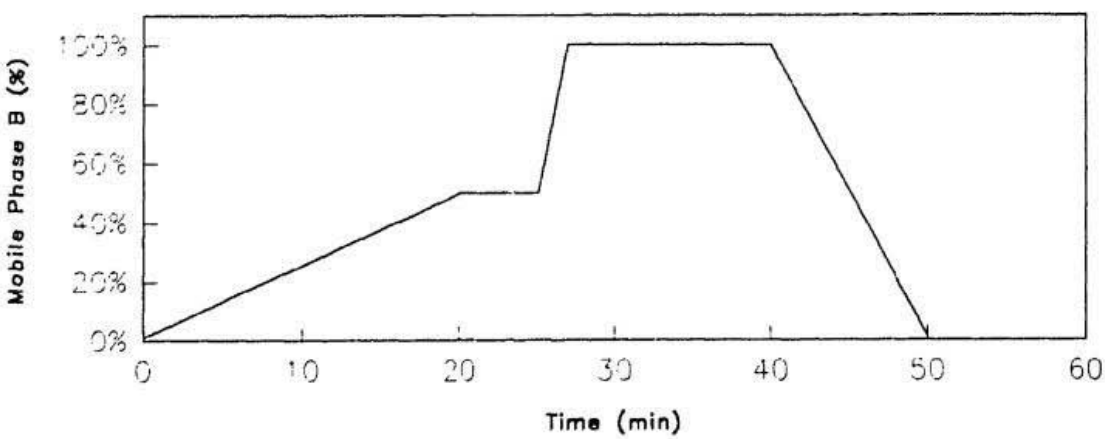
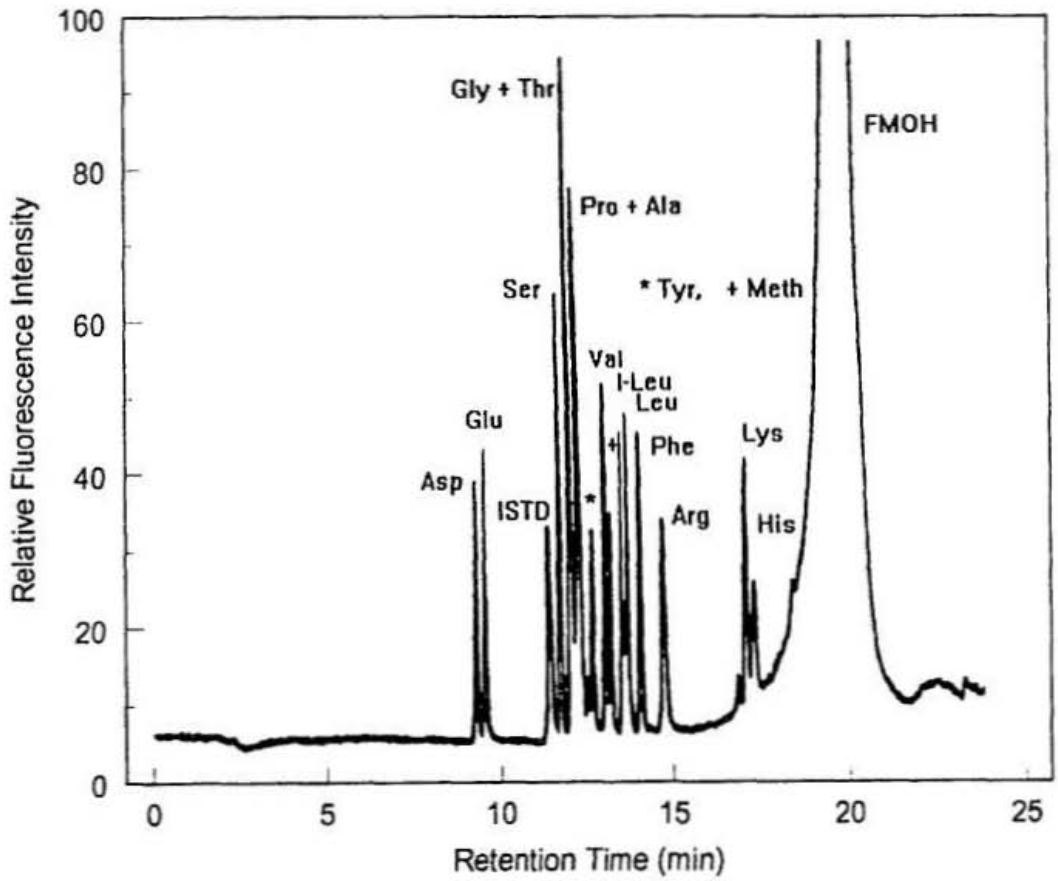


Figure 3.6. Optimized HPLC Gradient Elution Program for FMOC-Derivatives



Each Peak Represents 50 pmol of Individual Amino Acid

Figure 3.7. Optimized HPLC Chromatogram of FMOC Derivatives of Sixteen Amino Acids

3.2.7. Optimized HPLC Analysis of FMOC Derivatives

The optimized HPLC chromatogram for a standard aqueous solution containing 16 amino acids using FMOC derivatization technique is given in Figure 3.7. As expected, the FMOC hydrolyzed side-product, FMOH, produced a huge chromatographic peak at trace level analysis (e.g., 50 pmol of individual amino acids). Under optimized conditions however, FMOH eluted after all the amino acid derivatives and it's interference was significantly minimized.

Most FMOC derivatives separated to near base-line resolution with the exception of Gly co-eluting with Thr and Pro with Ala. In terms of fluorescence response sensitivity, most derivatives were of equal fluorescence intensity with the exception of His. The order of elution, by functionality of the amino acids, was found to be acidic < neutral < basic.

CHAPTER 4

COMPARISON OF PRE-COLUMN DERIVATIZATION METHODS FOR THE CHROMATOGRAPHIC ANALYSIS OF TRACE AMINO ACIDS

4.1. Introduction

The best chromatographic technique to date for the analysis of DFAA in seawater is pre-column OPA-derivatization combined with reversed-phase HPLC and fluorescence detection[1]. There are obvious advantages and disadvantages with the use of OPA as the derivatizing reagent and these were discussed in the Introduction Section. This chapter compares the performance of OPA-derivatives of amino acids with the newly-proposed derivatization reagent, FMOC. Comparisons are made in terms of stability of derivatives, and, sensitivity, precision and recovery of the method of derivatization. All studies involved the use of "aged" seawater spiked with standard solution of amino acids. Finally, a chromatographic comparison is made on a real seawater sample.

4.2. Stability of OPA versus FMOC Derivatives of Amino Acids

Compared to other chromatographic methods, HPLC using OPA as a precolumn fluorescence labelling reagent has many advantages. But, the major disadvantage is that OPA derivatives are unstable due to a sulfur-oxygen re-arrangement to produce a

