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A precolumn derivatization procedure for the analysis of marine amino acids with 9-fluorenylmethyl chloroformate and high performance liquid chromatography

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ABSTRACT

The separation of 20 amino acids has been achieved by gradient elution and reversed phase high performance liquid chromatography employing 9-fluorenylmethyl chloroformate as the precolumn derivatizing reagent. The application of this technique to assess the extent of marine bacterial uptake of amino acids released from kelp has also been determined. The problem of excess reagent reacting with water to form a hydrolysis product has largely been overcome. Pentane extraction of the reagent after amino acid reaction caused the loss of less polar amino acid derivatives. Other factors such as the formation of di-labelled products and the pH dependance of the derivatization reaction have been investigated. The reproducibility between-analyses had a percentage error of 2 - 6%. The stability of the derivatives is about 2 weeks at room temperature. The application to physiological samples and seawater has been demonstrated. The method was applied to the study of kelp release and bacterial uptake of marine amino acids. Other chemical profiles of ammonia, nitrate, total N, particulate C, together with bacterial activity and bacterial density (biomass) were determined to provide correlative profiles to the amino acid values. The experiment was set up with kelp fronds in buckets, some containing antibiotics to halt bacterial activity, and a control bucket with untreated seawater. Alanine is the most dominant amino acid (concentration between 5 and 100 $nmol.dm^{-3}$). Values of glycine, aspartic acid, glutamic acid and arginine have much lower levels. Traces of histidine, asparagine, cystine, serine and tyrosine appeared near the end of the experiment. It was found that the amino acid concentrations

were low compared with the inorganic nitrogen species. The flux of these species was found to be too low to create a substantial response, as the activities are also low compared with normal in-shore regions. In order to infer more from the processes occurring in this study, we would have to increase the experimental time to the order of days.

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1. INTRODUCTION

1.1 THE ORIGIN AND ROLE AMINO ACIDS PLAY IN THE WATER COLUMN: FACTORS CAUSING THEIR RELEASE, UPTAKE AND BREAKDOWN

Amino acid analyses are essential if we need to determine any nitrogenous exchange occuring in the sea. The lack of an adequate understanding of their importance has been limited by the complicated analytical approach involved.

Not only are amino acids the building blocks of life in the sea, but they also serve as indicators of the important processes of decomposition of organic species and the regeneration of nutrients. Nitrogen can be the critical limiting element in algal growth and uptake processes in coastal marine waters and the availability of N-containing nutrients possibly determines the rate of organic production in the sea (Ryther and Dunstan, 1971). The rate of primary production could be limited by the availability of these nitrogenous nutrients and therefore it is important to assess and quantify the process involved with these nitrogen compounds (Hollibaugh et al., 1980).

Important nutrients such as nitrate and nitrite arise from deeper waters mixing into the euphotic zone. Other nutrients such as ammonia and urea are released into the euphotic zone as waste products of secondary production. Organic nitrogen species decompose, mediated by marine bacteria, to give off this ammonia

and also carbon dioxide (Hollibaugh et al., 1980). Certain of these specific organic species originating from the upper layers, which are finally decomposed by chemical and bacterial pathways, can tell us much about the state of the system and whether any fluctuations in their distribution pattern occur in time and space. Few such studies of the relationship between these substances and development of the euphotic community have been done (Ittekkot, 1982).

Since amino acids are a major constituent of marine organisms they can play an important role in the cycling of organic nitrogenous material. The oxidation of amino acids by heterotrophs possibly accounts for a large amount of regenerated nitrogen. (Hollibaugh et al., 1980.) We therefore need an increased understanding of the specific roles that amino acids play in the marine environment (Siezen and Mague, 1978). By observing the amino acids which are a more specific section of the dissolved organic carbon pool, inferences can be made about subtle changes within the water column as regards uptake, release and breakdown processes. However, these inferences cannot be achieved with certainty when the analytical technique used is prone to contamination and or loss (Lee and Bada, 1975). Once we have developed an analytical technique that can allow a simple, rapid and accurate assessment of marine amino acids, we can attempt to explain the reasons for the qualitative and quantitative distribution of these compounds.

It is not only important to assess the types and concentrations of these components, but also the dynamic role they play. Concentration and flux data should complement one another to explain the importance of the processes involved in community energetics (Hobbie et al., 1968). We therefore would have to design experiments to monitor the role of these compounds in these processes. This could be a "before and after" type assessment, or a chemical labelling technique.

The amino acid composition of organisms is not necessarily related to those they may supply to their surrounding environment. It is quite possible that similarities are due to uptake by organisms (Hendrichs and Farrington, 1979). We should always question the distribution of these species in the surrounding waters and take into account their origin from all sources, be it bacteria, macrophyte, phytoplankton, zooplankton or from tertiary producers.

There is much evidence to suggest that phytoplankton remove amino acids from solution to supply their nitrogen need, and compete with other organisms such as bacteria and zooplankton for available free amino acids (Lu and Stephens, 1984). It has been found that restricting the nutrient nitrogen available to a phytoplankton culture decreases the nitrogen content of their cells and slows down the growth rate. This greatly increases the rate of amino acid uptake (Wheeler et al., 1974). Bacteria attached to particles of the same size fraction may be responsible for some uptake as well, as there is strong evidence to suggest that bacteria and

phytoplankton both compete for the uptake of some amino acids (e.g. glycine). The contribution of bacteria must be taken into account when inferences of nett plankton uptake are determined and vice versa (Wheeler et al., 1974). This inverse relationship is an important factor to consider when designing an experiment similar to the one conducted in this study of kelp release and bacterial uptake. Furthermore, it has been shown that large amounts of amino acids are released from zooplankton (Webb and Johannes, 1976). Rates of production and utilization of these amino acids by these organisms and their mechanisms of release are unknown and require study (Lee and Bada, 1975).

Studies of uptake and release must be reconciled in order to explain the relationship between bacteria, zooplankton and phytoplankton and the free amino acid pool in the sea. Release can arise from either excretory organs or from simple release of cell contents. There are two main theories to explain the apparently wasteful excretion of dissolved organics. Riley (1963) suggests that phytoplankton and aggregates of organic matter provide a better source of food for zooplankton than would phytoplankton alone, which stabilizes the community. Wangersky (1965) suggests that more energy would be lost if the dissolved organics present in the organism are retained by the biological system involved, rather than by allowing diffusion of compounds produced in excess of immediate needs (Lee and Bada, 1975). However, we would need both a good experimental design and an accurate technique of assessment to even begin to determine which approach is more meaningful.

In a study of breakdown of amino acids after they are released, it was found that there is little resemblance in the distribution of amino acids from the sediment and those from the water column (Lee and Bada, 1975). Furthermore, plankton and seawater amino acids have compositions different from those of the sediment surface. An intercomparison of the different types and amounts of amino acids from marine and terrestrial input in sediments, would give us a good indication of how environmental factors influence decomposition (Gonzales et al., 1983). Earlier studies have attempted to assess the origin and fate of these compounds and any differences occurring have been ascribed to environmental factors and stabilities towards chemical or bacterial breakdown. However, the use of amino acids for such indicators is in question when one considers that amino acids are common to all organisms. A more thorough investigation of the composition of organisms and related marine systems needs to be tackled before inferences can be made with some justification (Gonzales et al., 1983). Here the appearance of any non-common amino acids can tell us much more in terms of the processes occurring than any of the more common amino acids. This has been demonstrated by the emergence of β-alanine as an indicator of decomposition (Lee and Cronin, 1982).

Decompositional processes in the water column can alter the sedimentary input by influencing the nutritive value, amount and composition of sinking particulates originating from the euphotic zone. A knowledge and understanding of specific compounds provides more insight into decompositional mechanisms and nutritional value.

Amino acids comprise the largest pool of organic nitrogen in most organisms and therefore should be useful decompositional indicators, and indeed, specific amino acids have been studied and their distribution related to certain decompositional factors and mechanisms (Lee and Cronin, 1982).

1.2 BACKGROUND TO THE MARINE APPLICATION OF THIS STUDY: REASONS FOR MONITORING KELP RELEASE AND BACTERIAL UPTAKE.

One of the main sources of organic matter in the ocean comes about due to release from plankton. This is in the form of detrital, particulate matter and released dissolved compounds. In coastal waters this input would be dominated by larger macrophytic organisms such as seaweed and kelp, depending on their abundance (Linley and Newell, 1981). In studying the effects of coastal kelp release and subsequent bacterial uptake, sampling sites are easily accessed and concentrations are orders of magnitude higher than in open water systems. This would also permit a more elaborate experiment to be designed and controlled.

Much work has been done on the microbial communities associated with kelp mucilage (Linley et al., 1981; Linley and Newell, 1981; Reichardt and Dieckmann, 1985) and on the assessment of dissolved and particulate organic matter released from kelp (Newell et al., 1980; Lucas et al., 1981; Newell and Lucas, 1981; Carlson and Carlson, 1984). Less has been done on the analysis of specific chemical species. Studies of release of D-mannitol from kelp as an important substrate for the surrounding microbial community (Lucas et al., 1981) has inferred much more than a general compound analysis (e.g. total carbohydrates).

From specific amino acid studies of other marine samples (see section 1.3) it is possible that an analysis of specific free

amino acids can infer much about the processess of kelp release and microbial utilization. There is little known of the amino acids of kelp. Newell et al. (1980) determined the total combined amino acids (TCAA) after acid hydrolysis using an amino acid analyser based on the technique of Spackman et al. (1958). Percentage loss varied between 1 - 16% due to loss during acid hydrolysis. Interactions between mannitol and the amino acids cause interference in the chromatographic analysis. For two common kelp species (**Ecklonia maxima** and **Laminaria pallida**), the total combined amino acid profiles were very similar, with both having high concentrations of aspartate and glutamate, with alanine having the highest concentration (375nmol). They found that total combined amino acids comprised 3 - 3,5% of kelp mucilage.

A study devised to monitor the free amino acids after they are released from kelp will complement concurrent research work (J. Brauer, 1986). Here collaborative work on joint experimental design and independent assessment of subsequent results achieved, would far outweigh the individual uninterrelated interpretations of amino acids in inshore waters, or phenolic and nutrient release from kelp achieved by different experimental designs. Release from kelp would have to account for uptake by bacteria and other organisms, and need an explanation of the origin and fate of amino acid concentrations. It was therefore decided to pool resources on kelp release with this work on amino acid assessment, so as to achieve a wider picture of the dynamics of inshore communities. The results would be interpreted in terms of what hypotheses were

initially set up by each working group and jointly on the overall process of release and uptake.

The problem with designing an exudate-uptake study is that the two dynamic processes occur simultaneously. The individual rates of release and utilization would therefore be unknown. We would therefore have to devise an in situ experiment which would allow these two processes to be separated to enable a better assessment and the following method was adopted.

Amino acids were monitored and these profiles complemented by bacterial activity and biomass estimates to assess the bacterial uptake of these species. Chemical determination of ammonia, nitrate and total N values, together with particulate C assessment, complemented these amino acid values for a better understanding of the nett process of the important nitrogenous species. These nutrients are said to be end products of the breakdown of proteinaceous material and are regenerated by bacteria (Hollibaugh et al., 1980). These nutrient-N values would correlate well with amino acid profiles, especially if breakdown mechanisms are stressed.

Bacterial activity was calculated using the ^{3}H-thymidine incorporation method of Fuhrman and Azam (1980, 1982) modified by Lucas et al. (1984), rather than the predator-reduced method, which requires time-consuming filtration. Biomass was calculated from the Acridine orange method of Hobbie et al. (1977), modified by Painting et al. (1986).

1.3 REVIEW OF SOME PREVIOUS MARINE AMINO ACID ANALYSES AND MEASURED PROFILES

One of the earliest analytical techniques for amino acid analyses was developed by Chau and Riley (1966). This required preconcentration and desalting before analysis by thin layer chromatography (TLC). The salt crystals formed during rotary film evaporation of the seawater sample were periodically removed by filtration. Final salt removal was by ion exchange using piperidine as eluant. Analysis was by two-dimensional TLC on a semi-quantitative basis. Sources of error were large and accuracy of the method was poor. Best results were obtained with amino acid concentrations of the order of 0,5 - 1μg.dm^{-3}.

Hobbie et al. (1968) used chelating resins for desalting and subsequent analysis using a Technicon Auto Analyser by the method of Webb and Wood (1966). They observed the uptake of some common seawater amino acids by planktonic bacteria. Glycine, serine and ornithine were found to be in relatively high concentrations. Intermediate concentrations of aspartic acid, threonine, alanine and methionine were found. Glycine, methionine and serine had the highest flux with valine, alanine and aspartic acid having an intermediate flux.

An improved method for extraction of amino acids from lyophilized seawater combined with a desalting stage using a cation exchange resin was presented by Pocklington (1972). This was followed by a

one-step reaction to give trimethyl silyl derivatives for separation by gas chromatography. The procedure is lengthy, requiring too many clean-up stages to make it a feasible approach for routine analysis. Large concentrations of alanine were found in the surface waters, and large changes occurred with depth.

An interesting study of the distribution of dissolved free amino acids (DFAA) from sewage outfalls in southern Californian coastal waters showed the contributions by individual amino acids to the total amount of DFAA to be similar in all samples from surface, bottom and interstitial waters (Clark et al., 1972). The analytical methodology entailed the desalting of the seawater using ligand exchange columns and quantification by TLC. Ornithine, glycine, alanine, serine and aspartic acid contributed to more than 50% of the total DFAA, with glycine and serine being most abundant. Total DFAA averaged 500 nmoles.dm^{-3} (60 - 70 μg.dm^{-3}) for normal Atlantic waters. Detrital material is one of the major sources of DFAA in the marine environment (Clark et al., 1972). Various studies of aerobic sediment layers suggest that these upper sedimentary layers may be a major reservoir for amino acids in the marine environment (Degens, 1968). It was found that effluent input gives 200 - 300 times higher levels of DFAA than inshore waters in southern California. The dilution of this input was also monitored along the adjacent coastline. There is evidence to suggest that changes in DFAA and other important organic compounds may affect the stability of sublittoral ecosystems. North (1964) found that large populations of sea urchins present at outfalls

were considered a major cause of the decline of giant kelp beds. These urchins may well have received their nutrients from these outfalls.

Wheeler et al. (1974) studied the potential significance of amino acids as a nitrogen source for phytoplankton growth. Here common marine amino acids were added in high concentrations as well as 14C-labelled amino acids at low, natural concentrations to axenic phytoplankton cultures. They found that uptake was sufficient to support moderate growth rates in both cases. The ability to assimilate DFAA at low concentrations is characteristic of most marine algae, but heterotrophic utilization by these phytoplankton did not occur in the dark. Growth of the phytoplankton was much slower than when the medium contained an equal concentration of nitrogen in the form of nitrate. Amino acids used were: glycine, serine, which were found to be most abundant; with alanine, aspartate, threonine, valine, glutamate, ornithine and lysine being added at lower concentrations.

Schell(1974) conducted studies to compare uptake rates of nitrate, ammonia and amino acids at levels naturally occurring in the sea. He also set out to determine whether the C and N groups of the amino acids were assimilated at equal rates and to measure the excretion rate of DFAA by growing phytoplankton. He found, using ^{14}C and ^{15}N labelled glycine and glutamic acid, that the N of glycine is preferentially incorporated with the carbon being respired. The reverse was found to occur with glutamic acid.

After being assimilated, the amino acid molecule may be used as is, or partially decomposed and the by-products returned to the environment by excretion or respiration. It was found that glutamic acid and glycine were assimilated fastest in the dark, apparently due to photo-inhibition in the light samples. This is in direct contrast to what Wheeler et al.(1974) found. They observed no heterotrophic utilization of amino acid in the dark.

In a study of amino acids in a marine sediment, Morris (1975) found that the major changes in planktonic protein amino acids occur in the water column during their sedimentation or in the top 10 - 20cm layers of the sediment. The extent of these changes is determined by the conditions in the sediment such as microbial activity, redox potential, humic substances and trace metal composition of the sediment. Changes to these protein amino acids were found to be slower below the upper layers due to processes such as polymerization of organic species and possible formation of organo-metallic complexes (Degens, 1968). Major trends with respect to depth were observed for the common amino acids. There was a relative decrease in the amount of aspartic acid, serine and alanine; and a relative increase in amounts of glycine, lycine and arginine. It was found that large levels of ornithine in the sediment could be attributed to the breakdown of arginine, as ornithine is found in much lower concentration levels in plankton and upper waters. Degens (1968) found the presence of some non-protein amino acids in the upper sedimentary layers and this could be attributed to the breakdown of some protein amino acids.

The changes in the total amount of amino acids in the upper sediment layers, relative to a predicted input, could reflect the various degrees of stability of the amino acids or those amino acids preferred for biological activity within sediments.

There appears to be no difference in amino acid composition for a number of species of marine phytoplankton (Chau et al., 1967). However, other workers found major differences in amino acid composition between diatoms and other marine algae (Chuecas and Riley, 1969). There is a large input of protein amino acids from plankton and algae into the marine environment, and Morris (1975) set out to determine whether the amino acid composition from plankton (upper water layers) would be the same for those present in the sedimentary layers. He found that they had changed due to interactions with the water column prior to sedimentation.

Lee and Bada (1975) suggest that the combined amino acid fraction may be a useful water mass indicator. They found that the DFAA pool makes up only 0.3% of the total dissolved organic carbon (DOC), much less than that found before by other workers. Their experimental technique was prone to errors, making any deductions from their results rather suspect. Desalting was achieved using a ligand exchange chromatographic technique (Siegel and Degens, 1966) but it was found that glycine tends to bleed from the resin making an effective analysis of this compound impossible. Analysis of the components was carried out using an amino acid analyser. Final recovery of an internal standard, added at the start of the

procedure, ranged from 70 - 90% for DFAA and 40 - 75% for hydrolyzed combined amino acid fraction.

Crawford et al., (1974) found that glycine, serine and ornithine make up over half the DFAA pool in estuarine samples. Profiles Lee and Bada (1975) reported show large percentages of alanine, aspartic acud, glutamic acid and serine, and that DFAA is only present in substantial amounts in surface waters. Lower concentrations occur deeper down the water column, this is attributed to uptake by organisms or absorption onto particulate matter. The turnover time for amino acids was found to be of the order of several days (Lee and Bada, 1975).

Webb and Johannes (1976) found that taurine, glycine and alanine were released in large concentrations from marine zooplankton. Cysteic acid and cysteine were less common, and present in low concentrations. Amino acids accounted for about 12% of the nitrogenous release products from zoop|ankton, and this release of organics was found to increase with temperature. It was also discovered that there are similarities between the amino acid patterns in tissues and those found in release products. Ammonia was the major dissolved nitrogenous compound released from marine invertebrates. A variety of other release products were identified, such as creatine, creatinine, urea and purines (Nicol, 1960). Their technique of amino acid analysis was by semi-automatic ion exchange chromatography.

In Morris and Calvert's (1977) study of organic rich sediments from the Namibian shelf, the amino acid pattern was found to change both with depth and to decrease in amount. They found a close similarity between amino acids from plankton and those found in the sediments, suggesting a large input of amino acids from plankton into the sediments. For specific amino acids they found valine, arginine, phenylalanine and tyrosine to increase with depth and, compared to the predicted input from plankton, it was found that serine, proline, glycine and alanine levels were much higher. Values of glutamic acid, arginine and lysine were found to be less than the predicted input, possibly due to these compounds being more susceptible to breakdown in the sediment, but this is only a tentative explanation.

In a study of amino acids in a sediment core, it was again found that concentrations decrease rapidly with depth (Whelan, 1977). Non-protein amino acids β-alanine and γ-aminobutyric acid were predominant in the deeper section of the sediment and accounted for about 70% of the amino acids. The amino acids are tightly bound to the sediment and are only released on acid hydrolysis. They accounted for about 0,4% of the total nitrogen in the sediment. The method required a rather lengthy sample preparation of extraction and desalting before separation by gas-liquid chromatography (GLC). Analyses were compared with an amino acid analyser, which gave results for those amino acids which could not be done by GLC due to thermal decomposition. Histidine and other basic amino acids were found in higher concentrations. Aromatic

amino acids occurred in low levels. Traces of α,ϵ-diaminopimelic acid were present which are typical of bacterial cell walls suggesting a high bacteria involvement. Bacterial counts were found to be normally higher at the sediment surface than in the water or below the sediment. It was found that plankton and seawater amino acids have compositions different from those of the sediment surface. This is in direct contrast to the results of Morris and Calvert (1977). Considering the turnover time of amino acids it would be expected that a difference would occur. However the amino acid composition from one sediment type to the next was remarkably similar.

Lee and Bada (1977) analysed various seawater samples for dissolved free and combined amino acids by a ligand exchange chromatographic technique. Various depth profiles were set up and the concentrations were found to be similar. They also determined the enantiomeric ratios of the isolated amino acids and found that the higher ratios (larger amounts of D-amino acids) indicated that the amino acids come from other sources besides phytoplankton. Bacterial production must be considered, as D-amino acids have been found in bacteria. The high ratios could then be attributed to bacterial decomposition products. Since Whatman GF/C filters were used, the D-amino acids could be derived from bacteria passing through the filter. The high ratio could then not be attributed to breakdown products alone. Bacteria may release or remove amino acids from the water, so that an assessment of amino acids alone cannot determine which process is occurring.

Wheeler et al. (1977) studied the uptake of ^{14}C-glycine by different fractions of plankton and found that phytoplankton was responsible for at least 50% of the glycine uptake. It was found that inshore species utilize more amino acids than offshore species. Phytoplankton continue to grow in culture with the only source of nitrogen being amino acids. Values for uptake correlated well with pigment content and photosynthetic activity determinations. They found that more than 80% of the pigments and a similar amount of photosynthetic ^{14}C incorporation occurred in the 3-25μm phytoplankton size fraction. Nanoplankton hence play a major role in primary productivity. Glycine uptake exceeded 50%. Bacteria attached to particles may be responsible for some of this uptake.

Dawson and Pritchard, (1978) devised a method for the analysis of picomolar levels of amino acids using an amino acid analyser with fluorometric detection. Amino acid profiles from different depths in the open Baltic ocean and elsewhere have been given and concentrations were found to range from 4,5 to 84 $\mu g.dm^{-3}$. The method involved desalting on ion exchange resins and the disadvantages of this procedure were discussed. It is possible that under the mildly acidic conditions during desalting, labile peptides and metal chelates are dissociated and therefore contribute to the apparent free amino acid pool. Distinct differences exist between samples desalted and those injected without treatment. β-alanine appears to be a product of the preconcentration procedure from resin contamination, or hydrolysis of larger molecular weight column components. Assessment using the methods of desalting would give erroneous results.

In a comprehensive study of particulate amino acid (PAA) in coastal and oceanic Pacific waters (Seizen and Mague, 1978), interesting trends were observed. The concentration of particulate amino acids in these waters ranged from 370 to 2260 $nmol.dm^{-3}$ in coastal waters and from 90 to 260 $nmol.dm^{-3}$ in open ocean waters. Much information on particulate organic matter is lacking, especially the exact molecular composition of those particles. Analyses were done by GLC and some amino acids suffered decomposition due to the prior hydrolysis, ion exchange and complex derivatization procedures. Despite this, some interesting results were obtained. The highest PAA concentrations were found in equatorial open ocean surface waters. This is due to the increased phytoplankton growth from increased temperatures and upwelled nutrients. As colder waters are approached, the PAA values drop. The amino acid compositions for the different sampling sites were remarkably similar. Some significant trends were detected. The relative amounts of glycine and serine increase with respect to depth and these are also the most abundant PAA. Hydroxyproline, methionine and lycine decrease with depth. Amino acids undetected were ornithine, the 2-aminobutyric acids and α,ϵ-diamino-pimelic acid, which is, according to Siezen and Mague, (1978) present in algal cell walls. The planktonic proteinaceous material is more prone to degradation than other carbon rich constituents during sedimentation.

Garrasi et al. (1979) developed a method for the direct injection of seawater samples into an amino acid analyser without prior

desalting. Up to 2ml of average salinity seawater can be directly injected with little band broadening. This broadening can be minimized by the addition of a few drops of concentrated H_2SO_4. An inter-comparison study of two other common methods with the direct injection method gives some interesting results. The cation exchange method overestimates the dissolved free amino acid concentration. This method also gives high blank values and hence sample concentrations need to be high. The presence of taurine, β-alanine and histidine is solely due to contamination from the cation exchange resin. The copper Chelex desalting method under-estimates the amount of dissolved free amino acids. The latter method gives low blanks but with variable recoveries. The addition of a single internal standard to correct these losses would not be representative of all the amino acids. In the direct injection method the acidic medium probably causes an overestimation of the amino acids due to breakdown of labile peptides and complexes. Some interesting trends from analyses of water column depth profiles have been observed. Valine was present as the major amino acid in most samples. Aspartic and glutamic acid were also present as a major fraction in many samples. The profiles for deeper waters differ markedly from those in surface waters, and this is in agreement with Whelan (1977). Valine, alanine, glycine, histidine and lysine are present in significant amounts near the surface, but are much reduced in deeper waters. Arginine gives the reverse profile, accounting for more than 50% of the total in deeper waters.

Analyses on preserved and stored samples may not give the true values that would be experienced at the time of sampling. The turnabout time for each analysis is about four hours and this creates problems for **in situ** analyses.

Little is known of the nature of organic substances dissolved in the interstitial water of marine sediments. Hendrichs and Farrington (1979) analysed amino acid in a series of interstitial water samples by GLC and with nutrient-N data, attempted to correlate the results and discuss the certain trends observed. Concentration in the interstitial water ranged from 2 to 95 μmolar which is two to three orders of magnitude greater than in seawater. Alanine, glycine, aspartic acid, glutamic acid and β-aminobutyric acid are the major components in most samples. These trends suggest that the benthic community utilizing organics in aerobic environments produces more alanine, glycine and aspartic acid. Glutamic acid and β-amino glutamic acid are produced by fermenting and sulphate reducing bacteria. The high glutamic acid concentration could be attributed to bacteria.

In a study on planktonic cycling of organic nitrogen (Hollibaugh et al., 1980), the occurrence and pathways of nitrogenous material are discussed. Concentration and flux calculations were used to determine the extent of dissolved free amino acid utilization. Comparisons with changes in ammonia concentration gave an estimate of 60% for the amount of ammonia produced from amino acid breakdown. It was found that the type of primary producer present is

independent of bacterial heterotrophic activity. This activity is associated with the rate of carbon fixation.

Further insight into the process of sedimentation of particulate matter can be achieved by using sediment traps set at different depths. Wakeham et al. (1980) analysed sediment trap material for specific chemical species such as fatty acids, hydrocarbons, sterols, esters and amino acids. The vertical flux of particulates is controlled by involved decomposition mechanisms and transport processes. Amino acid analysis was by the orthopthalaldehyde (OPA) method after first being extracted into a saturated salt solution. The amino acid profile for the middle water traps have similar compositions to the amino acids of marine plankton. Glutamic acid, aspartic acid, alanine, serine and glycine were found in the deepwater traps. This reflects bacterial input as they are found in bacteria cell walls. Most of the organics are utilized in the middle waters during sedimentation as a very small amount of these species reach the bottom layers unaffected. It is possible that zooplankton and other specifically particulate materials find their way into the sediment traps. This could well have been a source of error. Interpretation of these results is difficult due to differences in settling rates, resuspension of sedimented material within the traps and further changes to particulates after entrapment.

The distribution profiles of total dissolved amino acids (TDAA), together with total dissolved sugar and organic carbon values, were

monitored during a spring plankton bloom (Ittekkot, 1982). Since dissolved organic matter is the major form of organic matter in the oceans, we find that phytoplankton is its chief source. The TDAA was determined by the direct injection method (Garrasi et al., 1979). Samples were hydrolyzed, hence no distinction between free and combined amino acids could be made. Levels of TDAA of the order of 100 - 850 $\mu g.dm^{-3}$ were found. The amino acid concentrations decreased near the end of the bloom and a sharp increase was observed during a second bloom. Major amino acids were glutamic acid, serine, glycine, histidine, aspartic acid, threonine and lysine. Glutamic acid is in a relatively high concentration at the early stages of the bloom, but decreases to much lower levels soon afterwards. Serine showed moderate fluctuations. Serine, glycine and alanine are quite stable in the marine environment and have been found to be major constituents of phytoplankton. Histidine was found in large amounts near the end of the bloom, possibly being a breakdown product of phytoplankton pigments. This amino acid is the only exception to the general trends of the individual amino acids. Histidine could be an important metal chelator during remineralization procedures which would regulate external conditions ie. the trace metals. Lysine was found in large amounts during the second bloom. The concentrations of these species are due to a nett result of processes of release by phytoplankton and detritus, and utilization by heterotrophic organisms. The increase in dissolved organic matter in the early stages of the bloom is attributed to release by phytoplankton. High dissolved organic concentrations again near the end of the

bloom are due to messy grazing by zooplankton and also release from physiologically old cells.

Further amino acid and lipid analyses of sediment trap material were undertaken by Lee et al. (1983). From previous sediment trap studies, the flux of the organic species on sedimentation is directly related to the surface productivity. Amino acids were measured by the OPA-method after first being extracted with a toluene-methanol mixture in order to first remove the lipids for other analyses. Both free and combined amino acids were analysed. The results were hampered by bacterial activity occurring in some of the traps due to ineffectual poisons. Muramic acid, another unique component of bacteria and blue-green algae, was found in these traps indicating the presence of bacteria. The studies of the exact amino acid composition of marine bacteria are limited, hence inferences on bacterial presence need more data for a conclusive assessment. The concentrations of β-alanine, β-amino-butyric acid, and arginine change with respect to time in the traps. β-alanine decreases with longer time span in the traps, indicating that it is possibly preferentially removed relative to other amino acids, but removal is slower. Micro-organisms could be responsible for this uptake. β-alanine increases with depth in the traps suggesting it is a major breakdown product. From this we can see the possibility of β-alanine used as an indication of the extent of decomposition, provided much more is known about the presence of this amino acid; its exact origin and fate in the marine environment.

In an analysis of sediments (Gonzales, 1983), the amino acids were analysed by pre-column dansyl chloride derivatization and separated by high performance liquid chromatography (HPLC). No free amino acids could be extracted from the sediment by an acid wash, so total combined amino acids (those hydrolysed from the sediment) were determined. The presence of some amino acids could prove useful indicators within the sedimentary environment. Large amounts of hydroxyproline were present and this amino acid is the most common cell wall constituent of algal cellular debris. Threonine, serine and glycine are main components of a protein-silica compound of diatom cell walls that is very resistant to attack. Hydroxyacids can complex with phenolics to give more resistant compounds. Valine, leucine and isoleucine are the least stable. It was found that glutamic acid is predominant in marine sediments and is also the most dominant amino acid in marine bacteria. Aspartic acid is dominant in terrestrial hydrolysates. Lysine and ornithine are common in offshore and coastal sediments respectively. Phenylalanine is relatively abundant in off-shore sediments and has been found to be the major amino acid in other sediments (Morris, 1975). Sulphur amino acids tend to disappear due to preferential geochemical degradation of sulphur containing sedimentary organic material. The influence of the input of amino acids from the terrestrial environment is limited in this study. Differences in amino acids could be due to differences in organic input with respect to time.

Amino acid analyses of gut material from some species of sea slugs,

from sediment trap material and from surrounding surface sediment has been studied by Alberic and Khripounoff (1984). Compositions of ingested material and sediment trap material are very similar with only the sediment surface material having minor differences. Glutamic acid and uncombined diaminopimelic acid predominant in the digestive tract may be due to excretion by the organism or a result of bacterial activity within the gut. The remnants from bacterial activities are more common in the gut than in the sediment or sinking particles. β-alanine and γ-aminobutyric acid account for a large proportion of the amino acids in the upper sediment layer. Their presence in the gut material may be due to decomposition of ingested organic material.

In a study of amino acids in an estuary system (Laane, 1983), the amino acids were determined by the fluorescamine method (North, 1975) and the amounts are expressed as glycine equivalents. Such an analysis is meaningless today as all amino acids were measured as glycine equivalents, and bearing in mind that far more advanced analytical techniques are available. The exact analysis of each amino acid can infer much more than an assumed amount of total amino acids. The different molecular weights of each amino acid would not therefore be considered as the concentration units are in mg.glycine.l^{-1}. Interpretation of results obtained with a reagent which reacts with a wide range of substances remains questionable because of the unknown composition of reactive materials present in natural seawater.

In a study of amino acid uptake by a phytoplankton species, Lu and Stephens (1984) used radiochemical labelled glycine, a fluorescamine technique and HPLC analysis of utilized amino acids in order to determine the extent of this uptake. There is much evidence to suggest that phytoplankton remove amino acids from solution and that they can utilize normal marine amino acid concentrations to supply their nitrogen need. They compete effectively with other organisms for available free amino acids (such as zooplankton and bacteria). Radiochemical techniques do not account for any excretion of the non-labelled compounds. The amino acid uptake was determined by analyses using OPA derivatization and separation by HPLC. A series of amino acids was added to this phytoplankton medium and analysed before and after a 60-minute exposure. They found that this phytoplankton is capable of removing all of the amino acids supplied to the medium. The results imply that the basic amino acids are utilised at the highest rate and dicarboxylic amino acids the lowest.

Lee and Cronin (1982) analysed particulate matter in sediment traps for both free and combined amino acids in order to determine the decompositional profiles for these amino acids with respect to depth. The amino acids were determined by derivatization with OPA and separation by HPLC. The amino acid fluxes were complemented with results on primary production, particulate organic carbon (POC) and particulate organic nitrogen (PON) flux and found to be interrelated. Results of the sediment trap material showed that the free amino acids decrease faster than the combined amino acids

due to the difference in utilization. They found these free amino acids to be similar in composition to those amino acids found in plankton. Amino acids undergo decarboxylation and deamination as major routes of metabolism in organisms to give an amine or an aliphatic acid as final products. Diacidic amino acids can undergo decarboxylation to form smaller molecular weight amino acids. Aspartic and glutamic acids decarboxylate to give β-alanine and γ-aminobutyric acid. Serine and threonine can undergo dehydration to form alanine and α-aminobutyric acid. All these products were found in the sediment traps, but whether or not the occurrence of these compounds is related to those reactions is not too clear. β-alanine and γ-aminobutyric acid are non-protein amino acids sometimes occurring as metabolic intermediates in organisms. Evidence from the concentrations of β-alanine show that this compound could be formed during sedimentation. The proportion of β-alanine increases with respect to depth, so if bacterial activity is responsible for the production of β-alanine and γ-aminobutyric acid, due to the higher temperatures and increased bacterial biomass at surface waters, the β-alanine is expected to be in higher concentrations at the surface and not at depth. This implies that within 6 - 12 hours bacteria do not affect the amino acid concentrations to any great extent.

1.4 CHROMATOGRAPHY THEORY APPLIED TO GRADIENT ELUTION OF THE FMOC-Cl DERIVATISED AMINO ACID SEPARATION

Reversed phase separations are used for distinguishing between relatively non-polar compounds, in our case the 9-fluorenylmethyl chloroformate (FMOC-Cl) derivatized amino acids, on a non-polar stationary phase composed of octadecyl silane (ODS) using a polar mobile phase. The more polar compounds will be eluted ahead of those less polar, as the latter favours the stationary phase more than the mobile phase.

1.4.1 Improving resolution in terms of the resolution expression

In order to selectively separate each of about 20 FMOC-Cl derivatized amino acids from each other, we can optimize certain instrumental and experimental conditions to improve the resolution. These conditions indirectly make up the terms of the common resolution expression which is important in discussing our basis for separation:

$$R_s = \frac{1}{4} \left| \frac{\alpha - 1}{\alpha} \right| \sqrt{N} \left| \frac{k'}{k' + 1} \right.$$

$$\qquad\qquad S \qquad\quad E \qquad\quad C$$

where S is the <u>selectivity term</u>, and α is the nett retention time ratio for two components separated and as calculated from the chromatogram (Snyder et al., 1979). Small changes in α can affect the resolution. In liquid chromatography the mobile phase

plays a major role in the thermodynamic distribution process of the components through selective interactions, in that each species will interact very differently as far as phase selectivity is concerned, where there is a slight variation in the mobile phase gradient.

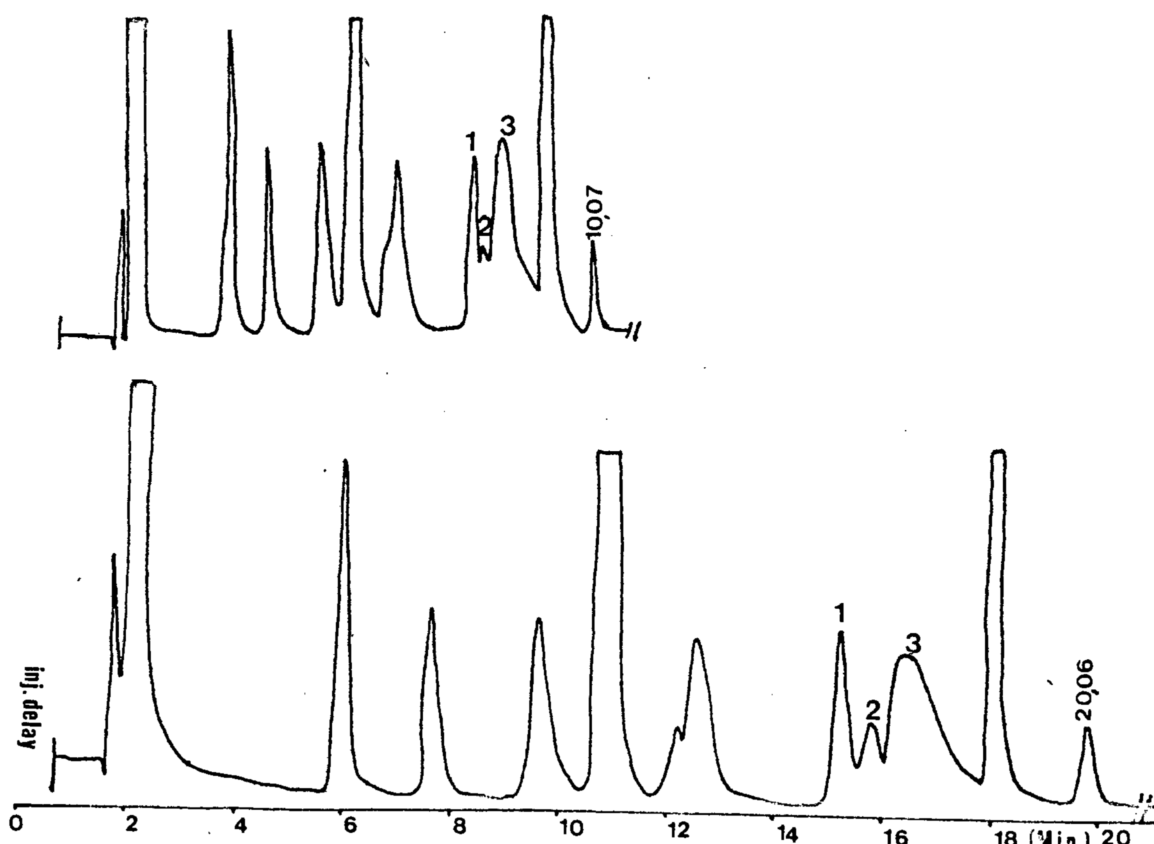


Fig. 1.1 and 1.2: Chromatograms showing the effect of different flow rates on the resolution. The flow rates are $2ml.min.^{-1}$ for the above chromatogram (1.1) and $1ml.min.^{-1}$ for the lower one (1.2). Gradient times are altered to accommodate this flow change.

N is the total number of theoretical plates of a column. Resolution is improved if N is increased, and this is achieved by reducing the flow rate or increasing the column length. Resolution changes when these parameters are altered, are marginal compared with other changes, such as mobile phase modification. The chromatograms

above show the marginal improvement in resolution achieved by reducing the flow by half. Note that the analysis time is in fact doubled.

Since the percentage composition at a particular time for each component will be different for the same gradient but different flow, the gradient has to be slowed down proportionately in order to have the same solvent conditions at any instantaneous time during separation. Failing this, resolution changes will be determined by the selectivity term as well as by N, and resolution changes due to flow alterations cannot be monitored. The gain in resolution by changing N (observe peaks 1 - 4 of each chromatogram to see a qualitative improvement in resolution) is offset by an increase in separation time and therefore a compromise must be reached between the resolution due to N and the analysis time. Increasing the flow rate with gradient elution can often improve resolution, provided none of the other parameters are changed. By increasing the flow rate, a larger volume of the weaker solvent passes through the column per time unit, effectively changing the selectivity term. The solvent composition in this case changes for each component along the column. This effect does not give marked resolution improvements, but only serves as a means to reduce the analysis time without sacrificing resolution.

C, the capacity factor, is influenced by the solvent strength of the mobile phase. The starting solvent used is normally chosen arbitrarily, depending on the type of separation required. For

reversed phase systems this solvent is usually water alone, or modified aqueous solutions (e.g. buffers). An increasing percentage of a water miscible organic solvent such as methanol, is then added to the starting solvent. This produces a solvent gradient.

The effect of solvent choice on resolution is seen Figs 1.3 and 1.4. The two organic solvents differing in polarities, affect different rates of elution. Holding the aqueous phase constant (reasons given later) and altering from methanol (fig. 1.3) to acetonitrile (fig. 1.4) gives marked resolution changes:

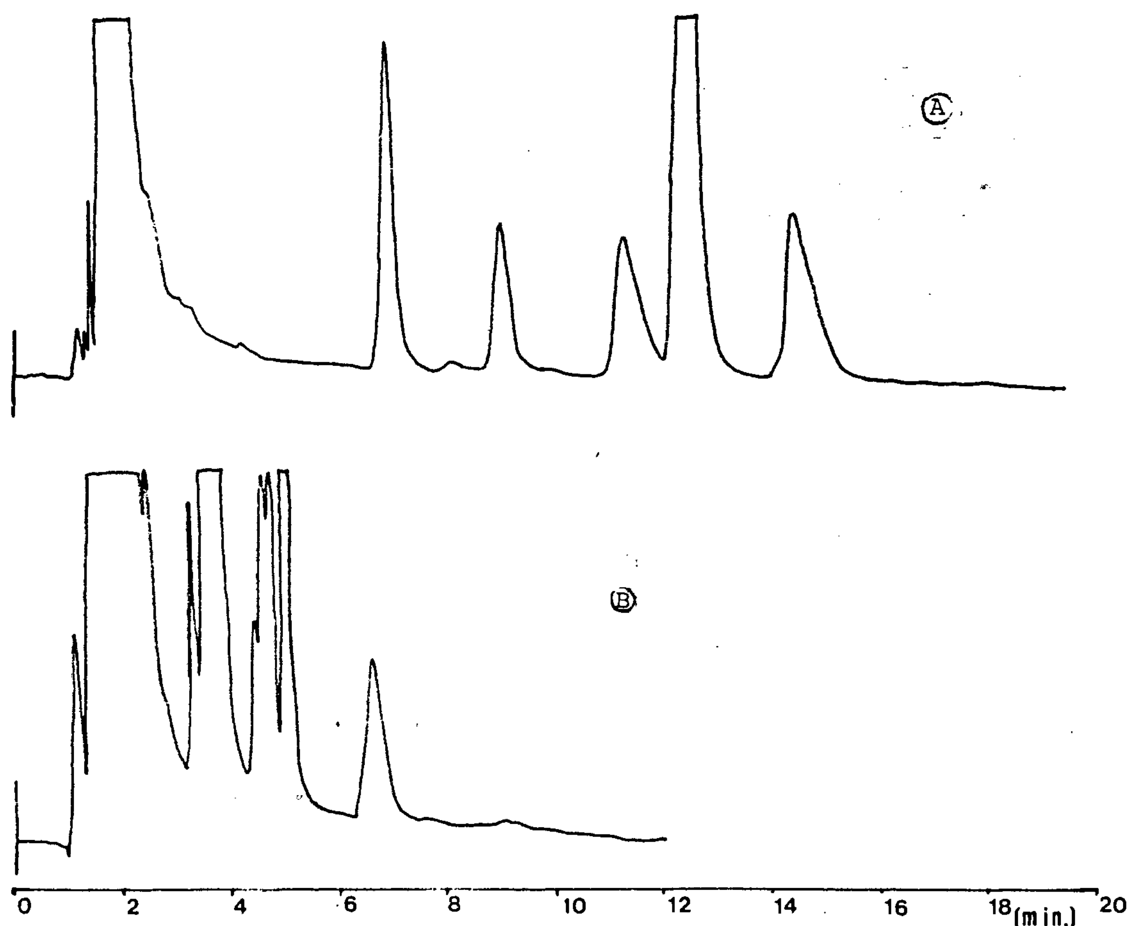


Fig. 1.3 and 1.4: Chromatograms of the separation of derivatized amino acids using different initial organic solvents. Methanol in above chromatogram, and acetonitrile in the one below.