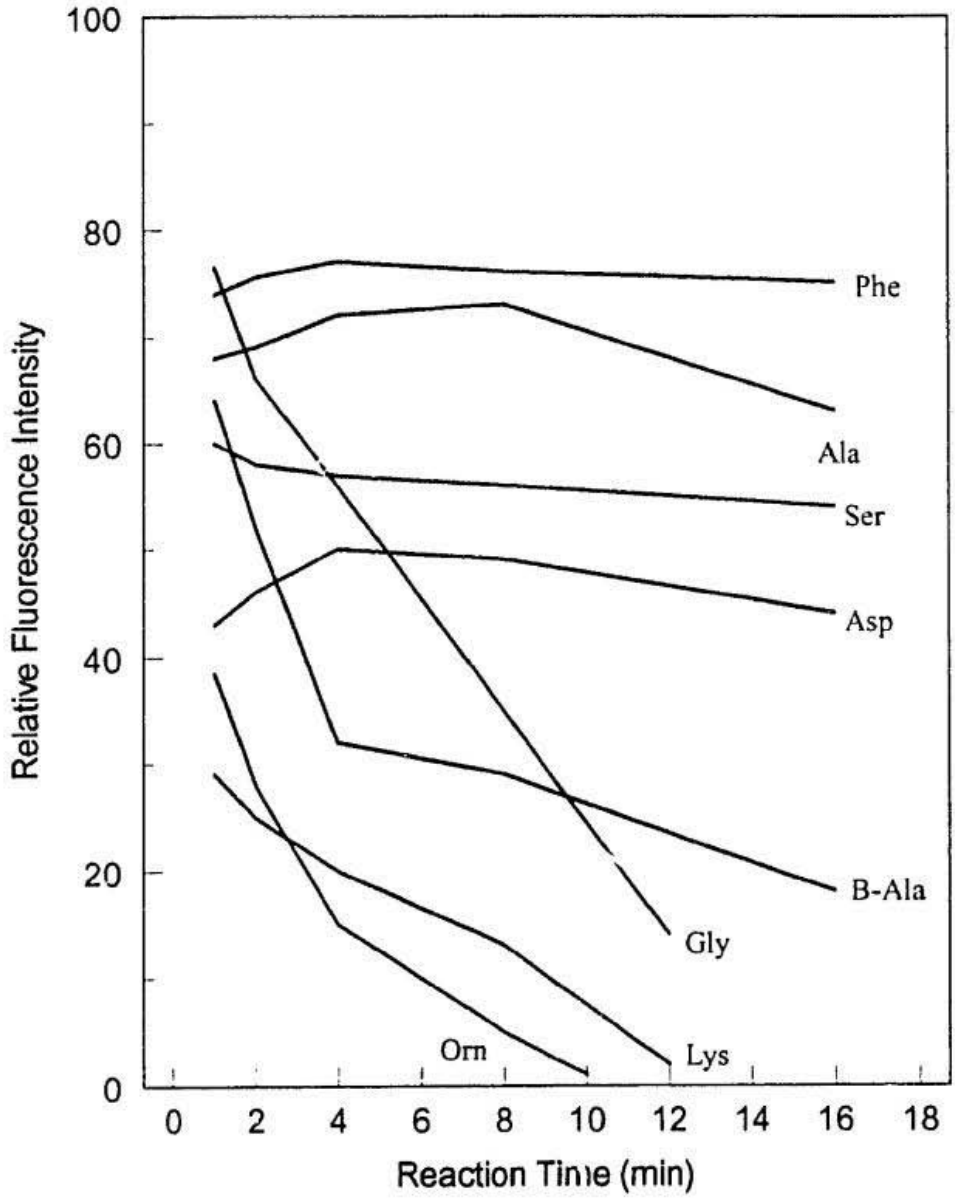
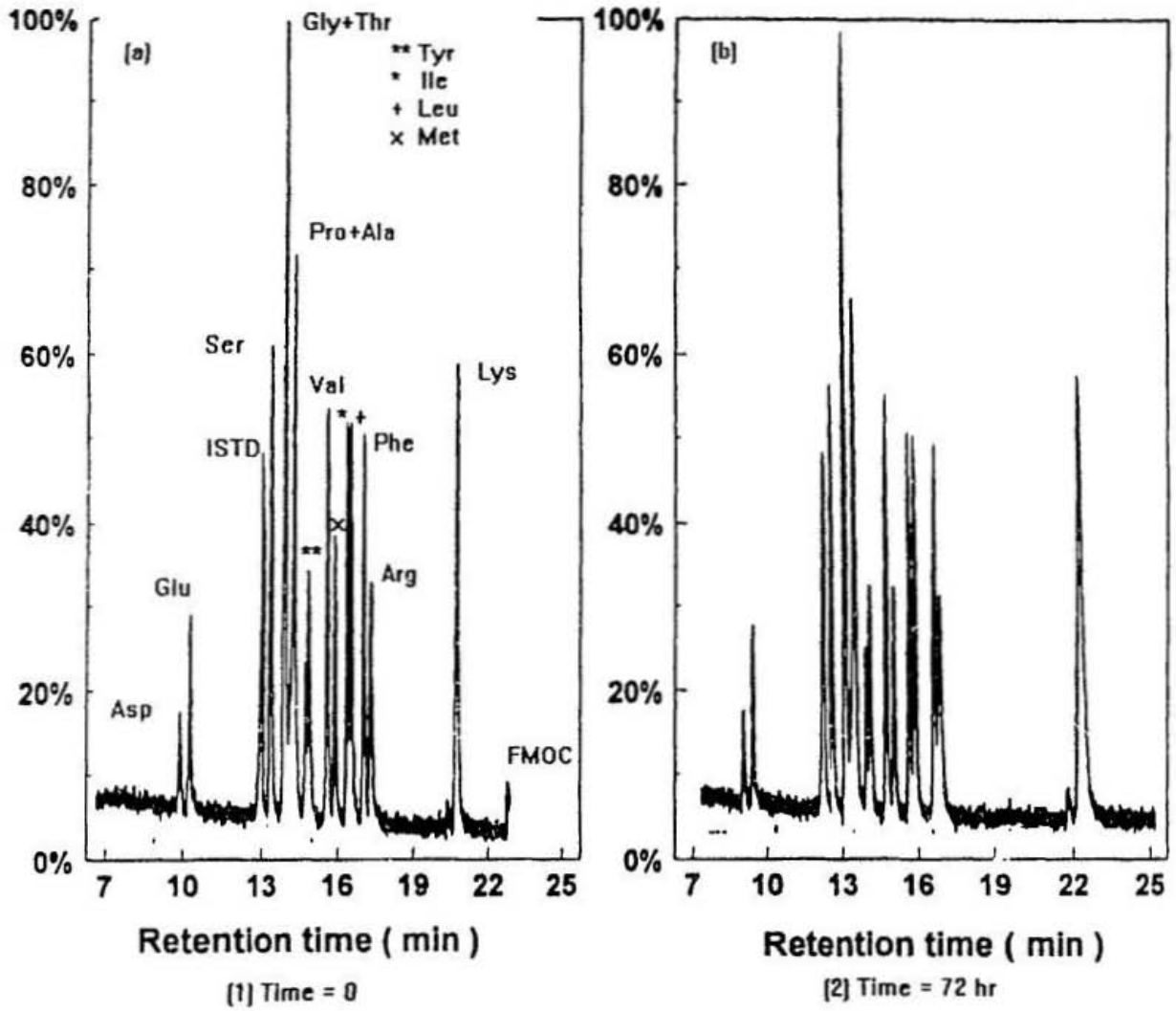


Figure 4.1. Stability of OPA Derivatives of Selected Amino Acids Measured by HPLC

nonfluorescent 2,3-dihydro-1 H-isoindole-1-one[114-115]. When a standard mixture of amino acids spiked in "aged" seawater are derivatized with OPA reagent and different waiting times are used before injection of the reaction mixture onto the HPLC column, the OPA derivatives displayed varying degrees of stability. In particular, the derivatives of glycine, ornithine and lysine were relatively unstable[114-115]. An additional stability study of OPA derivatives of selected amino acids was performed and the results are illustrated in graphical form (Figure 4.1). The fluorescence responses of other amino acid derivatives also exhibited small variations with reaction time. The instability of OPA derivatives thus prevents application of the method, in particular, to a HPLC system with manual injection.

In contrast, the major advantage of the precolumn derivatization of amino acids using FMOC reagent is that the FMOC-amino acid derivatives are very stable. At room temperature, the FMOC derivatives are stable for several days. Such stability is illustrated in Figure 4.2 which shows two HPLC chromatograms of the same FMOC-derivatized sample one with sample injection right after derivatization, and the other 3 days later. There is very good peak height similarity between the two chromatographic runs.

When the FMOC derivatives were stored at 4°C in the dark, they can be stable for weeks. A study was conducted to measure, by HPLC analysis, the stability of a selected number of amino acid FMOC-derivatives over 3 weeks. As seen in Figure 4.3,



Each Peak Represents 12.5 pmol of Individual Amino Acid

Figure 4.2. The Stability of FMOC Derivatives at Room Temperature. Chromatograms of FMOC Derivatized Standards (a), 5 min After Derivatization and (b), 3 Days After Derivatization