The mobile phase is favoured by the eluted components in (B), hence the much reduced retention times. Acetonitrile is less polar than methanol and the relatively non-polar derivatives begin to favour the non-polar stationary phase as the mobile phase becomes more polar (i.e. from acetonitrile to methanol).

The most common gradient system used is a two solvent gradient beginning with a weaker (more polar in the case of reversed phase systems) solvent A and then continuously adding increasing concentrations of a stronger solvent B to the solvent entering the column, finally ending up with pure B as the eluant. The initial and final concentrations of a gradient need not necessarily be pure A or pure B respectively. What is important is the solvent program, or how the solvent composition varies with time to achieve a well resolved separation of a series of components.

1.4.2 A mathematical approach to gradient elution

A rigorous mathematical approach for the initiation of a reversed phase gradient elution program has been published (Snyder et al., 1979). From these guidelines a simple approach to an initial gradient separation can be planned.

Consider an isocratic run of FMOC-Cl amino acid derivatives compared with a separation one achieved by gradient elution.

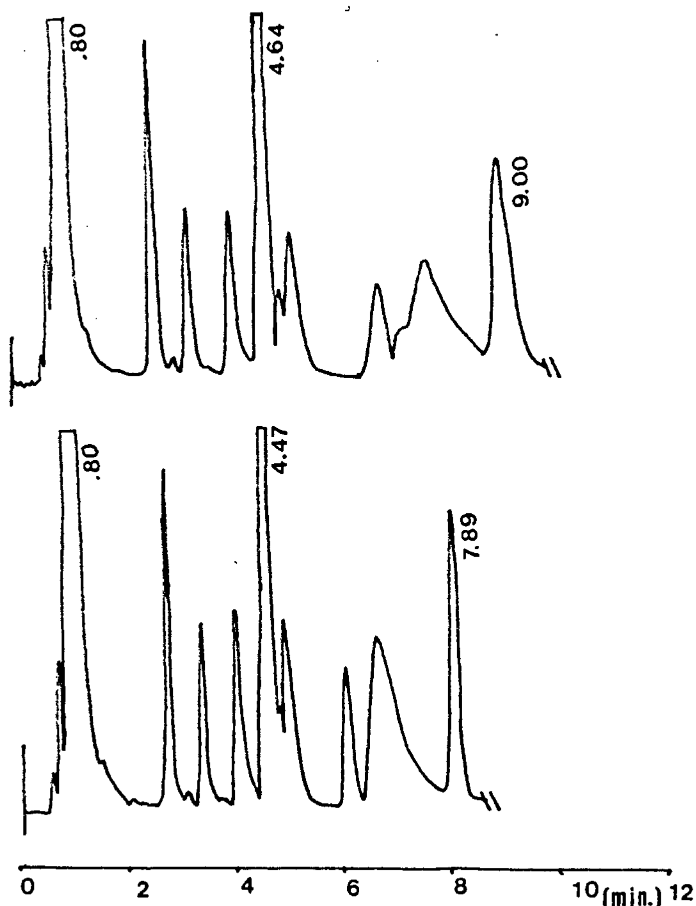


Fig. 1.5 and 1.6: Chromatograms showing the effects on the initial part of the chromatogram with isocratic and gradient elution having the same solvent conditions.

From Fig. 1.5 and 1.6, the first few peaks are of similar retention times. These peaks eluted before much change occurred in the mobile phase. Later in the programme, bands elute under essentially gradient type conditions. As stated before, the solvent program is the most important variable in gradient elution. A linear gradient (as opposed to a concave, convex or step-wise gradient) is the optimal theoretical gradient for separation (Snyder et al., 1979) and is termed the linear solvent strength program (LSS). The advantages of a LSS program are:

a) Approximately constant band widths for all the component peaks in the chromatogram exist. The band width of each peak is

affected by the normal broadening of the bands as they move along the column, and a "band compression" phenomenon arising from the faster migration of the tail of bands in gradient elution, compared to the uniform movement in isocratic elution. This is important in lowering the detection limit. Peaks become narrower due to more solute crossing the detection beam in less time resulting in a greater signal to noise ratio.

b) It gives comparable resolution for all the peaks, both those eluted early and those eluted later.

c) Regular spacing of the peaks occurs in the chromatogram.

d) It simplifies the interpretation of the separation process in terms of polarities, etc.

The "gradient steepness" (percentage organic phase increase per minute) should remain the same in a LSS program and be within limits depending on the nature of the solvents and the separation achieved.

The optimal gradient steepness can be calculated from equations given by Snyder et al. (1979) to give a percentage organic phase increase per minute for an initial separation. Improvements can be affected by other means once the gradient steepness has been calculated (e.g. initial and final solvent strengths and gradient duration).

It is recommended (Dolan et al., 1979) that the organic and aqueous solvents be pre-mixed by 5% water and 95% organic to avoid solvent

de-gassing and volume changes which occur when mixing pure solvents on line. Thus 0 - 100% organic gradients are actually 5 - 95% organic phases as programmed in the instrument. Since in the experimental work, the solvents were degassed by filtration on a Millipore vacuum filter and this pre-mixing step was not necessary. No problems due to solvent de-gassing occurred in this work.

1.4.3 The use of an aqueous buffer instead of water in elution

The starting mobile phase in reversed phase gradient elution is usually water. Because of the charged nature of some species in water, ionization is suppressed by driving the reaction towards a single uncharged species. This can be achieved by using a suitable buffer as the aqueous mobile phase, which will suppress ionization and decrease the polarity by providing a counter ion, enabling the compound to favour the stationary phase. Any hydrogen ions formed due to ionization of the species will react with the buffer and by this process the column will be protected from a too low pH upon each injection. The use of buffers in the mobile phase may cause quenching effects when fluorescence detection is used (Weinberger and Sapp, 1984).

1.4.4 The importance of gradient regeneration

Once the gradient has been set up it is important to re-equilibrate the column to initial gradient conditions before the next run. At the end of the gradient the column is saturated with a polar

organic phase and an immediate return to initial conditions will not re-equilibrate the column. This will occur only after running at the initial conditions for about 20 minutes (dependent on flow and difference between initial and final concentrations). The excess organic phase must be removed by decreasing the gradient in such a manner as to slowly unsaturate the column. A reverse gradient run for 10 - 15 minutes followed by a 10 minute run under initial conditions, is a generally accepted procedure (Dolan et al., 1979). If this is not done, each subsequent gradient run will increase the amount of polar organic phase on the column, resulting in decreasing retention times for each subsequent run.

Furthermore, if the final conditions of the gradient are unable to elute all compounds retained, each subsequent injection will elute these compounds on the column from the previous runs. After many samples the baseline will be affected and quantitative assessment will be in error. The solution would be to allow an almost pure final solvent composition (e.g. pure methanol) to wash the column free of strongly retained species. Thereafter, an adequate gradient regeneration would be required to return it to initial concentrations. This assessment became apparent while analyzing the low concentration seawater samples by fluorescence detection.

1.4.5 Simplified guidelines for achieving an initial separation

The procedure adopted in devising a suitable separation with regard to setting up a gradient was as follows.

1) Set the flow rate to the highest working flow, which is 2ml.min^{-1} on the instrument used due to a large back pressure. This is to reduce the analysis time since speed in achieving a suitable gradient is more important initially than is the improved resolution obtained from a low flow.

2) Start with 100% aqueous phase (0% organic) using a slightly less polar organic species than water and the more polar of the organic solvents commonly used. UV cut-off of the solvents must be measured when detecting at low wavelengths. For example, acetone as an organic modifier would be useless at a detector wavelength setting of about 260nm, since acetone has a UV cut-off at 330nm.

3) The gradient should be completed in about 20 minutes as an initial analysis time.

4) From the resulting chromatogram the initial and final solvent concentration differences can be reduced to improve elution if peaks are eluted near the end of a run. If the peaks are eluted beyond the end of the gradient, a more polar organic solvent must then be used. Once again, an adjustment of initial and final percentages can yield a well-separated chromatogram.

1.5 REVIEW OF THE HPLC TECHNIQUES USED TO SEPARATE AMINO ACIDS AND REASONS FOR CHOICE OF A TECHNIQUE

A review of past techniques showed (Section 1.3) that the major stumbling block in marine amino acid analyses was the non availability of an analytical technique free from the errors associated with sample clean up and desalting. Furthermore, the previous analysis times become too long for **in situ** type studies. HPLC techniques have been utilized in many ways for marine amino acid analyses. The aim was to select a technique which would meet the needs of simplicity and high sensitivity.

1.5.1 Precolumn or postcolumn HPLC?

The earlier HPLC systems were set up in a similar way to amino acid analysers, in that an ion exchange column was employed and a derivatizing reagent added by means of a pump between the column and detector. Amino acids alone are not readily measured by HPLC because of their high polarity and low response to ultraviolet or fluorescent detection, hence the need for derivatization to aid separation and allow detection. However, postcolumn derivatization systems suffer from several drawbacks:

1) Poor chromatographic performance is associated with these ion exchange columns (Umagat et al., 1982).

2) Complex eluant buffer systems are required.

3) Changes in the buffer gradient can cause a change in the baseline due to impurities in the buffers.

4) The dead volume of the instrument is increased with the inclusion of the postcolumn derivatizing stage. This results in a loss of peak resolution.

5) The postcolumn derivatizing stage dedicates the instrument to one type of analysis (Pfeifer and Hill, 1983).

6) Two columns are required to eliminate the loss of sensitivity and baseline fluctuations caused by impurities reacting with the reagent or having an absorbance at the detector wavelength setting.

7) Long analysis times, of the order of a few hours, and poor detection limits are often associated with this technique (Umagat et al., 1982).

8) Vigorous desalting techniques are required for seawater samples (Wilkinson, 1978).

A relatively new alternative to amino acid analysis utilizing HPLC is to employ precolumn derivatization and separation on reversed phase columns, and this is better, in most cases, than the

standard amino acid analyzer technique. This new development, eliminates the drawback of postcolumn derivatization, and is especially suited for marine applications, since:

1) A conventional HPLC instrument is used with no modifications and this frees the instrument for other types of analyses. Only minor changes are required and the instrument is operational within half an hour after the change (Pfeifer and Hill, 1983).

2) The technique has been successfully automated and can be taken on board ship for an **in situ** analysis.

3) Much shorter analysis times have been reported (typically 20 - 40 minutes) due to the increased flow rate.

4) Low detection limits at femtomole levels have been reported for some of these derivatizing reagents (Hill et al., 1979; Einarsson et al., 1983).

5) No sample cleanup other than filtration is necessary. Salts can be injected onto the column with no chromatographic band spreading (Lindroth and Mopper, 1979). This has also been tried for ion exchange resins using seawater with some success (Garrasi et al., 1979). It has been shown by Lindroth and Mopper (1979) that a seawater sample can be injected directly onto the column after filtration and derivatization. The

highly polar salts are not retained. To allow low concentration to be detected the injection of larger volumes (± 700 µl) of sample can be achieved. This results in a preconcentration of the solutes occurring at the top of the column. This preconcentration process can be utilized for trace analysis (Schauwecker et al., 1977). However, complex matrices such as physiological fluids may contain compounds which build up at the top of the column, therefore requiring a guard column between injector and main column.

1.5.2 Factors affecting the choice of derivatizing reagent

An ideal derivatizing reagent would have to fulfill certain prerequisites, but to date no reagent has been developed that fulfills them all. Recent techniques (Chang et al., 1981; Heinrikson and Meredith, 1984; Einarsson et al, 1983) are the closest approach to the ideal, and choice of reagent is dependent on these important requirements:

1) The derivatives must be stable.
2) The reaction must proceed rapidly.
3) Reagent should be able to react with all the amino acids.
4) The reagent itself should not fluoresce or absorb significantly.
5) A low detection limit is required.
6) The derivatization must be both reproducible and quantitative.
7) Reagent and amino acid should react to give only one product and hence only one peak.

In seawater analysis, it is most important to have a low detection limit, since expected concentrations are low. Stable derivatives and good reproducibility are also important.

1.5.3 Precolumn derivatizing reagents

Each past technique was characterized by the derivatizing reagent employed. The merits and drawbacks of these reagents will be discussed.

1.5.3.1 Ninhydrin

This classical postcolumn derivatizing reagent is not used in precolumn derivatization due to its low sensitivity. It reacts with primary and secondary amino acids yielding derivatives absorbing strongly at 570nm and 440nm - only one reaction product is formed. For complete amino acid assessment, 2 detectors or one variable wavelength programmable detector is required (Pfeifer and Hill, 1983).

1.5.3.2 Fluorescamine

This reagent is used for postcolumn detection with the amino acid analyzer developed from the chemistry of the ninhydrin-amino acid reaction. It provides a 10 - 100 fold increase in sensitivity over ninhydrin, but its major drawbacks are that 2 postcolumn pumps are required and that the reagent does not react with secondary amino

acids. The reaction products are not stable in the eluant buffer (Pfeifer and Hill, 1983). Reaction is rapid and quantitative, but the two reaction products formed are bulky. The derivatives are detected by a fluorescence detector.

1.5.3.3 Dansyl Chloride (DNS-Cl)

The use of dansyl chloride (DNS-Cl) as a derivatizing reagent in precolumn derivatization for the detection of amino acids has been reported by many workers (Wilkinson, 1978; De Jong et al., 1982; Oray et al., 1983). A typical DNS-Cl procedure entails preparation of the derivative in acetonitrile, with a borate buffer (pH = 9,5) added in the ratio of 1:2. The reagent is added to the amino acid sample (optimum ratio between 5:1 and 10:1) and left to react for 60 min. at 37°C, or for 10 min. at 65°C. It can now be injected or stored at -40°C until analysis is required. The reaction can be terminated by addition of methylamine (De Jong et al., 1982). The elution solvents used are normally variants of acetonitrile as one solvent and a buffer system as the other, dependent on which DNS-Cl procedure is adopted. Generally 2 columns are employed. The reagent does not give a very high quantum yield. The reagent and reaction byproducts fluoresce, and the technique subsequently requires their removal. This is further hampered by not having sufficient specificity on removal. A good sensitivity is lacking due to quenching effects of the aqueous buffer employed in elution. Difficulty exists in forming single reaction products reproducibly (Pfeifer and Hill, 1983), derivatizing at low concentrations and

reacting with samples in complex matrices, such as seawater (Gonzales, 1983), and physiological fluids (Lindroth and Mopper, 1979; Einarsson et al., 1983).

1.5.3.4 **Phenylthiohydantoin (PTH)**

This reagent is one of the earlier substances used for precolumn derivatization and the procedure is again time-consuming, limiting its usefulness for routine amino acid analysis (Pucci et al., 1983). It is used for the analysis of Edman degradation products as generated by peptide sequencing. It requires reaction with HCl and ethanethiol at 80° for 10 minutes. Although a very short analysis time of 14 minutes has recently been achieved (Pucci et al., 1983), the column has to be thermostatted at 35°C which may cause column bleed. Detection is by a UV detector set at 245nm, but may be subject to interferences in biological matrices. This procedure has a good reproducibility as its main advantage. It suffers from similar disadvantages to those associated with dansyl chloride.

1.5.3.5 **Orthophthalaldehyde (OPA)**

This was originally used as a postcolumn derivatizing reagent but is now used extensively for precolumn derivatization (Mell et al., 1978; Hill et al., 1979; Lindroth and Mopper, 1979; Gardner and Miller, 1980; Jones et al., 1981; Lookhart et al, 1982; Umagat et al., 1982; Hodgin, 1979; Fleury and Ashley, 1983). This non-

fluorescencing reagent reacts with primary amines in the presence of a reducing agent such as ethanethiol or 2-mercaptoethanol to give fluorescent derivatives. The choice of reducing agent is dependent on a variety of factors: Reduction with ethanethiol increases the stability of the derivative and if reacted in 95% ethanol, the relative fluorescence increases by about 60% compared with an increase of about 4% using 2-mercaptoethanol (Hill et al., 1979). The unpleasant odour of ethanethiol makes this reagent unpopular. This reaction proceeds within 1 minute at room temperature and has a detection limit of 50 femtomoles (Lindroth and Mopper, 1979). This technique has gained wide-spread support in many applications, particularly biochemistry (Mell et al., 1978; Jones et al., 1981; Lookhart et al, 1982; Fleury and Ashley, 1983), and marine chemistry to a lesser extent (Lindroth and Mopper, 1979; Gardner and Miller, 1980; Lee and Cronin, 1982; Lee et al., 1983). The important advantages are offset by some disadvantages. The fluorescence of the derivatives is not stable with respect to time and serious fluctuations can occur within a few minutes. This is eliminated by injection of the derivatized amino acids after the lapse of exactly one minute. This obviously reduces the reproducibility from one analysis to the next, but can be eliminated by the introduction of an intricate automation system (Hodgin et al., 1983; Fleury and Ashley, 1983). This is ideal for work on board ship where the reagent can be added to filtered sea-water directly by an automated system, and the analysis performed without subsequent clean-up. By choice of reducing agent and controlling certain conditions the stability is reproducible for a few

hours (Hearn, 1982). Another serious disadvantage is that secondary amino acid such as proline and hydroxyproline, and also cystine and cysteine to some extent do not form derivatives with OPA. However, this can be overcome by an oxidation step using performic acid for the sulphur amino acids and reaction with a suitable reagent, such as 4-chloro-7-nitrobenzofuran, for the secondary amines. The sensitivity of lysine and hydroxylysine can be improved by preventing their decomposition (Umagat et al., 1982). This is time consuming and offsets the initial advantage of ease and simplicity of the precolumn technique. Also, difficulties in quantitation may occur due to the use of quenchers (Heinrikson and Meredith, 1984).

1.5.2.6 Dimethylaminoazobenzenesulphonyl chloride (DABS-Cl)

Few applications of this method have appeared, although the method was developed some time ago (Chang et al., 1981, 1983).

The sample is evaporated under vacuum and the dried residue is dissolved in 10 µl of 0,2M sodium bicarbonate buffer, pH 9,0. 20 µl of DABS-Cl solution is added. The sample is heated at 70°C for 10-15 minutes after which about 300 µl of 70% ethanol is added, and the sample then injected. The reagent is hydrolyzed to DABS-ONa during derivatization and although this absorbs, it is fortunately eluted ahead of the amino acid peaks. The lengthy derivatization procedure and harsh reaction conditions reduce the possibility of using this reagent for simple routine analyses. A

good feature of this technique is that it has a low detection limit (0,5 - 1 pmol) using absorbance detection wavelength of 436nm. This reagent reacts with all the amino acids including proline and hydroxyproline. Furthermore, the derivative is stable and can be kept at -20°C for one year (Chang et al., 1983). It has been stated that this technique may suffer from the difficulties associated with dansylation (reaction of amino acids with dansyl chloride) in quantitative modification.

1.5.3.7 Phenylisothiocyanate (PTC)

This reagent has been used as a derivatizing reagent by Heinrikson and Meredith (1984), and has been used for protein sequencing using the Edman degradation scheme in biochemical studies. No marine applications have appeared, but the time consuming and complex procedure involved will certainly limit any possible routine marine analysis. The reagent itself fluoresces, and it and the reaction by-products are volatile enough to be evaporated off. Derivatization is complete in about 5 minutes. The column and injection port need to be thermostatted at 52°C which may affect the column life. Detection is by absorbance set at 254nm and the limits of detection are at the picomole level. The derivatization is complete for all amino acids, including proline, and is stable enough not to require in-line derivatization and hence the reproducibility is good.

1.5.3.8 9-Fluorenylmethyl chloroformate (FMOC-Cl)

This is a recent technique for precolumn derivatization developed by Einarsson et al. (1983). Its major advantage is that the derivatives are very stable and can be stored for about two weeks at room temperature before any significant breakdown occurs. The detection limit is very low, in the low femtomolar range when using fluorescence detection, but UV detection at 260nm is possible. It is characterized by a short reaction time of 40 seconds, and reacts with all the amino acids. The short reaction time certainly makes this reagent a viable alternative to the now common OPA technique. To 0,4ml of sample, 0,1ml of borate buffer (pH = 7,7) and 0,5ml of the reagent is added. After about 40 seconds the mixture was extracted twice with 2ml pentane to remove the excess reagent before it reacted significantly with the water. Effectiveness of removal of the reagent has been shown to be reproducible, but the possibility exists of removing derivatized amino acids as well. Because of these advantages and limited knowledge of any disadvantages, it was decided to develop and expand this technique for our purposes and to explore the characteristics and errors of this method.

1.5.4 Other reagents and some reviews

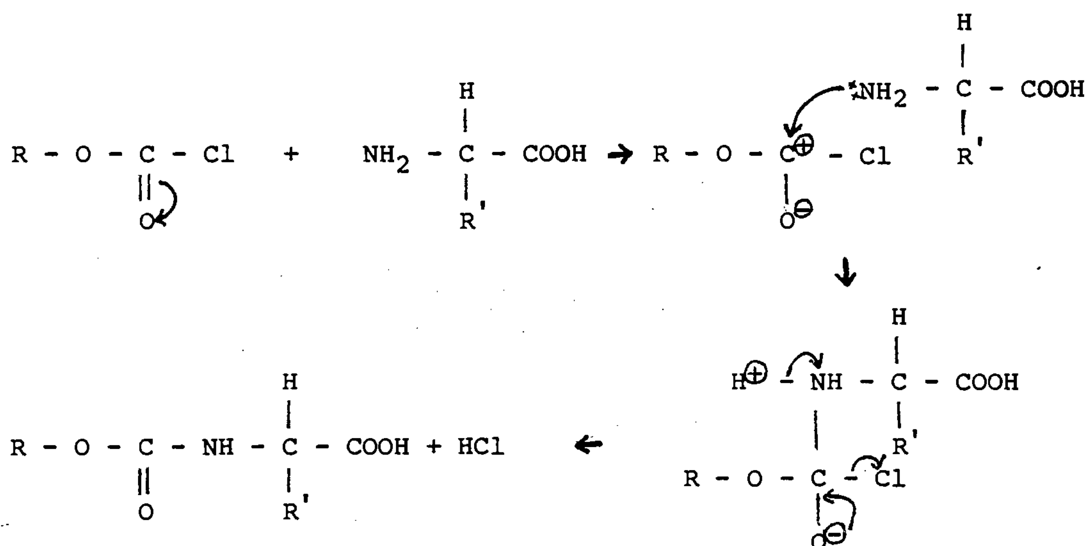
A recent review by Pfeifer and Hill (1983) of the state of the art of both post- and precolumn derivatization has been published. As can be expected, the more recent techniques are not mentioned, and

therefore this review is a bit dated. A good comparison of the workings of ion exchange and reversed phase strategies has been given, and also the reasons for choice of technique. Other reagents pertaining to Edman degradation and biochemical studies can be found in this review. Others such as dimethylaminoazobenzenethiohydantoin (DABTH) (Yang and Wakil, 1984) and phenylisothiocyanate (PTC) (Heinrikson and Meredith, 1984) are recent developments in biochemical studies not mentioned in this review. These have been discussed at some length in articles in the amino acid analyses by HPLC handbook by Hancock (1984). Here discussions of all forms of separation of amino acids and peptides have been given, together with a wide variety of practical applications. Both volumes are very comprehensive but have not reviewed the FMOC-Cl technique, possibly due to it being a later development.

1.6 WHY FMOC-Cl? - ITS ORIGIN AS DERIVATIZING REAGENT

The reaction of chloroformates with amino acids has been used both for the protection of the amine group during peptide synthesis (Matzner et al, 1964; Carpino and Han, 1972; Atherton et al, 1978a) and for analytical determination of primary and secondary amino acids (Anson Moye and Boning, 1979; Einarsson et al, 1983).

The general mechanistic chloroformate-amino acid reaction proceeds as follows:



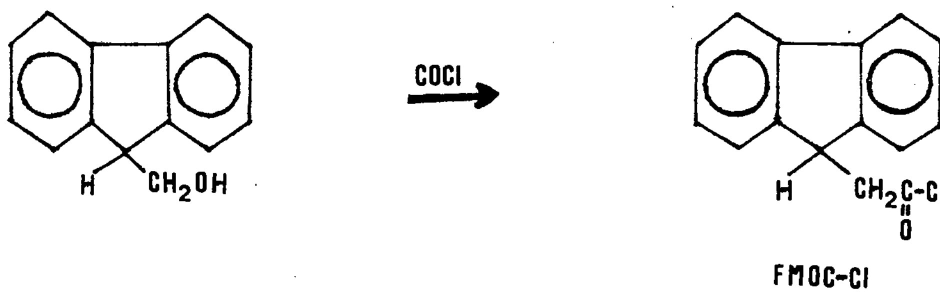
This condensation reaction illustrates the greater reactivity of the amine group towards the chloroformate rather than a carboxylic acid group of the amino acid, i.e. this reaction is favoured over peptide linkage (Matzner et al., 1964).

For analytical purposes we require a chloroformate that can easily

be detected, either by fluorescence or by UV absorbance, and the end product must be stable. The emergence of 9-fluorenylmethyl chloroformate (FMOC-Cl) as an amino acid labelling compound came about not from analytical necessity but, as mentioned before, as an amino protecting group during peptide synthesis.

1.6.1 The use of FMOC-Cl as an amino protecting group

9-Fluorenylmethanol, reacted with phosgene to give the chloroformate below:



This is a stable compound reacting with amino compounds to give carbamates in yields of 88 - 97% (Carpino and Han, 1972).

The amino protecting groups most commonly used, such as t-butoxycarbonyl (BOC) amino acids, are deblocked under various acidic conditions. FMOC-Cl as protecting group is stable towards acids and catalytic hydrogenation, but cleaved readily under mildly basic non-hydrolytic conditions. Liquid ammonia, piperidine or ethanolamine are used for deblocking (Carpino and Han, 1972).

FMOC-Cl has also been used for solid phase peptide synthesis using polar resins and mildly basic conditions for the final peptide cleavage (Atherton et al., 1978b). With conventional solid phase synthesis using t-BOC as protecting group, the vigorous acidic conditions, both during synthesis and cleavage from the solid support, cause the formation of major by-products of the synthesised peptide. Using FMOC-Cl as protecting group it was found that only one peak - the peptide product - was found by HPLC separation of end products after synthesis demonstrating the stability due to the mild basic conditions.

1.6.2 The separation of FMOC-amino acid-like compounds

It was found that chromatographic resolution of amino acid-like compounds by an ion exchange liquid chromatography is improved by reaction with FMOC-Cl. This produces a fluorphore with a large quantum yield which is unaffected by the different solute-solvent interactions (Anson Moye and Boning, 1979). The compound is dissolved in acetone and then added to the amino acid solution together with pH 9 borate buffer. This solution is incubated at 23° for 20 minutes and excess reagent is removed by an ethyl ether wash (Anson Moye and Boning, 1979). Since the carboxylic acid functional group does not react with the chloroformate, separation was achieved using anion exchange chromatography. Resolution could have been improved by ensuring more complete ionization of the carboxylic acid functional group by using a higher pH in the mobile phase i.e. pH 6.

Complete separation of FMOC-Cl derivatized amino acids on reversed phase HPLC was achieved by Einarsson et al. (1983). The reagent was dissolved in acetone and borate buffer pH 7,7 was added to the aqueous amino acid solution. The reaction is complete after 40 seconds, thereafter extraction with pentane removes the excess reagent before it has time to react with water. The chloroformate reacts with water to form a hydrolysis product peak which may swamp nearby amino acid peaks in the chromatogram. Separation was by gradient elution and the eluant varied linearly from acetonitrile - methanol - acetic acid buffer (10:40:50) to acetonitrile - acetic acid buffer (50:50) over 9 minutes. The gradient started 3 minutes after injection. Flow rate was at 1,3ml.min^{-1} and was increased to 2ml.min^{-1} after 12 minutes (Einarsson et al., 1983).

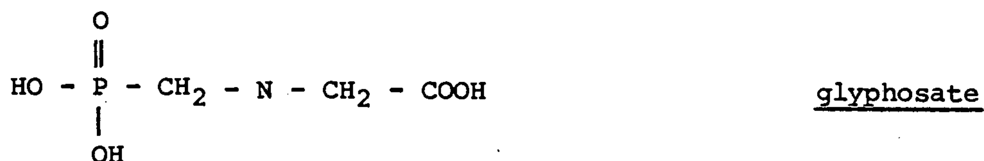
The problem with the derivatization step was that extraction with pentane removes some of the less polar amino acid derivatives from the aqueous solution.

1.6.3 Some reactions of FMOC-Cl

Reaction of amino acids with FMOC-Cl is rapid in slightly basic solutions. Reaction of tryptophan with FMOC-Cl, sodium bicarbonate solution and dioxane, after 4 hours in an ice bath and 8 hours at room temperature, gave a recrystallized product with a yield of 91% (Carpino and Han, 1972). Glycine and leucine reacted respectively giving yields of 88% and 90% after 2 hours reaction at room tem-

perature (Carpino and Han, 1972). These long times are to ensure complete reaction and are unnecessary when the reaction goes to completion in a few minutes, depending on the excess of reagent.

Reaction of FMOC-Cl and glyphosate show that the chloroformate bonds to the nitrogen rather than the carboxylic acid group:



Monitoring the reaction of FMOC-Cl and glyphosate with respect to time, Anson Moye and Boning (1979) found that the reaction was complete in about 5 minutes at room temperature using a sodium borate buffer of pH 9.

Simple derivatization using a borate buffer of pH 7.7 allows the reaction to proceed within a minute (Einarsson et al., 1983), but it has been found to be dependent on the concentrations of each species involved. Kinetic studies of this reaction were undertaken during this work and are elaborated on later (Section 2.1.7).

1.6.4 The absorbance spectra of FMOC-Cl

FMOC-Cl absorbs well in the UV region, but has even better fluorescent properties which are ideal for detection. Excitation and

emission wavelengths are quoted as 260nm and 313nm respectively for fluorescence detection. The UV-absorbance spectrum of FMOC-Cl in methanol is shown below.

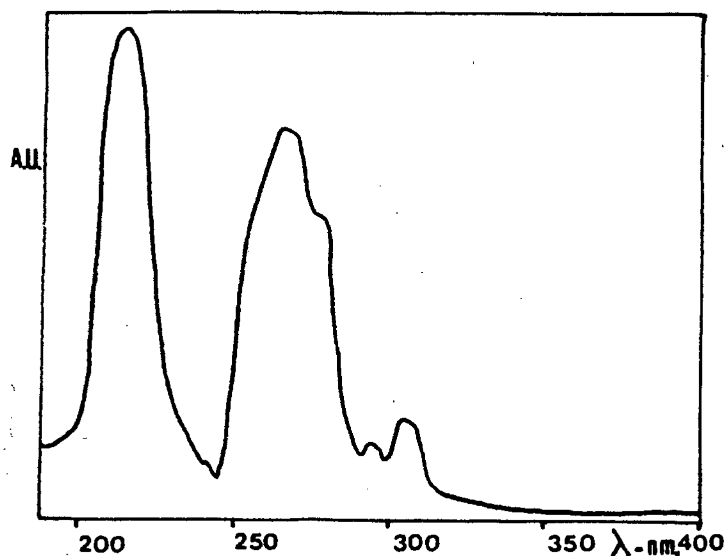


Fig. 1.7: UV-spectrum of 9 fluorenylmethyl chloroformate in methanol.

Although the reagent is dissolved in acetone for the derivatizations in the study, the UV-cutoff of acetone makes this solvent unsuitable for use when observing the spectra of FMOC-Cl below 330nm. Observing the chromatogram of the reagent alone shows peaks due to contaminated acetone reacting with the reagent. Dissolved in methanol, the spectrum is clear and the chromatogram of the reagent in methanol only gives one peak:

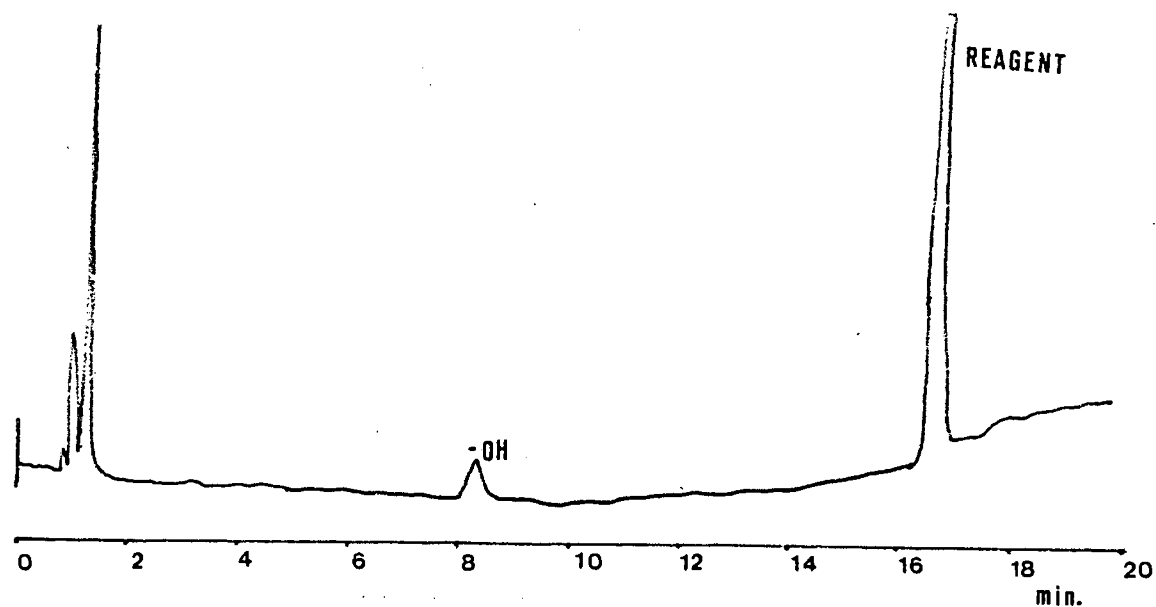


Fig. 1.8: Chromatograph of reagent in methanol

The unknown peaks at the start of the chromatograph could well be solvent impurities, but this is unknown.

1.7 THE EXPERIMENTAL APPROACH OUTLINED

The significance of an amino acid assessment in the marine environment and the errors associated with existing analytical techniques has prompted the development of this work. This is demonstrated from the review of previous amino acid analyses (Section 1.3). The choice of an adequate analytical HPLC technique was dependent on the assessment of the precolumn derivatizing reagents employed. The experimental work will involve the development of the existing FMOC-Cl technique (Einarsson et al., 1983) and the further assessment of certain aspects of the derivatization procedure.

The application of the method to a real-life marine system has come about from work being done on nitrogenous release from kelp (Brauer, 1986). Other chemical and bacterial profiles should complement the amino acid results and in so doing should demonstrate the feasibility of the analytical technique developed. Furthermore, the results can give a thorough interpretation of the processes of exudation from kelp and the subsequent uptake by bacteria.

2. EXPERIMENTAL METHODOLOGY

2.1 ANALYTICAL HPLC TECHNIQUE

2.1.1 Reagents, procedures and equipment

The method used to obtain a separation of amino acids through precolumn derivatization was based on the published procedure of Einarsson et al. (1983). The modifications and further testing carried out during the course of the study are discussed in detail below.

2.1.1.1 Chemicals and Reagent preparation

Reagents were prepared according to the procedure by Einarsson et al. (1983).

The derivatizing reagent was prepared by adding 388mg of 9-Fluorenylmethyl chloroformate (Fluka Chemicals) to 100ml of Analar® acetone (BDH chemicals). This gave a concentration of 15 mmol.dm^{-3}. This was then stored at -20°C.

Borate buffer was prepared by using 1M boric acid solution prepared from boric acid (Holpro chemicals). This solution was adjusted to pH 6,2 with 1M sodium hydroxide (Laschem) solution. This solution was then diluted five times with Milli Q water (Millipore), which was used in all preparations. This gave a pH of 7,7. This buffer

was used in the derivatizations.

The acetic acid buffer used as aqueous mobile phase was prepared by adding 9ml of glacial acetic acid (Laschem) and 3ml of triethylamine (Merck) to $3dm^{-3}$ of water. The pH was adjusted to pH = 4,20 with 1M sodium hydroxide solution.

2.1.1.2 Chemical procedures

For derivatizations, the amino acid standards (Sigma Chemicals) and samples were derivatized initially according to the procedure of Einarsson et al. (1983). 0,5ml of FMOC-Cl reagent and 0,1ml of borate buffer solution were added to 0,4ml of sample. After a minute the sample was extracted twice with 2ml pentane (Riedel-de Haen AG). This procedure was later disregarded and only 0,1ml of reagent and 0,1ml of borate buffer solution were added to 0,4ml of sample. The pentane extraction stage was omitted. Reasons for these changes are explained in a later section.

In order to determine the total combined amino acid (TCAA) fraction in various samples, the following hydrolysis procedure was adopted. 20 ml of 6M hydrochloric acid solution (AR, Pal chemicals) was added to 5ml of seawater sample (or 1g of solid sample) in a 100ml conical flask and heated in a Heraeus RT360 oven for 24 hours at 120°C. 1ml of this hydrolyzed sample was added to 20ml of water and the pH adjusted to ph = 7,7.

2.1.1.3 Instrumentation

The HPLC system used was constantly varying during the course of this work, as and when more equipment became available.

System 1: The initial separatory procedures and gradient development were achieved using a Varian 5000 liquid chromatograph coupled to a Varian UV-100 detector and Hewlett-Packard 3390A integrator/ recorder. A 10 μl sample injection loop was used and an Altex Ultrasphere ODS column, with 5 μm packing and an internal diameter of 4,6mm was used.

System 2: The reaction kinetics, reproducibility and other aspects of the derivatization procedure were carried out using a Beckman HPLC system, comprising of two model 112 pumps, a 340 organizer, a 420 controller, coupled to a Beckman model 165 variable wavelength detector. The recorder used was a Kipp and Zonen BD40 chart recorder, resulting in quantitation by peak heights. A 20 μl injection loop was used which alternated with a 100 μl loop for lower concentration samples. Columns used were the Altex 15cm column, then a 25cm Altex Ultrasphere ODS column, with 5 μm packing and an internal diameter of 4,6mm , when the resolution of the first began failing (see pg.78).

System 3: The exudate-uptake study samples were run on the Beckman HPLC system coupled to a Drew Scientific Chromatography interface and controlled by an Apple II computer with two disc drives, for

both gradient control and peak integration. This was coupled to an Epson FX80 printer. The detector used was a Perkin-Elmer 650-S fluorescence detector with an excitation wavelength set at 269nm and the emission wavelength set at 304nm for optimal results. Bandpass was 10nm. This detector was linked to the Kipp and Zonen recorder. A 20 µl injection loop was used and separation was achieved using a 7cm Altex Ultrasphere XL-ODS column with 3 µm packing and internal diameter of 4,6mm. The column was encased in a cartridge to accommodate the replaceable pre-column.

There was a simultaneous progression of work achieved and equipment acquisitioned for each HPLC system. The third system was used for the field study only.

Other HPLC related equipment used from time to time was a Knauer UV-Fluorezenz-detektor (fluorescence detector). This was not sensitive, due to the use of filters and a mercury lamp which are not ideal for near-UV work. Various recorders used were a Pedersen MR37 and a Perkin-Elmer recorder.

2.1.1.4 Apparatus

Derivatizations were originally done in 7ml glass scintillation vials with plastic screwcaps. These were later replaced by 6ml glass vials with plastic press-caps (see section 2.1.8). Volumes of sample, reagent and buffer were controlled using two 5000 Pipetman® adjustable volume pipettes (Gilson), one set at 0,4ml

and the other at 0,1ml. The plastic pipette tips (C200 polypropylene tips) were replaced after each sample, to prevent cross-contamination.

pH of the buffers and hydrolysed samples prior to derivatization were accurately assessed using a Radiometer PHM 84 research pH meter.

Injections were achieved using a Hamilton 10 µl syringe when using the 10 µl injection loop and a 100 µl syringe when using the other injection loops.

2.1.1.5 **Gradients**

Various gradients were developed for each column and the most effective in terms of separation and analysis times were chosen. Gradient development is discussed in sections 1.4 and 2.1.2.2.

Gradient 1: Separation was achieved using the Altex 15cm column. Flow rate was $2ml.min^{-1}$ and the remaining percentage mobile phase made up by the acetic acid buffer:

0 - 5 minutes, 55% Methanol
5 - 12 minutes, 55 - 90% Methanol
12 - 18 minutes, 90 - 55% Methanol

Separation from this gradient can be seen from fig. 2.15.

Gradient 2: Separation was by using the Altex 25cm column. Flow rate was 2ml.min^{-1}. Remaining percentage composition again made up by the acetic acid buffer:

0 - 5 minutes, 55% Methanol

5 - 8 minutes, 55 - 63% Methanol

8 - 16 minutes, 63 - 70% Methanol

16 - 21 minutes, 70 - 100% Methanol

21 - 25 minutes, 100 - 55% Methanol

The separation from this gradient can be seen from fig. 2.16.

Gradient 3: Separation was by using the 7cm Altex cartridge column. Flow rate was 2ml.min^{-1}. Aqueous phase remaining was again acetic acid buffer:

0 - 2 minutes, 40% Methanol

2 - 12 minutes, 40 - 60% Methanol

12 - 13 minutes, 60 - 100% Methanol

13 - 16 minutes, 100% Methanol

16 - 18 minutes, 100 - 40% Methanol

The separation from this gradient can be seen from fig. 3.4.

2.1.2 Initial chromatography procedures

2.1.2.1 Solvent Problems

Any solvent used for the mobile phase and which contains any absorbing impurity, will cause large baseline changes when the solvent gradient is changed. An example of baseline increase is shown by comparing an isocratic and gradient run of the same derivatized sample (Fig. 2.1).

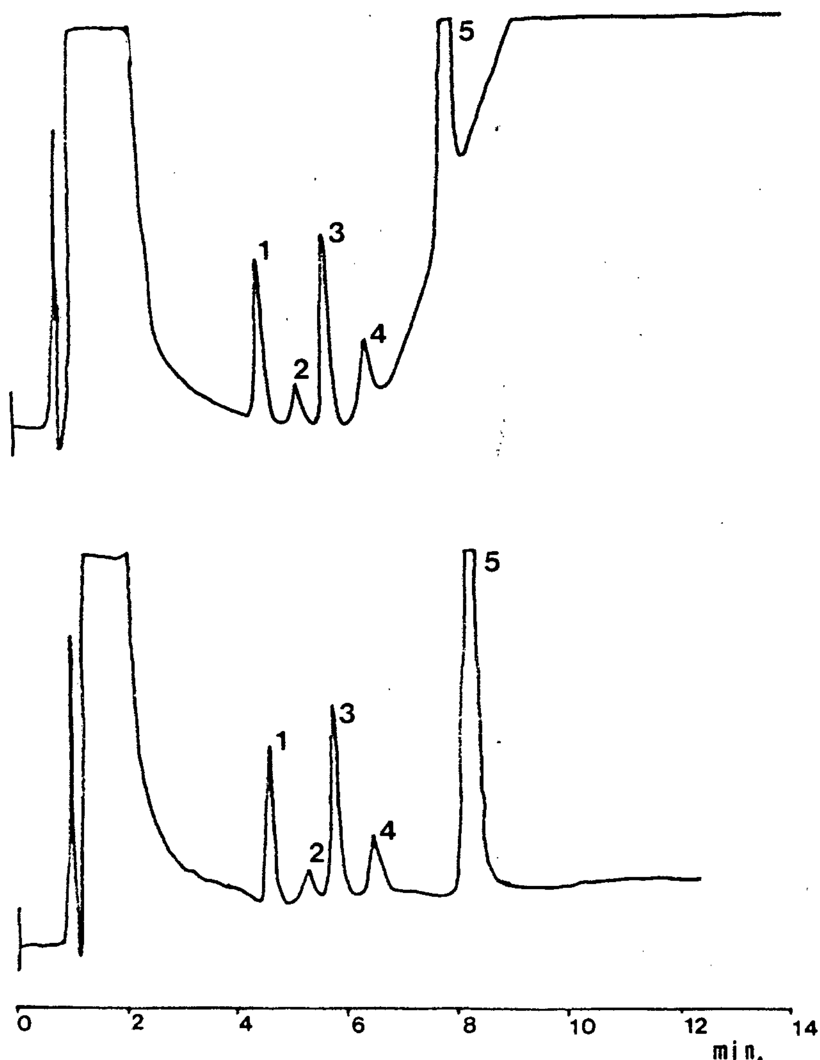


Fig. 2.1: Gradient run (top) and isocratic run (bottom) of 5 amino acids with contaminated mobile phase. Numbers correspond to the following: 1 - Asparagine; 2 - Aspartic acid; 3 - Serine; 4 - glutamic acid; 5 - glycine.

Initial organic solvents used in the separation were acetonitrile and methanol. This baseline increase was found to occur using reagent grade acetonitrile. The purity of these solvents, together with the acetic acid aqueous mobile phase, was checked by scanning the wavelengths close to that wavelength used for detection. These UV-absorbance spectras can be seen below:

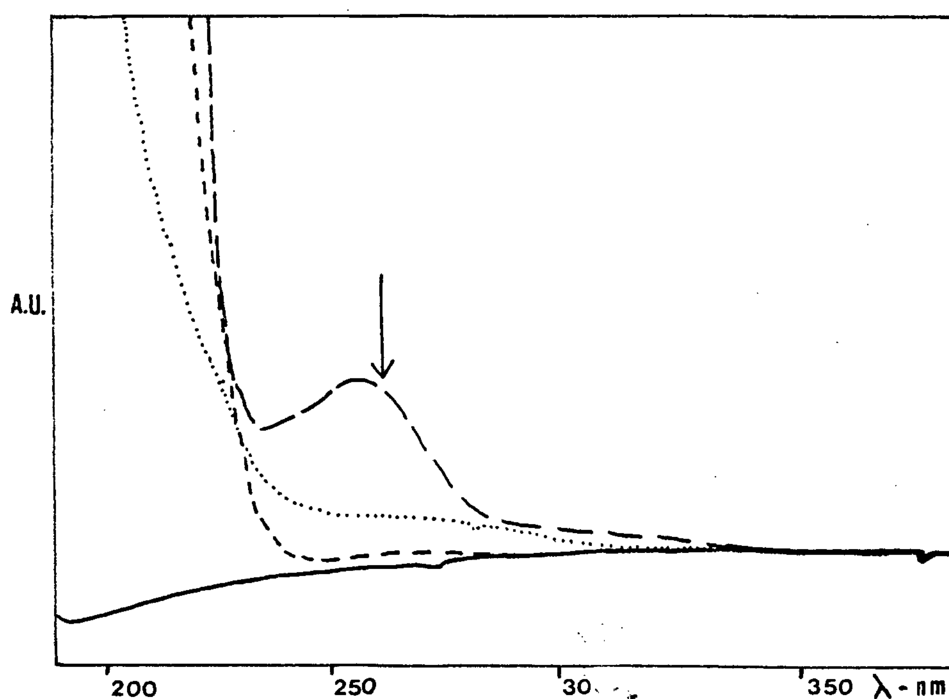


Fig.2.2: UV-absorbance scans of the solvents used in elution. Vertical arrow indicates the detector wavelength setting (265nm). Type of line corresponds to following solvents: bold line = water; broken line = buffer; dotted line = methanol; longer striped line = acetonitrile.

The normal spectrum of acetonitrile should resemble that of the buffer. This peak at 265nm is due to the unknown impurity. The difference in absorbance between acetonitrile and the buffer leads to this baseline increase.

It was decided to redistill the acetonitrile in an attempt at

purification. These attempts met with no success, since the impurity apparently distilled over, since it has a similar boiling point to acetonitrile. Passing the acetonitrile through a column of alumina in an attempt at purification, did not work. According to McCowan et al. (1984), batches of acetonitrile from various manufacturers all contain common absorbing impurities, the identity of some of which are unknown. Those known to be present are some imides, acetamide and ammoniacal nitrogen. A large amount of the impurities in analytical reagent grade acetonitrile react with FMOC-Cl giving many spurious peaks near the end of the chromatogram. This seen by comparing chromatograms of reagent in analytical grade and HPLC grade acetonitrile:

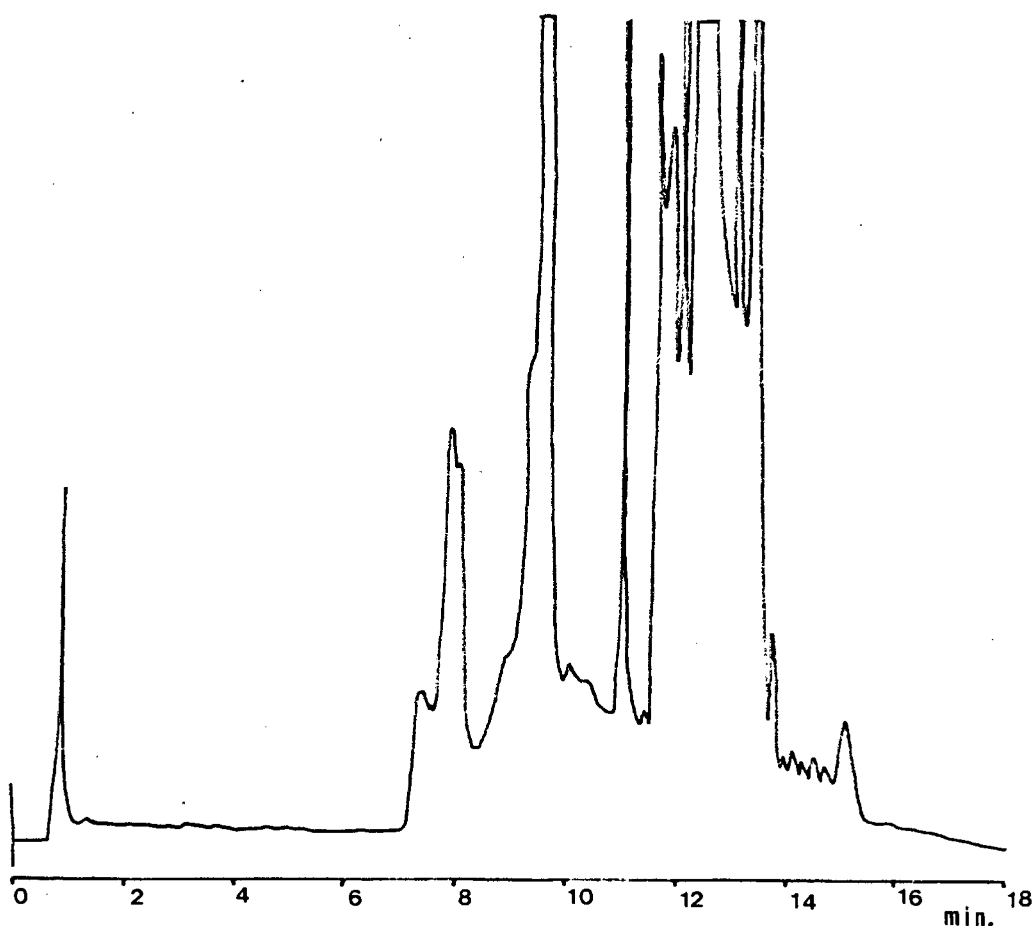


Figure 2.3: Chromatogram of FMOC-Cl dissolved in analytical reagent grade acetonitrile.

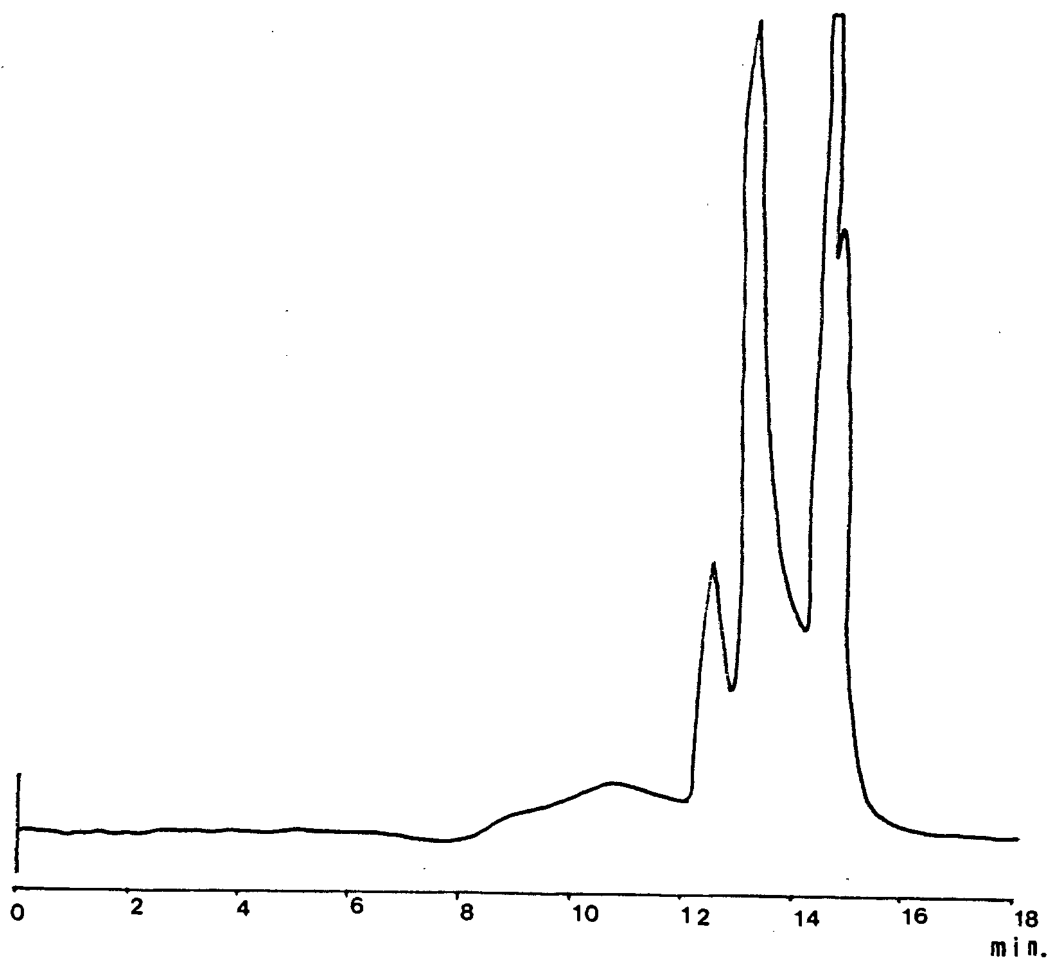


Figure 2.4 Chromatogram of FMOC-Cl dissolved in HPLC grade acetonitrile.

To overcome this baseline increase problem especially when working with low concentration samples, "HPLC grade" solvents were used throughout all separation (Section 2.1.1). UV-absorbance scans of HPLC grade acetonitrile and methanol, compared to the scan of the buffer, showed no nett spectral increase near the detector wavelength setting.

2.1.2.2 Gradient development

A total of 3 gradients were used in this study (see section 2.1.1.5) and each was developed according to the general procedure listed in section 1.4.5. As an example, the development of the first gradient, which was used for most of this section on HPLC technique, is discussed.

A derivatized amino acid standard was run on the HPLC system 1 (see section 2.1.1.3) using the 15cm column and the gradient of Einarsson et al. (1983), listed in section 1.6.2. Below are 2 chromatograms, one of our separation using the gradient of Einarsson et al. (1983) and the other of the chromatogram they finally achieved:

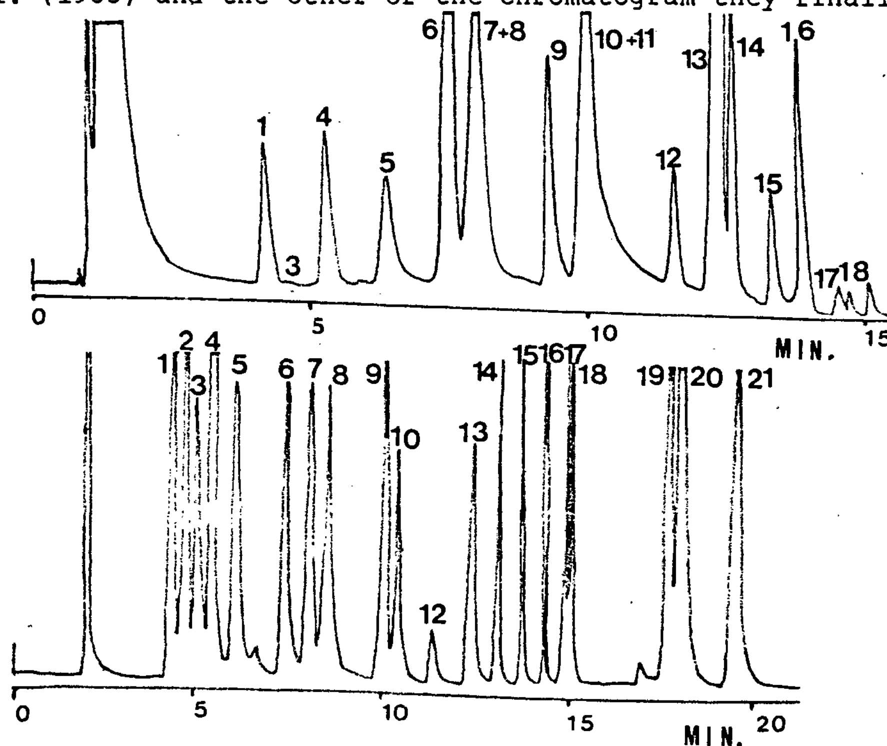


Fig. 2.5: Chromatograms from the same gradient, but from different workers. Numbers correspond to the following amino acids: 1 - asparagine; 2 - glutamine; 3 - aspartic acid; 4 - serine; 5 - glutamic acid; 6 - glycine; 7 - threonine; 8 - arginine; 9 - alanine; 10 - tyrosine; 11 - mono-histidine; 12 - water; 13 - proline; 14 - methionine; 15 - valine; 16 - phenylalanine; 17 - isoleucine; 18 - leucine; 19 - di-histidine; 20 - orthinine; 21 - di-lysine.

It can be seen from these chromatograms that separation cannot be achieved by merely duplicating gradients. The different columns used necessitate the development of a new gradient for each column to separate out these series of amino acids. The chromatograms demonstrate that the column plays the major role in separation. Certain amino acids co-elute in our chromatogram and those giving a weak response are due to loss by pentane extraction (isoleucine and leucine). A new gradient has to be devised which will separate out co-eluted peaks and bring peaks which are displaced far apart, closer together. This is not an easy task as will be seen in the subsequent gradient development and a compromise between the 2 must be made. This depends on whether analysis time or resolution is more important.

Initial attempts at improving this gradient were met with limited success as the acetonitrile-methanol-buffer gradient system does not separate out all the peaks effectively. It was therefore decided to use only one organic modifier and vary the percentage buffer. A good separation was achieved using an acetonitrile-buffer gradient after the solvent conditions had been optimized. Acetonitrile is expensive, is prone to contamination, gives a long analysis time of separation, and was therefore abandoned in favour of a gradient using methanol as the organic modifier. This gradient achieved an adequate separation within 12 minutes.

The procedure in developing a gradient is simple to follow (section 1.4.5). Gradients are best achieved systematically by changing one

variable at a time (Snyder et al., 1979). In setting up a gradient we keep the gradient time, flow rate and final solvent composition constant, changing only the initial solvent compositions. We start the first gradient using 100% buffer and no methanol. Subsequent gradients will have an increased methanol percentage as their starting compositions until the first peak elutes near the solvent front. The following gradients have been set up and the development of the separation is briefly discussed with each chromatogram. The remaining mobile phase percentage is made up from the acetic acid buffer:

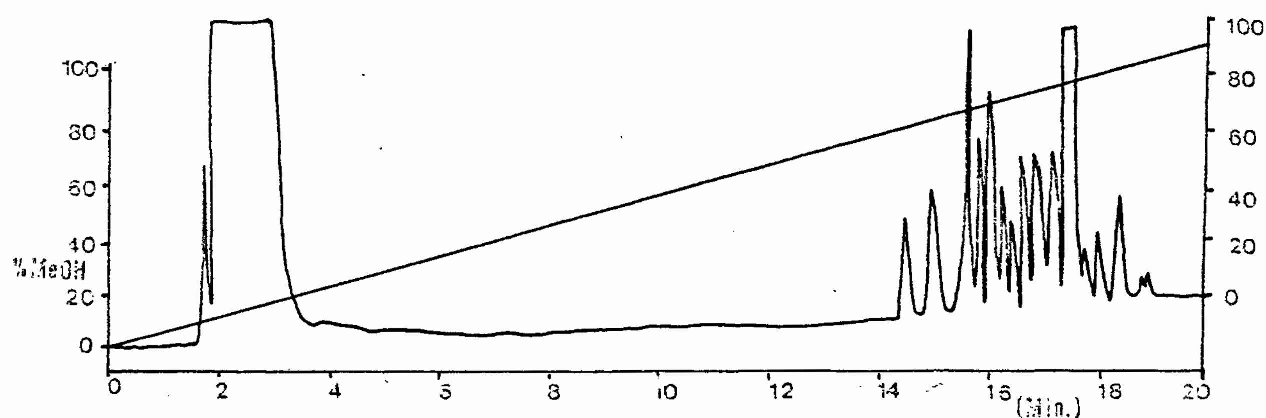


Fig. 2.6: Chromatogram achieved with the superimposed gradient (0-20 min; 0-90% methanol).

Peaks eluted near the end of the run in the above chromatogram require more methanol at the start of the gradient to allow earlier elution. Final composition of methanol and buffer is ideal at this stage as peaks elute before the end of the chromatogram.

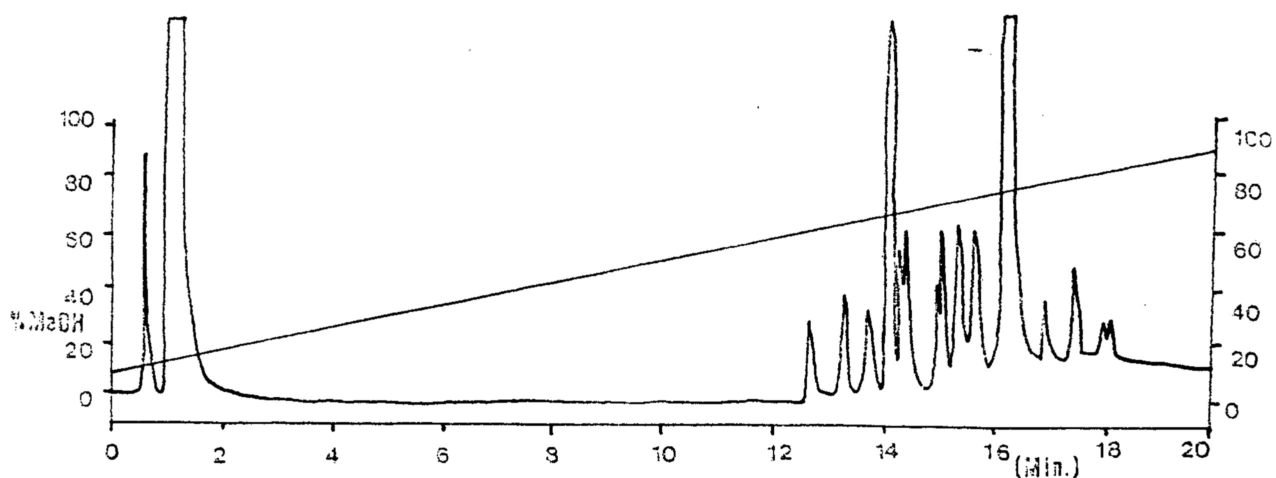


Fig. 2.7: Chromatogram achieved with the superimposed gradient (0-20 min; 10-90% methanol).

We can see above that the peaks slowly start to move towards the solvent front.

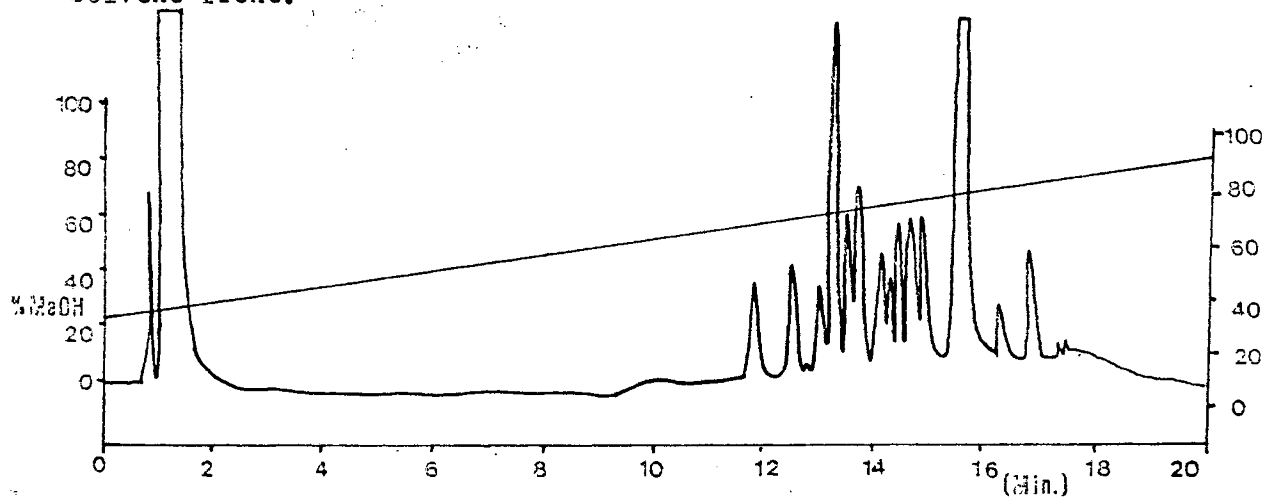


Fig. 2.8: Chromatogram achieved with the superimposed gradient (0-20 min; 20-90% methanol).

Here we see the closer migration of the early eluted bands to the solvent front. We now need to step up the initial methanol concentrations.

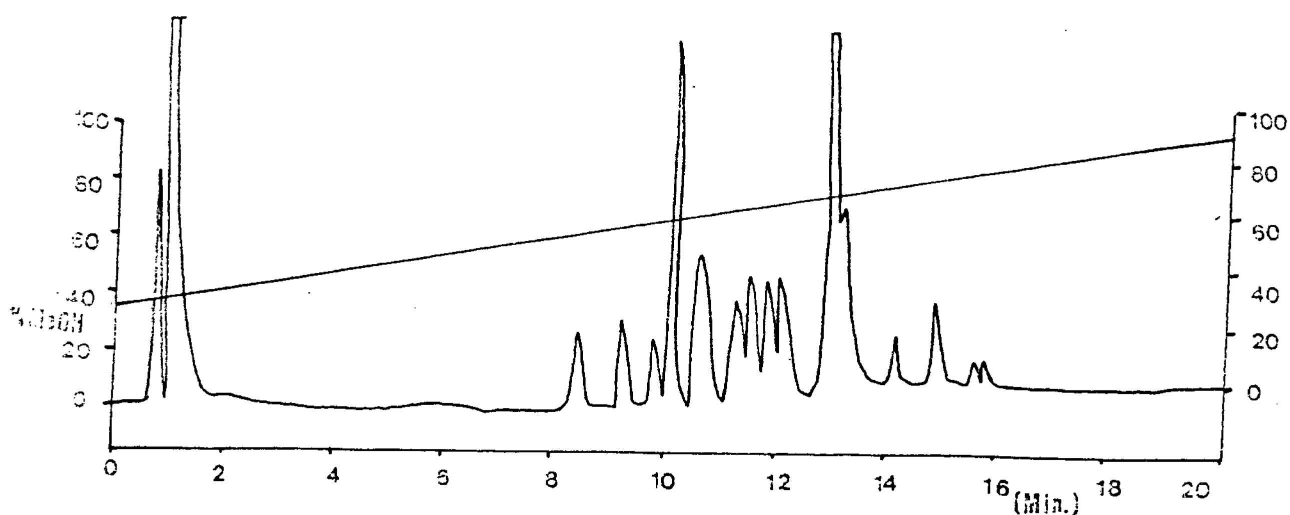


Fig. 2.9: Chromatogram achieved with the superimposed gradient (0-20 min; 35-90% methanol).

The chromatogram now begins to expand but also elution of the later bands is speeded up.

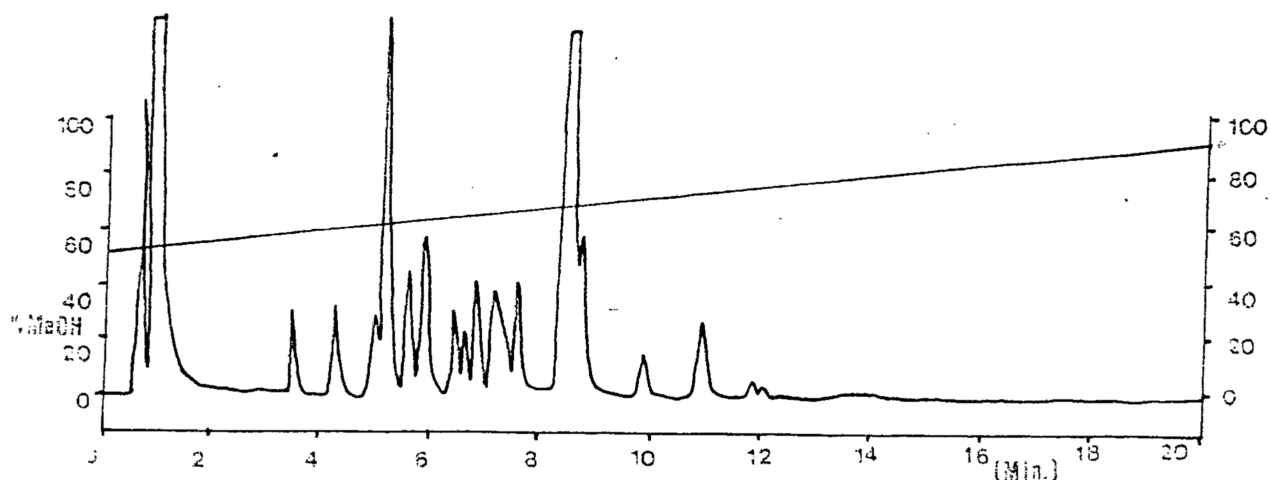


Fig. 2.10: Chromatogram achieved with the superimposed gradient (0-20 min; 50-90% methanol).

The initial peaks above are close to the solvent front and separation of the middle bands could be brought about by decreasing the amount of methanol at the end of the run.

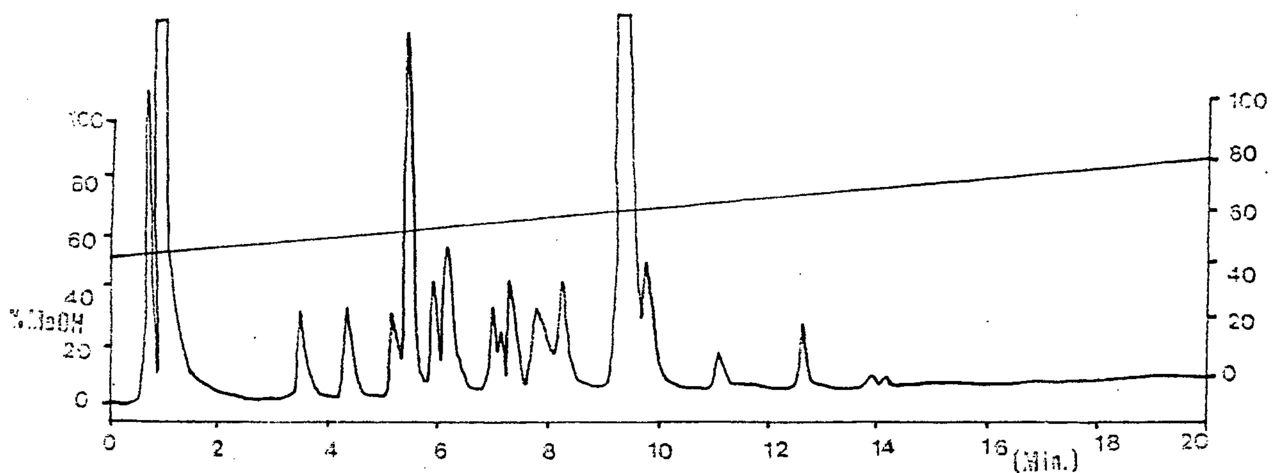


Fig. 2.11: Chromatogram achieved with the superimposed gradient (0-20 min; 50-80% methanol).

The gradient above becomes increasingly isocratic as final and initial concentrations tend toward each other.

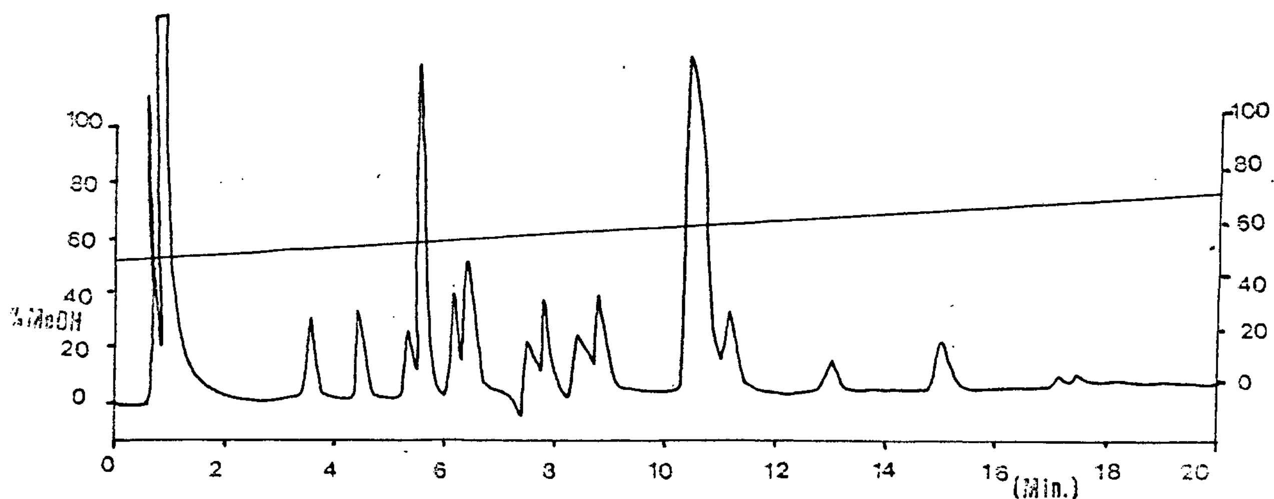


Fig. 2.12: Chromatogram achieved with the superimposed gradient (0-20 min; 50-70% methanol).

Separation above is still not perfect so we increase the initial methanol concentration again.

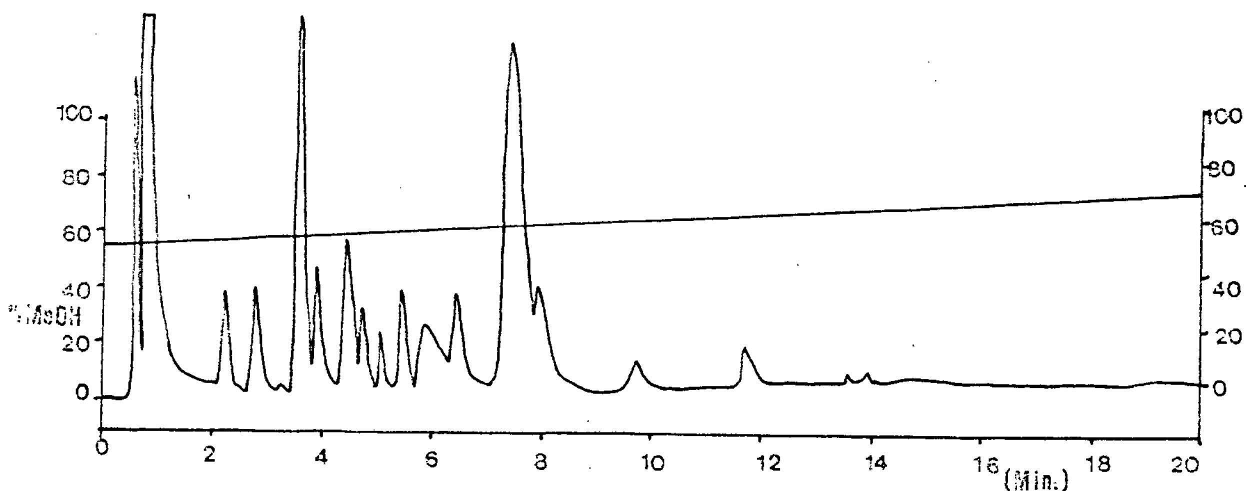


Fig. 2.13: Chromatogram achieved with the superimposed gradient (0-20 min; 55-70% methanol).

This separation above has good resolution for the middle eluted bands. This is a good basis from which to further achieve maximal separation. Attempts at increasing the resolution of each peak by further decreasing the final methanol concentration only increases the large separation between the final bands. An isocratic run of 55% methanol and 45% acetic acid buffer can serve as a basis on which to develop the final solvent composition.

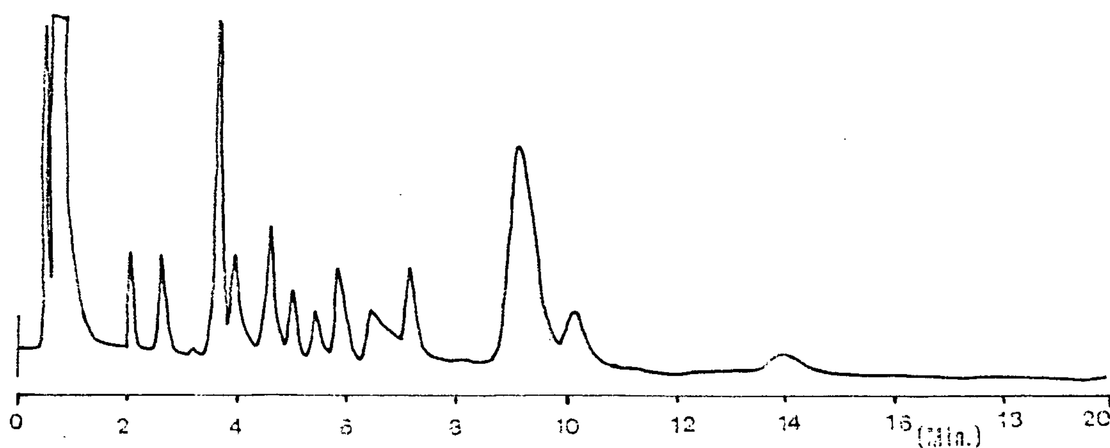


Fig. 2.14: Chromatogram from an isocratic run (55% methanol; 45% buffer).

From the above isocratic run we see the need to initiate the gradient at about t = 5 minutes to both retain early and middle resolution, but to speed up the later eluted peaks. We need to re-equilibrate the column to the initial solvent compositions after each run, so a 6-minute gradient regeneration stage is included. Final solvent composition should be at 90% methanol due to all the peaks being eluted at this composition.

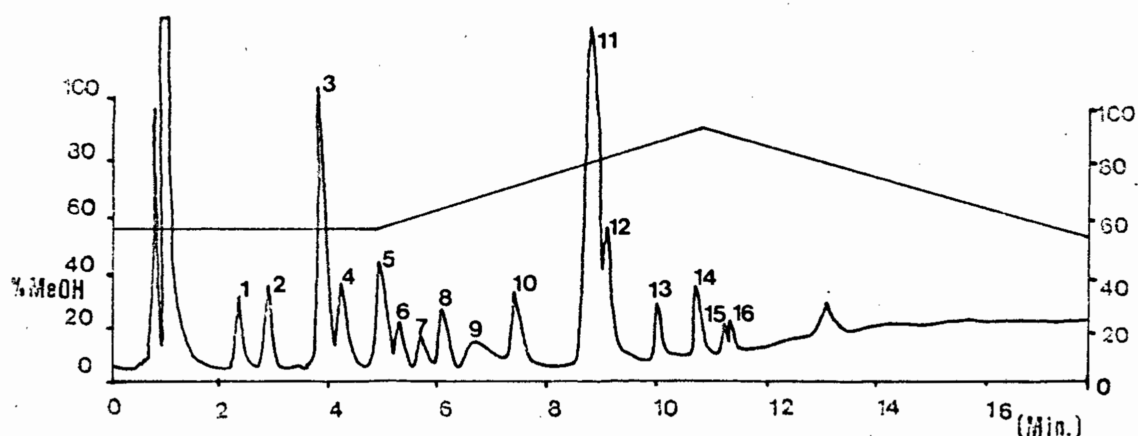


Fig. 2.15: Final chromatogram achieved from gradient development (gradient: 0-5 min, 55% methanol; 5-12 min. 55%-90% methanol; 12-18 min, 90-55% methanol). Numbers of the peaks correspond to the following amino acids: 1 - asparagine; 2 - serine; 3 - glutamic acid and glycine; 4 - threonine; 5 - arginine; 6 - alanine; 7 - tyrosine; 8 - mono-lysine; 9 - mono-histidine; 10 - water; 11 - proline; 12 - methionine; 13 - valine; 14 - phenylalanine; 15 - isoleucine; 16 - leucine.

This gradient gives us well separated peaks for a very short analysis time and was chosen for the initial work done. Methionine appears to co-elute with proline, due to the larger concentration of proline in the amino acid mixture. At equimolar concentrations they become separated. This final gradient is gradient 1 in section 2.1.1.5.

Once the resolution began failing on this 15cm column, the 25cm column was used and the gradient was developed similar to the procedure explained for gradient 1. The following gradient and separation for this column are shown below.

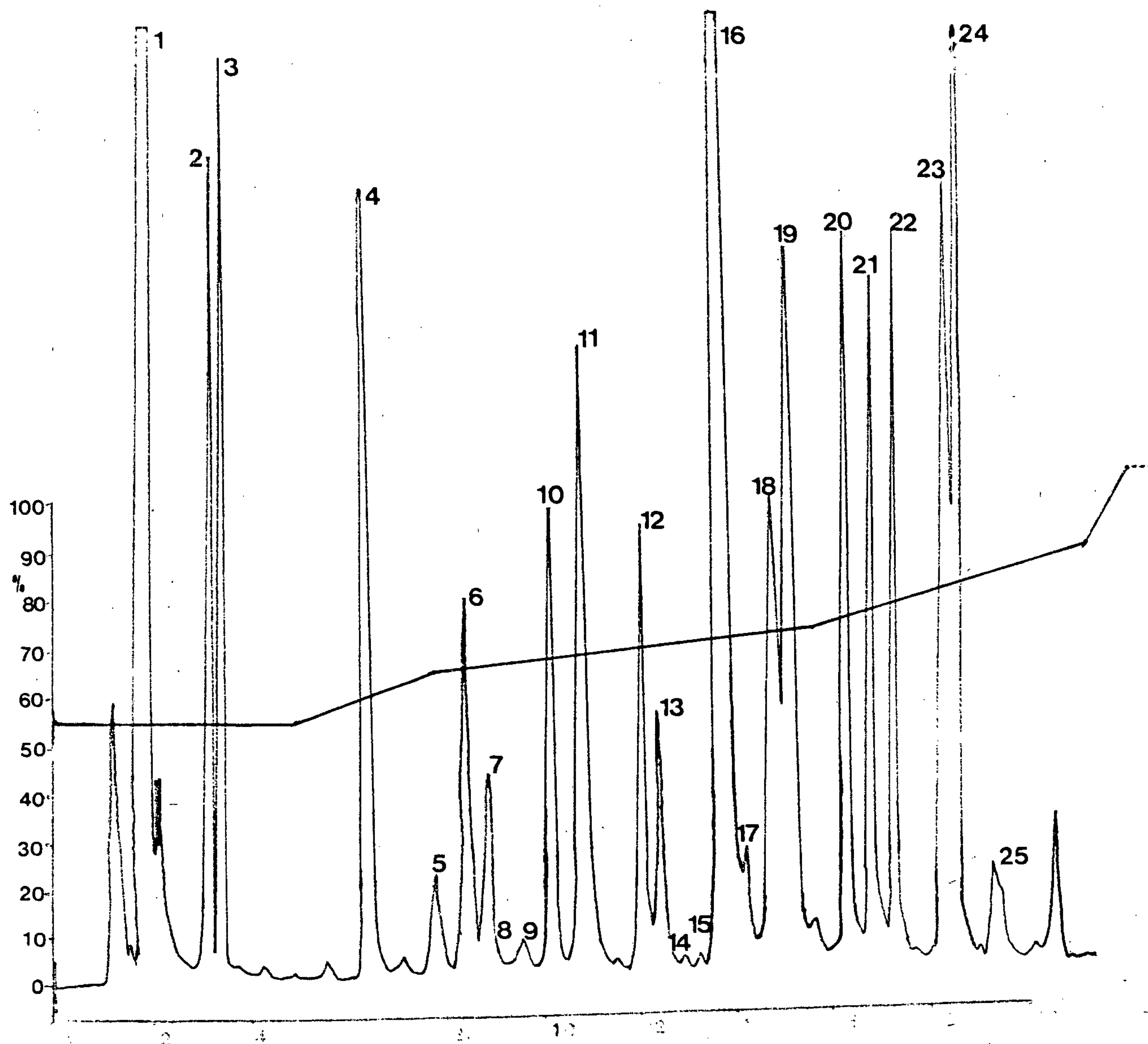


Fig. 2.16: Chromatogram achieved from the superimposed gradient of amino acid standard A2908 (Sigma). Numbers correspond to the following amino acids: 1 - acetone; 2 - cystine; 3 - cysteic acid; 4 - taurine and asparagine; 5 - aspartic acid; 6 - serine; 7 - glutamine; 8 - unknown; 9 - mono-histidine; 10 - glycine; 11 - threonine and arginine; 12 - alanine; 13 - tyrosine; 14 - mono-lysine; 15 - mono-histidine; 16 - water; 17 - ammonia; 18 - proline; 19 - methionine; 20 - valine; 21 - tryptophan; 22 - phenylalanine; 23 - isoleucine; 24 - leucine and norleucine; 25 - reagent peaks.

This is gradient 2 in section 2.1.1.5. It is unfortunate that some peaks still co-elute and as the column is used with time the resolution decreases. This 25cm column was extensively used before it was used for this work, so a newer column would give even better resolution. The large expense of these columns requires more than maximum use of the columns. Lack of resolution can be improved by newer gradients or by replacing some of the packing material. It would be ideal to devise a system for repacking the columns. After many runs with the longer column a resolution decrease was noticed. Replacing the upper 4-5mm of column packing with new, larger particle size packing material (Altex 10 µm packing), the resolution improved considerably.

Gradient 3 was developed along similar lines, but much more time was spent on "fine-tuning" the solvent conditions. This separation can be seen in section 3.1.1.

2.1.2.3 Peak identification

Single amino acids in solution were derivatized and run. From the position of the solvent front, the derivatized component, the hydrolysis product peak and a standard run superimposed upon this chromatogram, we can identify the peak from the retention times. This can be done for all the amino acids. Further identification by spiking is both necessary due to irreproducible retention times and the formation of more than one product. Identities in fig. 2.15 were done by retention times and those in fig. 2.16 by spiking.

Identification by spiking in fig. 2.16 proved difficult due to the large amount of derivatized amino acid added which swamped closer peaks. Contradictions in identification or possible superimposition of peaks occurred for taurine and asparagine, and serine and glutamic acid.