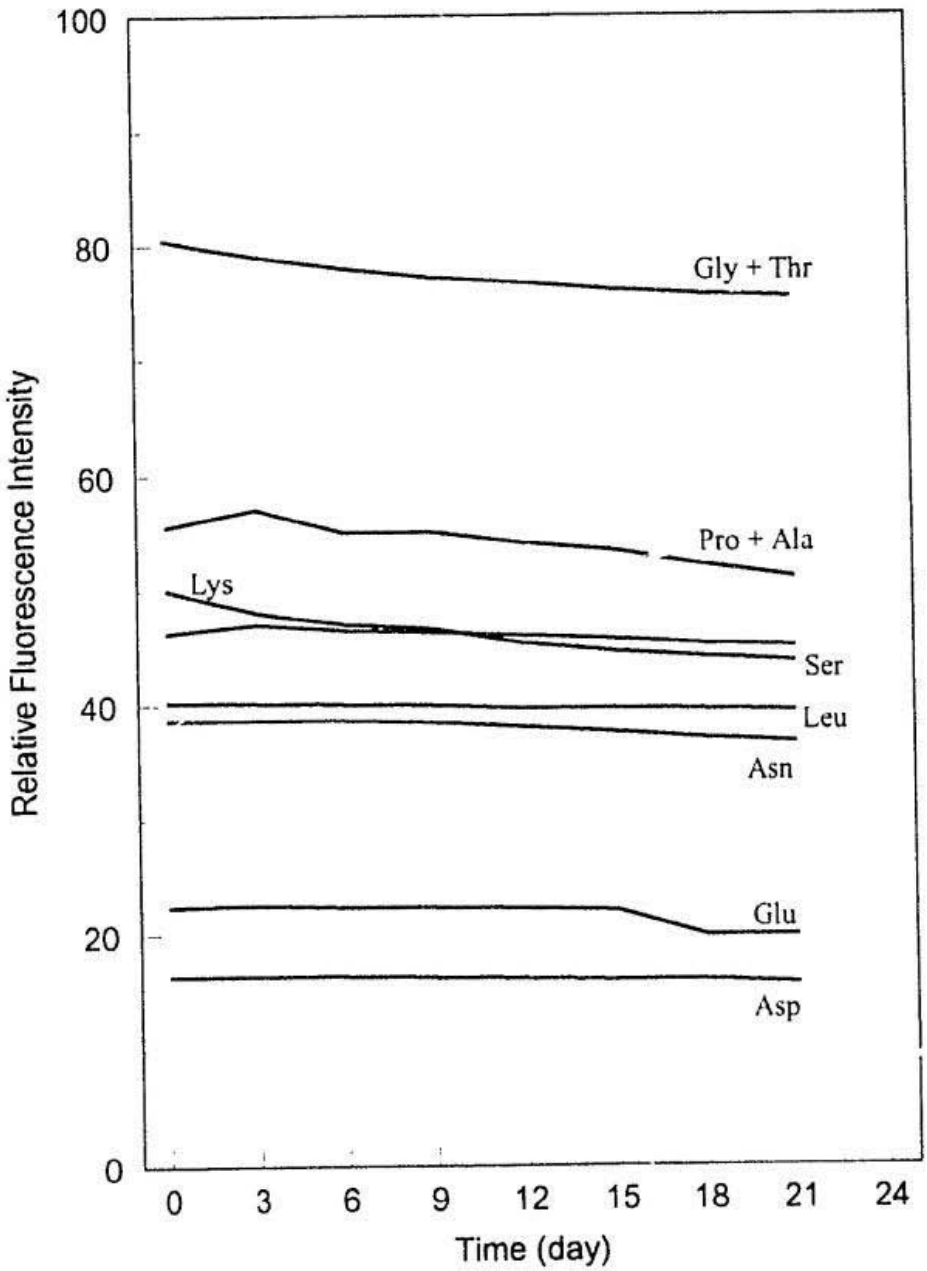


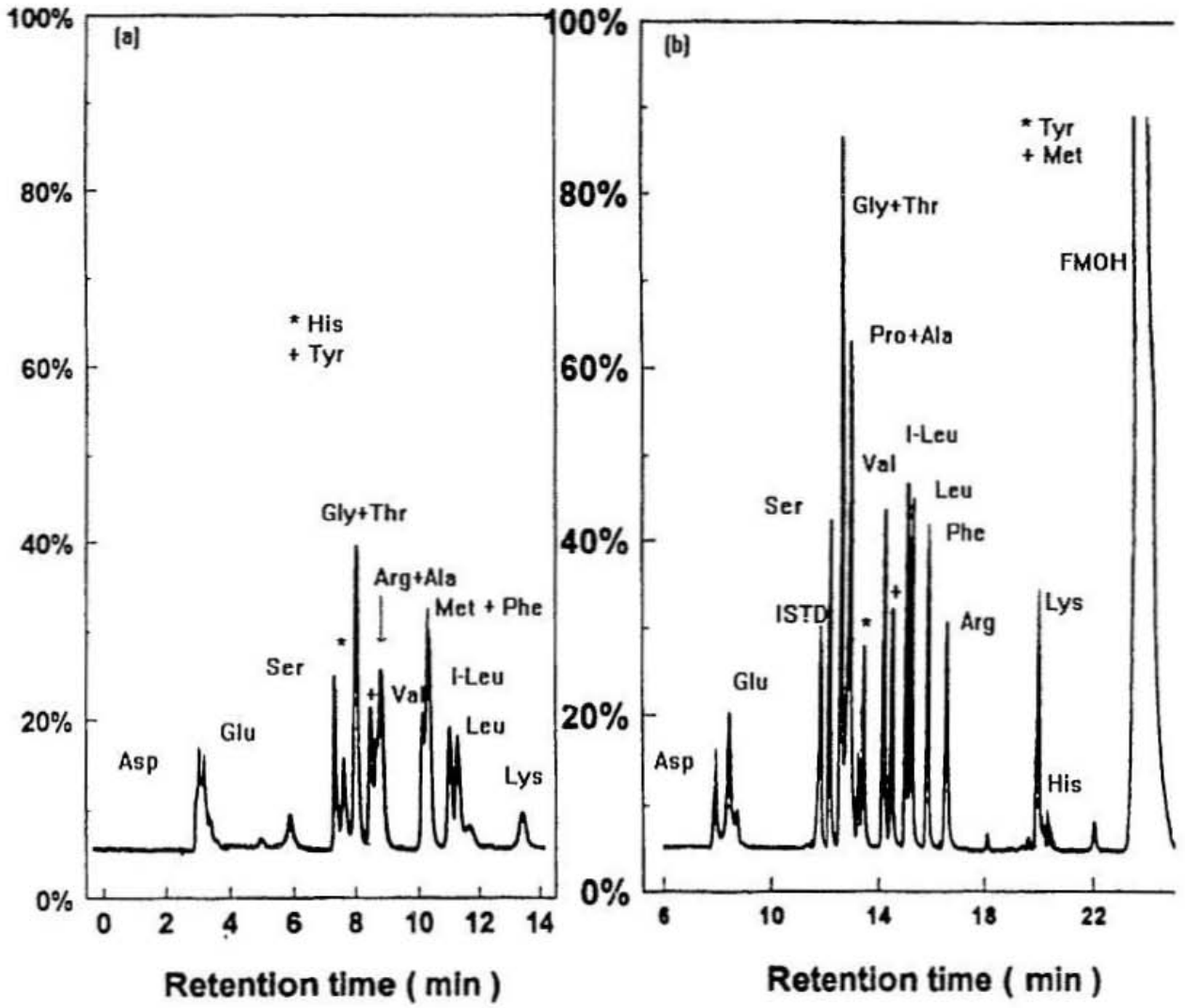
Figure 4.3. Long Term Stability of FMOC Derivatives Measured by HPLC

there is very little degradation in FMOC derivatives. This feature of long term stability enhances the application of the FMOC method in real seawater analysis. In field studies, FMOC reagent could be used as a means of preserving unstable DFAA in seawater as well as making the required fluorescence labelling reagent for precolumn derivatization in HPLC analysis. This promising application is discussed further in Chapter 5.

4.3. Overall Chromatographic Comparison of OPA vs. FMOC Derivatization Approach

An experiment to compare the overall chromatographic features of OPA vs. FMOC derivatization was performed. Conditions for OPA analysis were identical to that of FMOC analysis such as HPLC column, sample loop, and amino acid standard in "aged" seawater. Figure 4.4 compares the results. In general, the fluorescent responses of a specific amino acid varied between the two derivatives, but the FMOC approach showed overall higher sensitivity. The FMOC-derivatized amino acids were also better resolved chromatographically than the OPA derivatives.

In terms of sensitivity, histidine gave a much lower fluorescent response as a FMOC derivative than that as an OPA derivative. Aspartic acid, glutamic acid and methionine had relatively the same intense of fluorescence with both methods. The remaining of amino acid FMOC derivatives had obviously higher fluorescent responses.



Each Peak Represents 50 pmol of Individual Amino Acid

Figure 4.4. Chromatographic Comparison of OPA vs. FMOC-Derivatized Amino Acids. Chromatograms of (a) OPA-Derivatized Amino Acids and (b) FMOC-Derivatized Amino Acids

As expected the secondary amino acid Pro was not observed in the chromatogram of OPA-derivatives. As stated earlier, the OPA reagent is incapable of reacting with secondary amino acids.

4.4. Comparison of Linearity and Precision

Linearity The dynamic ranges (linearity) of the FMOC-derivatization and OPA-derivatization approach were compared for the analysis of total amino acid concentrations of standards spiked in "aged" seawater. Two sets of standards were tested: (1) low (total) concentration range 100, 200, 400 and 500 nM and (2) high (total) concentration range 5, 10, 25 and 50 μM. The dynamic range comparisons are illustrated in Figure 4.5 (low concentration range) and Figure 4.6 (high concentration range).

Under optimized derivatization and HPLC analysis conditions the FMOC approach has a significantly higher sensitivity in measuring total dissolved free amino acids as observed by the slopes of the response curves. The linearity of the two derivatization approaches for both concentration ranges is not significantly different thus indicating that FMOC-derivatives are as useful as OPA-derivatives for the analysis of DFAA over a wide range of concentration.

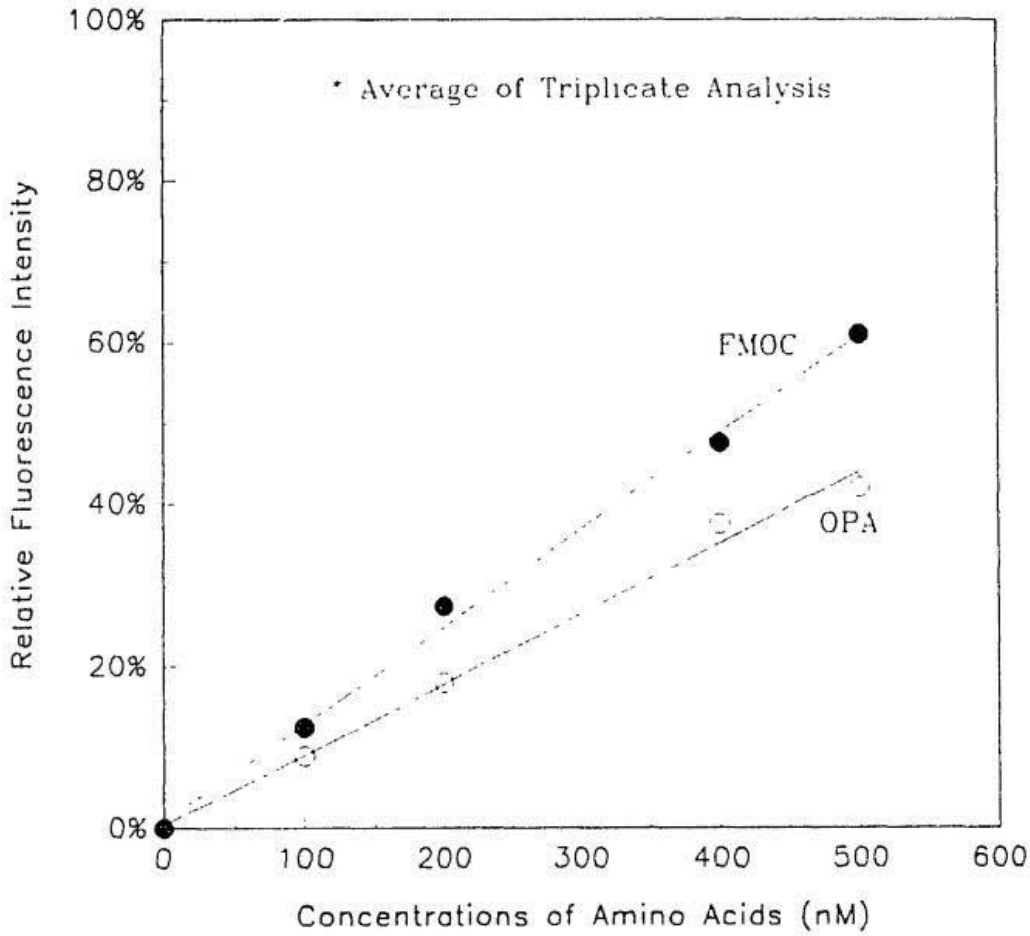


Figure 4.5. The Linearity Comparison (OPA vs FMOC) for the Measurement of Total Amino Acids at Low Concentration range