2.1.3 Formation of di-labelled products

Upon identification by retention times, it was found that histidine and lysine formed more than one derivative. According to Einarsson et al. (1983) these amino acids, as well as ornithine, should be eluted last as they form di-labelled derivatives. This is because of their structure - they have 2 reactive nitrogen sites on the molecule. It was also stated that histidine can form a mono-derivative which elutes between glycine and glutamic acid. The following chromatograms for derivatized histidine and lysine were obtained:

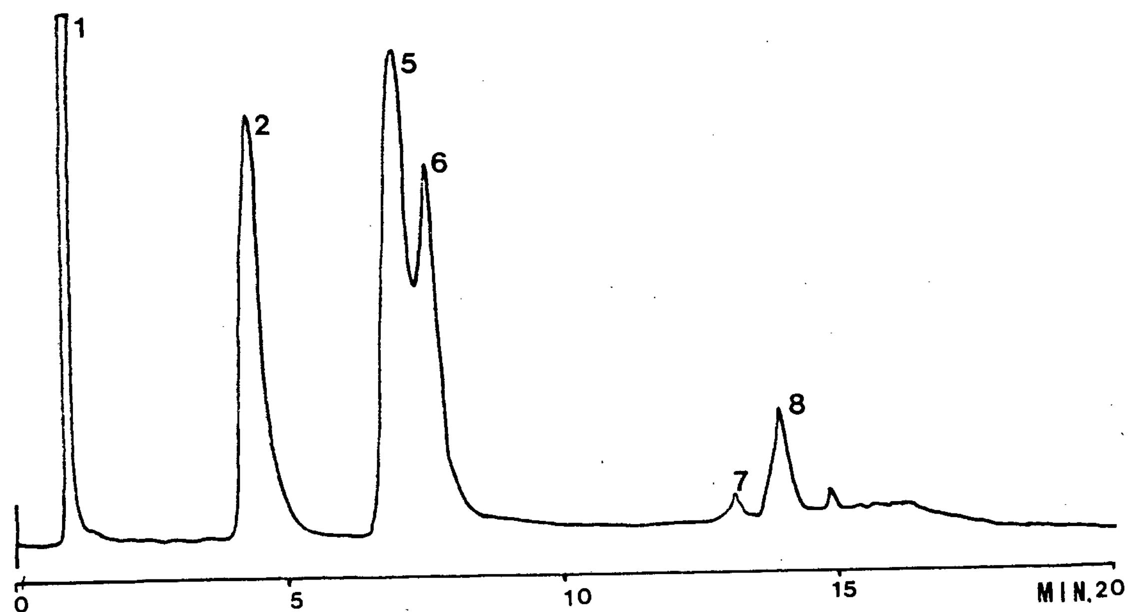


Fig. 2.17: Derivatized histidine peaks.

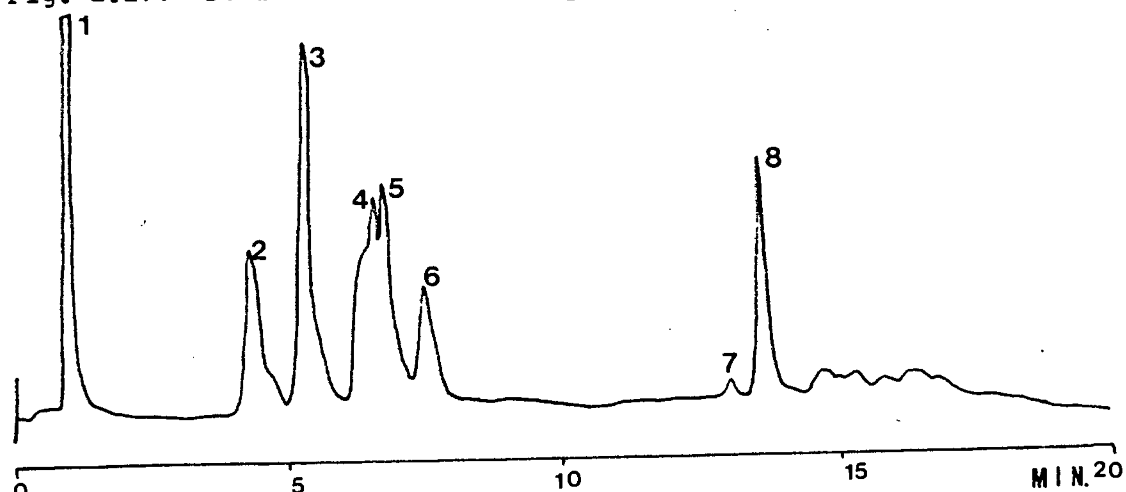


Fig. 2.18: Derivatized lysine and histidine peaks.

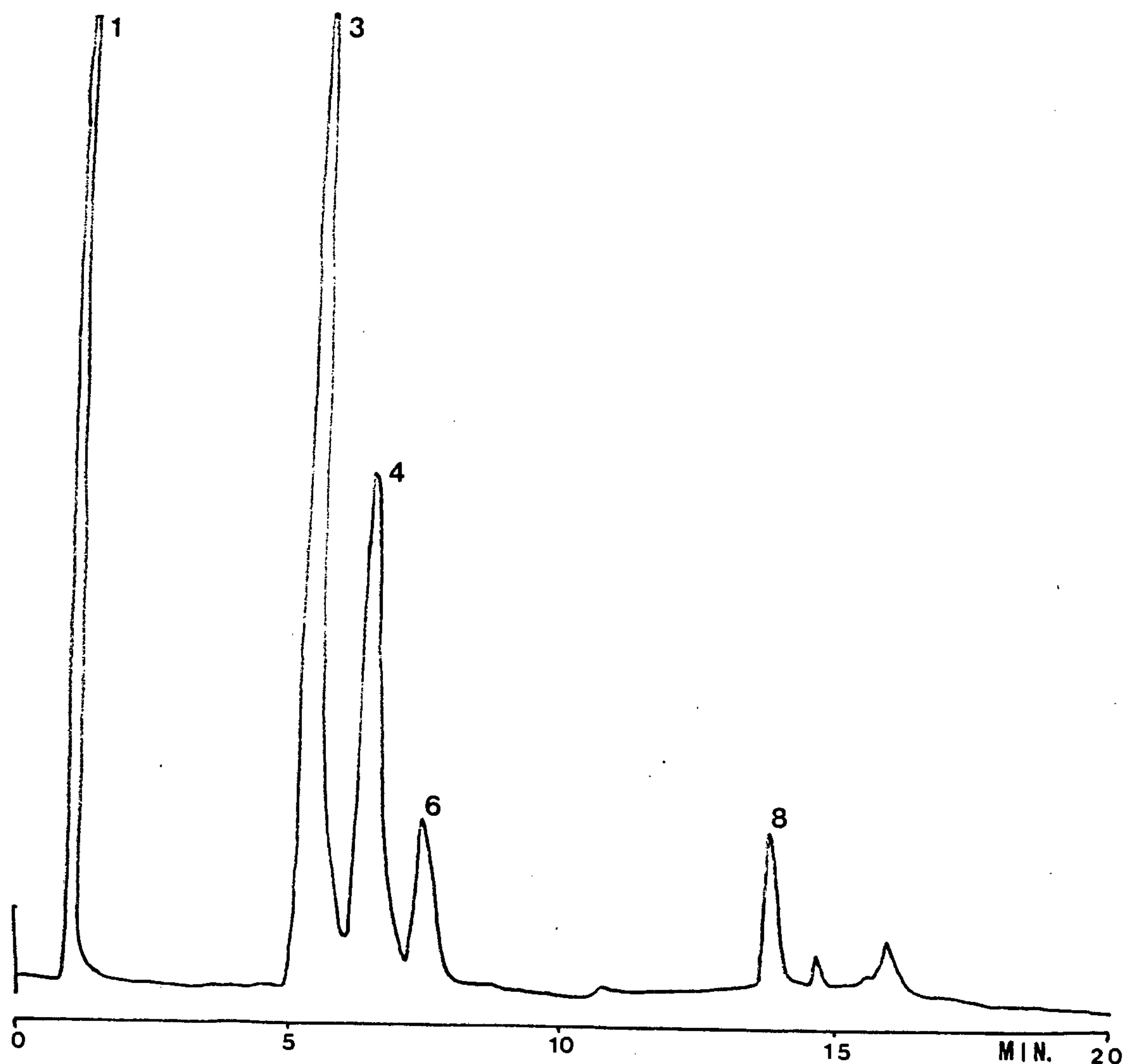


Fig. 2.19: Derivatized lysine peaks. Numbers for the above chromatograms correspond to the following amino acids and products: 1 - acetone; 2 - mono-histidine peak 2; 3 - mono-lysine peak 2; 4 - mono-lysine peak 1; 5 - mono-histidine peak 1; 6 - hydrolysis product; 7 - di-histidine or reagent peak; 8 - di-lysine or reagent peak.

We see that both histidine and lysine form peaks which elute before the hydrolysis product peak 6. They should in fact elute near our reagent peaks, if previous work is correct (Einarsson et al., 1983). Histidine and lysine under our reaction conditions (pH,

If we can find out at what conditions the di-labelled products are preferred, we could identify them amongst the reagent peaks. Runs of standards with and without histidine and lysine failed to identify any di-labelled products. Comparing lysine and histidine chromatograms to derivatized water runs, gave no identifiable di-labelled product peaks near the reagent peaks. The possibility of co-elution does exists.

The reason for the formation of 2 mono-products for these amino acid can be understood by observing their structures. The following reactions for both histidine and lycine are shown below:

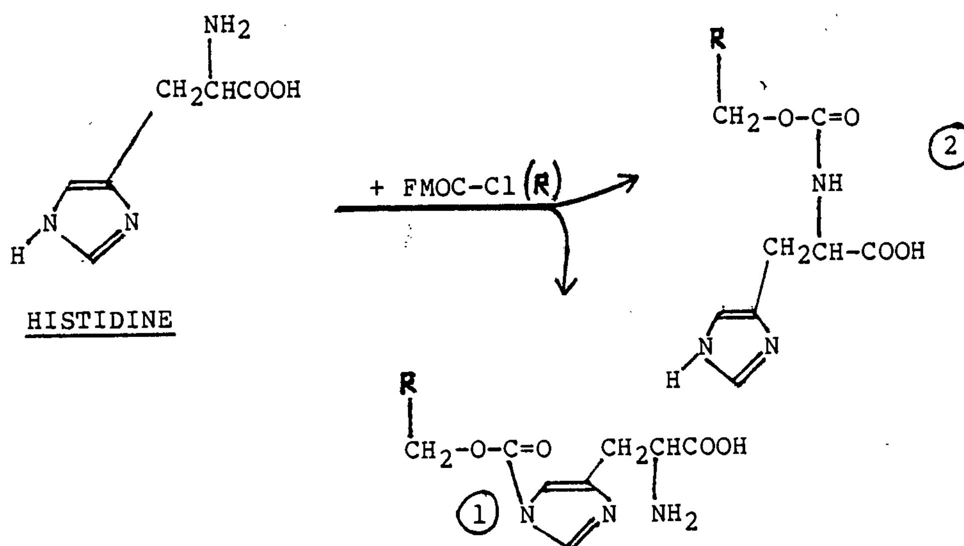


Fig. 2.20: Possible reaction pathways of histidine and FMOC-Cl.

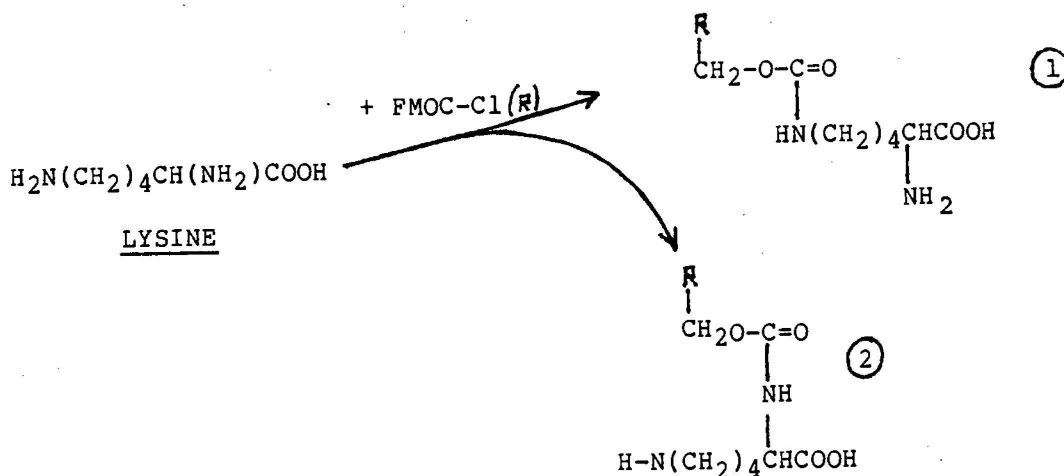


Fig. 2.21: Possible reaction pathways of lysine and FMOC-Cl.

These reactions are pH dependent as the reaction liberates hydrogen ions (see section 1.6). Furthermore we would assume that the formation of the di-labelled products would be dependent on the amount of reagent. The excess reagent may add on again to the mono-labelled product. It is possible that the above reactions are equilibrium reactions which favour one of the mono-products preferentially. This is shown by the differing peak intensities of the mono-products. Removal of the protons on the nitrogen reaction site would play a major role in this reaction.

The order of retention of these singly labelled products is another interesting factor. The polarity of the mono-labelled products is what would determine the retention time as their size and mass would be the same.

From the chromatograms we see a distinct difference in retention time for each mono-derivative due to the polarity difference. In

this case structural isomers are separated by HPLC. To understand the order of elution we require more knowledge of their polarities. We would also need to know which peak belongs to which structural isomer.

A standard containing only histidine and lycine was run at differing pH to determine the extent of reaction of the different products. A plot of peak height versus pH is shown below:

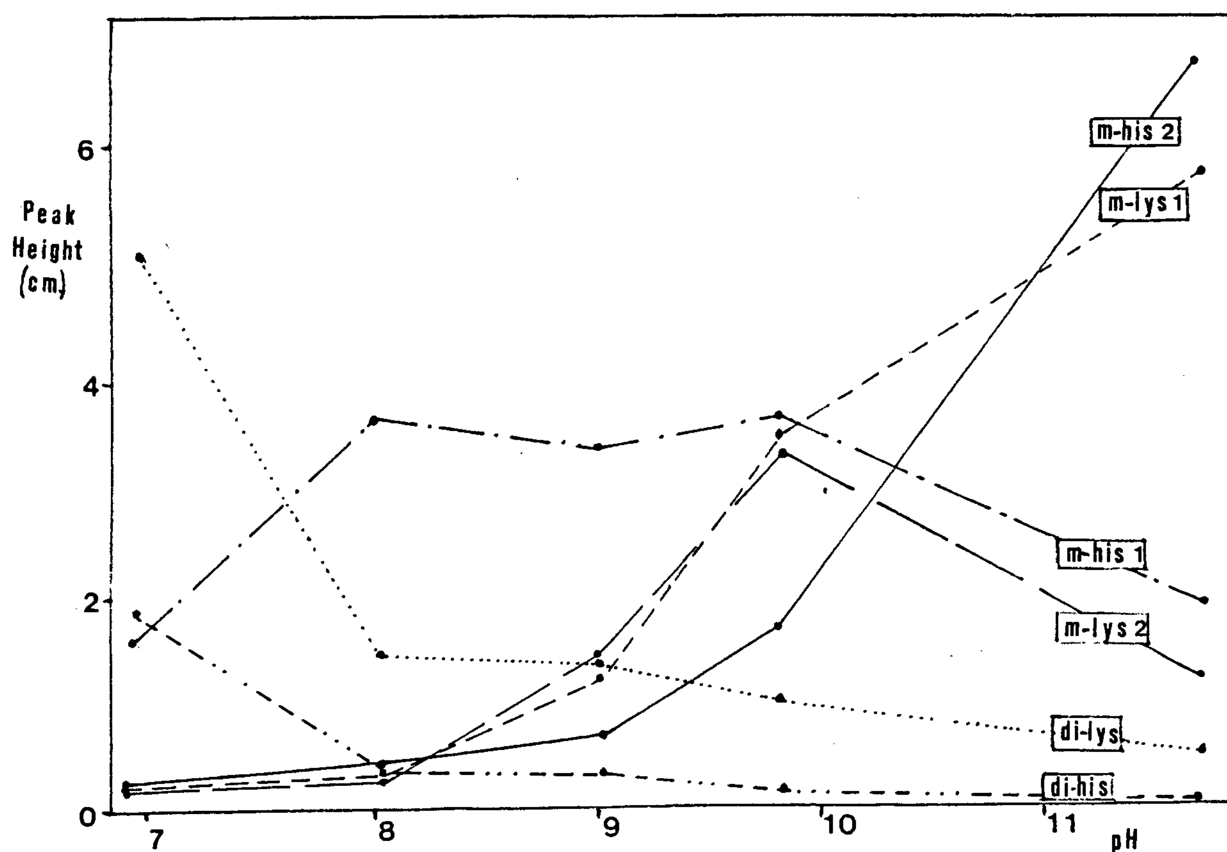


Fig. 2.22: Plots of peak height versus pH for histidine and lysine derivatives.

An increase of the mono-histidine 2 peak with respect to pH occurs. This reaction seems to be favoured as the hydrogen ions which are liberated can be accommodated at higher pH. Mono-histidine 1 in-

creases, then decreases as the pH is increased. At pH 11,68, the mono-histidine 2 increase is dramatic and at the same time there appears to be a proportionate decrease in mono-histidine 1. The di-histidine peak (which is most probably a reagent peak) decreases on increasing pH. If these peaks are reagent peaks, then their decrease on increasing pH is consistent with a larger product formation at higher pH.

The mono-lysine 1 peak increases on increased pH. This reaction favours a higher pH. The mono-lysine 2 peak decreases on higher pH, but up to pH 10 reacts in an equal proportion to mono-lysine 1. The increase of peak 1 is equivalent to the decrease of peak 2 at pH = 11,68.

It was assumed that an excess of reagent would drive the reaction towards the formation of the di-labelled products. A plot of peak height versus reagent volume (excess reagent) was drawn up. This can be seen overleaf:

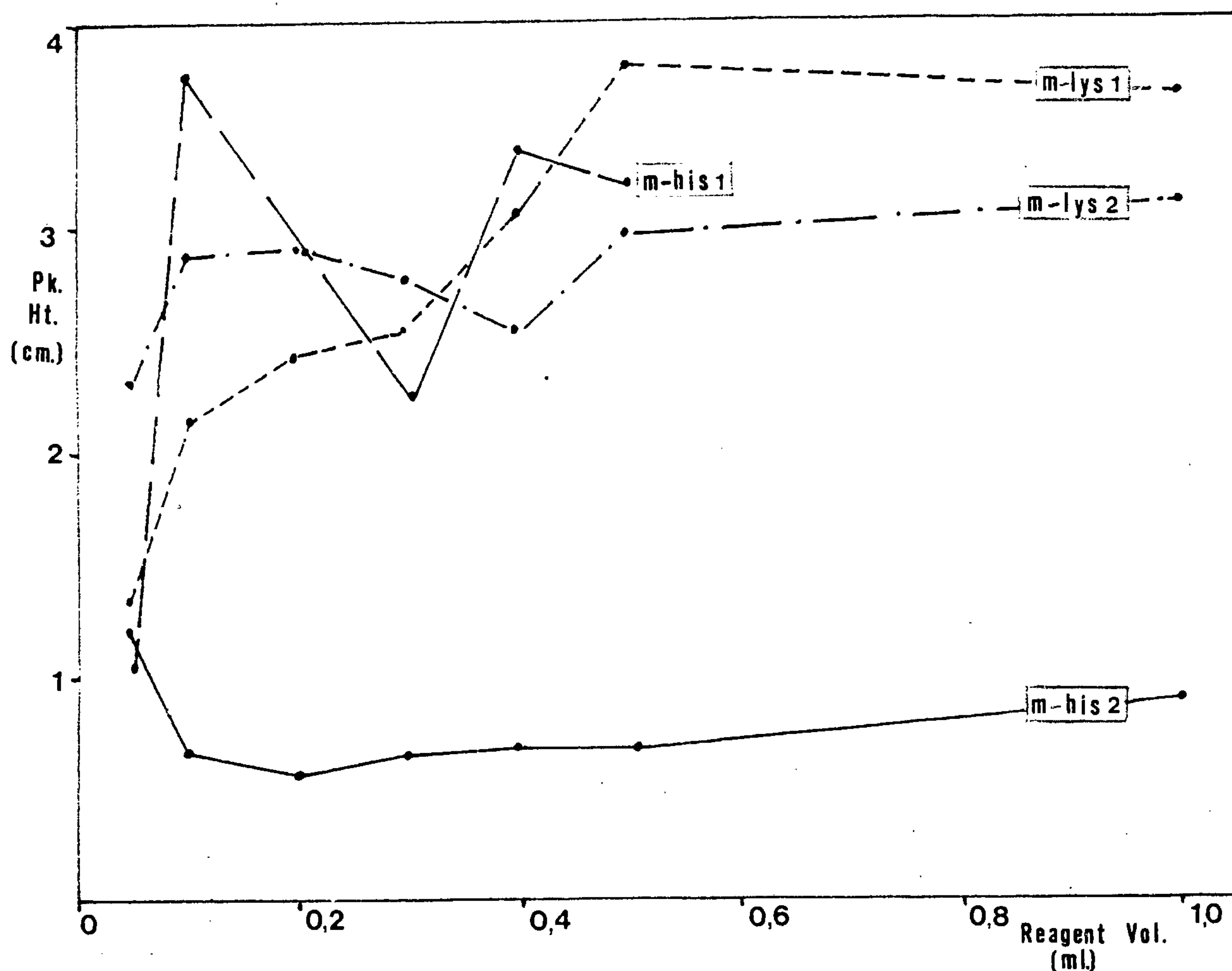


Fig. 2.23: Plots of peak height versus reagent volume for histidine and lysine derivatives.

These values have been corrected for the nett increase in volume of the standard as the reagent volume is increased. Any changes in peak height are therefore proportional to the original concentration. On increased reagent volume mono-lysine peak 1 increases. Mono-lysine 2 increases slightly and fluctuates about this increase. Mono-histidine 2 remains low and constant. Mono-histidine 1 fluctuates dramatically. The reason for this is not clear. From the chromatograms of this study we have observed no erroneous peaks near the reagent peaks that could be attributed to the di-labelled

products. We need more clarification on the reactivity of these amino acids that could form di-labelled products. It was thought that the di-labelled products were retained on the column and could not be eluted off under the final solvent conditions of the gradient. After running with pure methanol at the end of a gradient for a short while this did remove some column contaminants. Further runs with a strong final solvent strength after the column had been washed failed to produce peaks that could be singled out as di-labelled products.

We have observed the following points which Einarsson et al. (1983) have not made clear: (1) Histidine and lysine each form 2 peaks upon derivatization and separation. (2) No di- labelled products can be identified. (3) There is a pH and reagent concentration are important factors in determining which product would be favoured. (4) Possibility of quantitative error in some analytical assessments due to co-elution. (5) Tyrosine gives only one peak and a di-labelled product can not be identified.

2.1.4 The use of other solvents in the derivatization procedure

The method requires the use of acetone as solvent for FMOC-Cl when derivatizing a sample (Einarsson et al., 1983). This causes a large peak to appear at the start of the chromatogram due to acetone having a high absorbance at 265nm (acetone has a UV-cutoff of 330nm). The tail of this large peak can swamp earlier eluted peaks when the amino acids are in low concentrations, when using a high detector sensitivity and a low recorder attenuation. Attempts were made to use other solvents which would not give this large tailing peak and perhaps have some other merits, such as extraction selectivity associated with the pentane extraction stage. The choice would have to be a solvent having a UV-cutoff lower than the wavelength setting on the detector.

As a first attempt, the reagent was dissolved in pentane as this would not require further extraction. Since pentane removes excess reagent from solution, the relatively non-polar reagent will not pass from the non-polar pentane phase into the amino acid aqueous phase. The opposite in fact occurs. This can be seen chromatographically by having only one eluted peak, namely the reagent peak and no amino acid peaks.

Methanol and ethanol were used but due to their relatively non-polar nature, gave similar results as pentane. We therefore could not use these solvents.

Acetonitrile shows similar properties to acetone in that the derivatized amino acids remain in the injected aqueous phase, but whereas some acetone and reagent become extracted, acetonitrile does not become extracted, nor does it allow the reagent peaks to be extracted. These are only marginally extracted when a large volume of pentane is used. A study of the chromatograms of derivatized amino acids using acetonitrile as solvent showed hardly any peak height decrease upon extraction with increasing volumes of pentane. Compared with acetonitrile, acetone becomes extracted and also allows much more of the derivatized amino acids to become extracted, particularly those less polar eluting near the end of a run. It is possible that these species are "carried" by the acetone to the pentane phase, as acetone is removed from one phase to the other. The loss of these amino acids questions the efficiency and usefulness of the extraction step, which is discussed later.

Runs of the pentane extracted phase using acetone as solvent support these findings:

1. No amino acid peaks are found eluting before the hydrolysis product peak.
2. Large reagent peaks and smaller, later eluted amino acid peaks are found (i.e. relatively non-polar species are extracted).
3. Acetone forms a peak in this pentane run, confirming its extraction. This causes a pre-concentration of the aqueous phase and increased sensitivity.

Due to the presence of large impurity peaks in derivatized acetonitrile and the reduced sensitivity compared with acetone, it was decided to continue using acetone as a solvent. Furthermore, since fluorescence detection is finally used for the field study, the large acetone peak does not show as it does not fluoresce.

2.1.5. The feasibility of the extraction procedure

When FMOC-Cl is added to a sample, the time taken for complete reaction of the amino acids is primarily dependent on the concentration of the amino acids in solution and the amount (concentration) of reagent added, amongst other factors such as pH and temperature. The pentane extraction step removes the excess reagent before it has time to react with water, but after it has reacted with the amino acids. This time is crucial for optimum results if the water peak is to be kept to a minimum and all the amino acids have reacted.

The efficiency of extraction was investigated by plotting peak heights versus time of extracted and unextracted samples containing only asparagine and phenylalanine. These 2 amino acids were chosen because of their position on the chromatograms. The asparagine derivative is the most polar and elutes first of all the amino acid peaks, and phenylalanine, the least polar, elutes last. The size of the hydrolysis peak and reagent peaks could also be monitored.

The reaction times were 0,5; 1; 2; 18 and 36 minutes before injection for the unextracted species. The same times were used before extraction for the extracted species. The derivatized sample was injected after each of the above reaction times and thereafter each was immediately extracted with 1ml of pentane. This same sample, which was now extracted, was injected after its first unextracted run. The extracted sample runs had therefore a 20 minute lag behind the unextracted runs, which initially was not considered a source of

error. Further reaction would not have occurred within this time interval, as it was assumed that all the reagent was extracted. Each extracted sample was treated in the same manner so this lag possibly had the same effect. This is dependent on the amount of reagent left each time. The unextracted species have trends which are followed by the extracted species suggesting this lag had little effect on the determinations.

The trends of reactivity shown by these plots deviated from what was expected and certain errors become apparent. The asparagine peak was off-scale at the 18 minute reaction, and suffered split peaks at the 2 minute reaction. These plots can be seen below:

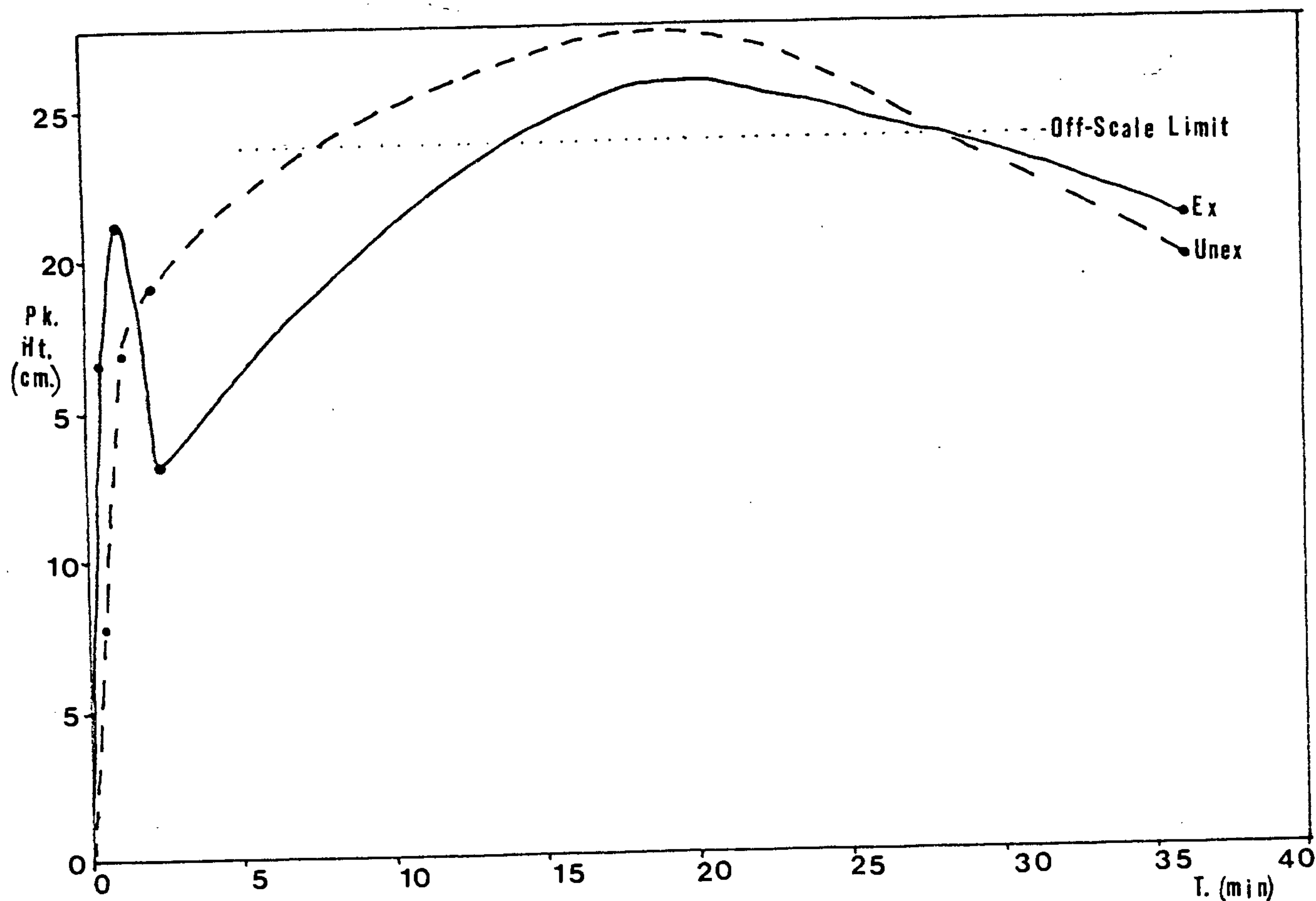


Fig. 2.24 Plots of peak height versus time for extracted and unextracted asparagine derivative.

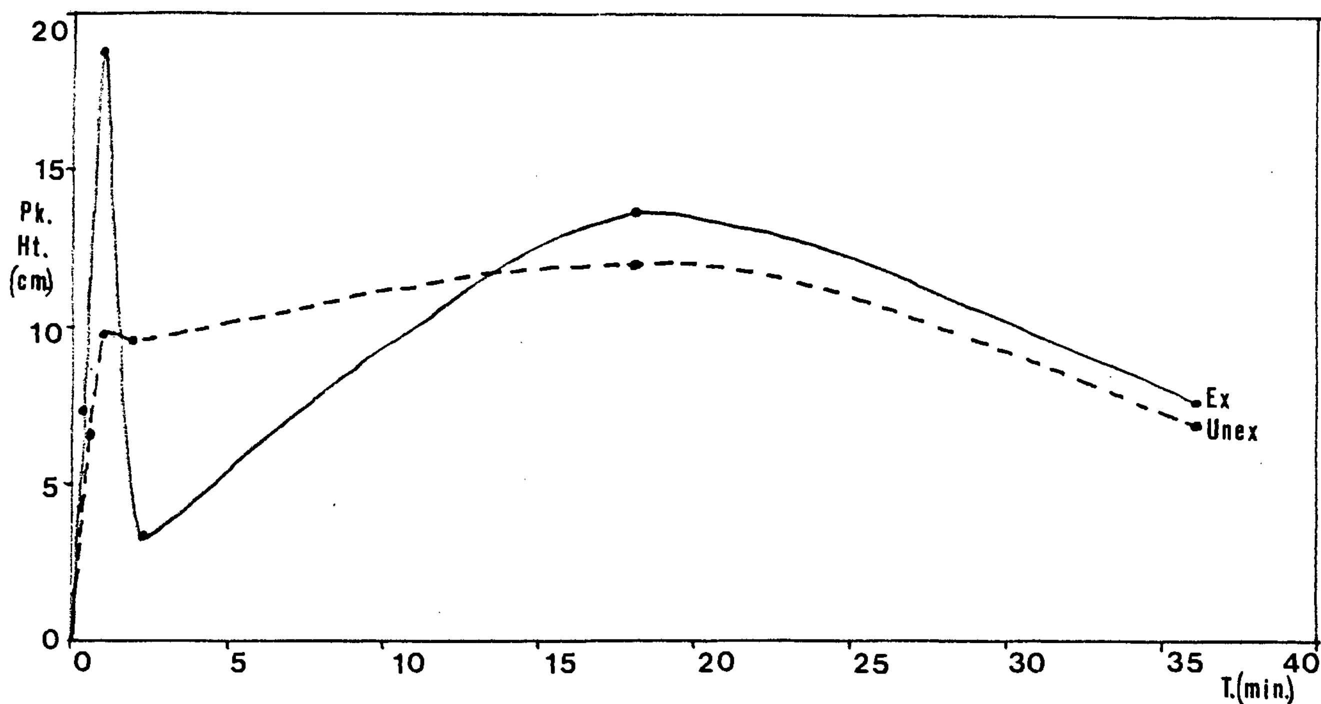


Figure 2.25 Plots of peak height versus time for extracted and unextracted phenylalanine derivative.

Observing the graphs it can be seen that the unextracted amino acid plots follow certain trends: maximum sized peak heights are reached very quickly and should remain constant. This is due to the amino acids reacting very quickly with the reagent so that the amino acid are totally consumed. The reason for the sudden decrease for the extracted species at the 2 minute reaction time is not too clear. This could be due to a slightly larger amount of pentane which was used in the extraction compared with the other extractions. This would explain the larger phenylalanine dip at the 2 minute interval, but the opposite effect would occur with asparagine - the increased pentane would have removed more acetone concentrating the solution.

The reagent and hydrolysis product profiles were so erratic that no expected trends could be seen. The large reagent peaks which were off-scale did not give sufficient information to explain their profiles. From the data it is clear that the pentane extraction stage is more prone to error than the unextracted solution. From previous data, it was shown that pentane can remove the less polar amino acid derivatives from the solution (Section 2.14). With this in mind a method was developed that would not require pentane extraction. By reducing the reagent volume and allowing a workable size hydrolysis product peak to form, we could overcome the problems of irreproducibility and error. This work led to the study of the kinetics of derivatization.

2.1.6 pH and derivatization

The reaction of FMOC-Cl and amino acids release protons (see section 1.6 for mechanism), and the effect of initial pH on derivatization should therefore be assessed. Samples having a large difference in pH from ideal reaction conditions (slightly basic medium) may adversely affect the quantum yield of the derivatized amino acids.

The buffer used in derivatization is important in maintaining a slightly basic medium to counteract the excess protons formed during reaction. The pH is kept at 7-8 to avoid tyrosine from forming a di-labelled product (Einarsson et al., 1983). The borate buffer used has a pH of exactly 7,7. The pH of hydrolysed samples is too low to allow the derivatives to be formed and the buffering capacity of the buffer is too weak at this very low pH. This is overcome by adding a few drops of sodium hydroxide solution to maintain a pH of 7,7. This is controlled by a pH meter. The buffer is then added before derivatization. If the reaction cannot proceed in an acidic medium, then it would be important to find out at what basic pH the reaction proceeds best.

In order to determine the effect of pH on the extent of derivative formation, a series of equimolar amino acid solutions from pH 6-11 were derivatized and plots of peak height versus pH were drawn up. The borate buffer was not added in case it affected the pH. The pH before derivatization was measured using a pH meter.

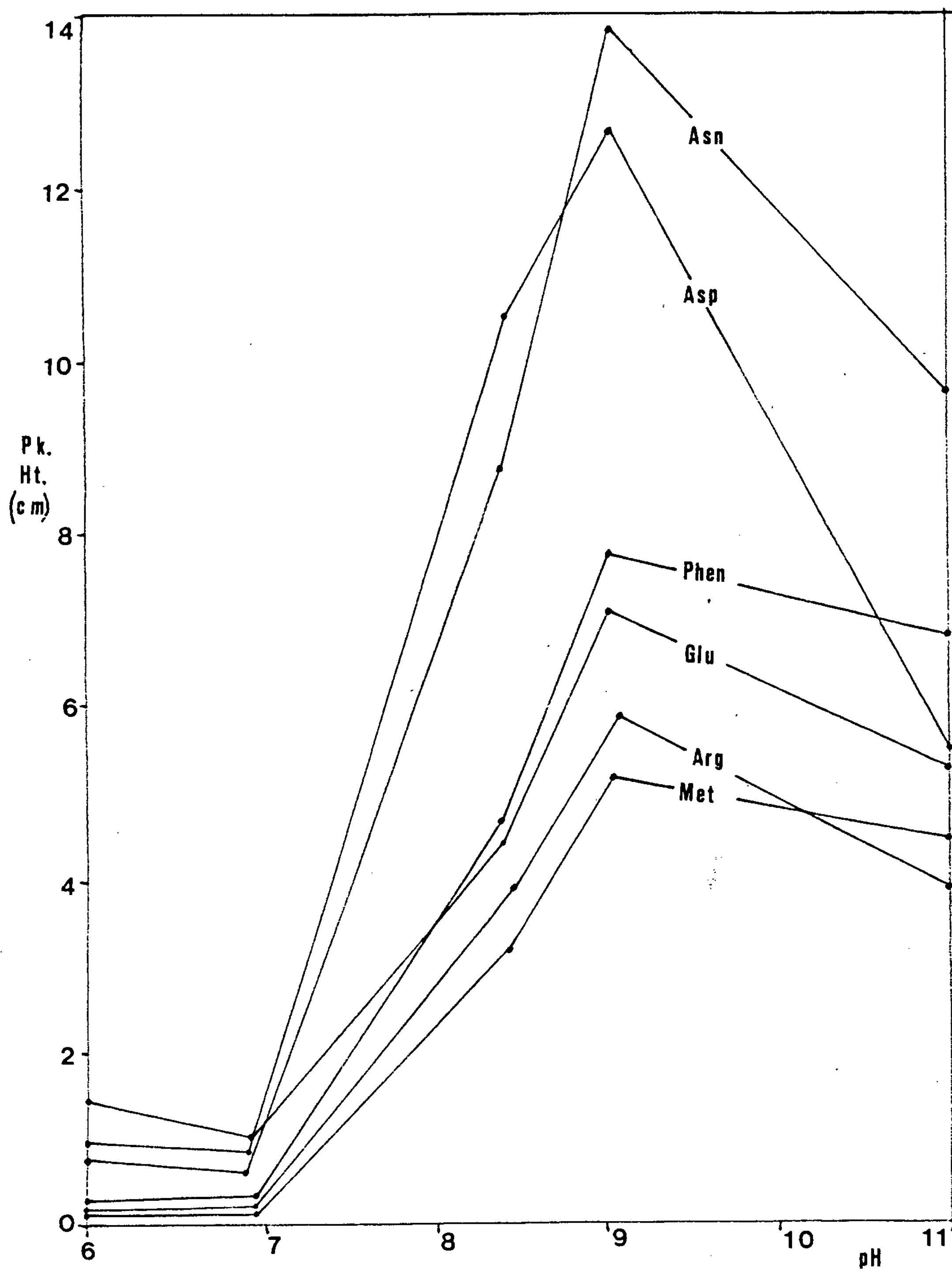


Fig. 2.26: Plot of peak height versus pH for some amino acids.

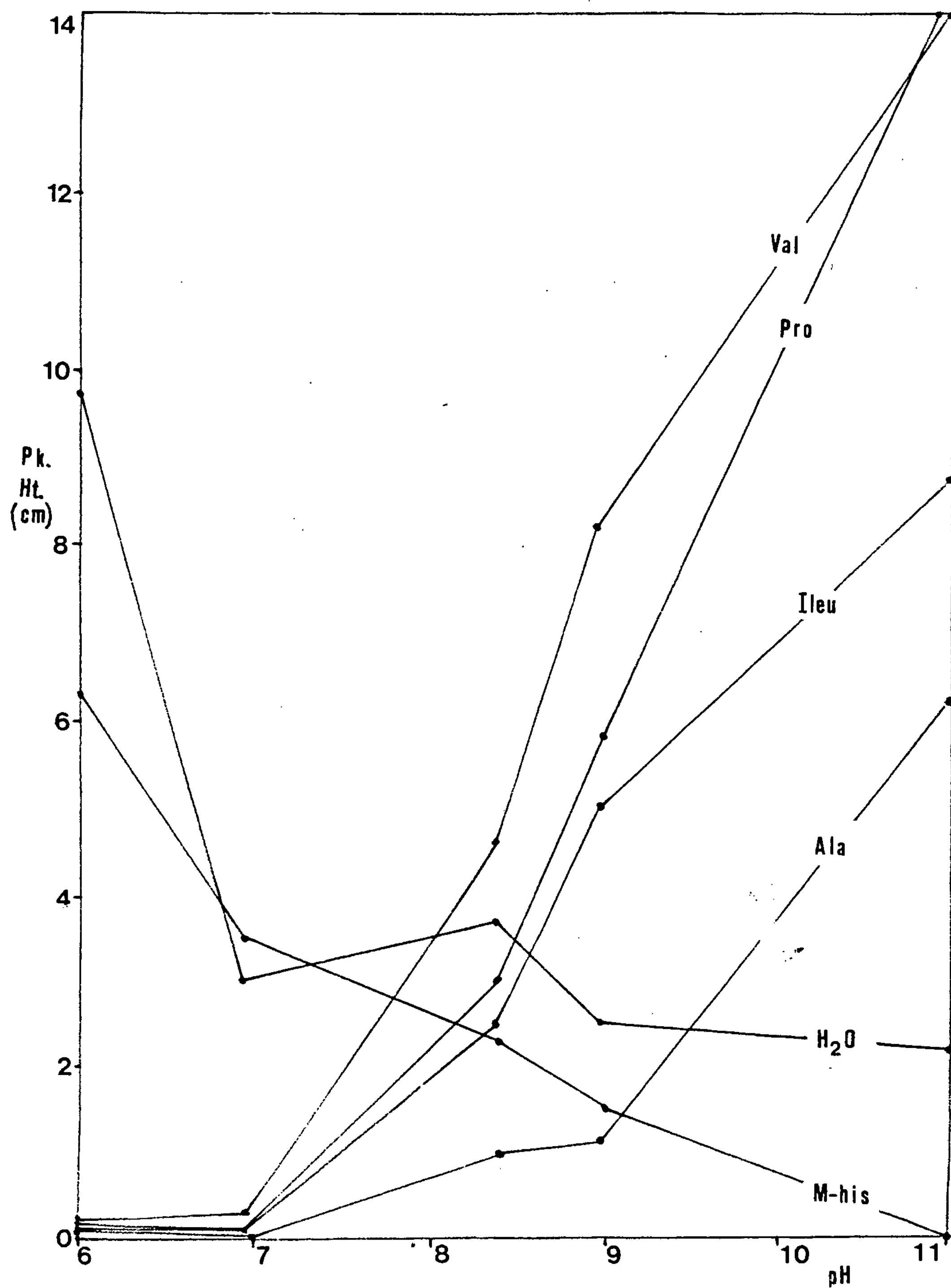


Fig. 2.27: Plot of peak height versus pH for some more amino acids.

From the graphs it becomes apparent that the reaction is definitely pH dependent. The error associated with a small pH change could be large: 1 pH unit would quantitatively give up to a factor of 4 difference, depending on the amino acid. The pH for future derivatizations would have to be carefully controlled, especially for samples with a large deviance from neutrality.

A higher sensitivity is achieved using a higher pH (more product is formed) and this must be weighed up against the maximum allowable pH of the HPLC column (for an ODS column, a pH of 8 is a workable maximum). However, at a much higher pH of derivatization, the low pH of the mobile phase buffer (pH of 4,2) would counteract this large pH upon injection. It was decided to maintain the pH of the derivatizing buffer and control this pH for the samples.

In fig. 2.26 we see the amino acids all follow the same trends - a maximum amount of product is formed at pH = 9 (in a further study of the pH between 9 and 11, it was found that this maximum is at pH = 10). In fig. 2.27 the amino acids valine and proline follow a different trend in that they form the maximum amount of derivative at pH = 11. At a higher pH for these species, accommodating the liberated protons seems to be favoured. The water peak is reduced on increased pH, possibly due to reagent loss from the formation of amino acid derivatives. Mono-histidine decreases with increasing pH, possibly due to the formation of di-histidine, but previous results cannot confirm this.