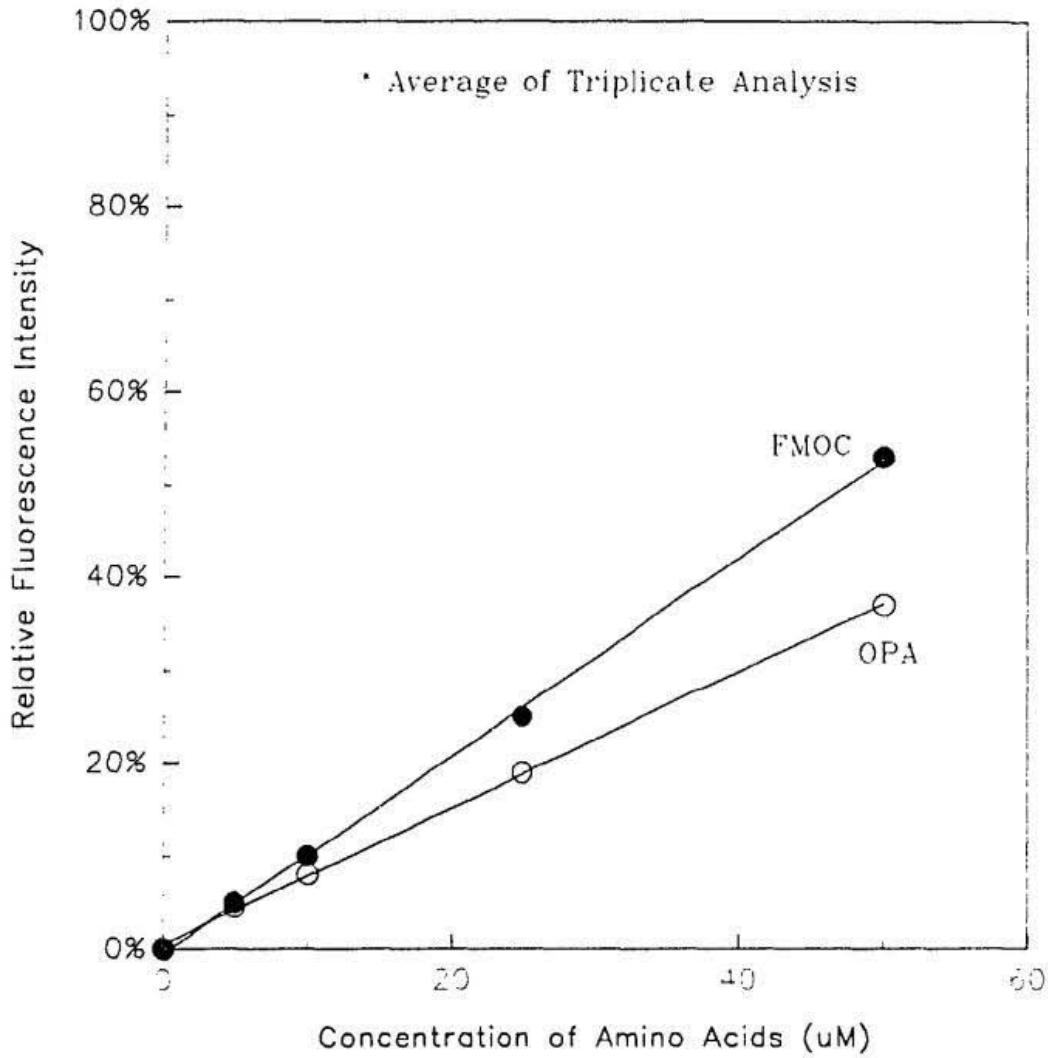


Figure 4.6. The Linearity Comparison (OPA vs FMOC) for the Measurement of Total Amino Acids at High Concentration Range

Precision A series of 11 separate derivatization and HPLC analyses of the same amino acid standard (in "aged" seawater) were conducted using the OPA and the FMOC approach. The precision of the two methods was measured using peak height of the amino acid derivatives and the relative standard deviations given in Table 4.1. The average deviation at the 100-pmol level was ± 3.1% for OPA while ± 3.4% for FMOC method indicating similar overall precision of analysis. For individual amino acids, there occurred a somewhat higher deviation in analysis of Lys using OPA, and, of Arg and Tyr for FMOC. Poorer reproducibility of tyrosine using FMOC derivatization was accounted for by interference from the impurity peak of FMOC reagent even after the FMOC was purified by re-crystallization.

4.5. Comparison of Recovery and Detection Limits

Recovery Experiments to examine and compare the recoveries of individual amino acids measured by both methods were conducted using a seawater sample spiked with amino acid standards. The % recovery was measured relative to the detection response of the individual amino acids dissolved in purified water. Recovery results are listed in Table 4.2. For FMOC-derivatization, % recovery ranged from 87 to 107% and is very comparable to % recovery range for OPA-derivatization results, i.e., 85 - 104%, thus, confirmed the usefulness of FMOC derivatives for the trace analysis of DFAA in seawater.

Table 4.1. Comparison of the Precision of the FMOC vs. the OPA Approach to the Determination of Individual Amino Acids Measured by Peak Height

AA's 100 pmol Each	Relative Standard Deviation (%) n = 11	Relative Standard Deviation (%) n = 11
	OPA Method	FMOC Method
Asp	2.3	3.5
Glu	3.4	2.8
Ser	1.8	2.9
Gly	2.8**	2.7**
Pro	n.d.	3.6***
Ala	4.6*	----
Tyr	1.5	6.1
Val	3.2	1.8
Met	2.6	4.2
Ile	3.4	1.1
Leu	3.2	2.4
Phe	1.2	3.9
Arg	----	5.6
Lys	6.5	2.5
His	3.8	4.3

* Ala + Arg
** Gly + Thr
*** Pro + Ala
n.d. not detected

Table 4.2. Comparison of the Recovery and Detection Limit of Individual Amino Acids Spiked in Aged Seawater

AA's 100pmol Each	a Recovery (%)	a Recovery (%)	b Detection Limit (pmol)	b Detection Limit (pmol)
	OPA Method	FMOC Method	OPA Method	FMOC Method
Asp	101	87	0.51	0.34
Glu	95	92	0.43	0.28
Ser	100.5	102	0.35	0.19
Gly	96**	98**	0.40**	0.12**
Pro	n.d.	105***	n.d.	0.15***
Ala	103*	----	0.14*	----
Tyr	96	93	0.38	0.40
Val	89	95	0.50	0.24
Met	104	89	0.39	0.37
Ile	99	103	0.36	0.28
Leu	100	105	0.36	0.26
Phe	100.8	94	0.73	0.28
Arg	----	101	----	0.40
Lys	89	107	0.40	0.28
His	95	91	0.88	3.0

* Ala + Arg
** Gly + Thr
*** Pro + Ala
n.d. not detected
a relative to amino acids dissolved in purified water
b signal to noise ratio of 5 to 1

Detection Limits The detection limits of individual amino acids in seawater of both methods were measured and calculated with signal-to-noise ratio N = 5 (Table 4.2). In general, the detection limits of most amino acids using the FMOC approach were relatively lower than that of the OPA approach, some amino acids by as much as 2 fold. One exception is histidine where the detection limit by FMOC is very poor. The reason for the poor sensitivity for histidine using FMOC reagent has not been found. The overall higher sensitivity using FMOC approach makes it possible to detect most major amino acids in seawater at lower concentrations including possible secondary amino acids such as proline.

4.6. Real Seawater Sample Comparison

An experiment was setup whereby a representative seawater sample from Conception Bay would be analyzed for DFAA. The objective was to compare the chromatographic results of DFAA analysis between stored and unstored seawater and to show the advantages of FMOC derivatization. First, the unstored sample was quickly derivatized with FMOC right after filtration and analyzed by HPLC. This represents a "fixed at t=0 " amino acid analysis. Later, the stored seawater (frozen, 3 days) was derivatized by both OPA and FMOC and each analyzed. This storage/ derivatization scheme for seawater is more clearly illustrated in Figure 4.7. This scheme was also adopted in seawater analysis in Chapter 5.

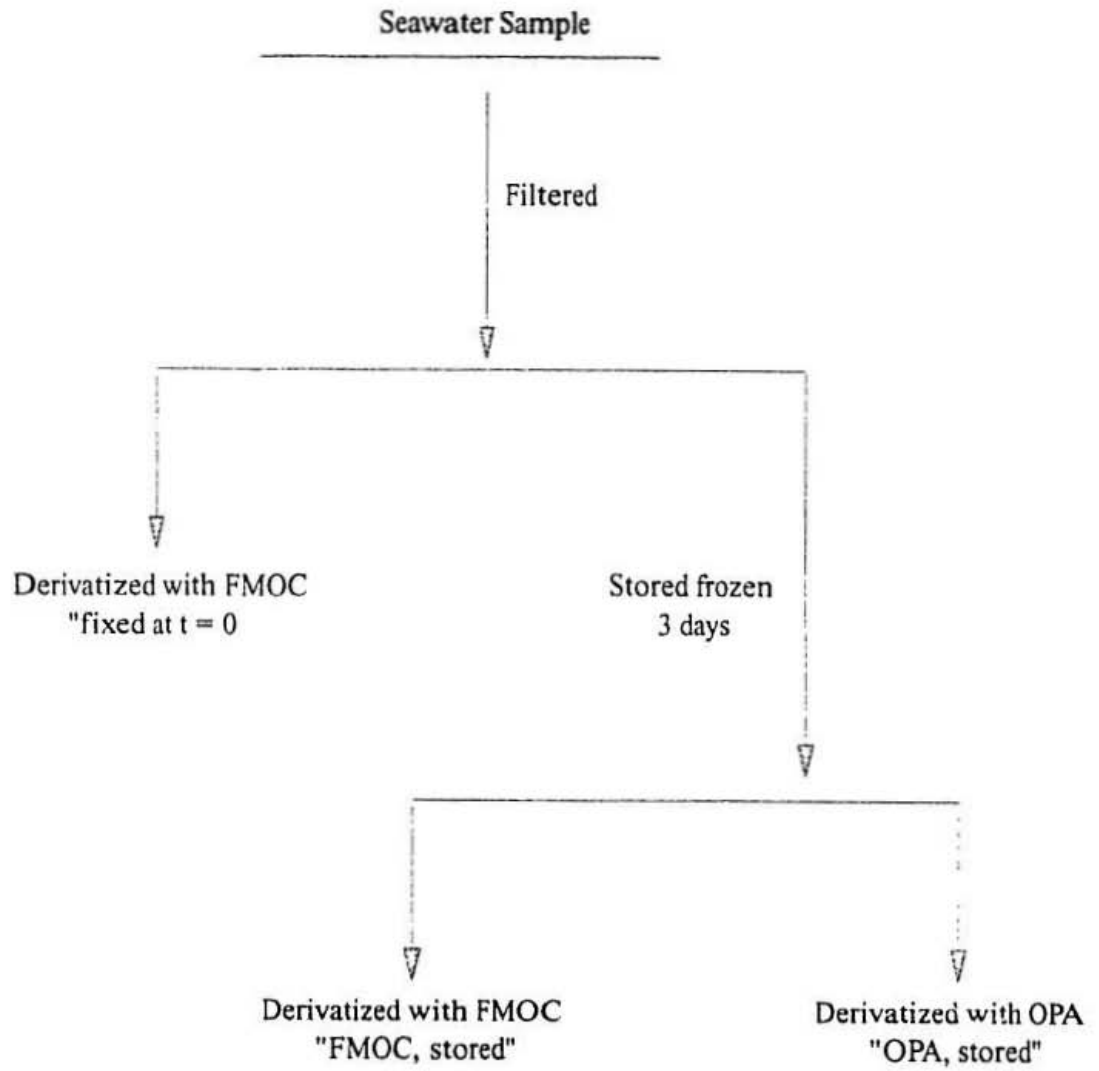


Figure 4.7. Storage/Derivatization Scheme for Comparison of DFAA Analysis of Seawater

By way of comparing the results , chromatograms and calculated DFAA levels (written on each chromatogram) for each set of derivatives are given in Figure 4.8. As expected, the highest levels of DFAA are obtained when the seawater samples are

quickly derivatized (Figure 4.8c). For this particular seawater sample, DFAA levels are more than 50% higher than that measured in "FMOC, stored" (Figure 4.8b). All individual amino acids were substantially lower in stored seawater, particularly the amino acids Arg and Gly.

Comparing the chromatograms of stored seawater analysis it can be seen that there is higher sensitivity for trace amino acids by FMOC derivatization. The "FMOC, stored" chromatogram (Figure 4.8b) has a much better profile of trace DFAA than "OPA, stored" (Figure 4.8a). Unexpectedly, the concentration of DFAA in stored seawater measured by OPA derivatization was significantly lower (20%) than that measured by FMOC derivatization. This result is also observed in a selected number of other seawater samples discussed suggesting that DFAA levels in samples measured by OPA derivatization are underestimated.

Finally, to confirm the advantage of using FMOC reagent to "fix " amino acids at time of sampling , another seawater sample was quickly derivatized with FMOC while, at the same time, the same water was stored on ice and quickly brought back to the lab. Within 20 min the DFAA were derivatized with OPA reagent and analyzed. Again, concentrations of DFAA had dropped in stored seawater, this time by 35% . Although storage time was kept at a minimum and the sample not frozen , there was still loss of DFAA .

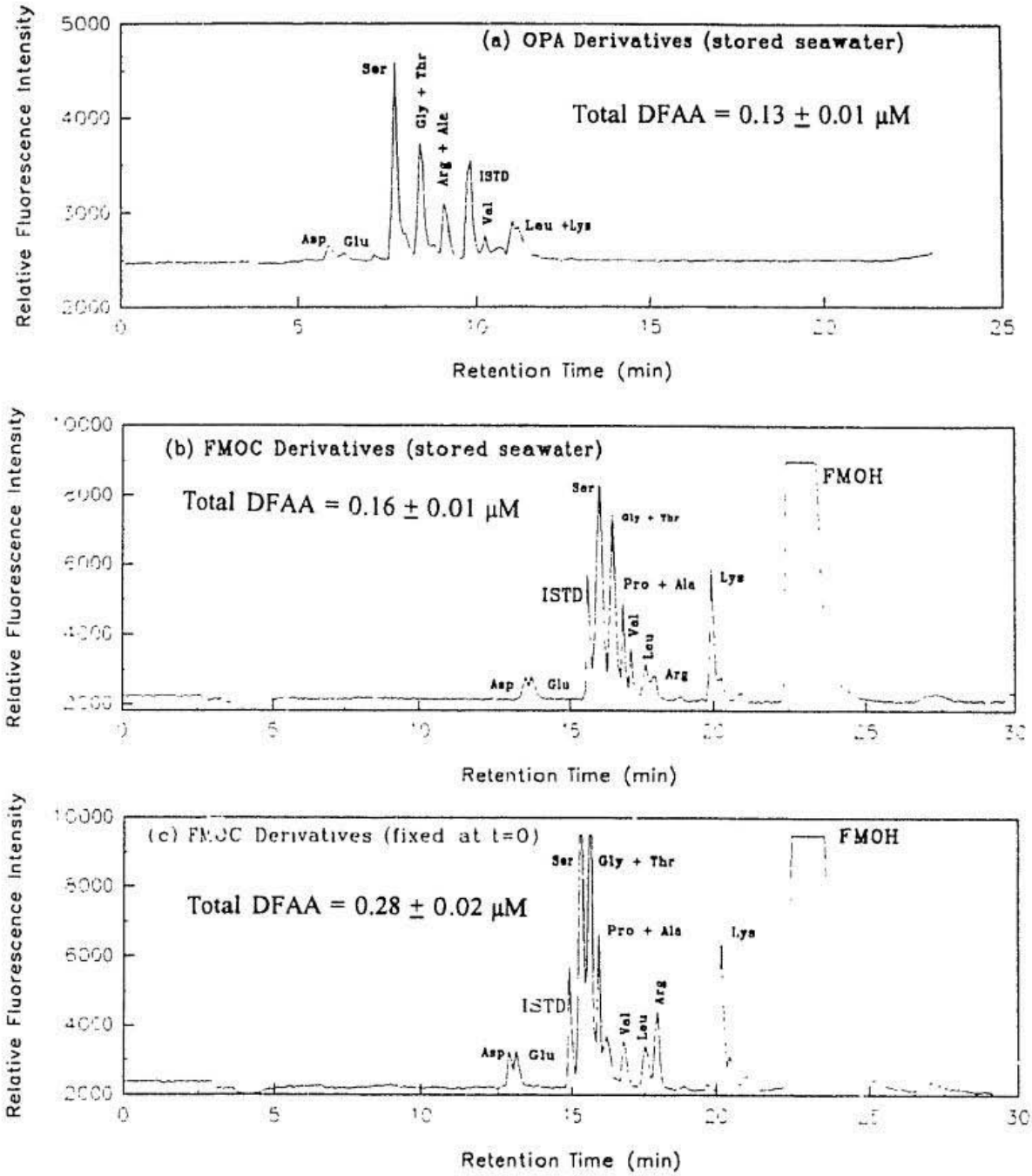


Figure 4.8. The Real Seawater Comparison of FMOC vs. OPA Derivatized Amino Acids. Chromatogram of (a) OPA-Derivatized Amino Acids (stored seawater), (b) FMOC-Derivatized Amino Acids (stored seawater) and (c) FMOC-Derivatized Amino Acids (fixed at t = 0).

4.7. The of OPA-Derivatization Approach

Compared to the classic IC approach, HPLC using OPA as a reagent is fast, sensitive and reproducible. Since its introduction in 1979[11], OPA derivatization has been successfully applied to seawater analyses[7-9,113]. But the major limitation with the method is that the OPA-amino acid derivatives were unstable.

DFAA in seawater are very dynamic. The uptake and release of DFAA by marine organisms in seawater can be very fast with turnover times in the range of minutes to hours[7, 10-11]. This suggests that in order to obtain accurate concentrations at time of sampling, HPLC analyses should be performed immediately onboard ship. In field studies this is often not possible. The loss of DFAA during the storage can be very significant. In terms of analytical requirements owing to the instability of OPA derivatives and to the variation in fluorescent responses with reaction time, each derivatization and HPLC injection must be precisely controlled. This introduces potential error in analysis.

In summary, the disadvantages of OPA derivatization are, (1) OPA derivatives are not stable and variation in fluorescent responses with reaction time is observed, (2) it is impossible to derivatize DFAA at time of sampling, unless HPLC is available, (3) in field studies significant changes in DFAA concentrations are observed because of storage, and, (4) secondary amino acids can not be derivatized.

4.8. The of FMOC-Derivatization Approach

In contrast, the major advantages of the FMOC approach to derivatization are that FMOC derivatives of amino acids are very stable and that the method is more sensitive. Using optimized chromatographic conditions, most amino acids can be resolved, even in the presence of a huge FMOH reagent peak. The excellent stability of FMOC derivatives is particularly important for the analysis of DFAA in seawater at time of sampling where the DFAA concentration can be fixed at time = 0 through derivatization. Results more closely reflect the real DFAA values in seawater and sample contamination is reduced.

In summary, the advantages of FMOC derivatization are, (1) FMOC derivatives are stable for weeks, thus allowing for automated HPLC analysis, (2) FMOC derivatives are more sensitive than those of OPA in trace analysis, (3) in field studies, FMOC reagent can be used both as a means of preserving DFAA in seawater and as the fluorescence labelling reagent, and, (4) FMOC can react with both primary and secondary amino acids.

CHAPTER 5

FIELD APPLICATIONS OF SEAWATER ANALYSIS

5.1. General Considerations

Sampling

In field studies, sampling is the most important step to obtain accurate and reliable results, especially in the trace level analysis of DFAA. There are two critical aspects in sampling, representation of the sample and contamination. The concentration and compositional information of DFAA in the sea varies from place to place, with time and depth, due to chemical, physical and biochemical processes. It is obvious that improvements in the analytical measurement are useless if it is uncertain whether the sample is representative of the body of water sampled.

During sampling, seawater samples can be contaminated from several sources such as the sampler, the boat, and the hands of the operator. Great care must be taken to prevent the risk of contamination. Samplers should be precleaned, then flushed with seawater before collection. Operators should be very careful not to touch the sample or apparatus and plastic gloves are recommended.

Samplers

An important decision in sampling of seawater is the choice of the appropriate type of sampler. The basic purpose of a sampler is to enclose a representative sample from the prescribed sampling depth and to leave the sample unaltered until the water is retrieved and subsampled. The requirements for an ideal seawater sampler are, that it rapidly fills with seawater at a specific depth, seals in the water completely, minimizes chemical and biological change within the sampler, and, is free from contamination.

There are several types of samplers commercially available for specific purposes, i.e., for the microlayer, for shallow depths and deep water samples, and for bottom waters. The most common sampler is the Niskin sampler. In field work performed in this study a 5 L Niskin sampler was used. The Niskin is inexpensive, convenient and meets the requirements for sample collection in the depth range from sub-surface down to 400 m in the water column.