The reactivity at different pH of these amino acids may be dependent on a number of factors such as the removal of the proton on the amine group by a basic medium to allow reaction, or the ability of the basic medium to react with the protons formed during the derivatization. This must involve the dissociation constants (pKa) for the amino acids, but the trends experienced in the graphs could not be correlated with their dissociation constants. It was also found that the type of amino acid, whether basic, neutral or acidic was independent of the shape of the pH plot. There must be some other factor or combination of factors that can account for the shape of these plots. There is a possibility that the reagent is consumed before it reacts with all the amino acids, but evidence from a similar study of pH 9 to 11 show the same trends experienced for all the amino acids. The chromatograms of this experiment can be seen at the end of this section.

The effect of pH on the derivatization was much larger than was previously thought, so it is quite possible that the reproducibility will be affected on slightly differing pH. The buffering capacity of the borate buffer is quite weak (pH = 7,7), so for acid hydrolysed samples, the pH should be brought as close as possible to pH 7,7 in order that the buffering capacity can be effective. All unknown samples have their pH determined before derivatization takes place. We need to investigate whether or not the pH changes upon reaction (due to loss of protons) without the buffer being present. Furthermore, all acid washed glass vials

used for derivatization release a large amount of protons from the glass walls sufficient to actually change the pH considerably. All vials used in future work were rather washed with methanol after the pre-washes.

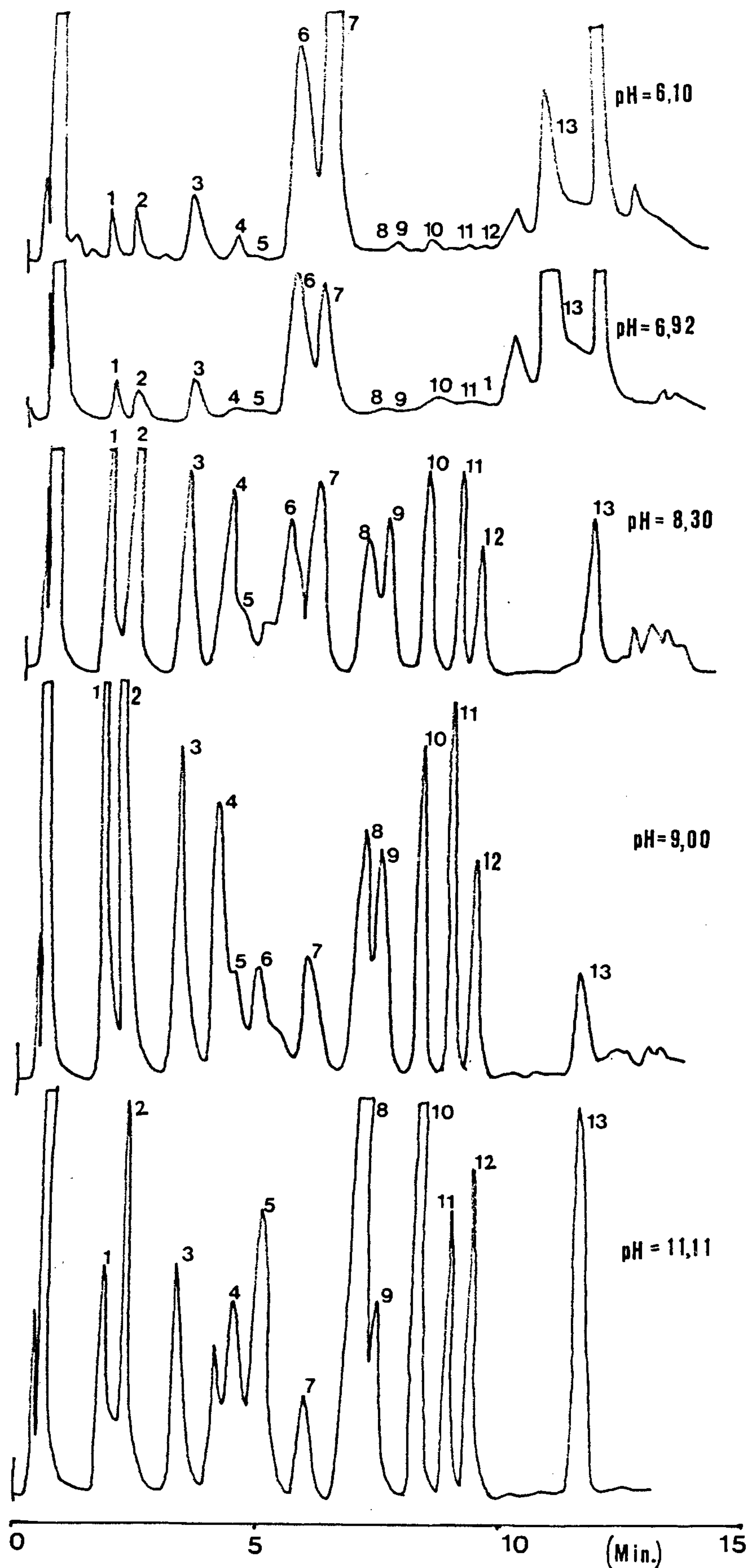


Fig. 2.28: Derivative formation w.r.t. pH. Numbers correspond to the following: 1 - asparagine; 2 - aspartic acid; 3 - glutamic acid; 4 - arginine; 5 - alanine; 6 - mono-histidine; 7 - water; 8 - proline; 9 - methionine; 10 - valine; 11 - phenylalanine; 12 - isoleucine; 13 - reagent peaks.

2.1.7. Reaction kinetics

Since we have omitted the pentane extraction stage, we now have to determine the rate of amino acid reaction, and the rate of hydrolysis product formation for different reagent volumes (which is proportional to reagent concentration). If we use too much reagent, the water peak could swamp closer important peaks such as the mono-histidine derivative (the only effective histidine peak for quantitative assessment) and ammonia, which lies as a shoulder on the water peak (see fig. 2.16). If we use too little reagent, not all the amino acids will be able to react before the reagent is used up. We have to work out a comfortable excess of reagent to add for the range of concentrations of standards and samples we are working with. From the data of reactivity and time, we can work out some simple kinetics for the reactions concerned.

A time series study of the reactivity of reagent and the amino acids was set up. This study compared reagent volumes (0,05ml, 0,1ml and 0,2ml) and reaction times (3 min, 23 min, 44 min and 180 min) as a function of peak height. It was found that for each reagent volume added, the reaction proceeded after the first 3 minutes. Only the size of the hydrolysis product peak increased after this time. Taking the 20 minute reaction (choice purely arbitrary) we can look at the effect of reactivity (peak height) with increasing reagent volume. We again choose the 2 amino acids phenylalanine and asparagine. The following graph can be seen overleaf:

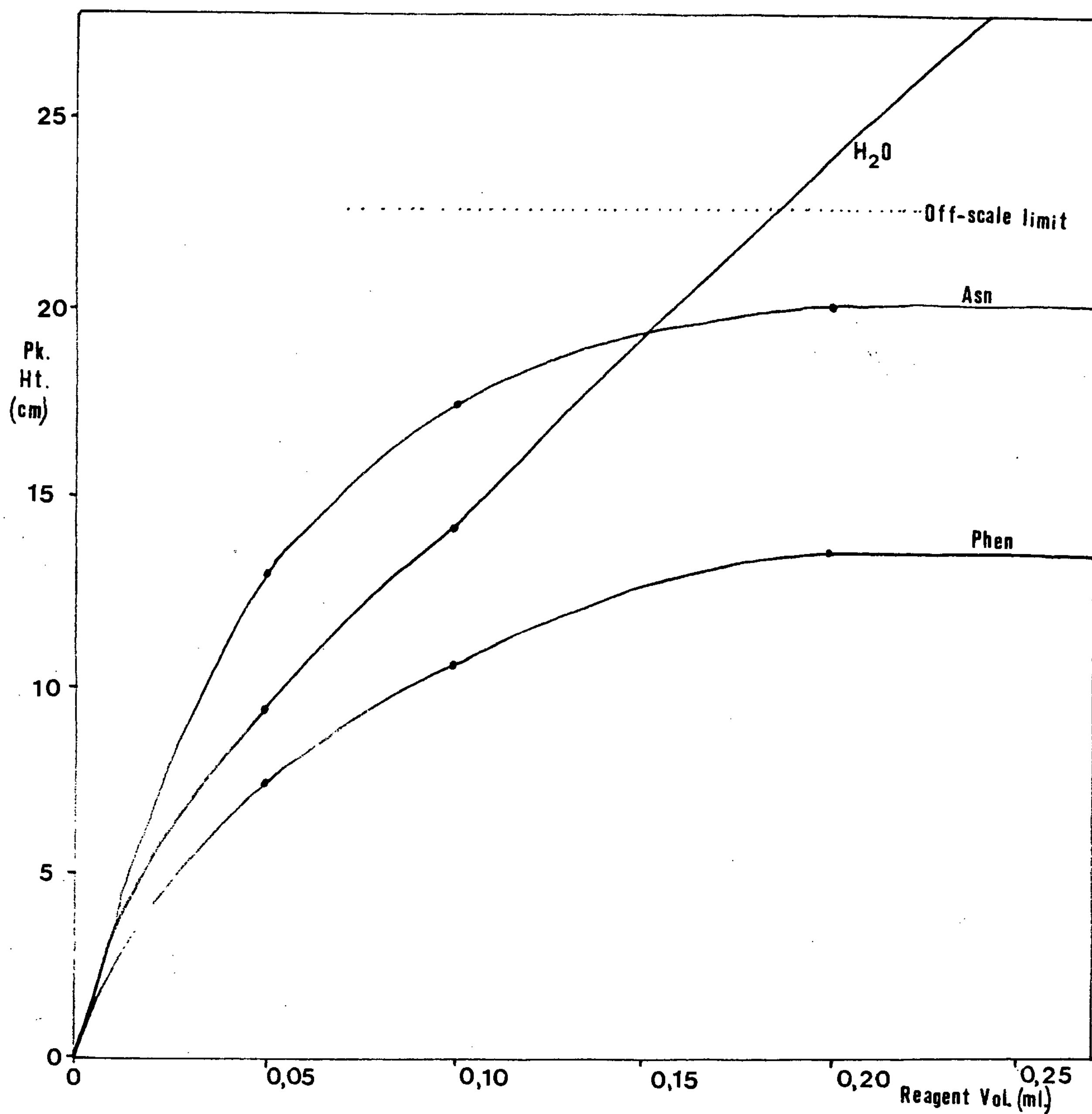


Fig. 2.29: Peak height versus volume of reagent for a 20 min reaction.

From the graph, most of the amino acids have reacted after 0,2ml of reagent has been added. At 0,1ml of reagent, the water peak and amino acid peaks are of the same order of magnitude. These amino acid peaks are not much lower than those for 0,2ml of reagent (peak height is lower by 15% for asparagine and 21% for phenylalanine). Bearing in mind that this is a very concentrated standard ($\pm$ 10mmol.l^{-1}), we would be dealing with very low concentrations in marine samples, having a almost non-existent percentage error (less amino acids to react with the reagent). Reproducibility at lower concentrations would not be in doubt when using a reagent volume of 0,1ml. Adding further amounts of reagent would have the effect of only increasing the hydrolysis product peak as can be seen from the linear extrapolation, as all the amino acids would have reacted and only water would be left to consume the reagent.

To determine the extent of reagent consumption by water alone, 0,1ml of reagent was added to 0,4ml of pure water according to the procedure developed. The ratio of reagent loss to hydrolysis product formation was plotted as a function of time. Due to the possibility of non-reproducibility upon each derivatization at the short reaction times used, it was decided to work with peak height ratios, so any nett difference due to an aspect of the derivatization procedure would be cancelled out (e.g. injection volume difference). The following graph was obtained:

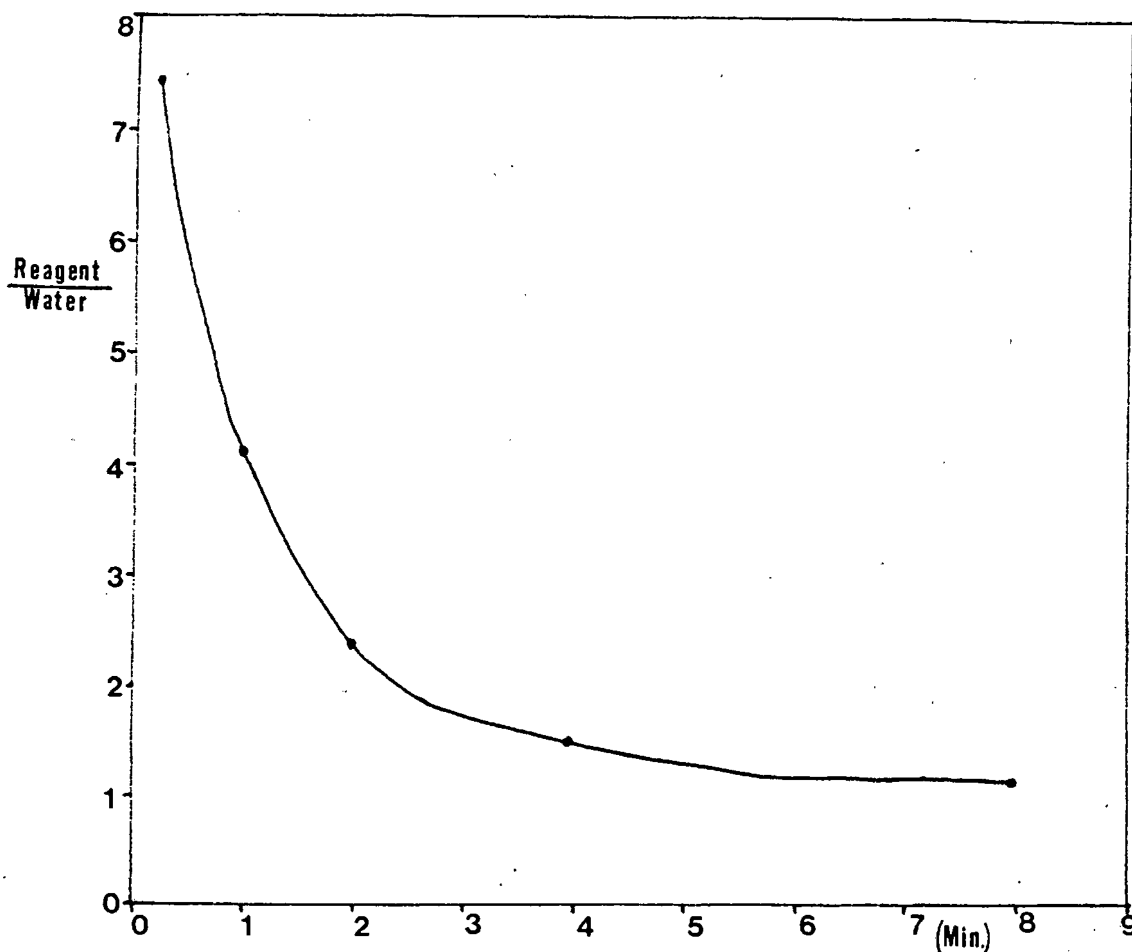


Fig. 2.30: Peak height ratio of reagent and hydrolysis product peaks versus time of reaction.

We can see that the uptake of reagent by water is fairly rapid, and slows down as more reagent is consumed. Compared with amino acid uptake, as we will see in detail later, this rate is very slow and this can be utilized to keep the hydrolysis product peak to a minimum. Once the amino acids have reacted and the hydrolysis product begins to form, we can inject the sample within a fair margin of time before it becomes too large. This graph shows that reagent will consume the water at a rate which is dependent on the concentration of the reagent (water is in a great excess because

of the aqueous medium of the samples). As more reagent is consumed the reaction becomes slower and this implies an equilibrium reaction which is concentration dependent.

If we now observe asparagine and phenylalanine together with the water peak in a single run, we can see the relative rates of product formation. From this we can draw up an ideal explanation of the rates of reagent loss and product formation. Below are the graphs of amino acid and water peak heights due to reactivity with respect to time:

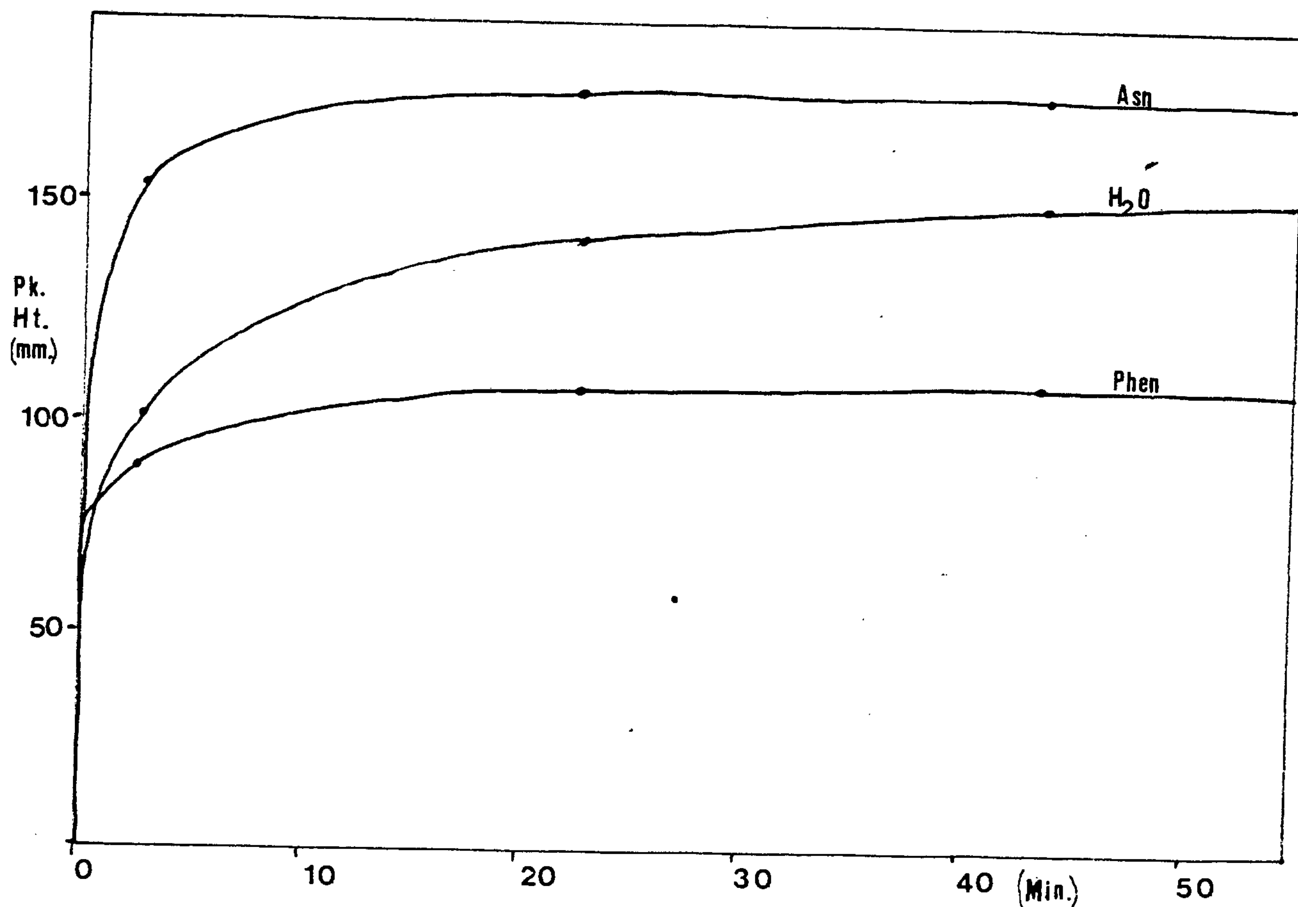


Fig. 2.31: Reactivity of the amino acids and water showing plots of peak heights versus reaction time.

The above graph is for a reagent volume of 0,1ml. A further point at 180 min (off-scale on above graph) maintained the horizontal position of the amino acids having the same order of magnitude as the points at 22 and 44 minutes. The hydrolysis product peak maintained a steady increase. We can see that the reaction proceeds rapidly within the first few minutes, and this reaction is too fast to monitor by using HPLC. This is obvious when considering the turnabout time between the injections and possibility of further reaction within the chromatograph before the amino acids become separated from the reagent (within the dead volume of the chromatograph). Furthermore, adequate assessment of the hydrolysis product peak formation is prone to further error as the reagent continues reacting with the aqueous mobile phase during separation. Since this affects each injection, it would not allow a large error between runs. The effect of reaction with the mobile phase can easily be seen by injecting pure reagent and observing the hydrolysis peak that has formed.

The graphs all confirm that reaction with water is slower than reaction with the amino acids. The reaction is definitely concentration dependent, and has been shown to be pH-dependent. This would explain an equilibrium reaction between reagent and amino acids, and would therefore account for the shape of the graphs given.

2.1.8 Reproducibility and peak stability

A series of 6 derivatizations of an amino acid standard was run to determine the reproducibility of this technique. The derivatizations followed the procedure of reduced reagent volume (0,1ml) and no pentane extraction. Qualitatively the chromatograms appeared identical with only the water peak increasing between each run. One such chromatogram is shown in fig. 2.32. This increase was due to the reagent further reacting with water with respect to time. This did not affect the intensity of the other peaks. On a quantitative basis the mean values and error of the peak heights are given below (concentrations = 2 $\mu mol.dm^{-3}$):

amino acid	mean	% error
asparagine	9,06 ± 0,56	6,18
aspartic acid	8,00 ± 0,40	5,00
glutamic acid	4,19 ± 0,17	4,00
arginine	4,01 ± 0,24	6,00
alanine	1,30 ± 0,11	8,00
mono-histidine	1,60 ± 0,32	20,00
proline	2,81 ± 0,13	4,60
methionine	3,16 ± 0,09	2,85
valine	4,32 ± 1,18	2,73
phenylalanine	4,62 ± 0,09	1,95
isoleucine	2,55 ± 0,15	5,88

The large errors of mono-histidine and alanine are due to these amino acids being in lower concentrations resulting in smaller peaks. The percentage error varies for each amino acid and ranges between 2 and 6%. Values given by Einarsson et al. (1983) range between 2,4 and 6,4%. These results agree well even though this procedure of theirs would be prone to a larger error due to the

pentane extraction stage. Our percentage error is very good considering the peak heights had to be calculated manually, as no form of integration system was available at the time of calculating reproducibility. The large fluctuation in peak height for differing pH would give much larger errors, so this reproducibility calculated gives us an indication of the relatively good pH stability of derivatization. These percentage errors are within acceptable limits.

In a test of reproducibility with respect to time, a derivatized amino acid standard from the above study was analysed periodically up to 330 hours after derivatization. A chromatogram of a derivatized sample after a few minutes and one of the same derivatized solution some 330 hours later can be seen below:

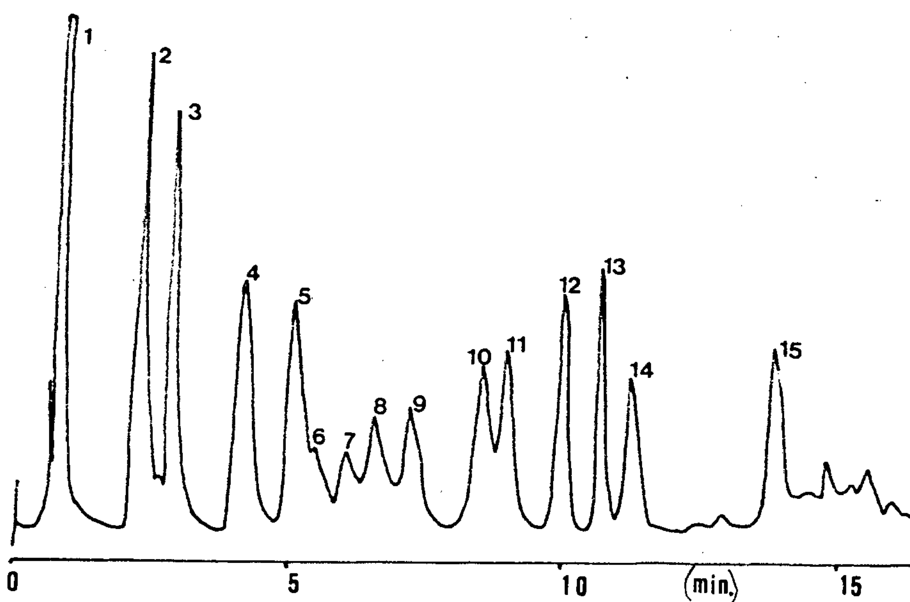


Figure 2.32: Run of an amino acid standard after a few minutes.

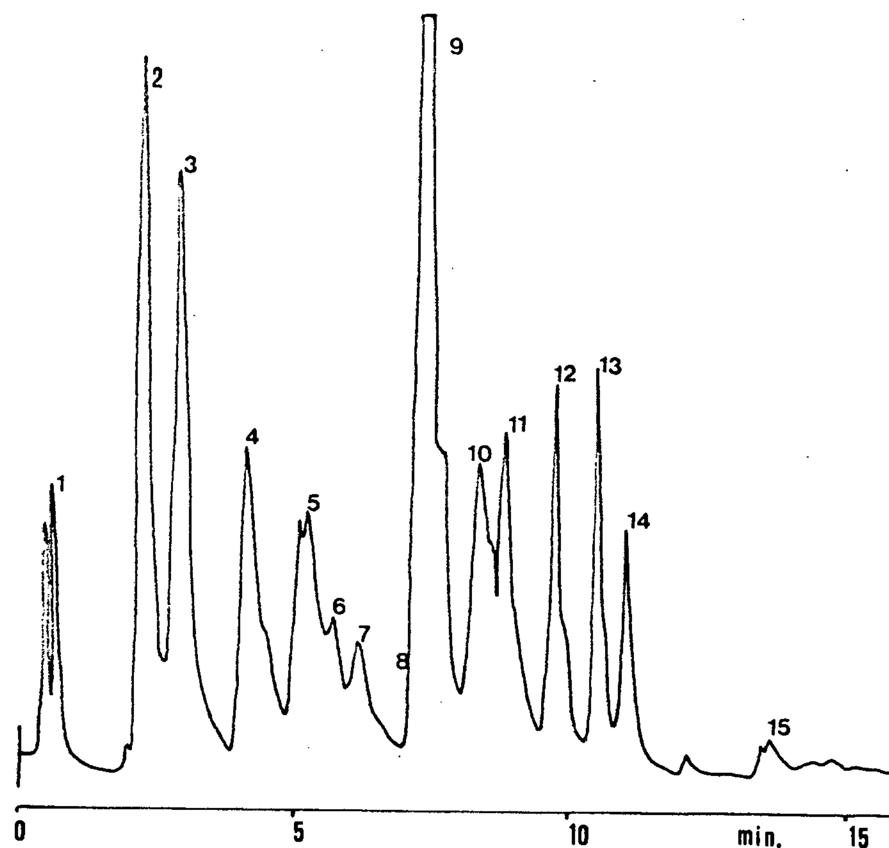


Fig. 2.33: Run of same standard after 330 hours. Peaks correspond to the following amino acids: 1 - acetone; 2 - asparagine; 3 - aspartic acid; 4 - glycine; 5 - arginine; 6 - alanine; 7 - tyrosine; 8 - mono-histidine; 9 - water; 10 - proline; 11 - methionine; 12 - valine; 13 - phenylalanine; 14 - isoleucine; 15 - reagent peak.

From the above 2 chromatograms certain factors become apparent. The acetone peak 1 is almost entirely reduced in the later chromatogram; this is due to the acetone evaporating through the screw-caps of the vials. These vials had the cork removed from their plastic caps to avoid contamination, and so doing lost the airtight seal. Later press-cap vials proved very effective in stopping this acetone evaporation. This evaporation has also led to an increase in the intensity of the peaks due to pre-concentration. The water has consumed most of the reagent in the later chromatogram and hence shows comparatively no reagent peaks 15 and a very large water peak 9.

The size of the amino acid peaks remain the same demonstrating the good stability of the derivatives. A graphical representation of the stability over this 330 hour period can be seen below:

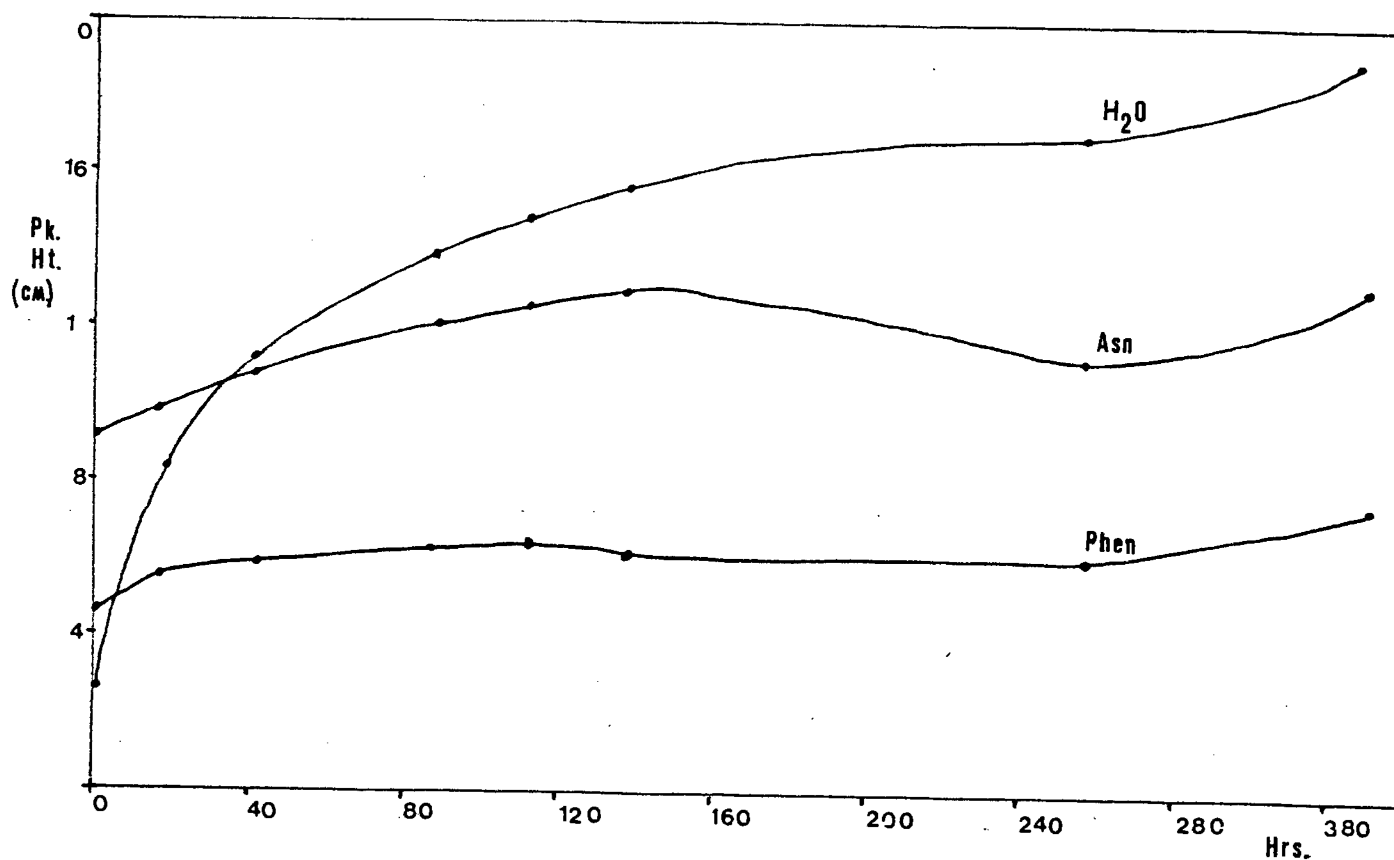


Fig. 2.34: Stability of peak heights with respect to time.

The above graphs show this stability and that the derivatives react totally within the first few minutes. This stability is quantitative either once the acetone is prevented from evaporating, or has evaporated totally. There is still the gradual increase of the hydrolysis product peak compared with the almost instantaneous amino acid reaction. From this and data on the reaction kinetics we need not worry about reagent consumption by water before all amino acids have reacted.

We have discussed derivative stability over periods of minutes and hours. We now observe the stability in sunlight for a period of about seven months. Below is a series of chromatograms from one derivatized solution under different conditions with respect to time:

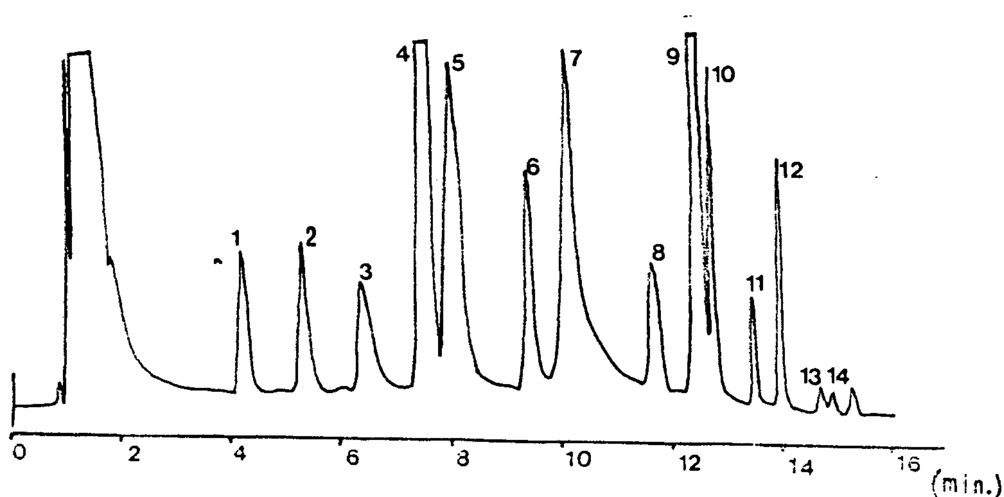


Fig. 2.35 Amino acid standard run after 2 days kept in the dark.

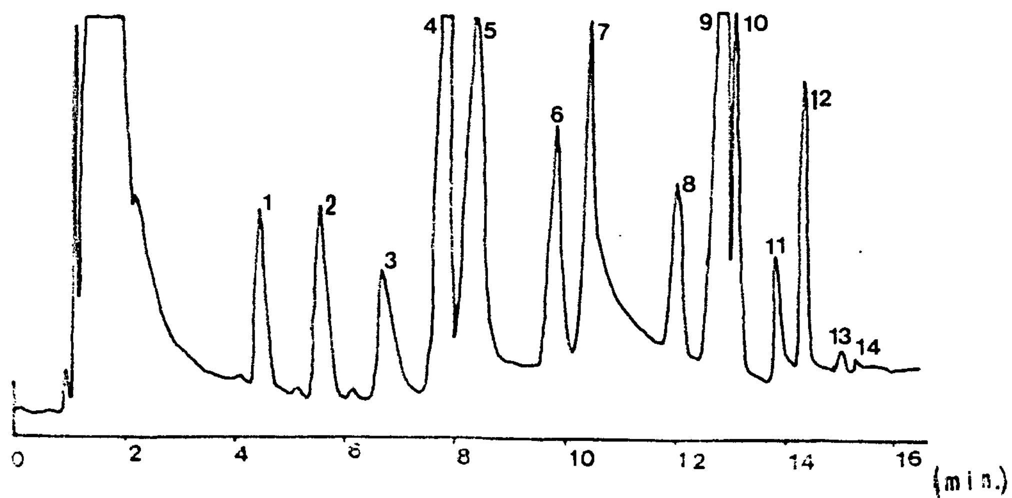


Fig. 2.36 Same standard after 63 days at room temperature and in the freezer.

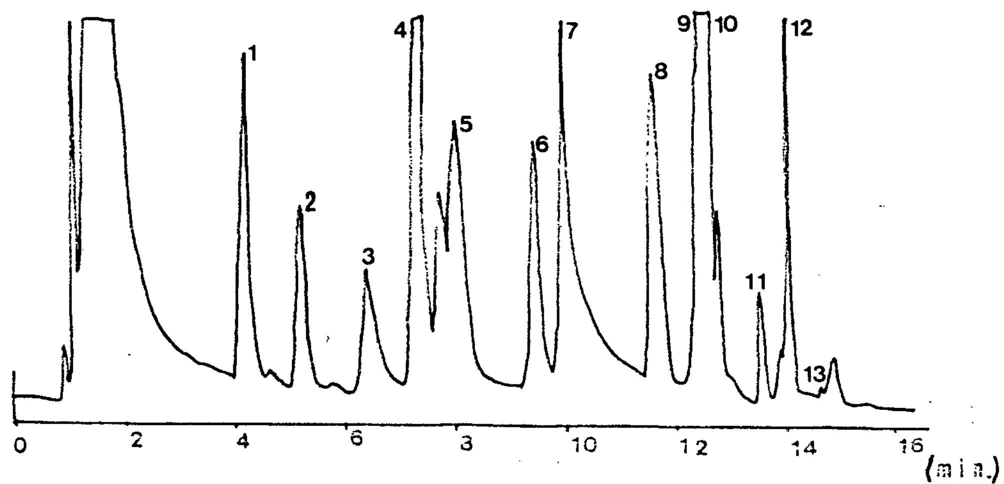


Fig. 2.37 Same standard after 66 days of which 3 were in sunlight.

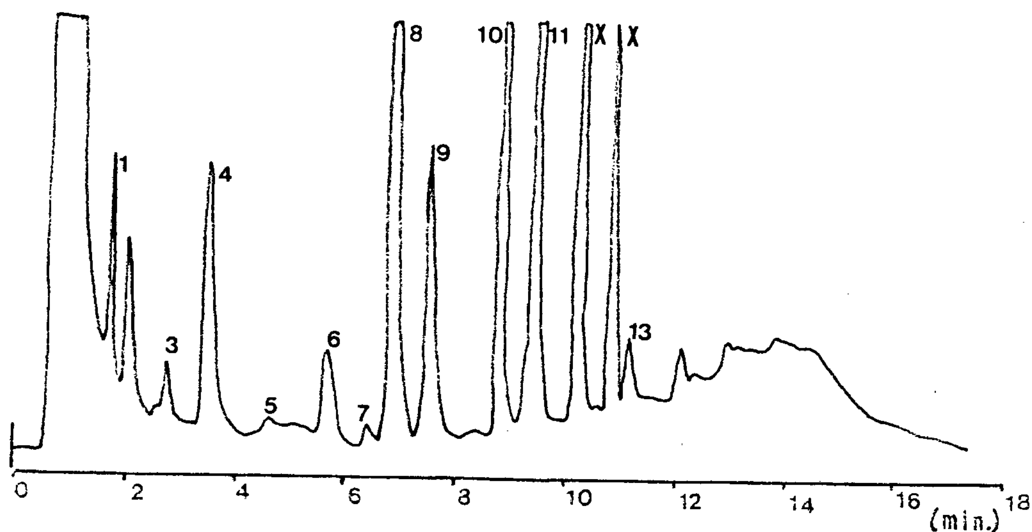


Fig. 2.38 Same standard run after 202 days of which 139 were in sunlight.

Figs 2.35 - 2.38: Time series study on peak stability. Numbers correspond to following amino acids: 1 - asparagine; 2 - serine; 3 - glutamic acid; 4 - glycine; 5 - threonine and arginine; 6 - alanine; 7 - tyrosine and mono-histidine; 8 - hydrolysis product; 9 - proline; 10 - methionine; 11 - valine; 12 - phenylalanine; 13 - isoleucine; 14 - leucine; X - unknowns.

We can see that there is relatively no change in the size and shape of the first 2 chromatograms after 63 days of periods in the freezer, at room temperature and in the dark. We notice some minor changes after the solution is left in intense sunlight for a period of 3 days (chromatogram 7.5). Certain peaks increase and others decrease slightly under these conditions. There is no nett increase due to evaporation of acetone as the vial used was an airtight scintillation counter vial with rubber stopper and screw-cap. Major changes occur after 202 days in sunlight. Some unknown peaks X are formed and some peaks have increased dramatically in size (e.g. peak 11 - Valine) or been reduced entirely (10 - methio-

nine). This chromatogram was run using a modified methanol-buffer gradient, whilst the other three chromatograms were run by the acetonitrile-methanol-buffer gradient of Einarsson et al. (1983). Despite the changes in the last chromatogram, it becomes clear that the derivatives are so stable that normal exposure to heat and light between derivatization and injection would not affect the derivative in the slightest. Derivatization can be done **in situ** and left in the sun for a few days before proper storage is available. They can be left in the freezer and in the dark for very lengthy periods before injection. Care must be taken to prevent acetone evaporation.

2.1.9 Summary of the experimental factors and some kelp physiological applications

From these previous studies of important experimental factors, certain points have become apparent.

In observing the pH of reaction, the pH of the sample must be brought as close as possible to the operating pH of 7,7 before the buffer and derivatizing reagent are added. Highly acidic or basic samples need to be neutralized before derivatization. Failing this the reproducibility could be affected.

Water reacts with the reagent and may swamp the closer amino acid peaks, so the reagent volume and time of injection must be controlled to some extent. The reaction of water with the reagent is slower than the reaction with amino acids and this is used to advantage in reducing the amount of hydrolysis product formed.

Certain amino acids form 2 mono-labelled products, the chemistry of their reaction is little understood. Further investigation into a method for the conversion of mono-labelled products to one di-labelled product is required. Interference from the reagent peaks must be overcome.

Either the pentane extraction stage is improved to reduce loss of amino acid on reagent removal, or other means of removing the reagent need to be developed. Removal of excess reagent after

amino acid reaction would be important in halting hydrolysis product formation. Some reproducibility has to be sacrificed in utilizing the pentane extraction stage. On the other hand, some resolution has to be sacrificed to keep the reproducibility by eliminating extraction. These have to be weighed up in deciding which is required.

Acetone forms a large absorbance peak on elution that swamps closer amino acid peaks. Reducing the amount of added acetone and using a fluorescence detector overcomes this interference.

This technique is characterized by an almost instantaneous amino acid derivative formation and a subsequently impressive product stability. The reproducibility is well within limits provided the pH of the sample can be brought to reaction requirements.

A series of trial applications of the methodology to physiological samples were undertaken to both determine the amino acid profiles of kelp matter and to check for any matrix interferences normally associated with physiological fluids. Frozen and dried portions of kelp frond were extracted with ethanol for a day to determine the amount of free amino acids present. More samples were hydrolysed to determine the amount of total combined amino acids.

A portion of the ethanol used for kelp extraction was derivatized according to the procedure and then separated by HPLC. The following chromatograms were obtained.

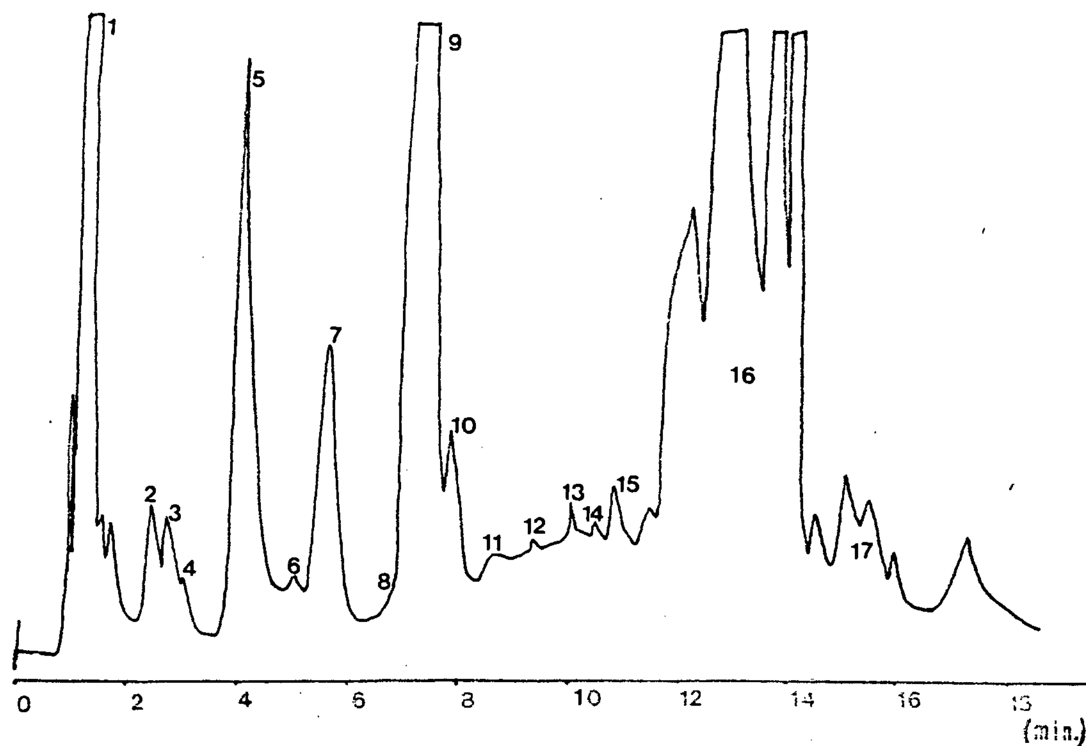


Fig. 2.39 Chromatogram of an ethanol extract from a frozen kelp frond section. Numbers correspond to the following amino acids: 1 - acetone; 2 - asparagine; 3 - aspartic acid; 4 - serine; 5 - glutamic acid and glycine; 6 - arginine; 7 - alanine; 8 - mono-histidine; 9 - water; 10 - ammonia; 11 - proline; 12 - methionine; 13 - valine; 14 - phenylalanine; 15 - isoleucine and leucine; 16 - reagent peaks; 17 - unknowns, possibly some peptides.