Filtration Procedures

As mentioned earlier, the distinction between dissolved and particulate amino acids in seawater is by filtration using a 0.45 μm filter. This definition is not strictly correct because there are a variety of very small particles such as colloids and bacteria

which may pass through the 0.45 μm filter[12-13]. The filters used in most experiments were Whatman GF/C glass fibre filters (nominal pore size 1.2 μm). These filters have been accepted for general filtration. In order to reduce possible contamination, filters were precombusted at 450° overnight and cleaned with methanol and purified water. Clean tweezers were used for mounting the filter.

The effect of pore size of the filter was investigated employing three types of filters: GF/C, and 0.2 μm and 0.02 μm Nanopore filters. Results suggest that the higher values of DFAA using 0.02 μm Nanopore filters was mainly due to the higher vacuum (or pressure) required to filter which probably break th· cell membranes of filtered plankton thus releasing DFAA. It was impossible to filter seawater through a 0.02 μm Nanopore filter without the employment of high vacuum (or pressure when using a syringe).

There was relatively good agreement between results for GF/C and 0.2 μm Nanopore filtration indicating that the concentration of particulate amino acids between the two filter sizes is very small. The GF/C filters were, however, used in most filtrations because little or no pressure was required and the filters are inexpensive.

Slightly higher DFAA values for GF/C-filtered seawater versus those from unfiltered seawater is interesting. The GF/C filters may be a possible source of contamination, or any type of filtration will inevitably release some DFAA.

Results suggest that it may be better to analyze DFAA without filtration. Lindroth and Mopper used unfiltered seawater to study depth variation in DFAA in the Baltic Sea. The reason not to filter was to minimize the risk of contamination[7].

In order to reduce the artificial increase in DFAA concentration due to cell release, gravity filtration was used with large volume seawater samples. In the case of small samples, a minimum pressure was employed to facilitate filtration where a 50 mL glass syringe was employed. The first 10 mL of seawater filtrate was always discarded to further minimize contamination.

Storage and Preservation

After a seawater sample is collected and filtered it continues to be a biologically active system since the bacteria which passes through the filter continue to take up and release DFAA. The best method for the analysis of DFAA in seawater is an *in-situ* method, or, analysis onboard ship. In the absence of HPLC, storage and preservation of seawater samples is very important to obtain reliable results. Due to the instability of OPA derivatives, seawater samples must be stored. The acceptable method for storing and preserving samples is at -20°C in the dark. The storage containers can either be pre-cleaned glass sample vials or polyethylene bottles. From experimental results however, changes of DFAA concentrations were always observed in stored samples after any length of time and storage temperature.

Sampling Sites

Two sampling areas in this study were the eastern Newfoundland coast and Gulf of St. Lawrence. Samples along the coast were taken from Logy Bay (Station LB) and Conception Bay (Station CB). In general, the concentrations of DFAA at station LB were relatively lower than those at station CB. The levels in the Gulf of St. Lawrence were highest. Further discussion of DFAA concentrations in different waters is discussed later in this Chapter.

Sampling Time

In order to get comparable concentration data for a series of different depths (depth profile) or different months (time-course), sampling was always carried out during the same time of day. Samples were usually collected between 11:30 a.m. and 12:30 p.m. Large changes of DFAA concentrations in seawater have been observed during a 24 hour period by Lindroth and Mopper[7]. In the time-course studies, samples were collected weekly or monthly from Station LB.

5.2. Comparison of DFAA Measurements by OPA-Derivatization (stored samples) Versus FMOC-Derivatization (fixed at t = 0) in Field Studies

Field studies were conducted whereby seawater samples were collected from

Newfoundland coastal waters and from the Gulf of St. Lawrence. DFAA measurements were obtained in two ways, 1) by using FMOC- derivatization on freshly collected seawater and, 2) storing the seawater frozen and measuring the DFAA by OPA - derivatization back in the lab. The objective of the study was to compare the differences in DFAA results between our novel approach to DFAA analysis (i.e., fixing DFAA at time of sampling with FMOC reagent), and the more established approach involving the storage of seawater followed by OPA-derivatization. Comparisons were made between total DFAA values and concentrations of individual DFAA in selected waters.

5.2.1 Comparisons of Total DFAA Concentrations

The comparison of total DFAA concentrations with the time of year (1992) as measured by FMOC (fixed at t = 0) and by OPA (stored seawater) methods is given in Table 5.1. Substantial differences in total DFAA were observed between the two methods where higher levels were obtained when seawater samples were immediately derivatized with FMOC reagent. Differences as high as 240% were recorded (Table 5.2) in the Gulf of St. Lawrence. These results suggest that significant changes occured during the storage period and that the storage/OPA-derivatization approach grossly underestimates the actual concentrations of total DFAA in seawater.

Table 5.1. Seasonal Comparison of Total DFAA Concentrations Measured by FMOC (fixed at t = 0) and by OPA (stored seawater) Methods

Location: Logy Bay, Newfoundland
Depth: 15 m Year: 1992

Date	6/3	23/3	3/4	17/4	7/5	21/7	16/9	22/10
OPA[1]	0.054	0.15	0.12	0.042	0.13	0.22	0.16	0.11
FMOC[2]	0.072	0.18	0.19	0.056	0.19	0.27	0.21	0.14
Δ%[3]	33%	20%	58%	33%	46%	45%	31%	27%

1. DFAA, μM (C.V. = 5 - 8%, n = 3), measured by OPA-derivatization on stored seawater (-20°C, 3 days).

2. DFAA, μM (C.V. = 4 - 6%, n = 3), measured by FMOC-derivatization and fixed at time of sampling.

3. $\Delta = \frac{FMOC - OPA}{OPA} X100\%,$ (C.V. = 9 - 14%)

Table 5.2. Comparison of Total DFAA Concentrations Measured at Different Depths by FMOC (fixed at t = 0) and by OPA (stored seawater) Methods

Location: Conception Bay, Newfoundland

Date	April 6, 1992		
Depth (m)	10	25	50
OPA[1]	0.14	0.07	0.08
FMOC[2]	0.22	0.13	0.16
Δ%[3]	57%	85%	100%

Table 5.2. (continued)

Location: Conception Bay, Newfoundland

Date	May 7, 1992		
Depth (m)	10	25	50
OPA[1]	0.05	0.06	0.04
FMOC[2]	0.11	0.09	0.08
Δ%[3]	120%	50%	100%

Location: Gulf of St. Lawrence

Date	December 12, 1992		
Depth (m)	10	40	65
OPA[1]	0.25	0.18	0.23
FMOC[2]	0.26	0.20	0.27
Δ%[3]	4%	11%	17%

Location: Gulf of St. Lawrence

Date	December 15, 1992		
Depth (m)	10	40	65
OPA[1]	0.087	0.12	0.16
FMOC[2]	0.31	0.15	0.38
Δ%[3]	240%	25%	100%

1. DFAA, μM (C.V. = 5 - 8%, n = 3), measured by OPA-derivatization on stored seawater (-20°C, 3 days).

2. DFAA, μM (C.V. = 4 - 6%, n = 3), measured by FMOC-derivatization and fixed at time of sampling.

3. $\Delta = \frac{FMOC - OPA}{OPA} X100\%$, (C.V. = 9 - 14%)

The DFAA concentrations change not only with time of year but also with depth of the water sampled. Table 5.2 gives the comparison of total DFAA concentrations measured by FMOC (fixed at t = 0) and by OPA (stored seawater) methods with the depth of water. In most water, higher levels of DFAA concentrations were observed using the FMOC technique in the surface water (10 m) and deeper down (50-65 m). The data (in Table 5.2) obtained by the OPA approach does not reflect this amino acid-depth relationship. In fact a very low DFAA concentration at 10 m (December 15, 1992 from Gulf of St. Lawence sample) suggests a very significant error of analysis resulting from sample storage. It is interesting, but unexplainable, how the value for Δ% between waters sampled on December 12 and December 15 from the Gulf of St. Lawrence are so much different. It is possible that the samples collected on December 15 were not stored properly before OPA-derivatization.

5.2.2. Comparisons of Individual DFAA Concentrations

Figure 5.1 shows the comparison of individual DFAA measured by FMOC (fixed at t = 0) and by OPA (stored seawater) methods during different seasons on one site. As expected, higher concentrations of individual DFAA are obtained using the FMOC-derivatization approach for almost all amino acids. The biggest differences in the individual DFAA were the neutral amino acids Ser and Gly, and the acidic amino acids Asp and Glu. The concentrations of the basic amino acids (Arg and Lys) are only slightly different between the two techniques. Similar results were obtained from the