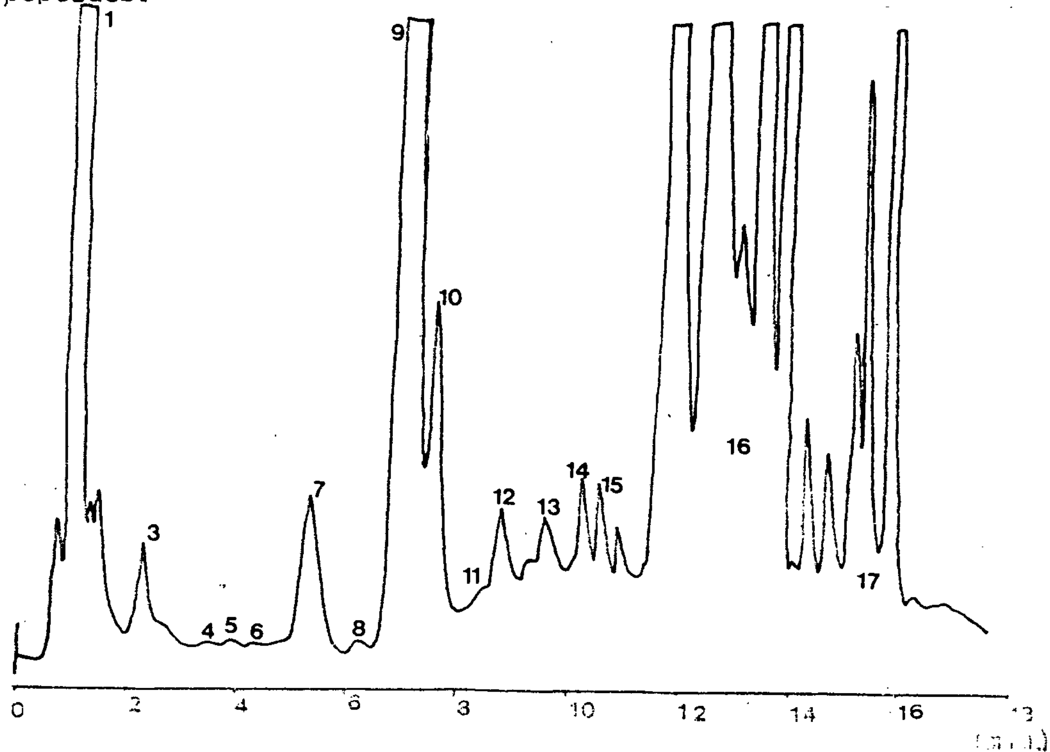


Fig. 2.40 Chromatogram of an ethanol extract from a dried kelp frond section. Identities of amino acids in above caption.

The ethanol releases the dissolved free amino acids from the kelp into solution. No bacterial breakdown occurs in this ethanol medium. A large amount of free alanine is found in kelp judging by the relatively large peak 7 for both of the samples. The difference in amino acid profiles between the dried and frozen kelp species is interesting. The largest amount of change would have occurred in the dried kelp species, this can be seen by the large amount of ammonia present (peak 10), which is a known breakdown product of amino acids (Hollibaugh et al., 1980). The glycine peak 5 for the dried kelp species is non-existent. It will suffice to say at this stage that biochemical changes of the free amino acids in kelp are very rapid and samples should either be treated immediately or preserved until analyses can be done. There are no other physiological compounds that form interfering peaks within the run. There are unidentified peaks (peaks no 17) eluted last that could be due to peptides or other less polar amino acid derivatives.

To determine the total combined amino acids, the hydrolysis procedure in section 2.1.1.2 was applied to the frozen and dried sections of frond. The following chromatograms were achieved:

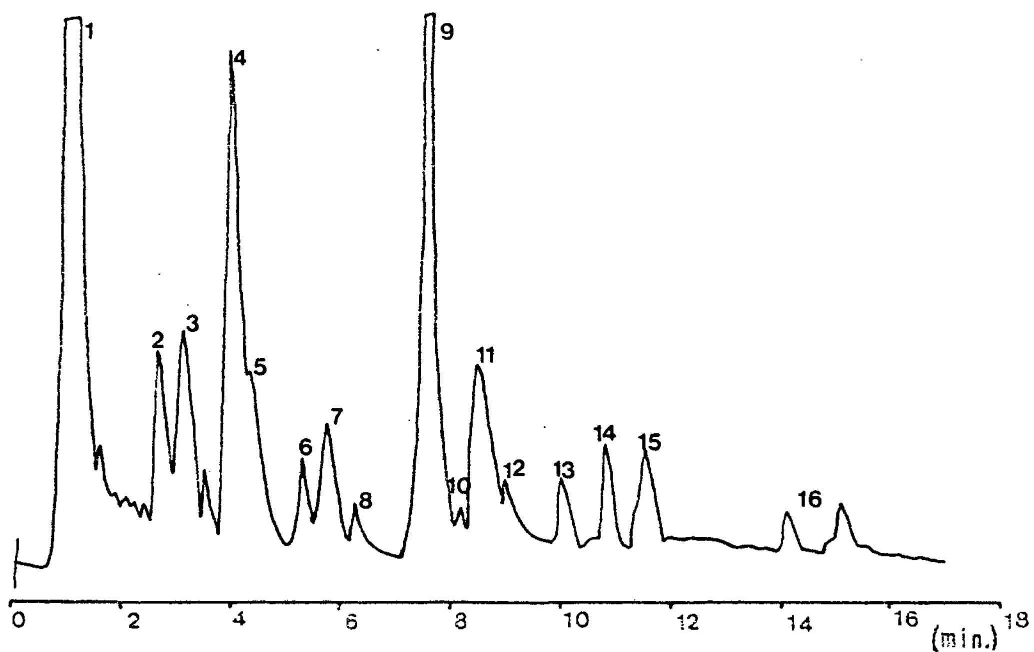


Fig. 2.41: Chromatograms of hydrolysed amino acids from a frozen kelp frond. Numbers of amino acids have been given in fig. 2.39.

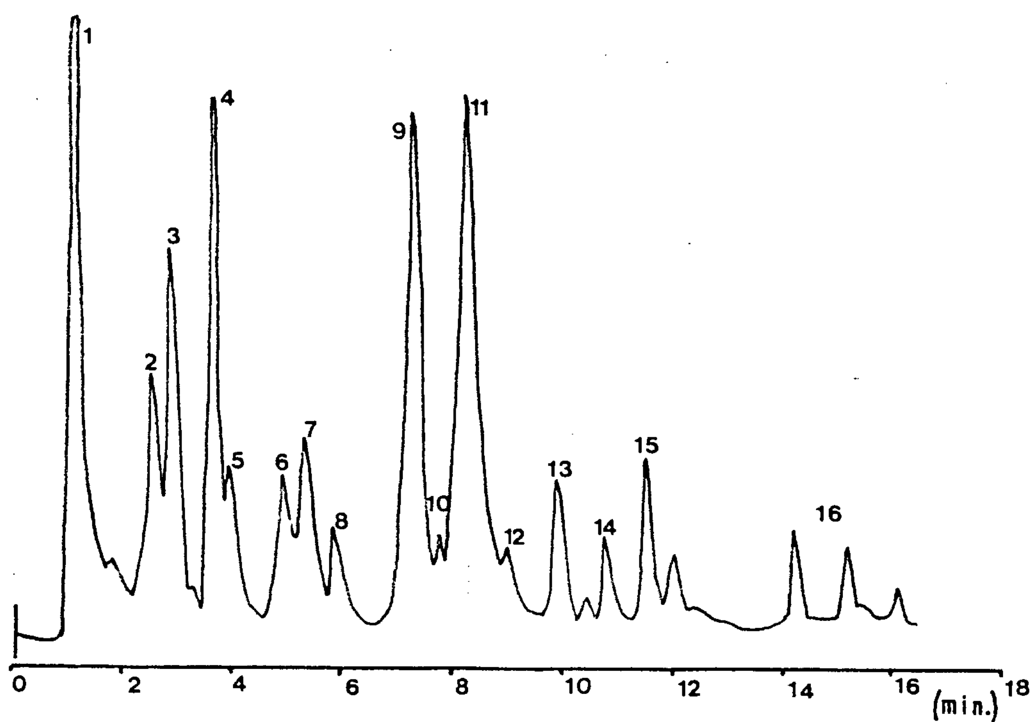


Fig. 2.42: Chromatograms of hydrolysed amino acids from a dried kelp frond. Numbers of the amino acids are given in fig.2.39.

The above 2 chromatograms are qualitatively very similar, except for a large amount of proline for the dried hydrolysate. It is possible that physiological changes occur due to stress of some sort (e.g. temperature, dehydration). This may affect the amino acid profiles. Monitoring these changes by controlling one variable in a time-series study would give us a better understanding of amino acid profiles in kelp. The concentrations of the total combined fraction are about five times larger than the free amino acid fraction.

2.2 FIELD STUDY METHODS

2.2.1 Devising the experiment

Having developed a suitable analytical technique to determine amino acids in seawater at the concentrations expected, it was decided to plan a field exercise in which these methods would be used to attempt to quantify the nett process of exudation from kelp in a simulated natural environment. The experiment was designed in a simple way to minimise any extraneous effects. Unfortunately, the results were far more complex than were anticipated and obviously even such simple experiments in the marine environment lead to results which are difficult to interpret (see Discussion). Nevertheless, the study is reported on here since some insight into exudation and bacterial action can be gained. To observe the nett process of exudation and uptake, a kelp frond was placed in a bucket of previously unfiltered seawater and sampled at various time intervals for 240 minutes. At 120 minutes the kelp was removed to monitor the nett uptake of the previously exuded species. For reproducibility three such experiments were run simultaneously. To observe exudation on its own, antibiotics were added to three further buckets and sampled at the same time intervals. The chemical and bacterial baselines of the system were determined by a control model set up in unfiltered seawater without a kelp frond.

Bacterial activity determination during this study required a concurrent experiment at each sampling time. Due to the impossibility of monitoring activity for each bucket at each time, only one

experimental bucket (A_1) and the control bucket (C) were chosen for activity studies. The reasoning for this was that the experimental buckets were all assumed to be similar and only one would be necessary for activity determinations; the antibiotic buckets would have a very low level of activity due to the added antibiotics suppressing this activity, therefore determinations are not required; the control bucket will have active bacteria present and therefore would be required for activity assessment.

2.2.2 Equipment, apparatus and procedures

Seven 20dm3 plastic buckets were used as containment vessels. 15dm^3 of seawater was added to each at time t = 0. Sampling was done by using seven 50ml plastic syringes (Millipore). 60ml of this sampled seawater was placed in each of four 10ml plastic scintillation sampling vials, after the sample had been filtered through one pre-combusted 25mm diameter GF/F® filter (Whatman) and a 25mm diameter, 0,2 µm pore size Nucleopore PC filter both in series, contained in a 25mm internal diameter plastic Swinnex® W/O filter holder (Millipore), to remove both particulate matter and then bacteria. The sealed vials were placed in a 15dm^3 thermostatted flask containing liquid nitrogen to prevent further bacterial activity. After each sampling time the GF/F® filter was removed and placed in a 47mm disposable plastic petri dish® (Millipore), which was sealed with the lid and placed in ice for later CHN-analysis. The filters were replaced before the next sampling time interval. At about the same sampling time after the

above procedure was completed, exactly 10ml of unfiltered seawater was sampled from the buckets using a 10ml plastic syringe (Millipore). This was added to a 10ml plastic scintillation counter vial containing 1ml of 25% glutaraldehyde (electron microscopy grade, Merck) for fixing the bacteria for counting at a later stage.

After sampling for time t = 0, three kelp fronds were added to each of the experimental buckets A and three kelp fronds were added to each of the antibiotic buckets B together with 110ml of $10g.dm^{-3}$ antibiotic solution containing chloramphenicol, erythromycin and tetracycline. The control bucket C was not treated.

Sampling times were at time = 0 without kelp, then 5; 10; 14; 30; 60; 90; and 120 minutes after the kelp was added. Thereafter the kelp was removed and sampling continued for 150; 180; 210; and 240 minutes elapsed time. The experiment was then stopped and the kelps taken back to the laboratory for dry weight assessment.

In order to determine the bacterial activity at each sampling time, 50ml of seawater from bucket A_1 and 50ml of seawater from bucket C were each added to a 100ml Xactics plastic bottle with press-cap lid and placed in a constant temperature water bath (same temperature as the seawater). Using a P20 pipetman® (Gilson), 12 μl of ^3H-thymidine solution (49 $Ci.mmol.dm^{-3}$, cat. no.TRK637, Amersham) was added to this seawater. 5ml of this seawater was immediately added to a 10ml disposable plastic stoppered test tube (Weil Organization) and was cooled on ice in a cooler box for 1 minute.

5ml of ice-cold 10% (v/v) trichloro-acetic acid (TCA) was added by means of an Oxford lab pipettor automatic dispenser. The test tube was returned to the ice bath for futher analysis in the laboratory. The above procedure was applied every hour to each incubation initiated from the original experimental and control buckets' sampling times. Furthermore 1ml from each incubation flask was frozen to measure the background radioactivity as well. The total activity experiment lasted 453 minutes and was cut short due to failing light with only three more subsamples to do.

2.2.3 Methods of the chemical profiles

2.2.3.1 Amino acid methods

The amino acid sampling vials were pre-cleaned by a procedure to eliminate possible contamination. The 10ml plastic vials were initially washed in 2% (v/v) Contrad® (Merck) for three hours then left in 6M HCl for 24 hours after they were rinsed three times to remove excess Contrad®. After the acid wash they were again rinsed with Milli-Q® water and shaken with 2ml HPLC grade methanol for a few minutes. They were left this way for a further 24 hours. They were then dried, sealed and labelled with a red indelible ink cross on the cap, signifying amino acid analysis only.

The filtered samples stored in the liquid nitrogen container were,

taken to the laboratory where they were stored in the freezer at -20°C until they were required for derivatization. Due to the delay in securing a fluorescence detector the samples' amino acid composition may have been affected through being stored in the freezer, but results suggest otherwise (Dawson and Gocke, 1978).

Once the HPLC system 3 (Beckman HPLC, Apple controller, Drew interface and Perkin Elmer detector) was operational, the samples were thawed in batches and derivatized according to the procedure listed in Section 2.1.1. The derivatization vials were cleaned in a similar manner to the sample vials. The derivatized samples were injected after a maximum delay of 24 hours. This would not have affected the stability, as has been shown (Section 2.1.8).

2.2.3.2 N-nutrient methods

The nutrient-N analyses were organized by Mr Brauer from the Botany Department, University of Cape Town. These analyses were done on the Technicon AutoAnalyser at the Department of Sea Fisheries' chemical laboratory (Ebenezer Road, Cape Town). Nitrate was analysed using a modified EDDH-sulphanilamide method after Ca-reduction (Strickland and Parsons, 1972). Ammonia was analyzed according to the phenol-hypochloride method (Strickland and Parsons, 1972). Total N was calculated using the peroxo-disulphate oxidation method according to Nydahl (1978).

2.2.3.3 CHN analysis method

In order to determine the amount of particulate C matter in each of these buckets, the pre-combusted GF/F® filters in the frozen petri dishes® used to filter the samples, were used for CHN analysis. After being stored in the freezer for a few weeks the samples were thawed and the filters were removed from the slides and placed in a vacuum dessicator containing silica gel until they were completely dried. Prior to this some of the more moist filters were pre-dried in a laminar flow chamber, normally used for dust-free chemical preparations. The clean air moving over them speeded up the drying process. Due to some leakage through the petri slides from improper closure before placing in the ice-bath, it is possible that some of the particulate matter may have been washed off the filter. This loss would have incurred an error in some of the results and it is probable that these analyses would be the least reliable in all the analyses done in this study.

After drying, the 25mm filters were folded using stainless steel tweezers and placed in 12 x 6 x 5mm tin boats. These boats were then folded closed with the filter inside, flattened along the edges and pierced with about six pin holes along each side to aid final combustion. These samples were then analysed by Mr P. Fielding of the Zoology Department, University of Cape Town, using the Heraeus universal combustion analyser, according to a modified method of Monar (1972). These results are discussed later.

2.2.4 Methods of the bacterial profiles

2.2.4.1 Bacterial activity methods

The frozen test tubes in the cooler box were thawed out in the laboratory and the samples were each filtered through a 25mm diameter 0,22 µm pore size GS filter (Millipore) in batches using a Millipore multiple filter holder connected to a vacuum pump. The filters were rinsed twice with 5ml 5% TCA with each washing first added to the empty test tube so no residual bacteria was lost. Thereafter the filter paper was placed in a 10dm^3 scintillation counter vial and stored until the scintillation counter became available for use. Before counting, 10ml of Filtercount® scintillation cocktail (Packard) was added to the vial containing the filter. These were counted on a 460C Packard liquid scintillation counter after an appropriate quench curve was set up. Calculations and conversions of these results appear in a later section (Fuhrman and Azam,1982).

2.2.4.2 Bacterial counting procedures

The bacterial counting samples fixed with 25% glutaraldehyde were counted by Mr I. Humphries of the Zoology Department, University of Cape Town, according to the Acridine orange method of Hobbie et al. (1977), modified by Painting et al. (1986).

This entailed staining the 25mm diameter 0,2 µm pore size nucleopore

filters with an irgalan black solution before filtration to reduce the background fluorescence. The samples were stained with Acridine orange reagent (Merck) prior to filtering through the stained filters at low vacuum pressure (0,8atm). A portion of the filter was cut out for observation and counted using a Zeiss standard 18 microscope fitted with an epi-fluorescent condenser, HBO 50W Mercury lamp , 455 - 500 bandpass excitation filter 510 beam splitter and LP520 barrier filter. Magnification was 1000x. Bacterial counts were divided into six size and shape categories (small cocci - Sc; large cocci - Lc; small rods - SR; large rods - LR; small U-shaped - SU, and screw-shaped -∿) and the total counts per size field and number of fields were given too. Calculations of bacterial density and bacterial biomass from these counts are shown in Section 3.2.2.

3. RESULTS

3.1 CHEMICAL RESULTS OF THE FIELD STUDY

3.1.1 Amino acid results

A typical chromatogram from the field study samples (bucket A_1, time interval 210 minutes), together with the integrated results and concentration calculations can be seen below:

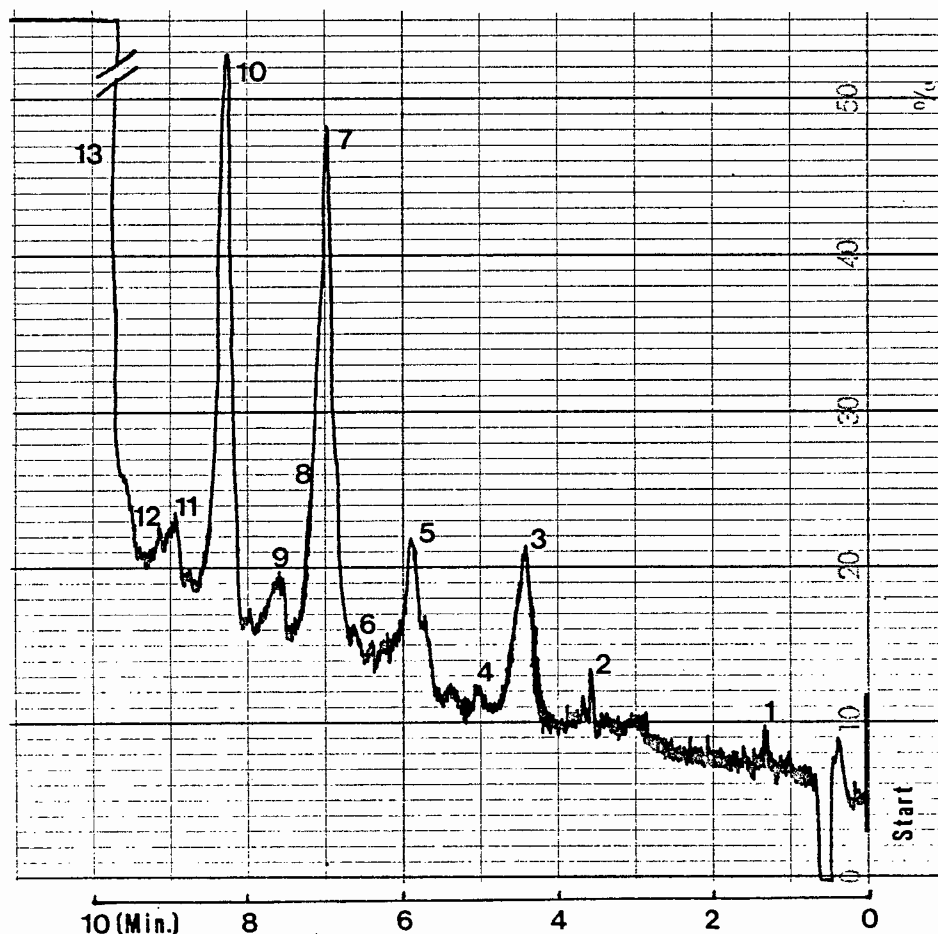
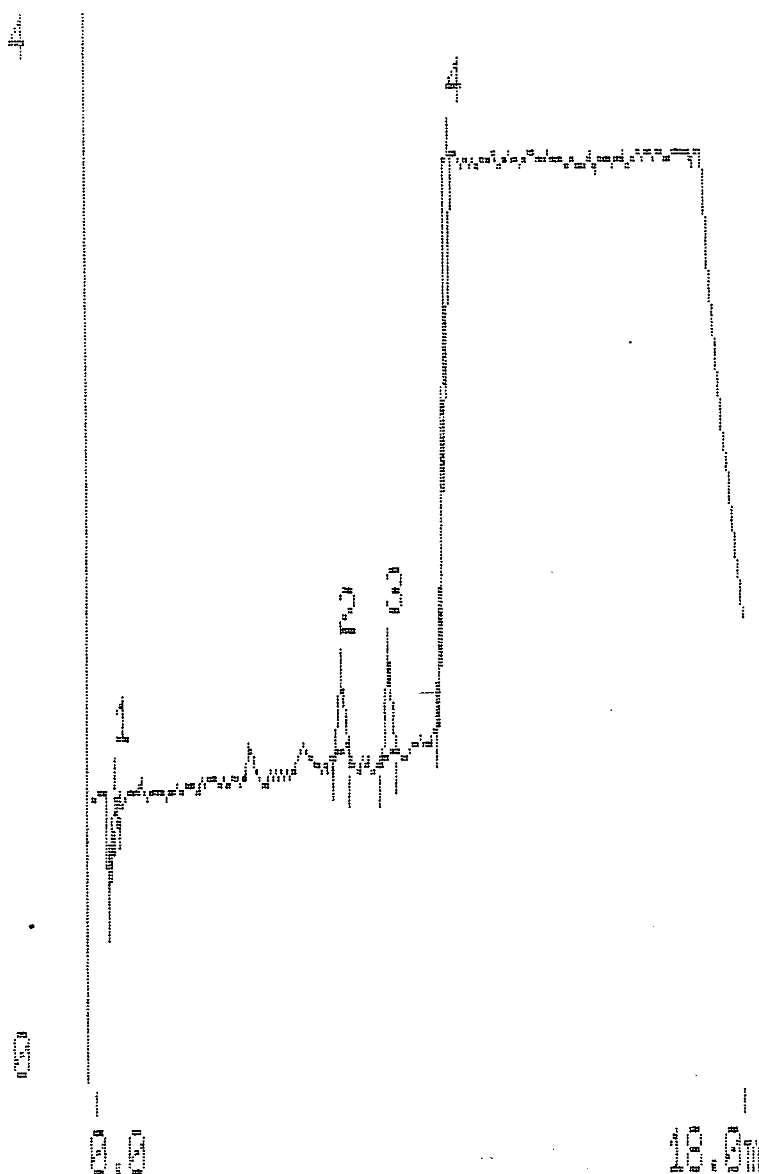


Fig.3.1: Chromatogram of an amino acid sample from the field study (sample A_{1210}). Detector range = 10. Numbers correspond to the following peaks: 1 - cystine; 2 - asparagine; 3 - aspartic acid; 4 - serine; 5 - glutamic acid; 6 - mono-histidine; 7 - glycine; 8 - threonine; 9 - arginine; 10 - alanine; 11 - tyrosine; 12 - mono-histidine; 13 - water.



AMINO ACIDS F.D Sample : 3 Date : 06/02/86 Channel : A

Peak	RT(mins)	Area	Height	Base	%Area
1	0.8	3.109	0.233	0.856	13.444
2	7.1	3.920	0.335	1.221	16.948
3	8.3	4.633	0.440	1.208	20.031
4	10.0	11.466	0.510	2.934	49.577

Fig.3.2: Integrated chromatogram of same sample. Peaks correspond to the following amino acids: 1 - inverted acetone peak; 2 - alanine; 3 - glycine; 4 - water.

Peak	Identity	Area	Concentration (nmol.dm^{-3})
2	asparagine	0,284	0,87
3	aspartic acid	1,366	6,28
5	glutamic acid	0,992	5,41
7	glycine	3,920	4,42
9	arginine	0,510	2,19
10	alanine	4,633	18,65
11	tyrosine	0,454	1,13

Fig.3.3: Table of corrected areas and calculated concentrations for same sample.

3.1.1.1 Qualitative assessment of chromatograms

Observing the above chromatograms, after about 9,5 minutes the baseline increases due to the water peak and remains off-scale masking the further amino acid peaks. This is most unfortunate as valuable information on the other amino acids is lost. We see here the necessity to halt the excess reagent reacting with water, especially at low concentrations. If the access to a fluorescence detector had been available during the development of the method, the hydrolysis product formation at these low concentrations could have been accounted for in some manner, as it was for higher concentrations.

Due to the limited time available using the fluorescence detector, certain important features of this method could not be explored.

The most important of these is the low detection limit. By optimizing excitation and emission wavelengths and using the correct bandpass we would be able to calculate the detection limits and optimize the greatest signal-to-noise ratio for our experimental results. Integration parameters could also have been accurately tested to ensure integration of all significant peaks. From the present results the detection limit is estimated at 10^{-11} M.

3.1.1.2 Quantitative assessment of chromatograms

Most large peaks in the chromatograms were integrated, but some of the peaks with a large enough signal-to-noise ratio were not integrated by the Apple computer integration system (see fig. 3.2 for such a run). It is unknown whether this is due to an interface error or the signal not being large enough for the detector's range. This would mean that valuable information on lower concentration amino acids is lost unless a means of peak quantitation could be found. Using their measured peak heights to calculate their concentrations would not correlate with those concentrations calculated from peak areas. Therefore the peak heights for those integrated peaks were measured as well and an area conversion factor was calculated by averaging the ratio of integrated area to measured peak height. This value was multiplied with the measured peak heights of unintegrated peaks to give an area very close to that had it been integrated.

This area conversion factor was found to be the same for all the

peaks regardless of their identification. This value, which is 0,567 ± 0,038, was calculated from 25 such peaks and the percentage error (6,7%) was in acceptable limits. This error must be weighed up against the possible loss of information by ignoring unintegrated peaks. The error becomes more tolerable when considering the very low concentrations we are working with.

A typical standard run used for quantitative assessment is shown below:

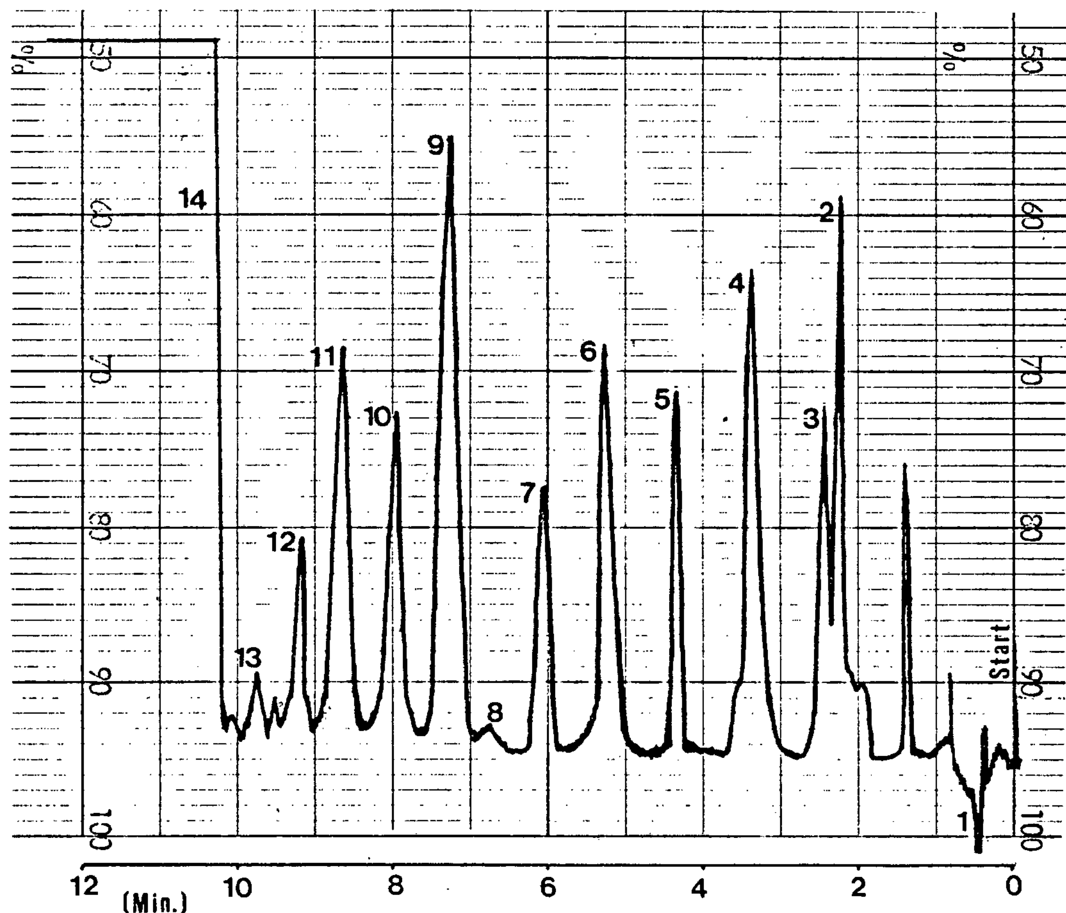


Fig.3.4: Chromatograph of an amino acid standard (A2908). Detector range = 0,1. Numbers correspond to the following amino acids: 1 - acetone; 2 - cysteic acid; 3 - cystine; 4 - asparagine; 5 - aspartic acid; 6 - serine; 7 - glutamic acid; 8 - mono-histidine; 9 - glycine; 10 - arginine; 11 - alanine; 12 - tyrosine; 13 - mono-histidine; 14 - water.

Four such runs were undertaken and the mean areas are shown below. Errors were very low, of the order of the normal reproducibility experienced (2,6%).

Amino Acid	Integrated Area	Conversion Factor (x10^{-3})
asparagine	3,253	3,07
serine	3,334	2,99
aspartic acid	2,174	4,60
glutamic acid	1,832	5,45
glycine	8,867	1,12
arginine	2,319	4,31
alanine	2,484	4,02
lysine	1,158	8,63
tyrosine	3,988	2,50

Fig. 3.5: Table of the mean amino acid concentration conversion factors.

These concentration conversion values were multiplied by each sample peak area for the corresponding amino acid to give the concentration values shown in fig. 3.3. Concentration of each amino acid in the standard was 1 μmol.dm^{-3} after approximate dilution of the standard. This was about 100 times less sensitive than the samples. The calculated concentrations for each amino acid in each bucket were plotted as a function of sampling times. Certain low concentration amino acid profiles were omitted because of the error associated with measuring very small peaks (peaks

below 0,5mm were regarded as noise level). Profiles of asparagine, histidine, lysine and tyrosine were so low as not to give any concrete variability and were therefore not drawn up. Concentrations for each amino acid were very varied with alanine having the largest concentration (between 2 and 170 nmol.dm-3). These profiles are shown below and are discussed in a later section.

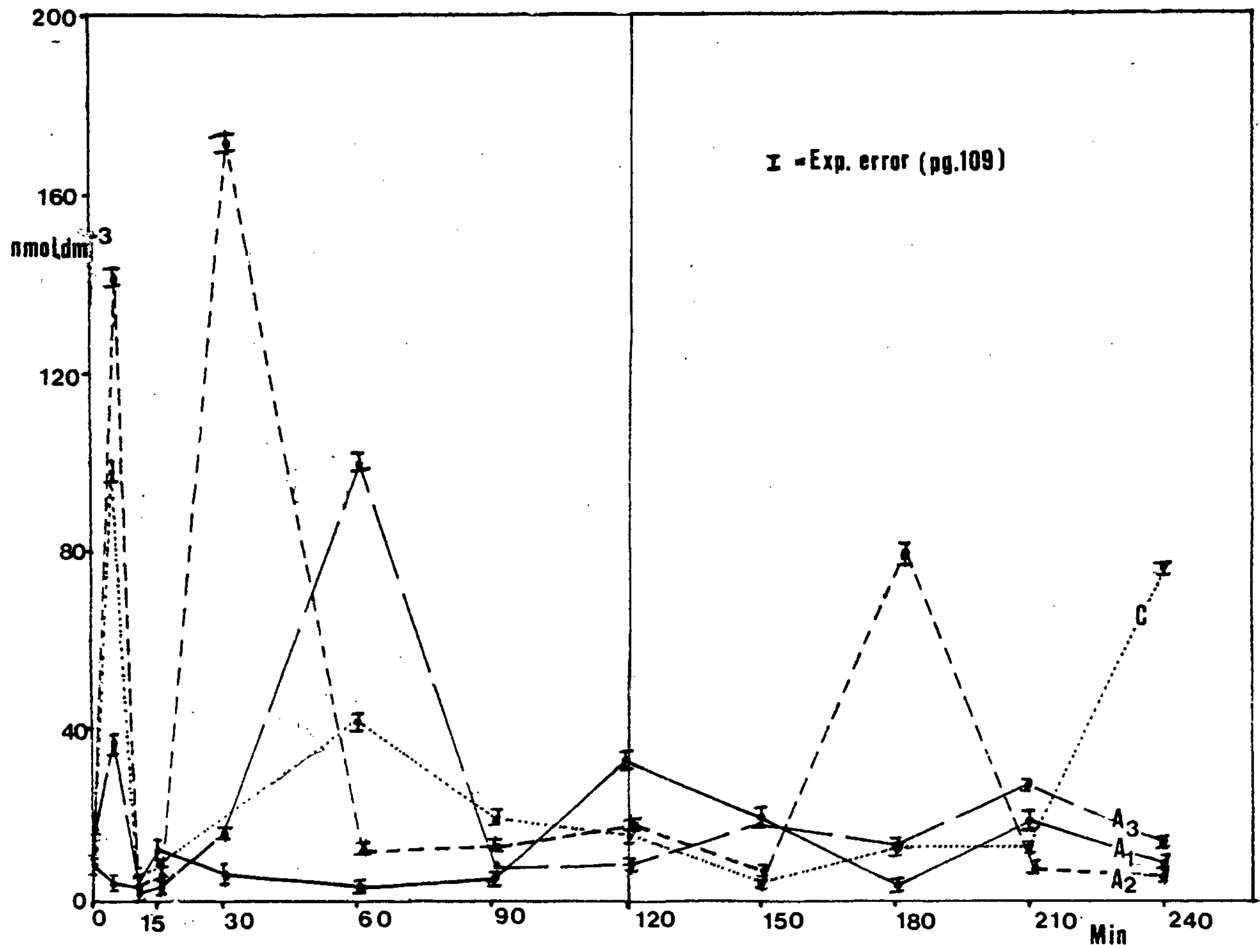


Fig. 3.6: Graphs of the alanine profile for the experimental and control buckets.

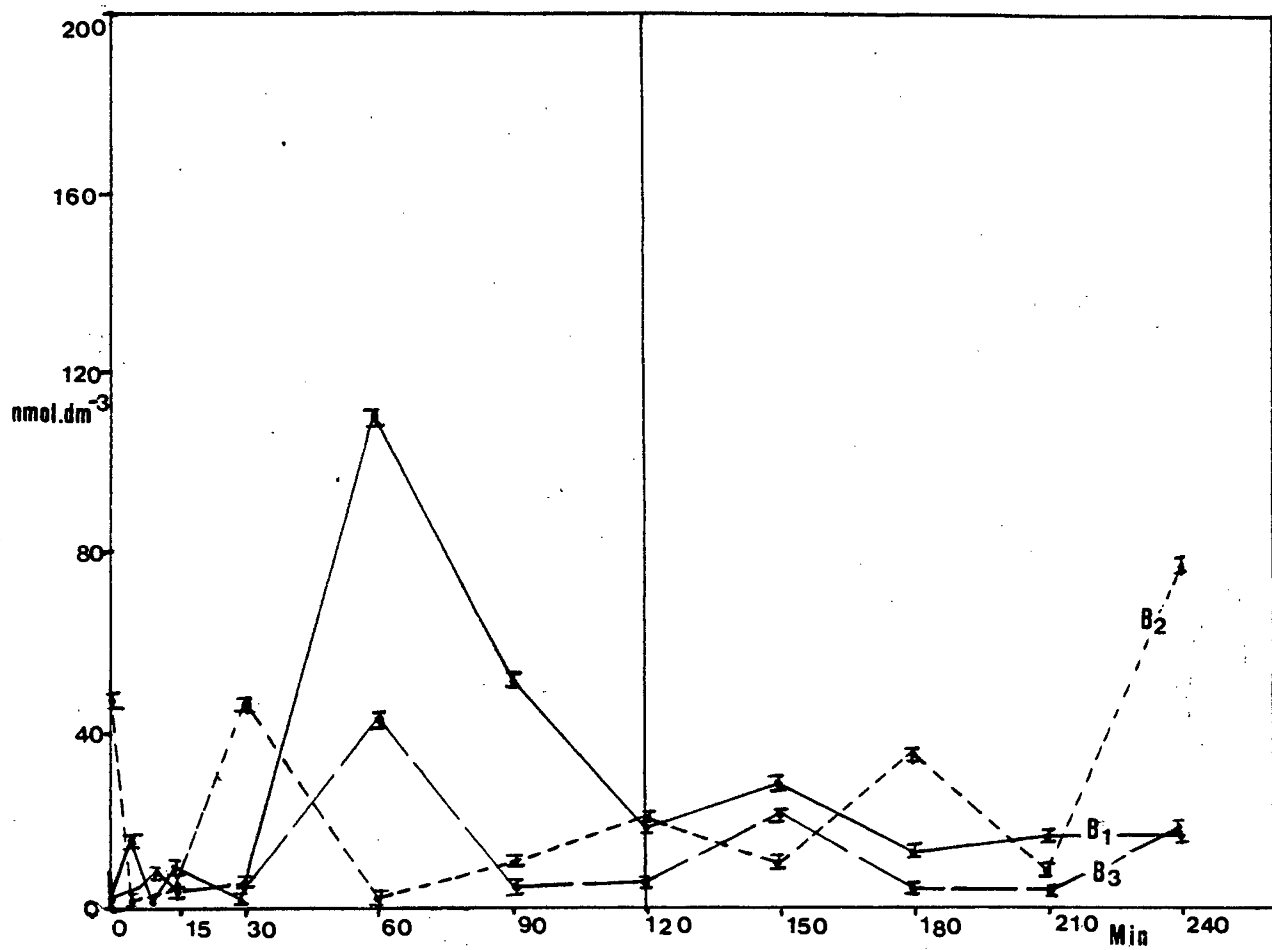


Fig. 3.7: Graphs of the alanine profile for the antibiotic buckets.

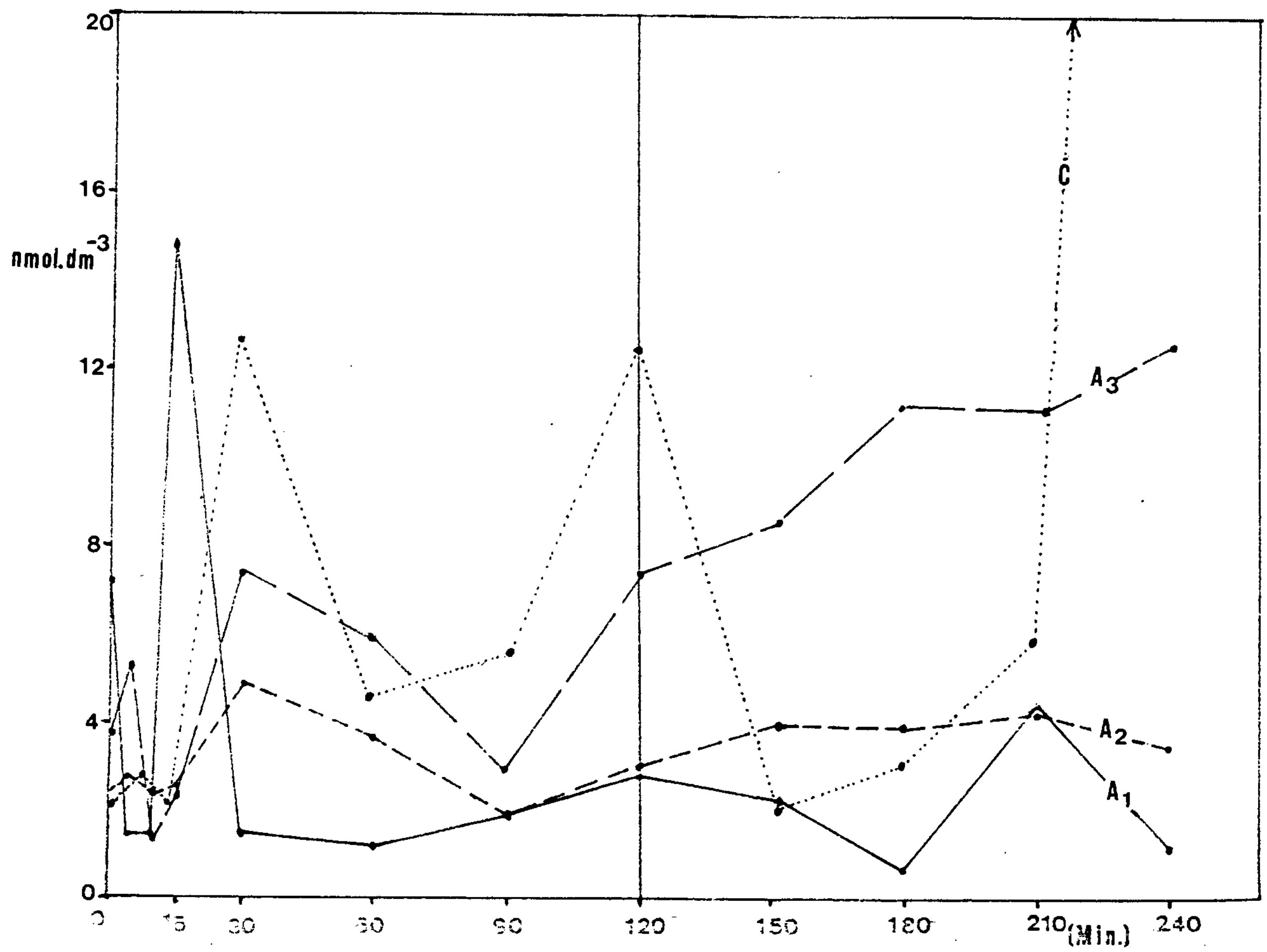


Fig. 3.8: Graphs of the glycine profile for the experimental and control buckets.

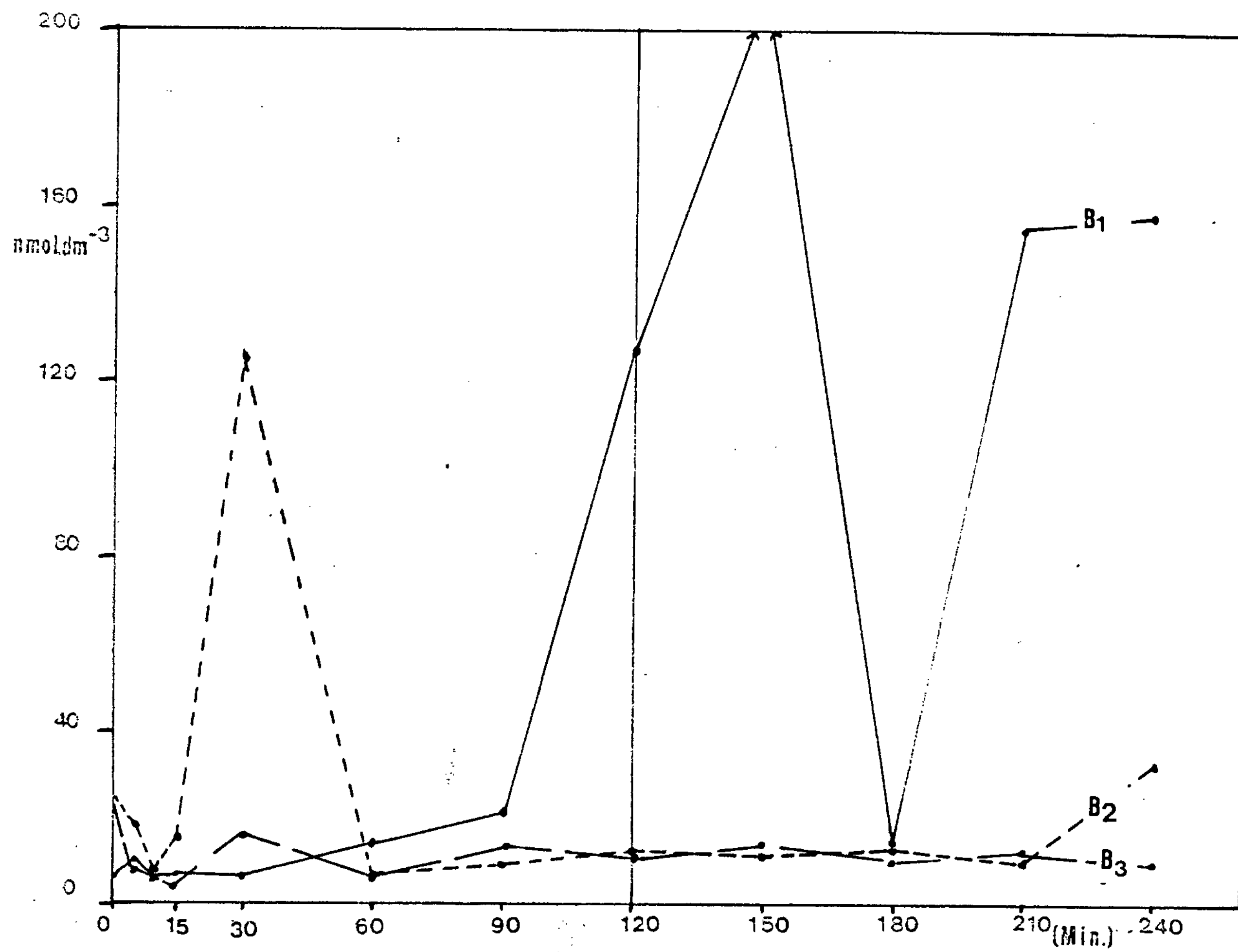


Fig.3.9: Graphs of the glycine profile for the antibiotic buckets.

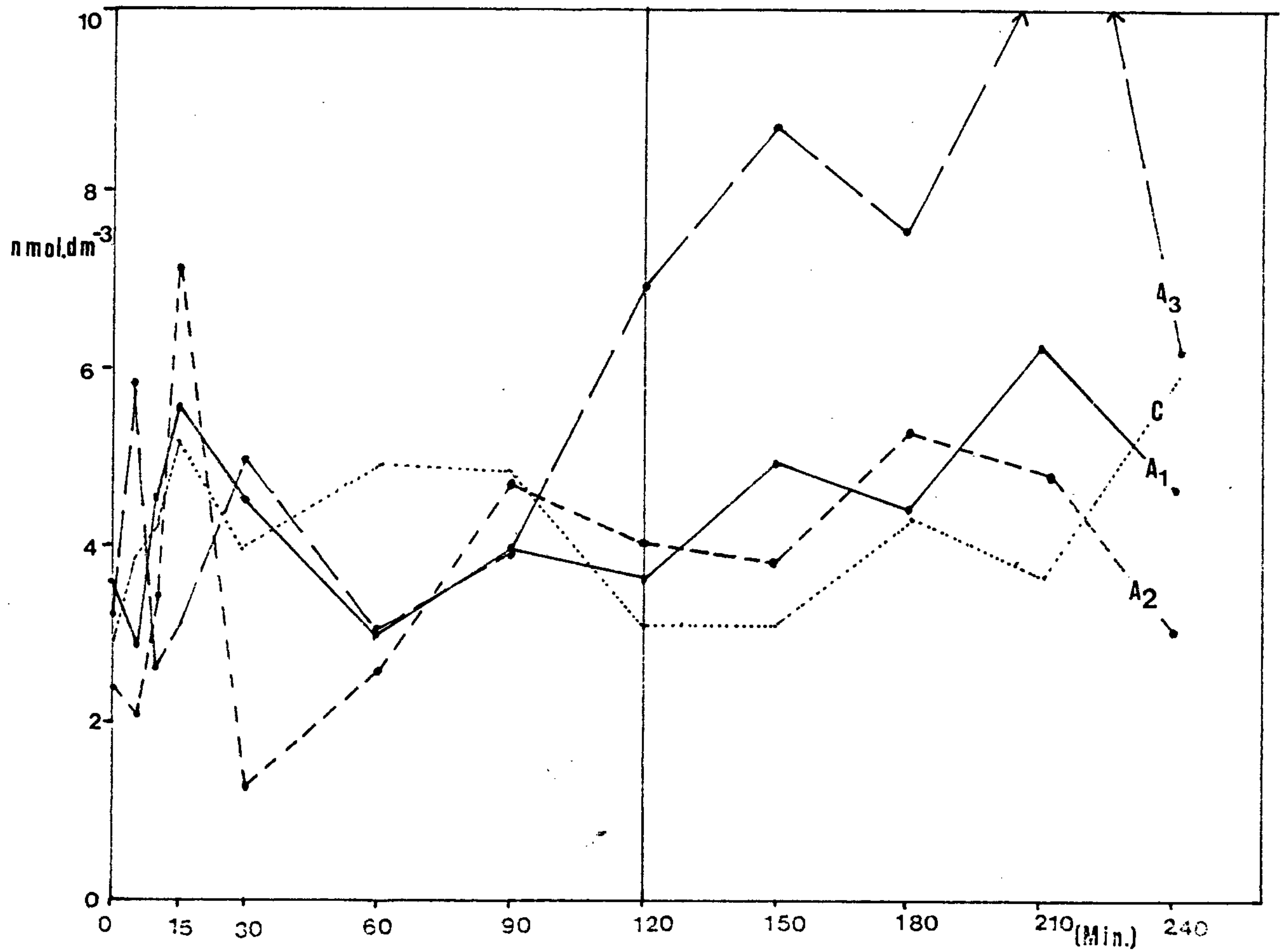


Fig. 3.10: Graphs of the aspartic acid profiles for the experimental and control buckets.

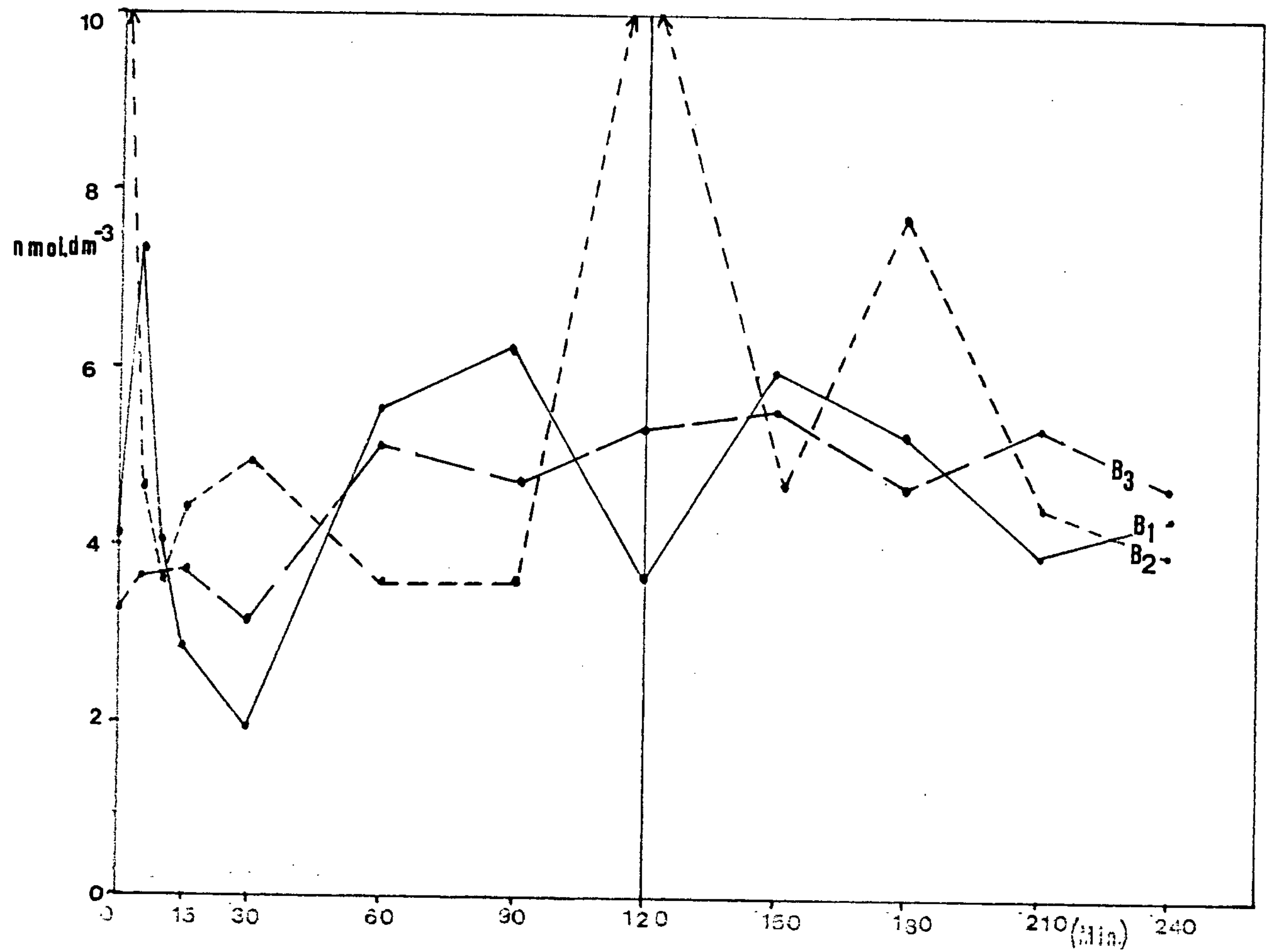


Fig. 3.11: Graphs of the aspartic acid profiles for the antibiotic buckets.

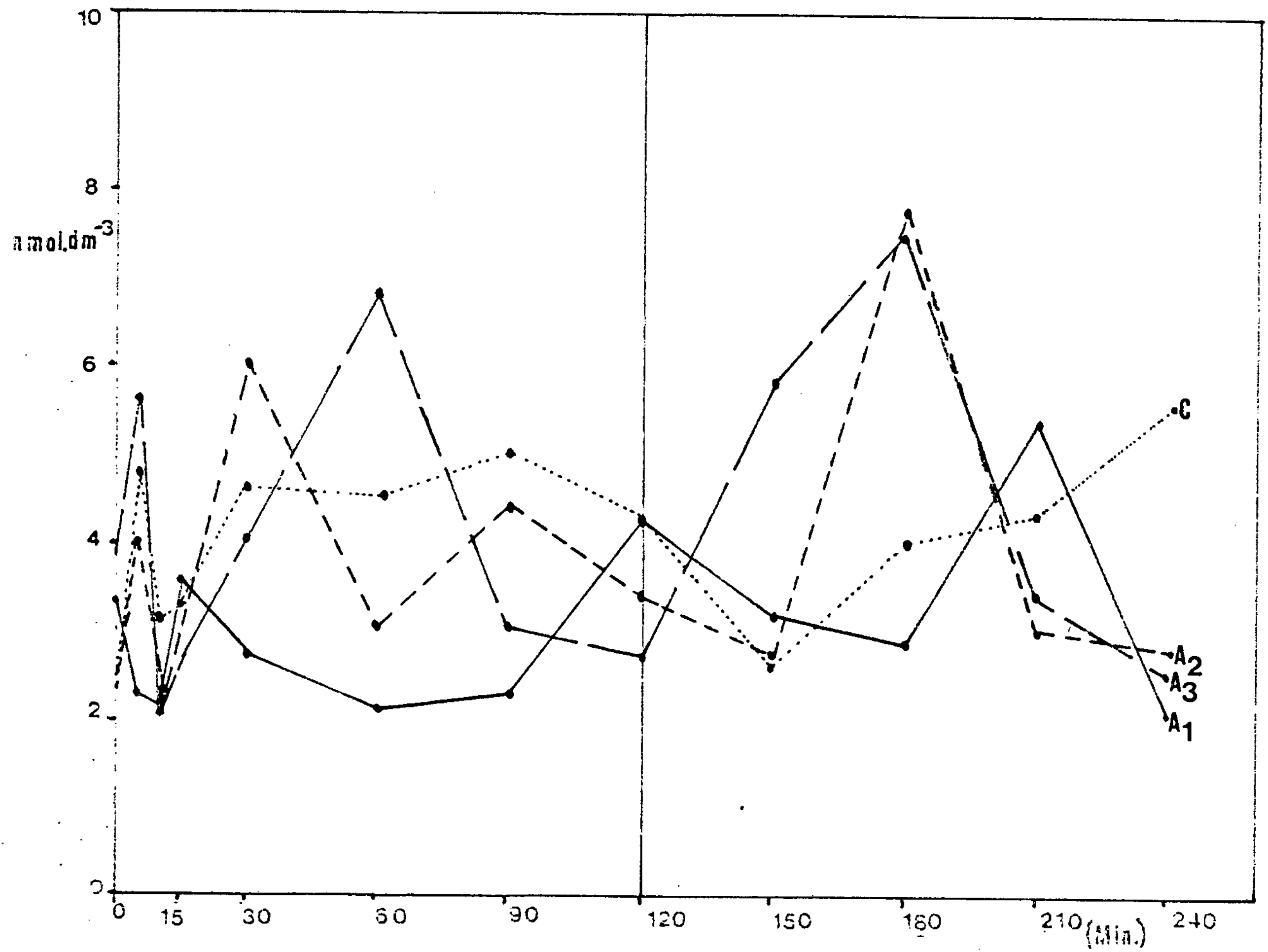


Fig. 3.12: Graphs of the glutamic acid profiles for the experimental and control buckets.

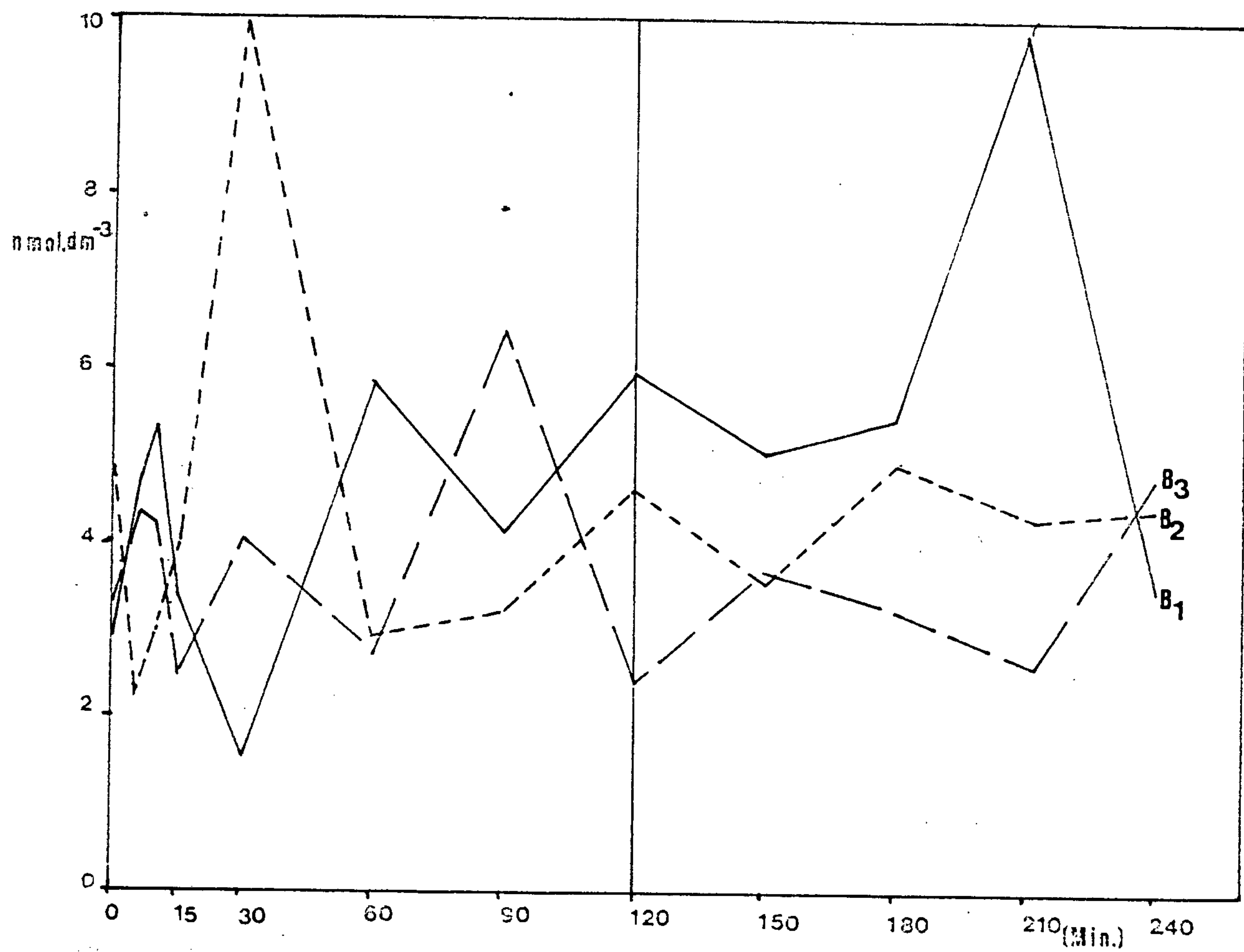


Fig. 3.13: Graphs of the glutamic acid profiles for the antibiotic buckets.

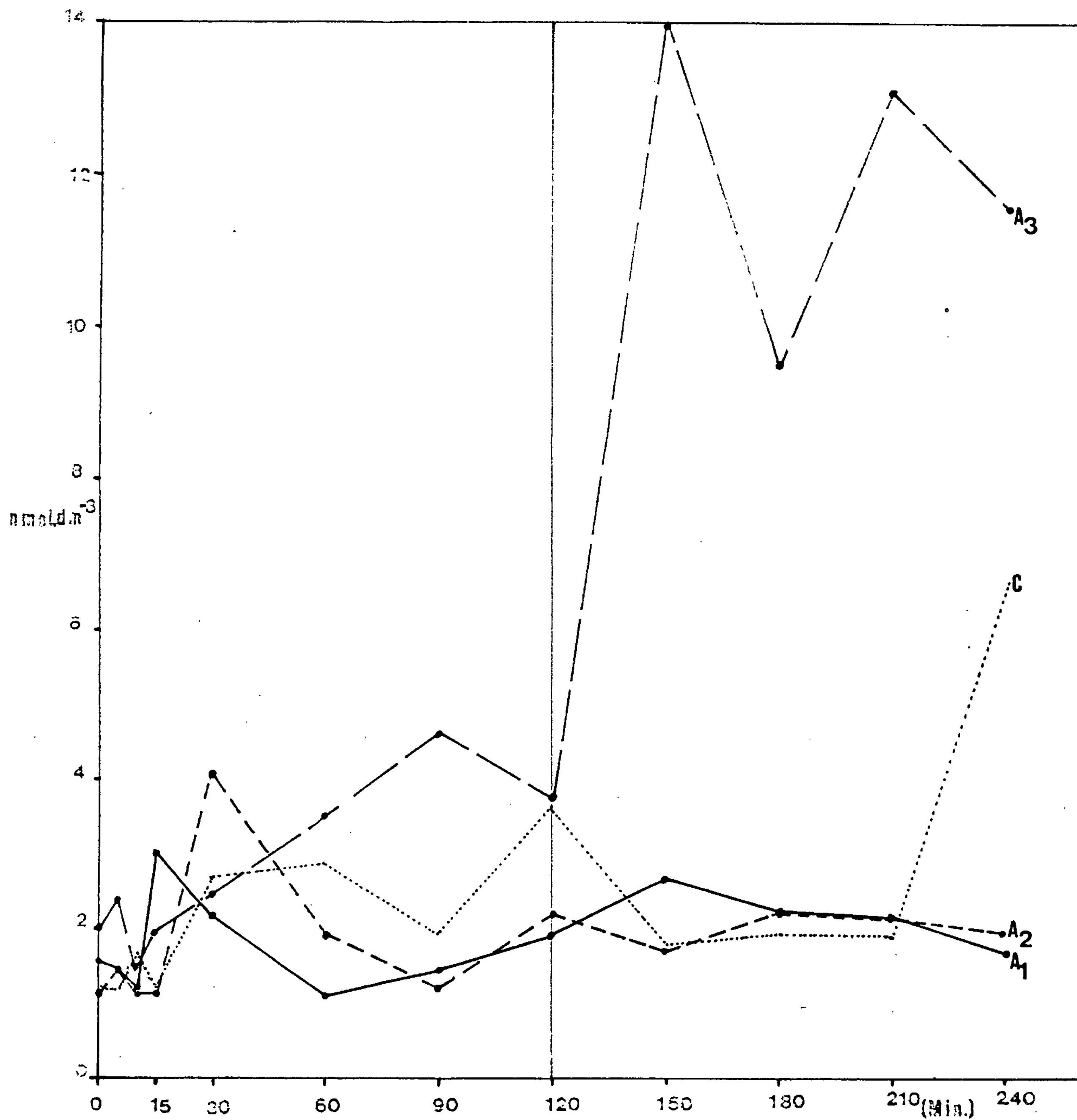


Fig. 3.14: Graphs of the arginine profiles for the experimental and control buckets.

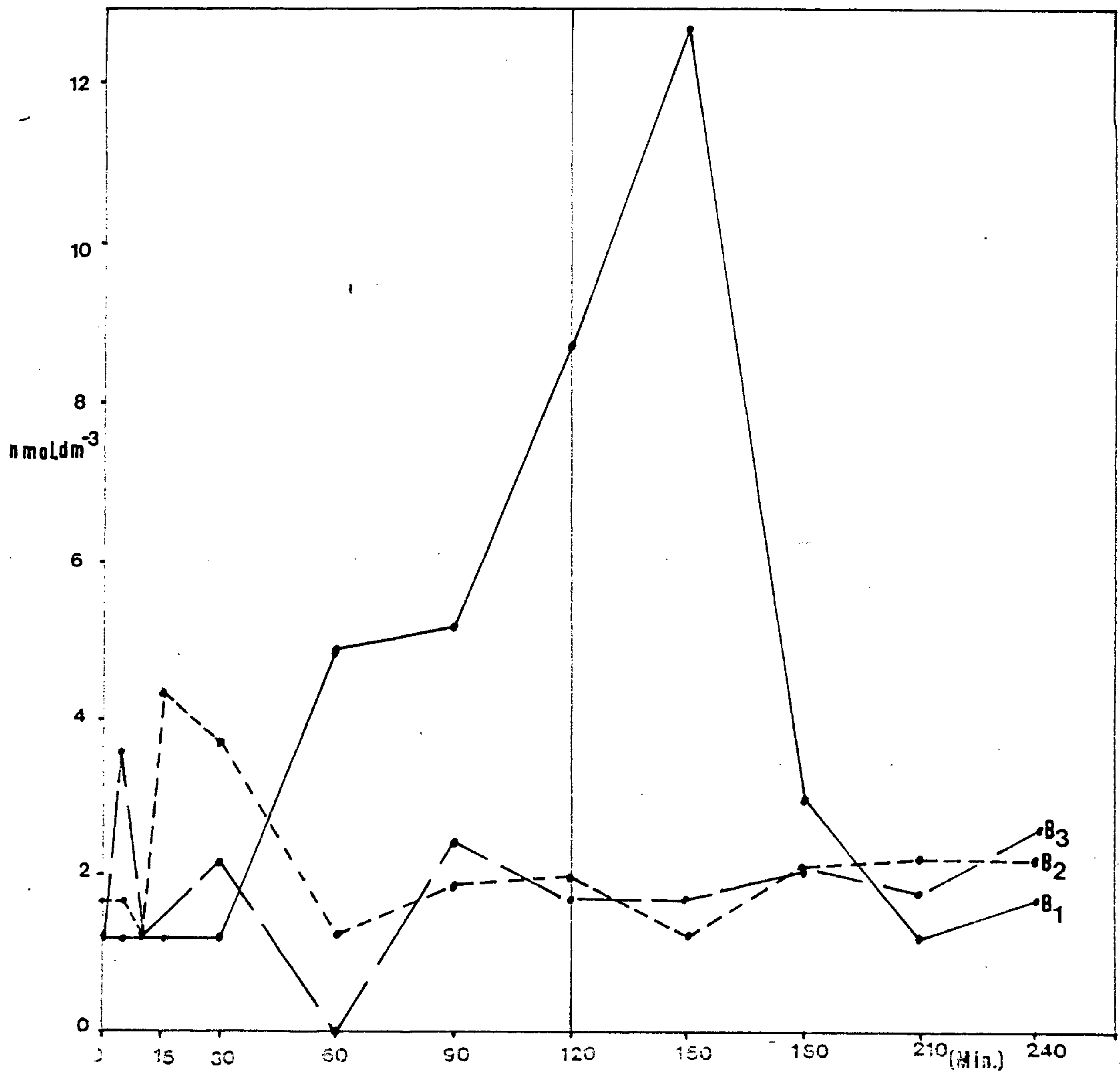


Fig. 3.15: Graphs of the arginine profiles for the antibiotic buckets.

3.1.2 Nutrient results

Concentration values for the analysed results are in $\mu g\ N.dm^{-3}$, which must be converted to $\mu moles.dm^{-3}$ by dividing these results by the molar mass of Nitrogen (N = 14). These new concentration values for ammonia and total N are tabulated in the appendix section. The profiles of these are shown below:

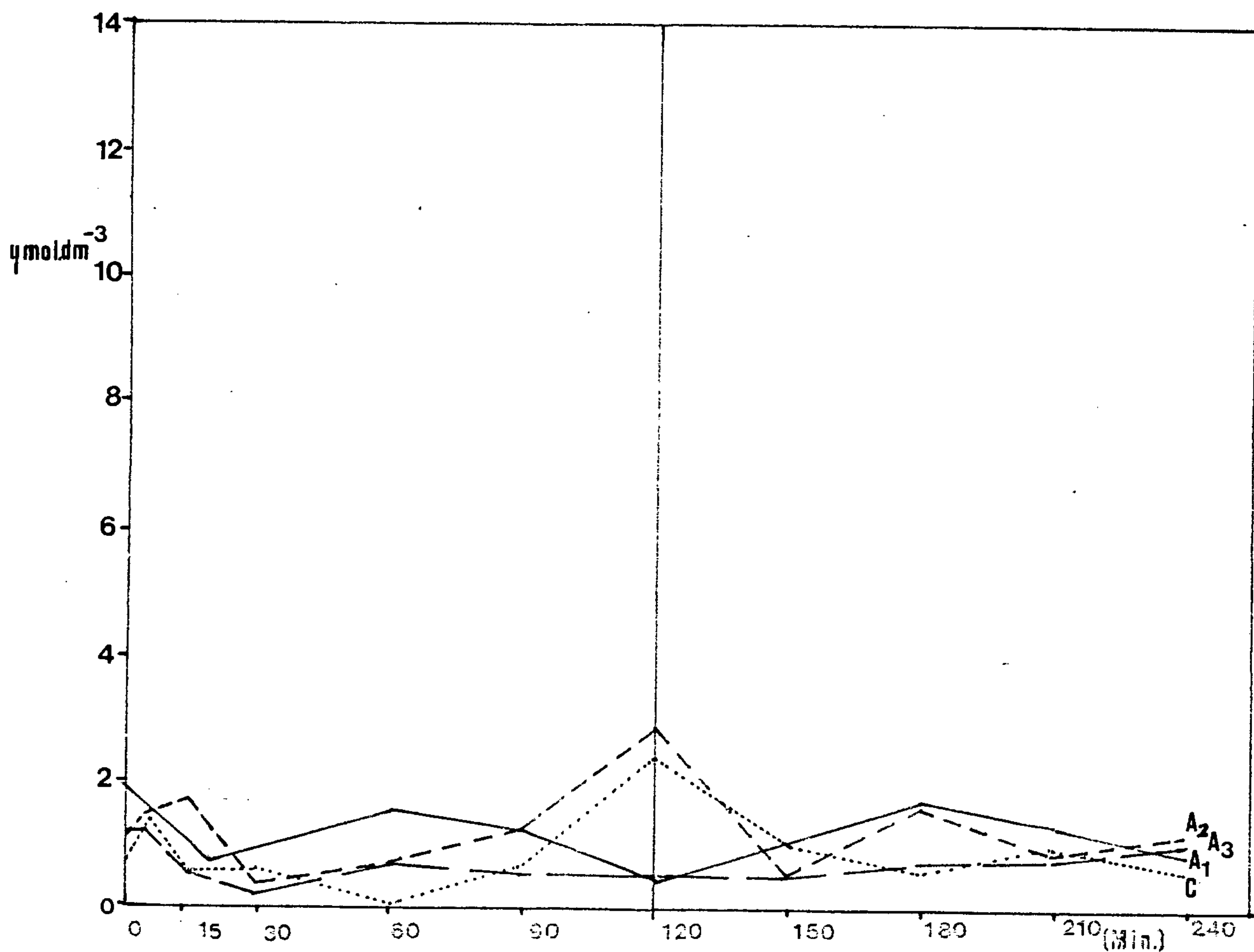


Fig. 3.16: Ammonia values for the Experimental and Control buckets with respect to time.

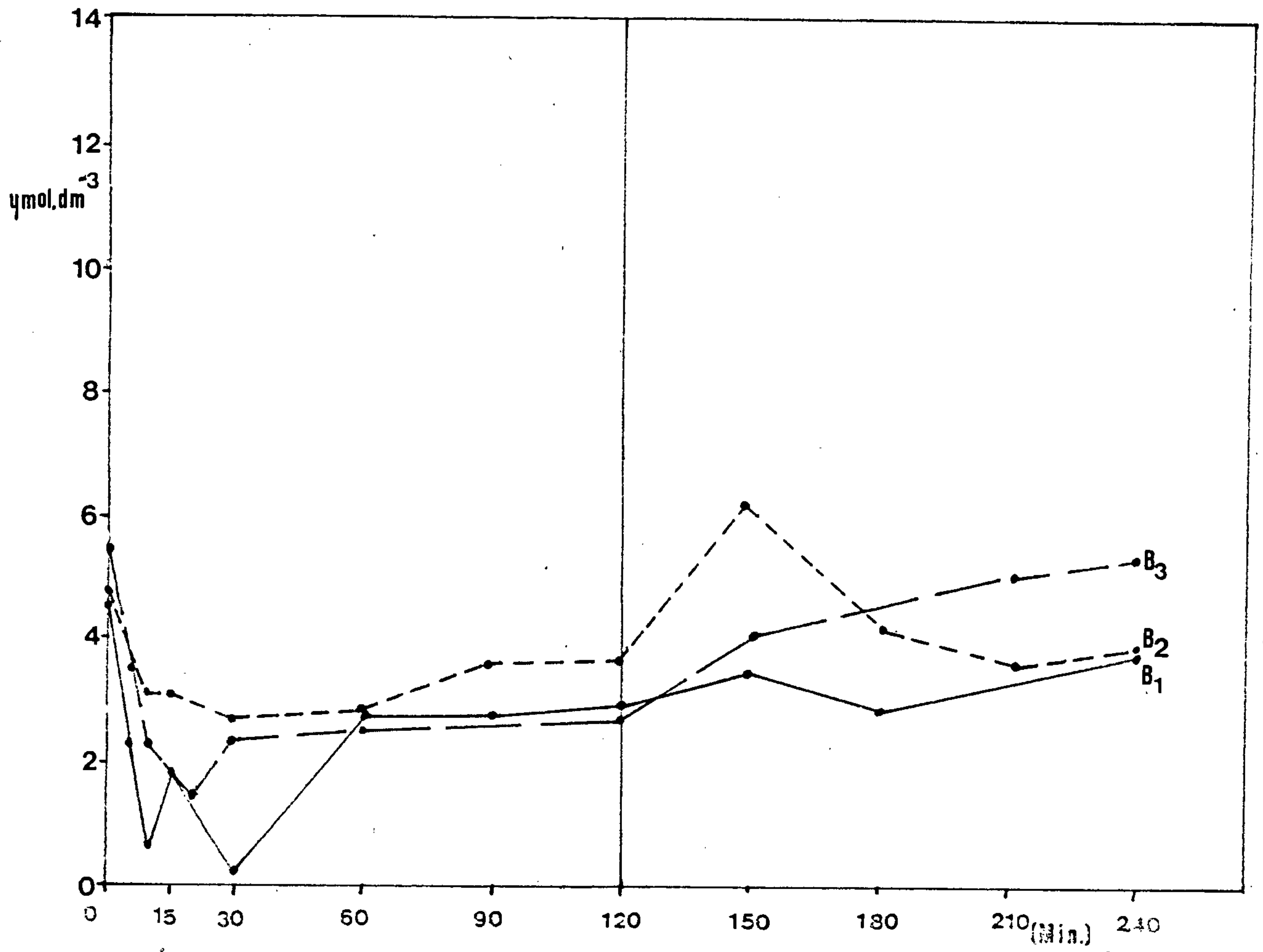


Fig.3.17: Ammonia values for the Antiobiotic buckets with respect to time.

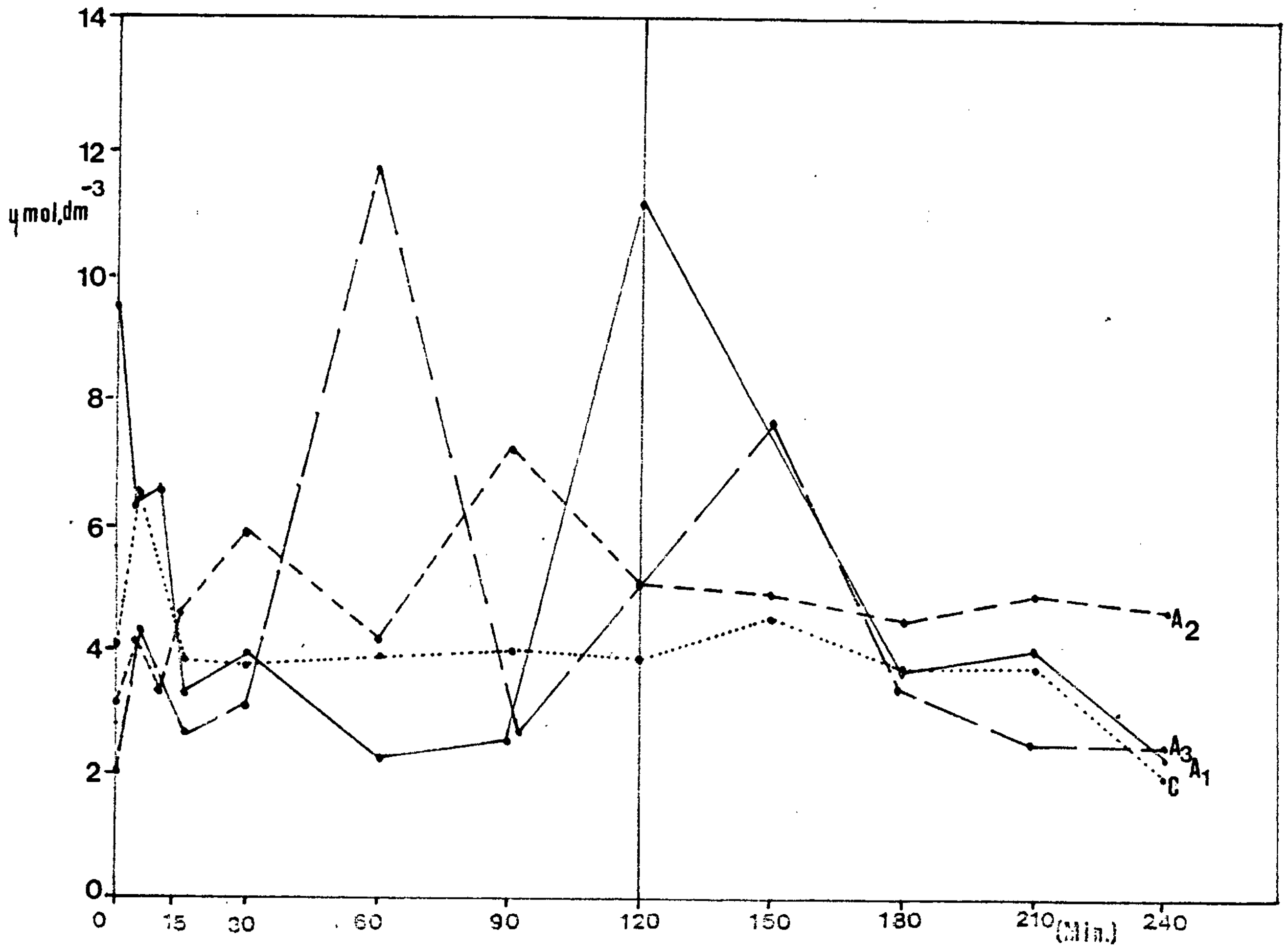


Fig. 3.18: Nitrate values for the Experimental and Control buckets with respect to time.

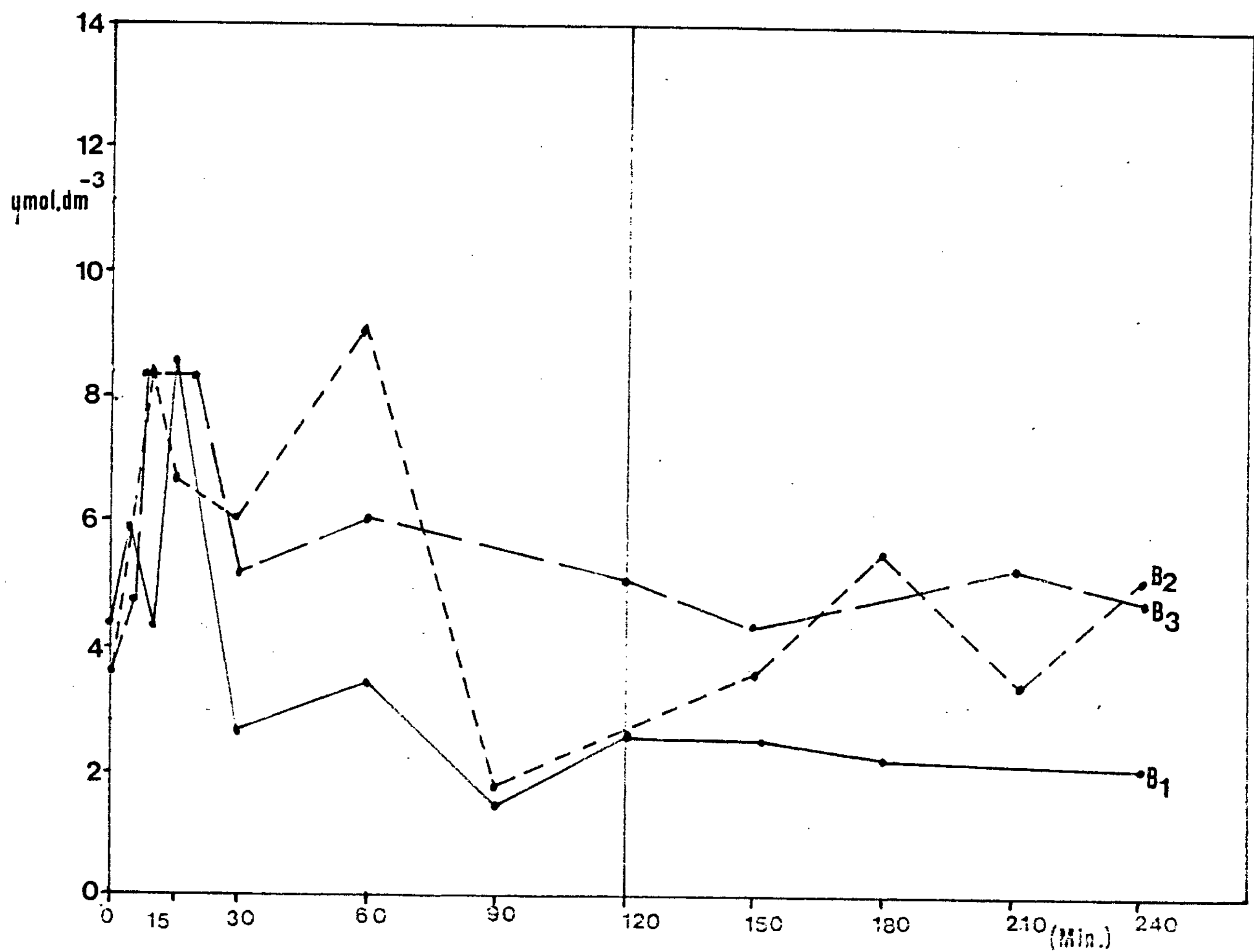


Fig. 3.19: Nitrate values for the Antiobiotic buckets with respect to time.

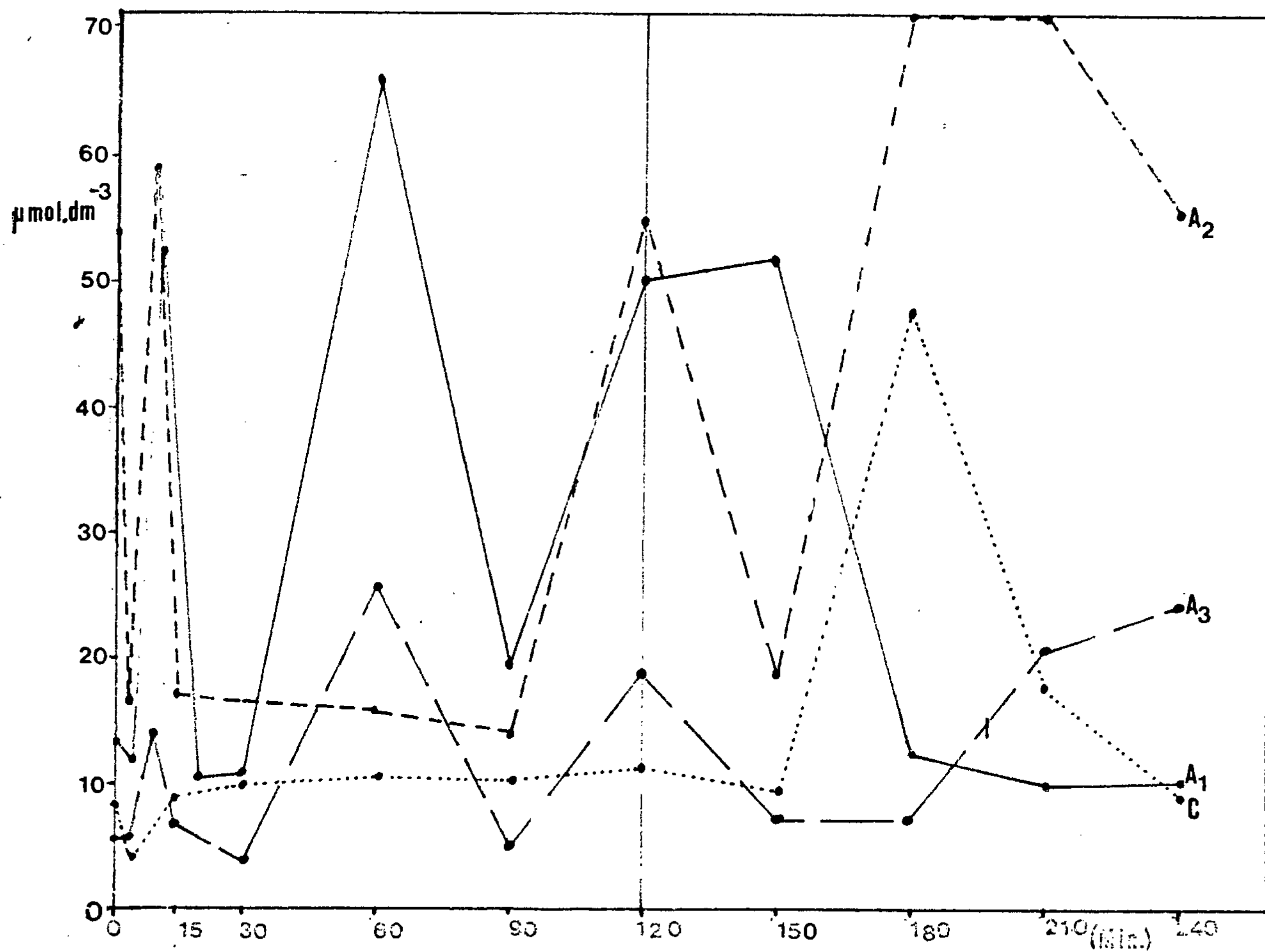


Fig. 3.20: Total N values for the Experimental and Control buckets with respect to time.