Location: Logy Bay, Newfoundland
Depth: 15 m

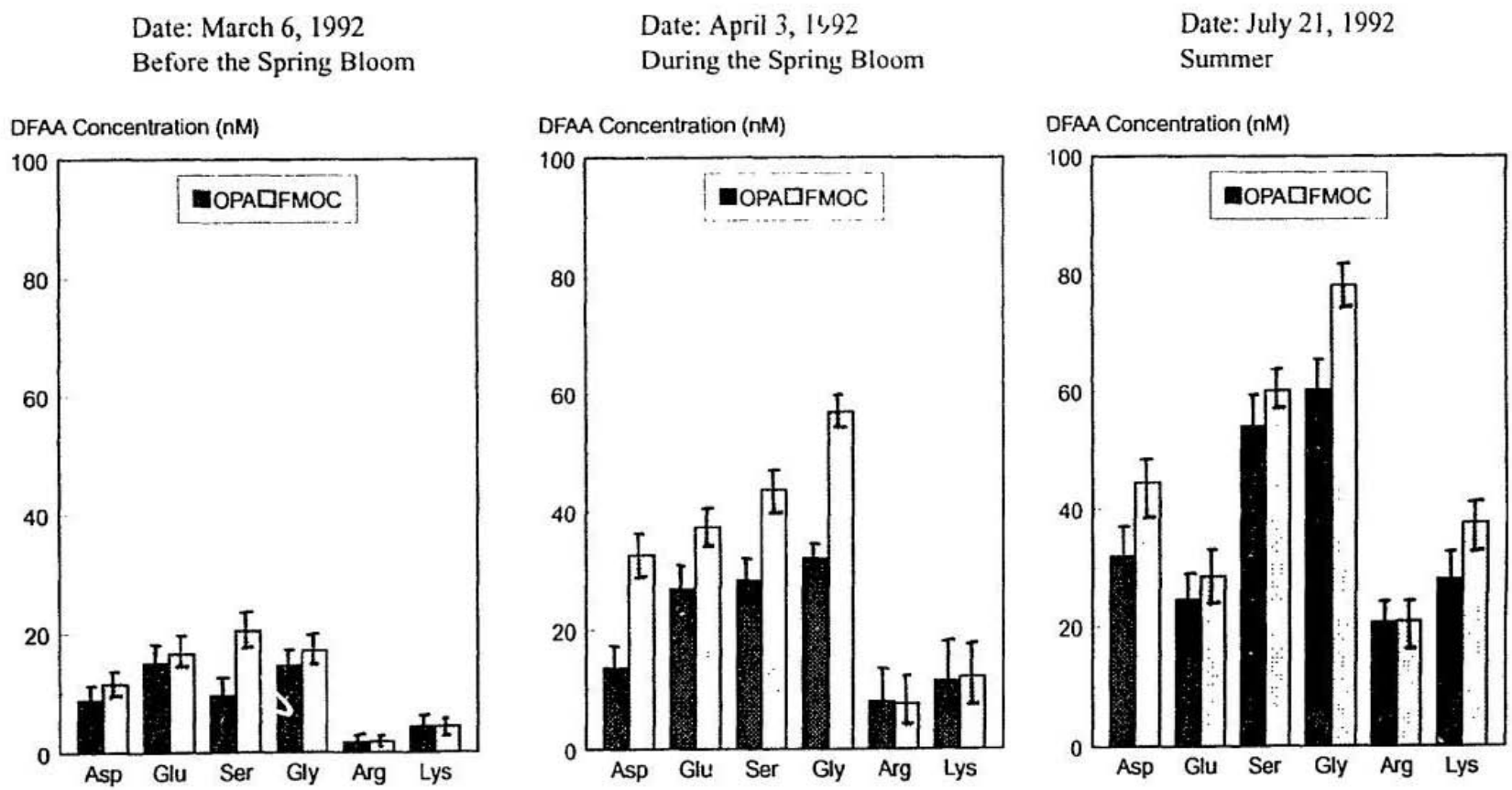


Figure 5.1. Comparison of Individual DFAA with Time of Year Measured by FMOC (fixed at t = 0) and by OPA (stored seawater) Methods.

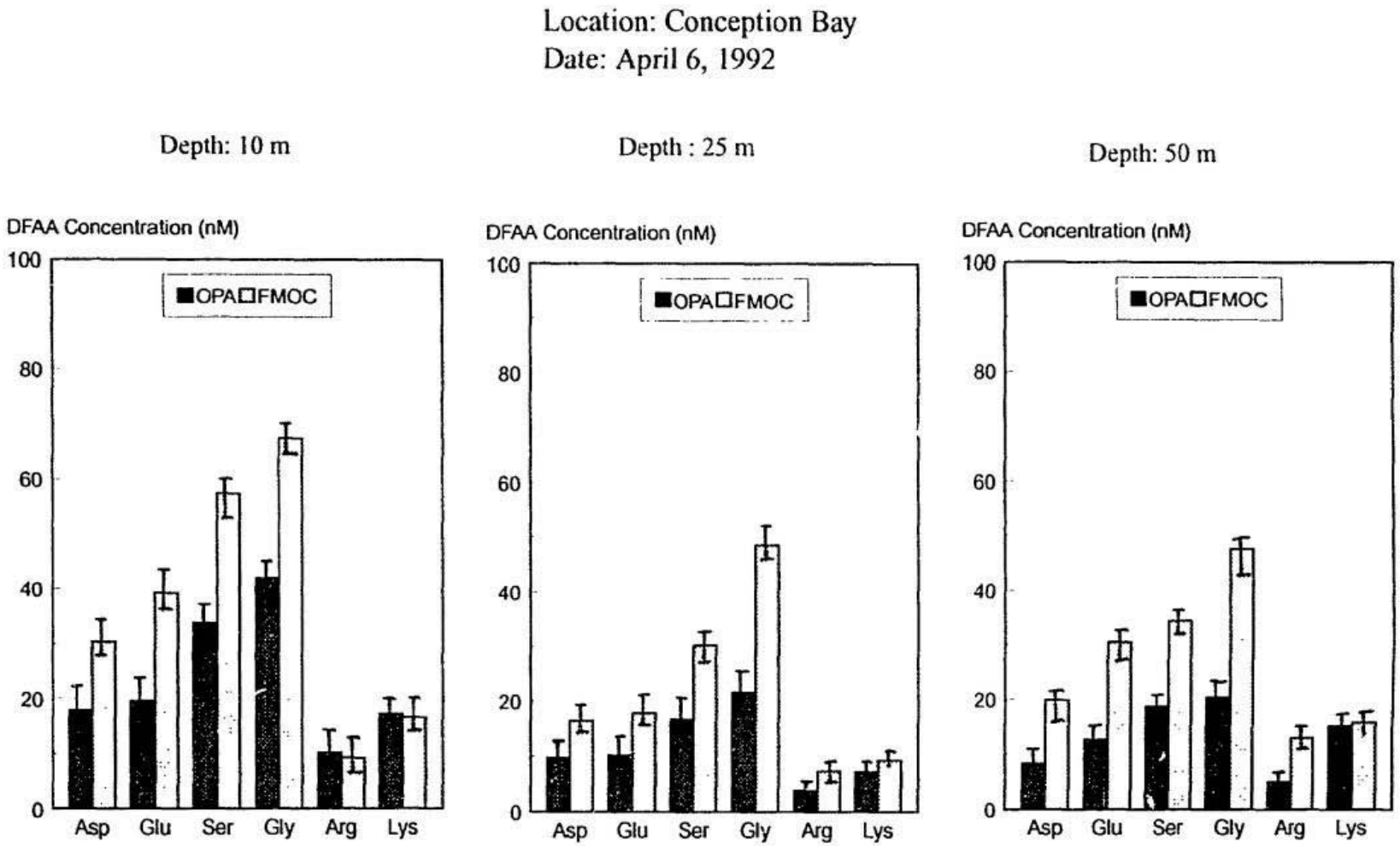


Figure 5.2. Comparison of Individual DFAA at Different Depths Measured by FMOC (fixed at t = 0) and by OPA (stored seawater) Methods.

comparison of individual DFAA with depth measured by FMOC (fixed at t = 0) and by OPA (stored seawater) methods (Figure 5.2). These results would indicate that neutral and acidic DFAA are more prone to loss during storage than basic DFAA.

5.3. Use of the FMOC-Derivatization Approach to Study a Date Profile of DFAA Concentrations

Field studies were conducted to obtain a time-course of total DFAA concentrations along the Newfoundland coast from February to October, 1992. The results are illustrated in Figure 5.3 and Figure 5.4. All DFAA concentrations were obtained using our FMOC-derivatization approach (fixed at t = 0). Seawater samples were collected from Logy Bay between 11:30 a.m. and 12:30 p.m. at a depth of 15 m.

In Figure 5.3 an increase of total DFAA concentration on March 23 possibly indicates the start of spring bloom of phytoplankton off the Newfoundland coast. A decrease of total DFAA concentration on April 17 may be the result of increased bacterial utilization of DFAA. The highest level of total DFAA measured was in water collected on July 21 suggesting a minor summer bloom.

Figure 5.4 is an expanded time-course of total DFAA concentrations during the period of the spring bloom along the Newfoundland coast in 1992. Although there are

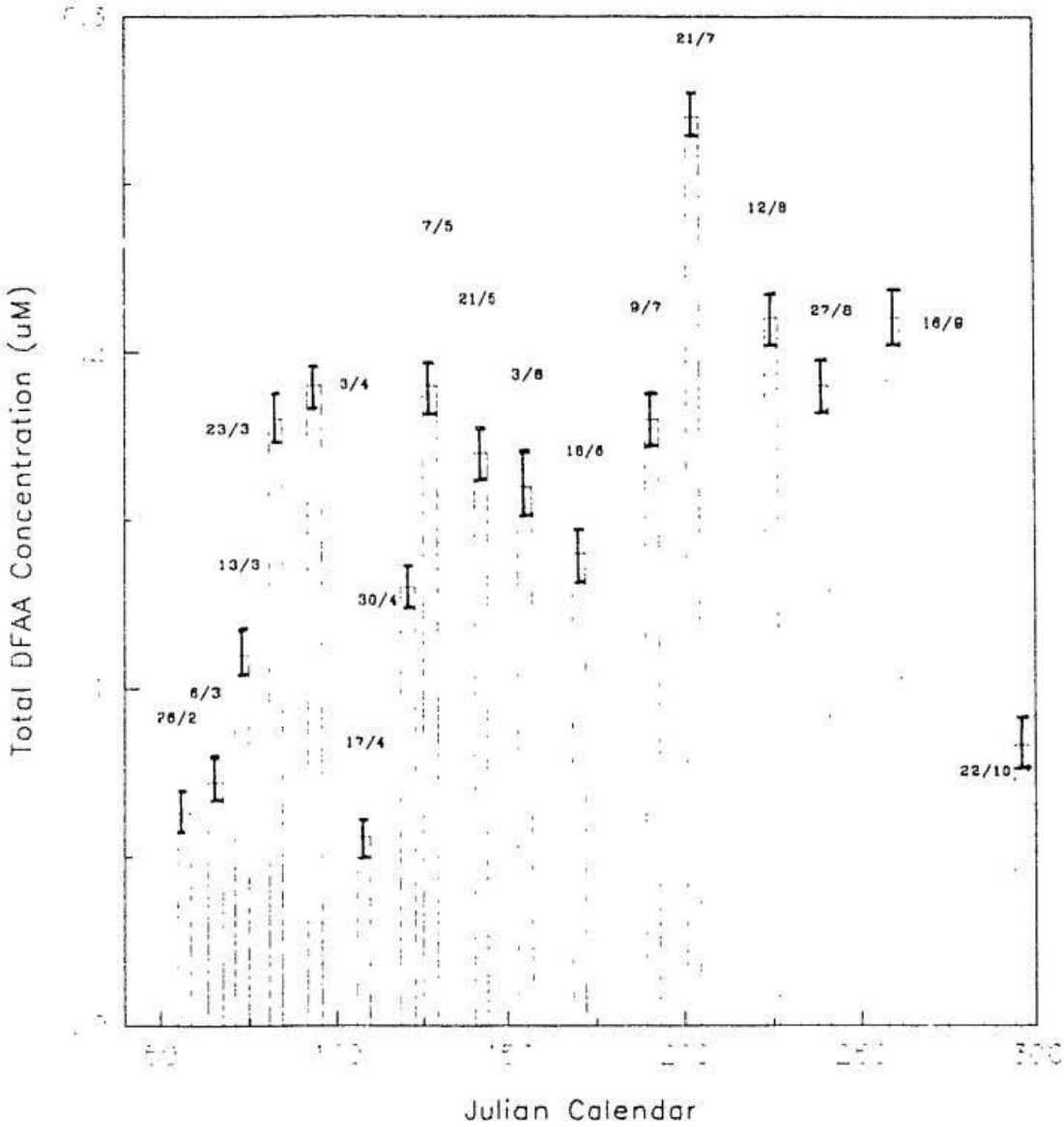


Figure 5.3. Time- course of Total DFAA Concentrations Measured by FMOC-Derivatization Approach (fixed at T= 0) from February to October , 1992

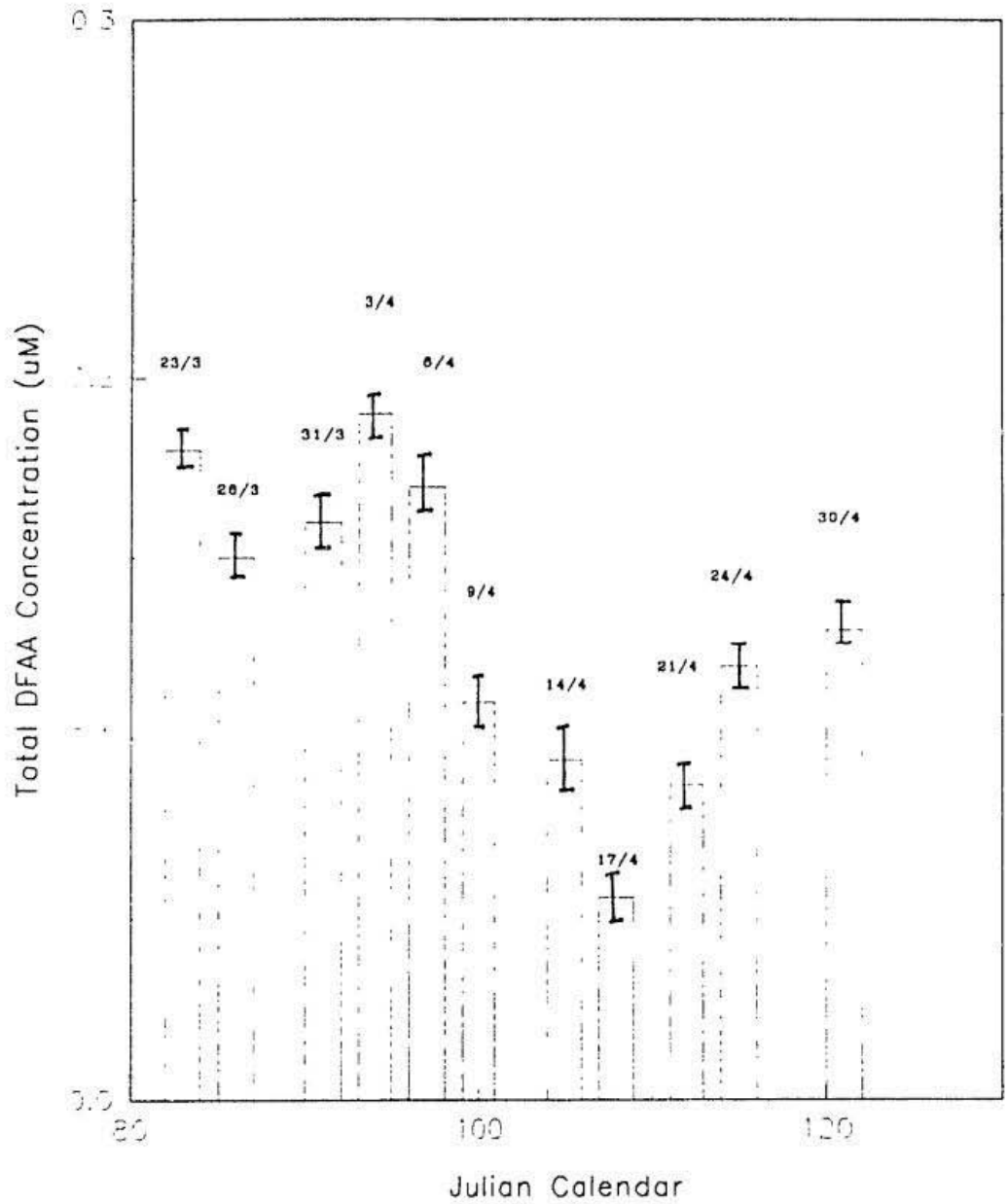


Figure 5.4. Expanded Time- Course of Total DFAA Concentrations Measured by FMOC-Derivatization Approach (fixed at T= 0) During a Spring Bloom of Phytoplankton off the Newfoundland Coast in 1992

slight differences in total DFAA concentration from March 23 to April 6, 1992, the average DFAA for this period was around 0.17 μM. This is much higher than the DFAA average of the April 14, 17 and 21 sampling dates (average 0.079 μM).

CHAPTER 6

CONCLUSIONS

The objective of this project was to develop a sensitive and accurate analytical method for the trace analysis of dissolved free amino acids (DFAA) in seawater. The newly proposed HPLC method using 9-fluorenylmethyl chloroformate (FMOC) as a fluorescence labelling reagent has been demonstrated to be not only a sensitive technique for trace analysis of DFAA, but also a convenient approach to derivatize the DFAA at the time of sampling and for stablizing the amino acids for further analysis by HPLC.

Initial studies concentrated on optimizing the chromatographic separation of FMOC-derivatized amino acids. Using the established chromatographic procedure described in the literature[2-3], it was found that FMOC hydrolysis-product, 9-fluorenylmethanol (FMOH), eluted among the amino acid derivatives in the HPLC profile thus interfering with the analysis, especially at the trace level amino acids are found in seawater. In order to overcome this problem, the effect of pH of the mobile phase on the separation of the amino acid derivatives was investigated. It was found that mobile phase pH was critical in the elution profile of both the amino acid derivatives and FMOH. By increasing the pH to 6.5 and higher all sixteen standard amino acids eluted before FMOH. An ionic medium pH of 7.5 was selected for optimum resolution of the amino acid derivatives.

Other chromatographic mobile phase parameters were studied. The effect of acetonitrile concentration in mobile phase B was examined. Acetonitrile concentration was found not only to affect the total analysis time but also to influence the resolution of amino acid derivatives. The optimum conditions which were selected for mobile phase B composition were 80% acetonitrile and 20% sodium acetate (pH 7.5). The effect of the chemical nature and concentration of the ionic medium were also studied. Under optimized chromatographic conditions, most amino acid derivatives were satisfactories resolved using a standard reversed-phase column.

Attention was then turned to a comparison of newly-developed FMOC-derivatization approach with the well-established procedure of using *o*-phthaldialdehyde (OPA)-derivatives. The OPA-derivatization approach has many advantages compared to classical IC analysis. The major disadvantage, however, is the instability of OPA-derivatives of amino acids. Seawater DFAA concentrations at "time of sampling" cannot be obtained unless a HPLC is on board. Even after a short period of storage, a significant drop of DFAA levels was observed. In contrast, the major advantage of derivatizing amino acids using FMOC reagent is that the FMOC-amino acid derivatives are stable for weeks. This feature of long term stability enhances the application of FMOC-derivatization in real seawater analyses. In field studies, the FMOC reagent was successfully used as a means of preserving unstable DFAA in seawater samples as well as being used as a sensitive fluorescence labelling reagent for precolumn derivatization in HPLC analysis. Comparisons of sensitivity, detection limit and recovery between the

two methods were conducted. In general, FMOC-derivatives are more sensitive (and lower detection limit) than that of OPA. In terms of recovery, the FMOC approach is very comparable.

The ultimate objective of this research was to apply the newly-developed FMOC method for the analysis of DFAA in seawater. Owing to instability and dynamics of DFAA in seawater, sample handling is very important to obtain accurate and reliable results. Thorough studies were performed in terms of sampling, filtration and storage. A critical step in sampling was found to be filtration. It was established that the use of Whatman GF/C glass fibre filters were quite suitable and most convenient.

Finally, comparisons were made of DFAA analyses in real seawater samples. Three analytical approaches were selected; FMOC-derivatization (fixed at t = 0), OPA-derivatization (stored seawater) and FMOC-derivatization (stored seawater). As expected, the highest levels of DFAA were obtained using FMOC-derivatization (fixed at t = 0), in some waters, 100 to 200% higher than those found in stored seawater. The excellent stability of FMOC-derivatives of amino acids makes it now possible to obtain "time of sampling" information on DFAA concentrations in seawater.

In 1992, various field studies were carried out where numerous seawater samples were obtained to test and support the FMOC-method of analysis. The FMOC-derivatization approach showed very good reproducibility in measurement (C.V. 4-6%).

An extensive investigation was performed to study the pattern of total DFAA concentrations at one site over 9 months. An increase of total DFAA in late March and in the summer was observed suggesting a possible spring bloom and a minor summer bloom respectively.

In summary, HPLC analysis using 9-fluorenylmethyl chloroformate (FMOC) as a fluorescence labelling reagent for the trace analysis of dissolved free amino acids (DFAA) in seawater is sensitive, accurate and convenient. In field studies, FMOC reagent can also be used as a means of preserving unstable DFAA in seawater. The long term stability of FMOC-derivatives of amino acids makes it possible to obtained reliable results of DFAA concentrations at the "time of sampling" in the absence of HPLC onboard ship.

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