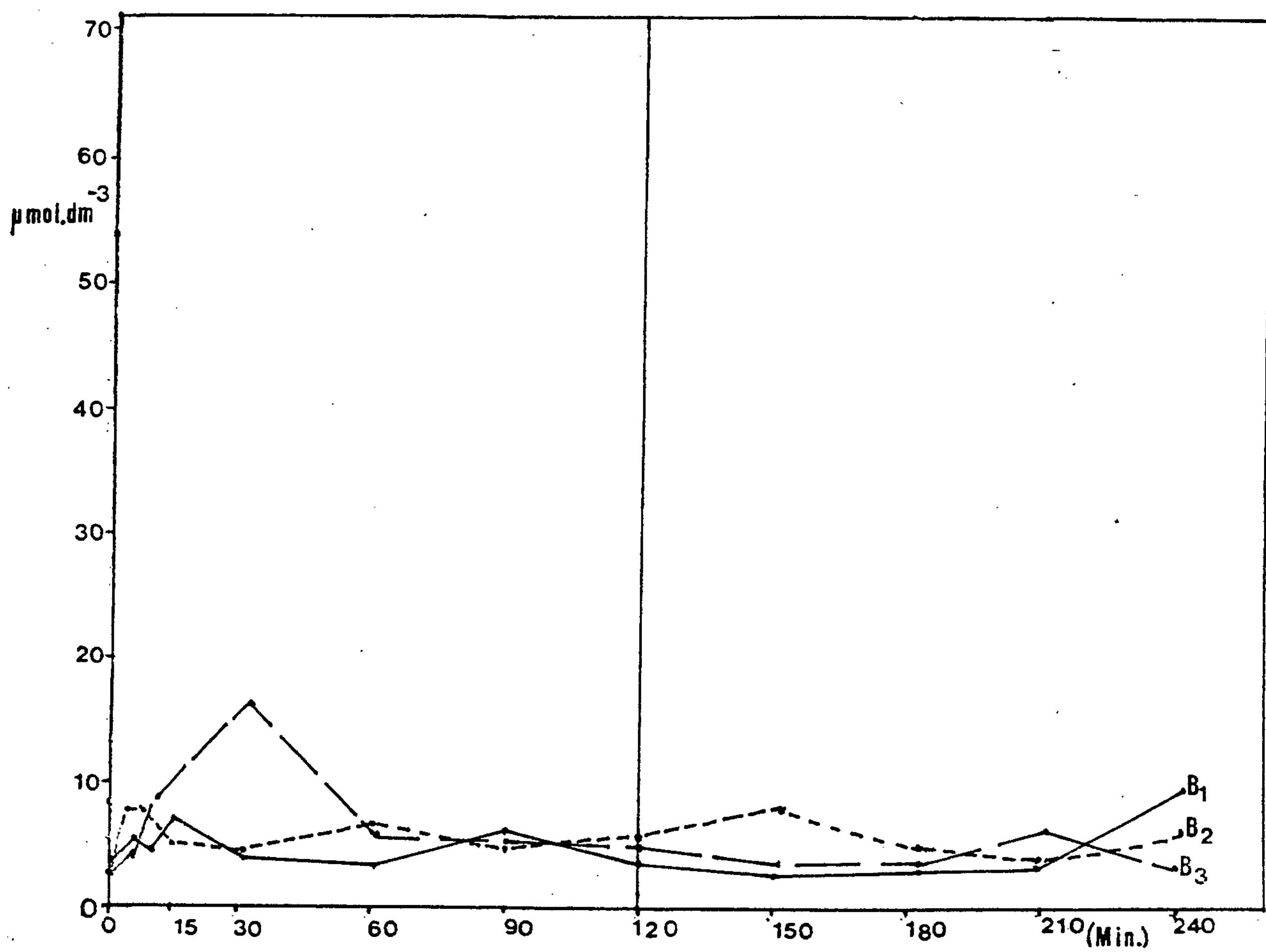


Fig.3.21: Total N values for the Antiobiotic buckets with respect to time.

3.1.3 CHN-analysis result

From the results of the elemental analyser, certain H-determinations were above the instrument's calibration range and some of the N-determinations were below this range. We could therefore not use these determinations with confidence, so only the C-determinations were used as a measure of particulate carbon in the seawater. The C:N ratios have been included in the Appendix, but are not discussed. Values were given as μg per filter after analysis and the following calculation was applied to convert to $\mu mol.dm^{-3}$:

$$\text{concentration of particulate C} = \frac{\text{analysed value}}{\text{molar mass of C}} \times \frac{1}{\text{volume sampled } (dm^{-3})}$$

$$= \frac{c}{12} \times \frac{1}{0{,}060}$$

$$= 1{,}388 \times c \ \mu mol.dm^{-3}$$

The concentration values have been tabulated in the Appendix section. Concentrations range from 40 - 267 $\mu mol.dm^{-3}$ with an average value of about 85 $\mu mol.dm^{-3}$ for all the results. The various buckets' profiles can be seen overleaf.

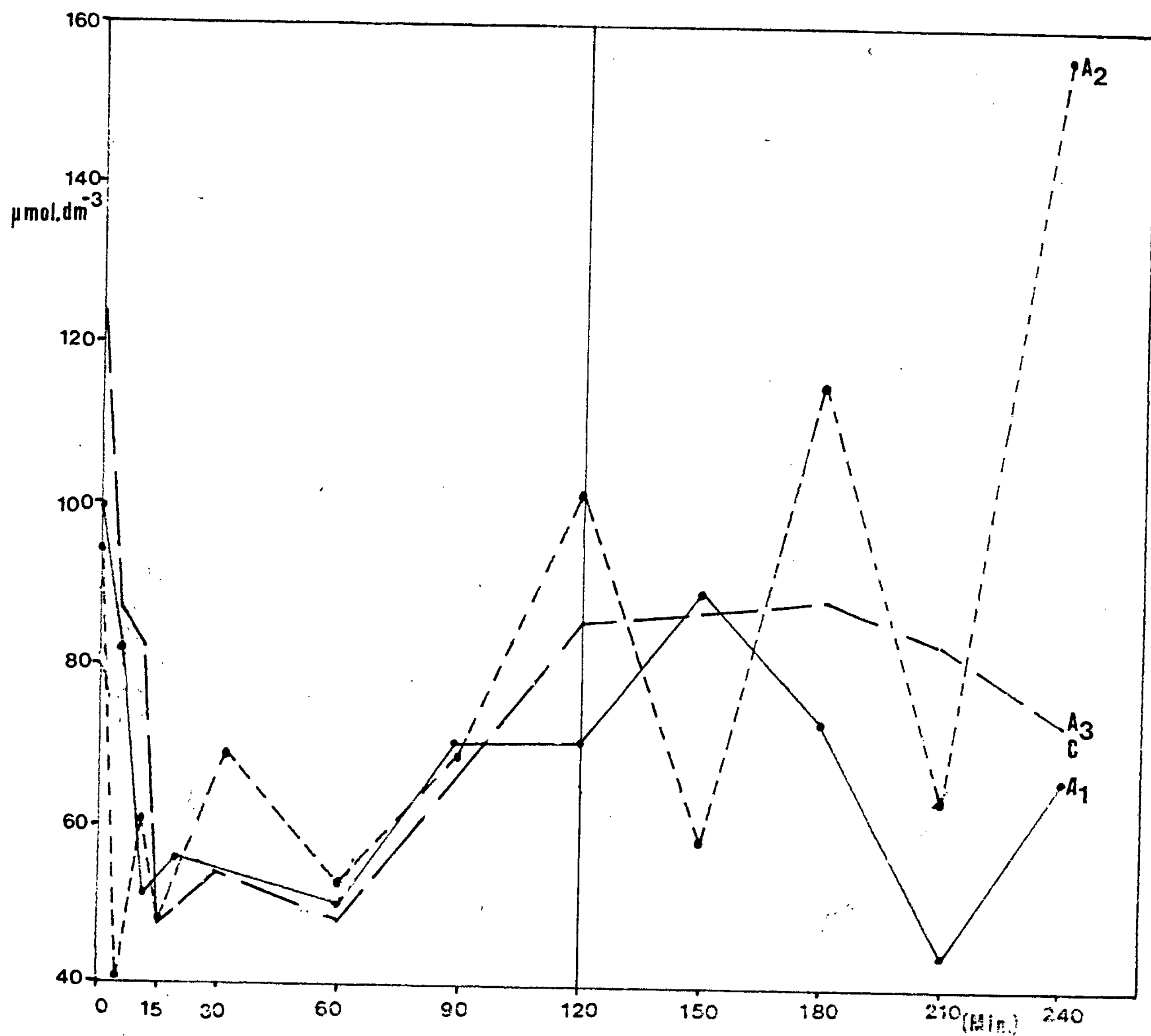


Fig.3.22: Particulate C profile for the Experimental bucket.

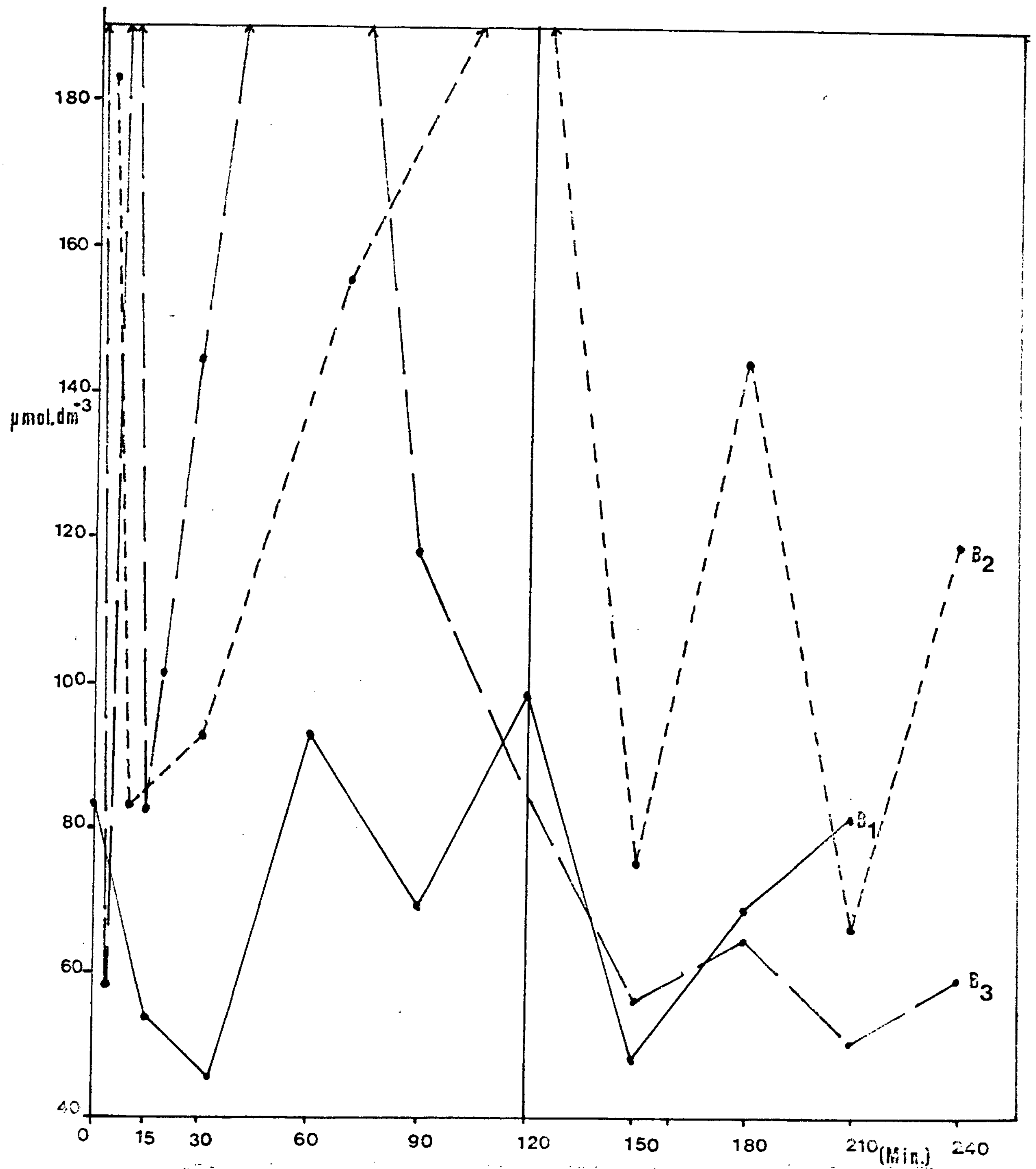


Fig.3.23: Particulate C profile for the Antibiotic buckets.

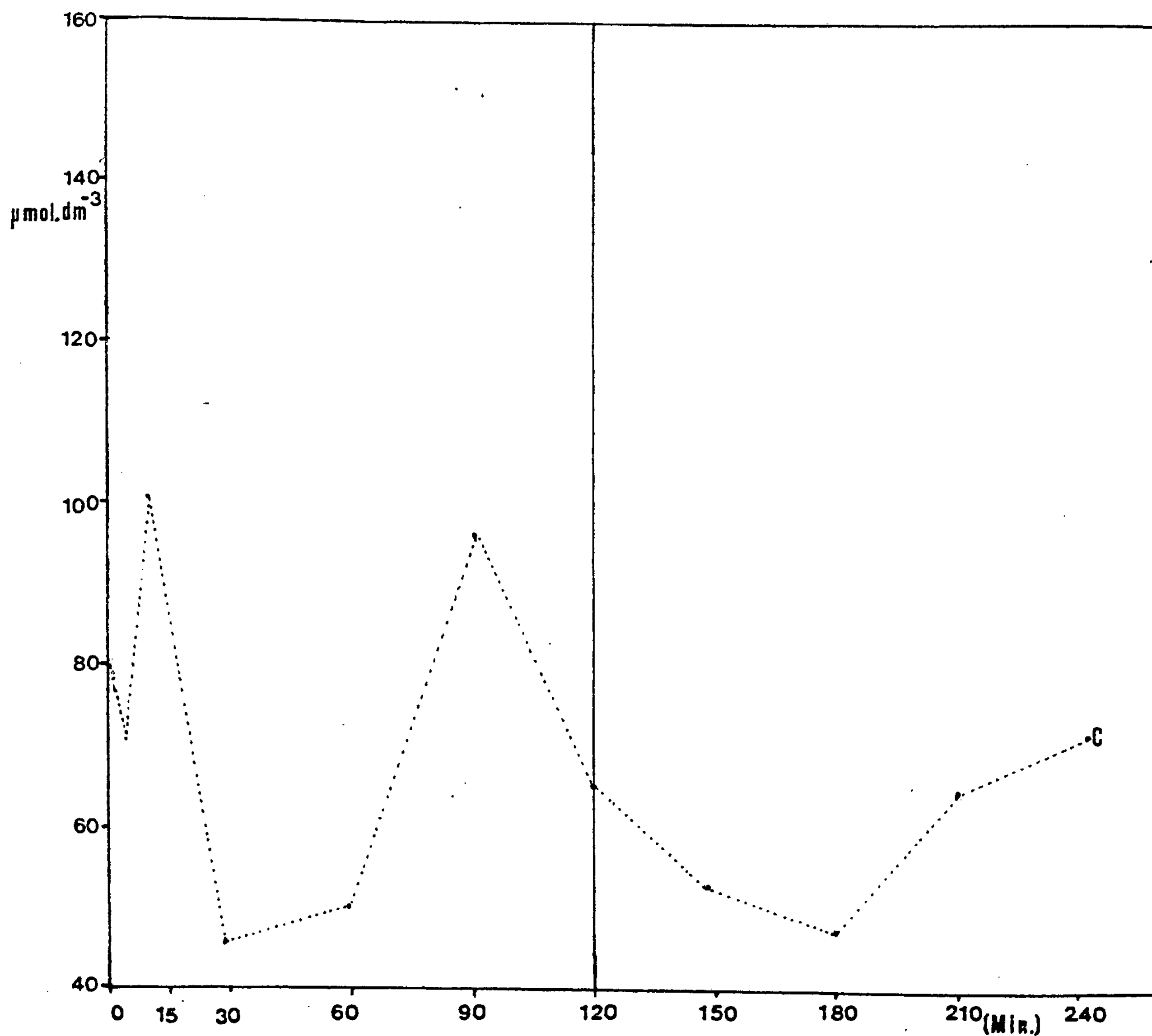


Fig.3.24: Particulate C profile for the Control bucket.

3.2 Bacterial results of the field study

3.2.1 Activity results

The disintegrations per minute (DPM) values analysed from the scintillation counter were plotted as a function of time to enable the activities to be calculated from their slopes by regression.

These results appear in the Appendix section and the graphs are shown below:

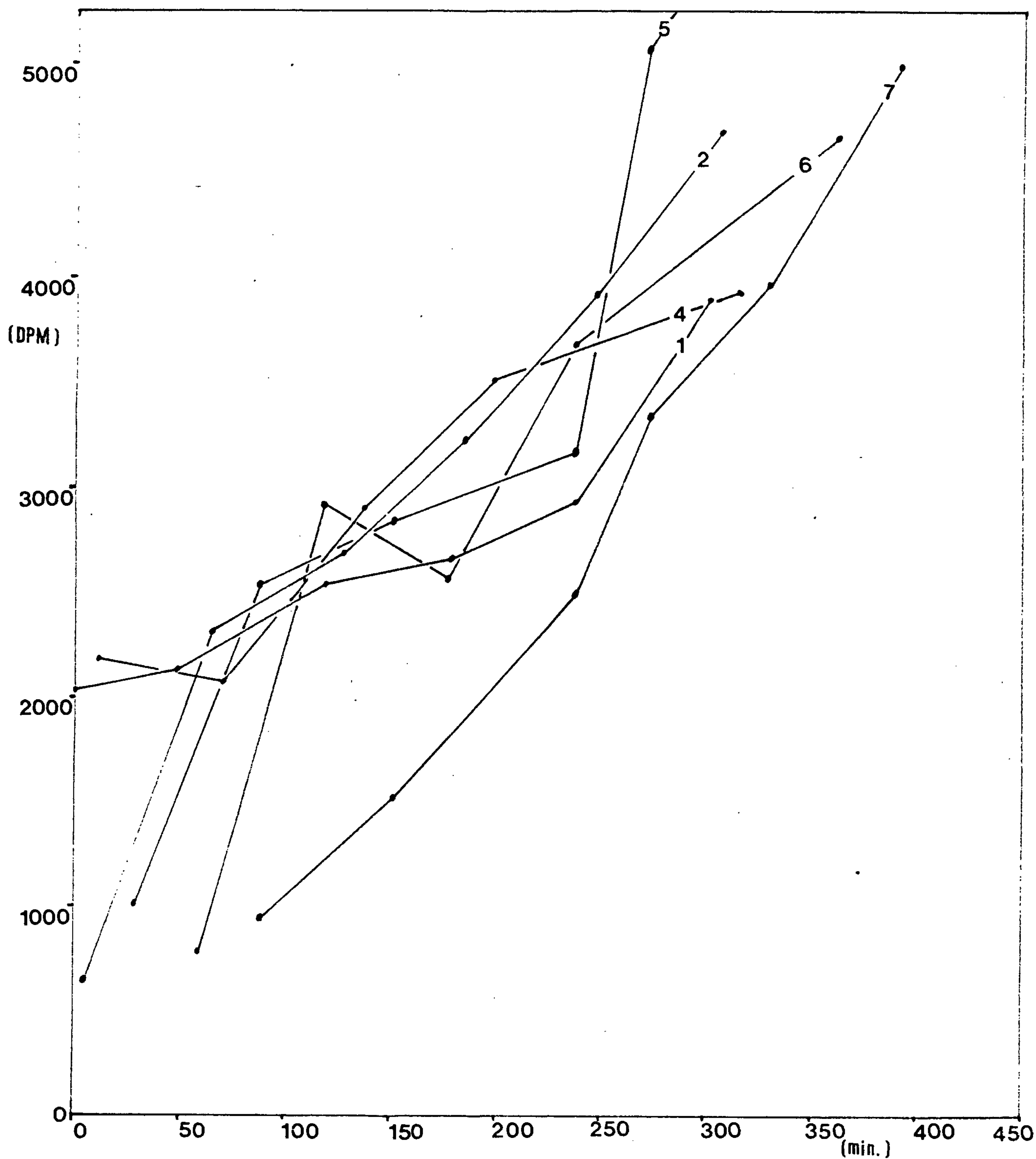


Fig. 3.25: DPM values for each subsampling time (vertical axis) for the first six experimental bucket sampling times. Numbers of the graphs correspond to the experimental bucket sampling times.

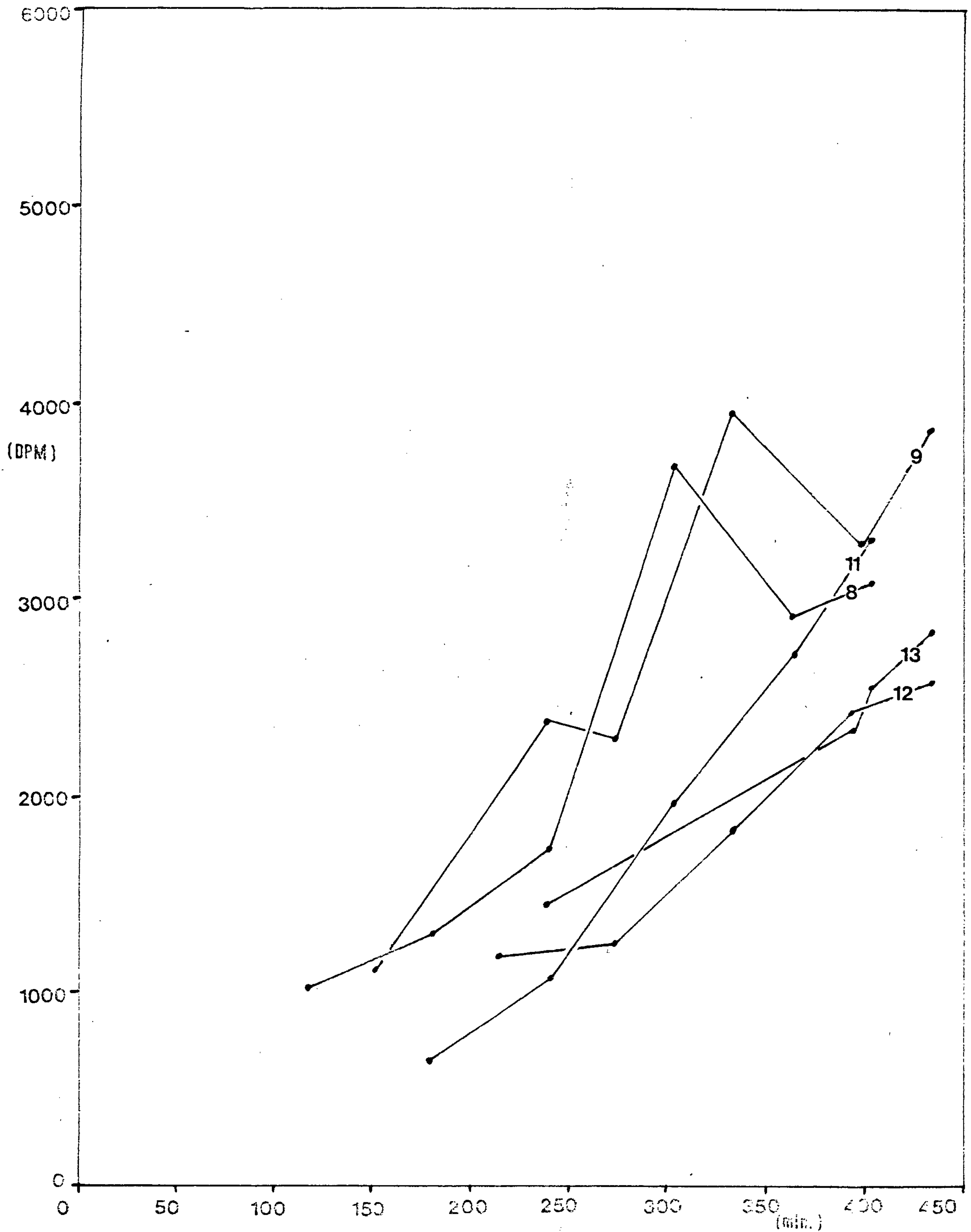


Fig. 3.26: DPM values for each subsampling time for the last five experimental bucket sampling times. Numbers correspond to the experimental bucket sampling times.

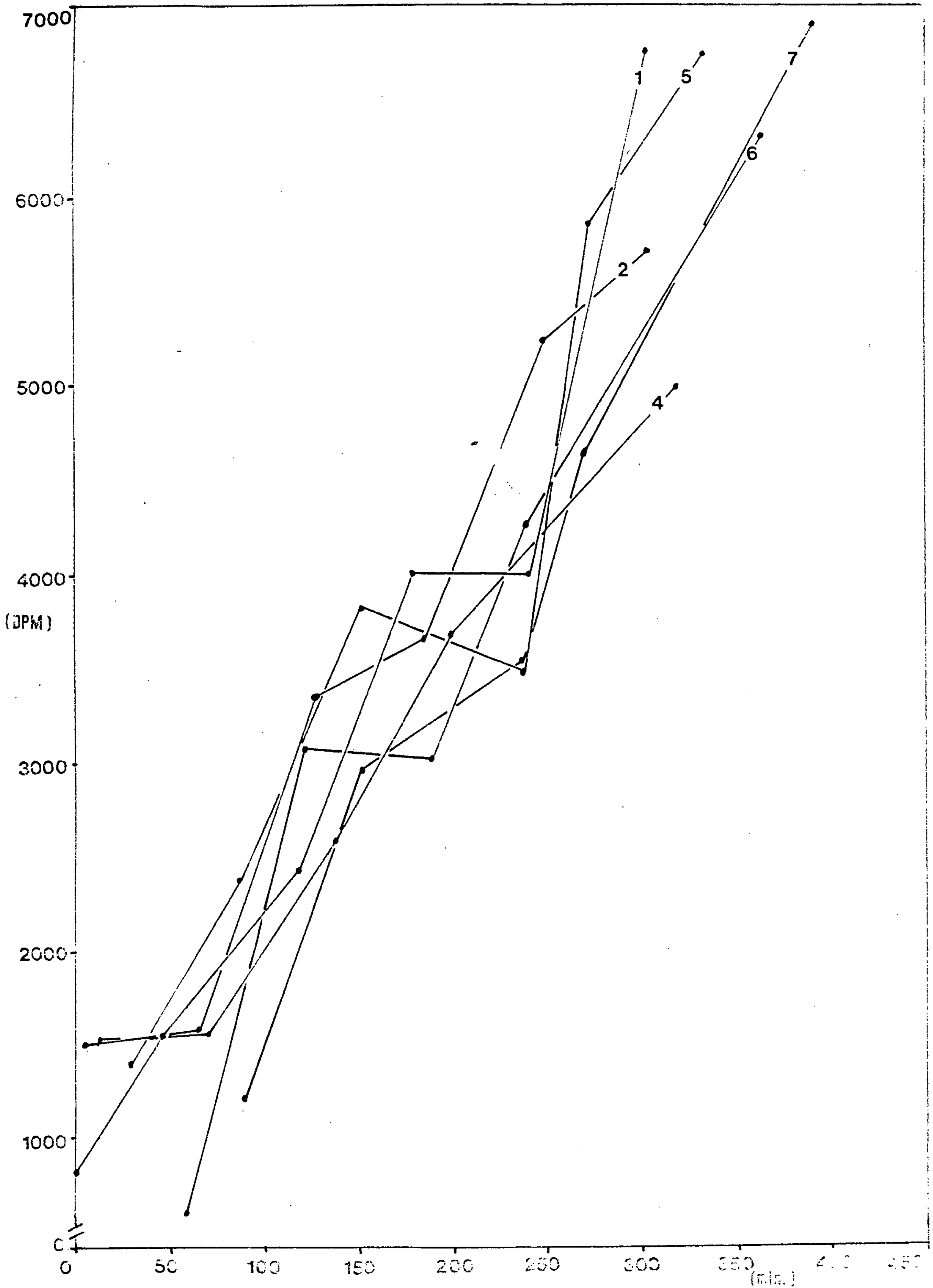


Fig. 3.27: DPM values for first six subsampling times for control bucket, numbers corresponding to control buckets sampling times.

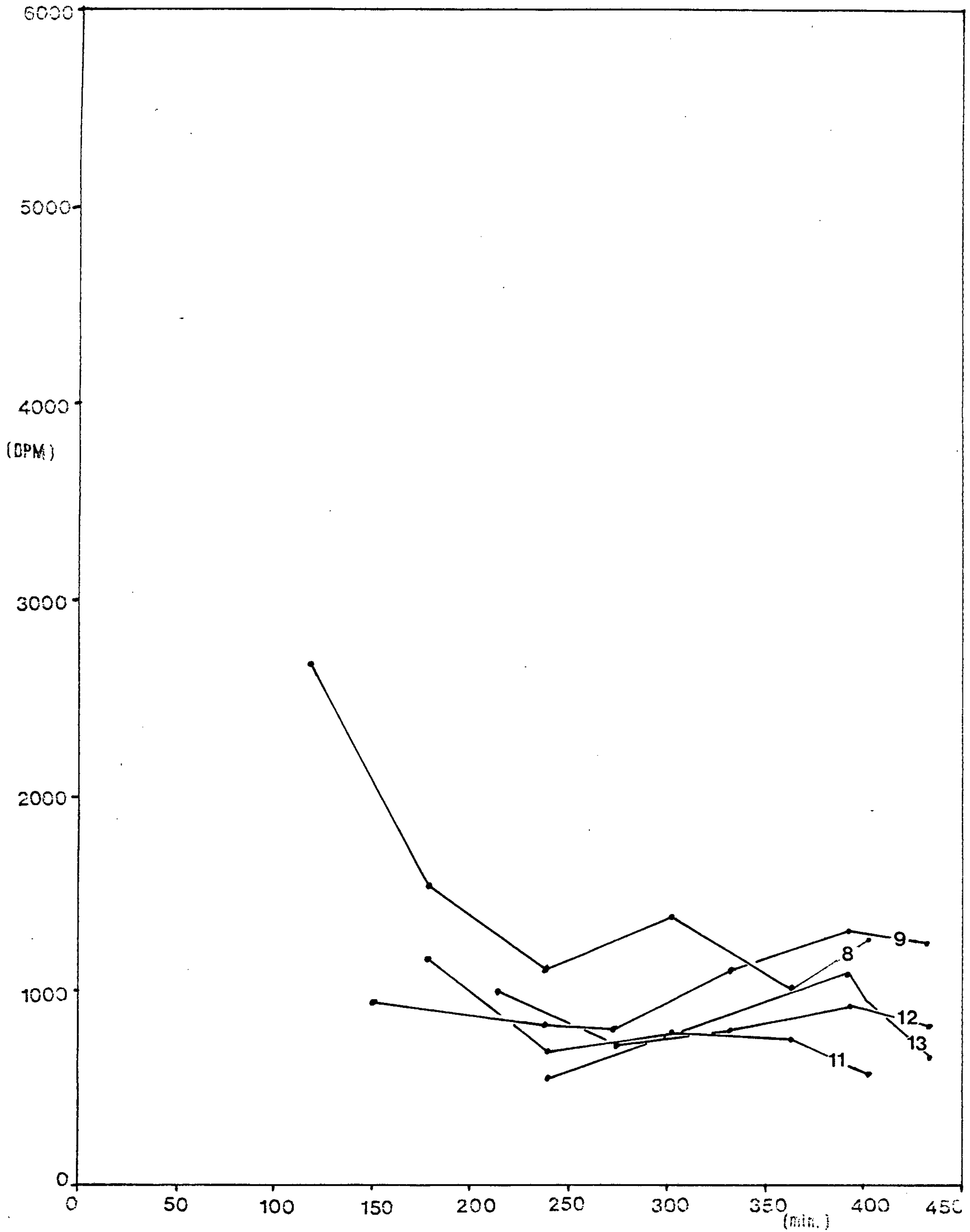


Fig.3.28: DPM values for each subsampling time for the last five control buckets sampling times. Numbers correspond to the control bucket's sampling times.

The slopes from the above plots of DPM versus time were calculated from the regression formula. Blank values are normally subtracted from each DPM value, but since this blank value is the same for all points, it would not affect the slope whether we subtract it or not. The slope values (DPM.min-1) were used to calculate the carbon production per day according to the following series of calculations:

1. Moles of thymidine incorporated per minute
   $= DPM.min^{-1} \times (SA)^{-1} \times C$

   where $DPM.min^{-1}$ = Slope from DPM versus time graph

   SA = Specific activity of isotope ($4,9 \times 10^4$Ci.mol)

   C = Number of curies per DPM ($4,5 \times 10^{-3}$)

2. Moles incorporated per litre per day
   $= mol.min^{-1} \times (\text{Volume filtered in } dm^{-3})^{-1} \times (\text{minutes per day})$

3. Equivalent production of cells
   $= mol.dm^{-3}.d^{-1} \times (0,2 \times 10^{18})$ to $(1,3 \times 10^{18})$

4. Carbon production $= cells.dm^{-3}.d^{-1} \times b$

   where b = average cell biomass (15fg of C)

This gives a range of carbon production values (µgC.dm-3.d-1) for each time interval which we need to plot. We therefore use their mean value for plots of carbon production versus time. This gives us the activity curve set up for the Experimental bucket A_1 and the Control bucket C. These tabulated results can be seen in the appendix section, and the graphs are shown overleaf:

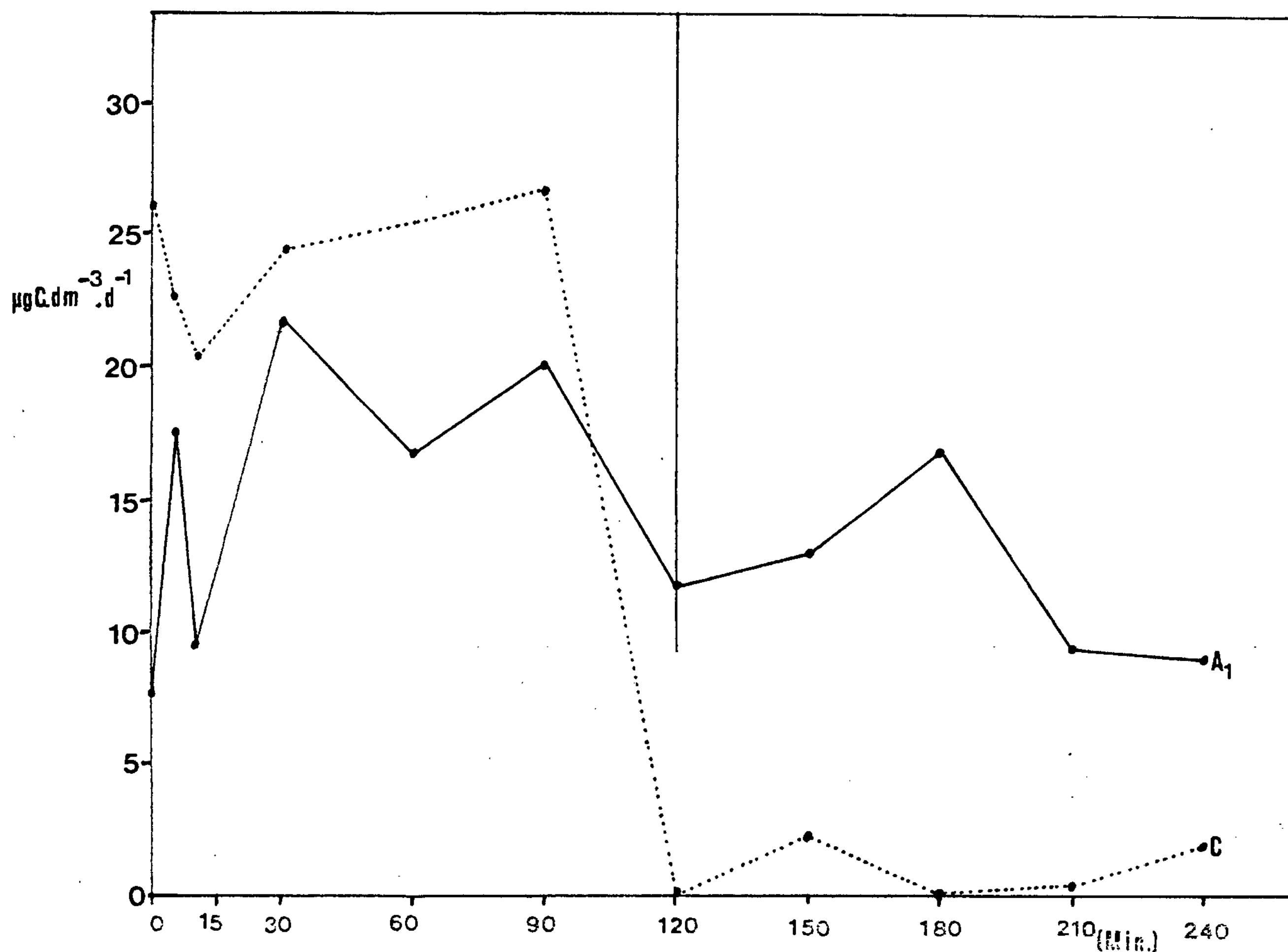


Fig. 3.29: Activity profiles of the experimental bucket A_1 (bold line) and the control bucket C (dotted line). Plots of mean carbon production versus time.

Two thymidine controls were set up to monitor the incorporation experiment. Control A was set up with 50ml of seawater with 0,5ml lugol (bacterial fixative, cross-linking the polymer chains of bacteria) and 12 µl ^{3}H-thymidine. Control B was set up with 50ml of seawater only with no thymidine. Both controls were duplicated and sampled at the same subtimes as the incubation flasks. Values of control A remained low between 1000 and 2000 DPM, while control B averaged below 500 DPM. These values are low compared with the

DPM values of the buckets (see figs. 3.25 - 3.28). These low values together with the low blanks (average = 120 DPM) are indicative of an effectively functioning experiment.

3.2.2 Biomass Results

Bacterial density ($cells.dm^{-3}$) for the total amount of free-living bacteria in the buckets, regardless of their dimensions of shape and size, can be calculated from the following expression (Hobbie et al., 1977):

$$N = \frac{n \times S \times 10^6}{s \times V}$$

where N = Bacterial density in $cells.ml^{-1}$.

n = mean bacterial count per field (total).

S = area of filter paper in mm2 (diameter = 17mm).

s = area of microscope field in μm^2.

$$= \text{field size} \times \left(\frac{\text{microscope unit}}{2}\right)^2 \times \pi$$

$$= 10 \times \left(\frac{7,69}{2}\right)^2 . \pi$$

V = Volume filtered in ml.

We then convert this $cells.ml^{-1}$ value to $cells.dm^{-3}$ to bring it to the same quantitative unit (dm^{-3}) as the other chemical and bacterial profiles. These values for bacterial density can be found in the appendix section, and the graphical profiles for these values are shown overleaf.

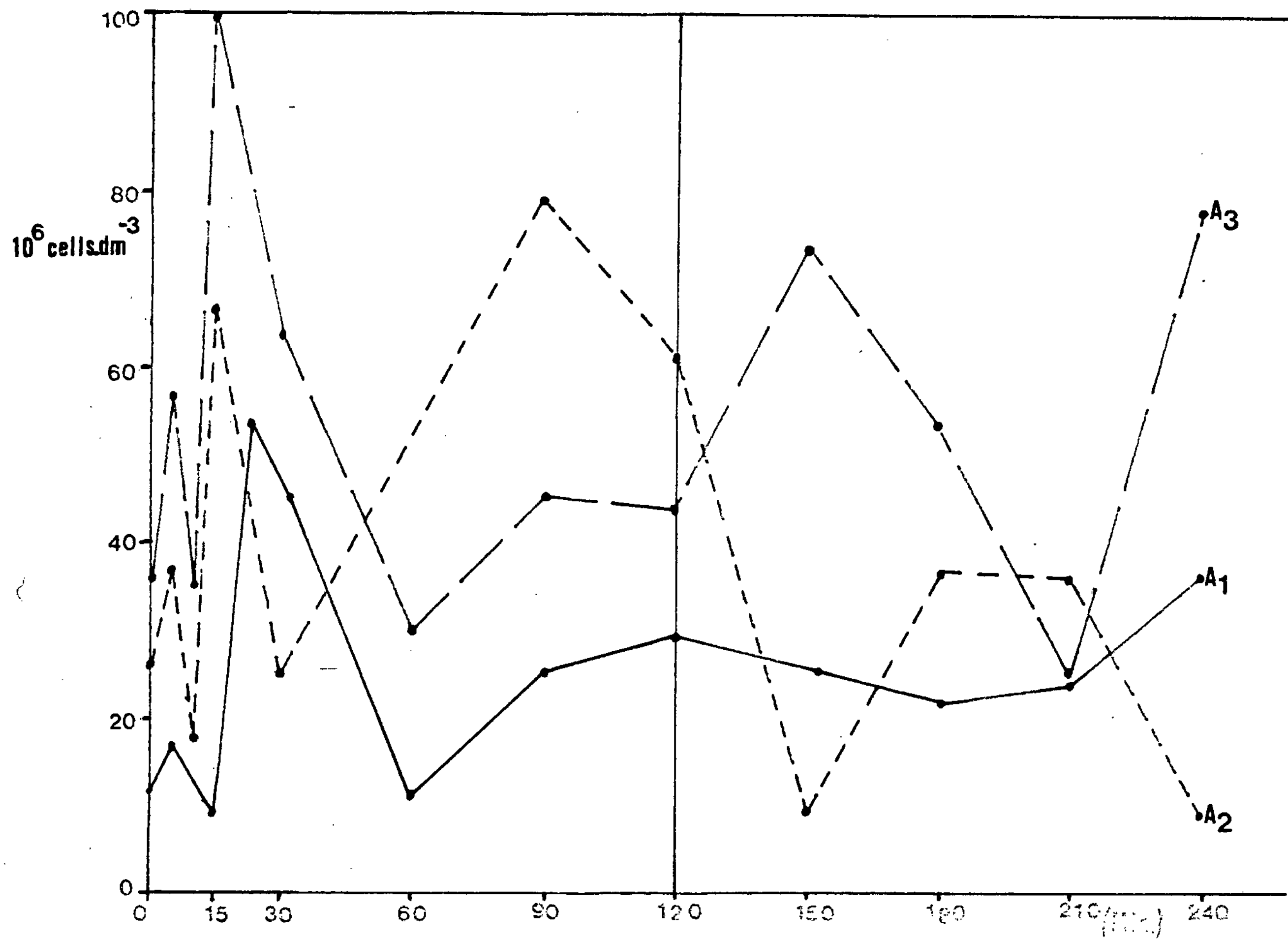


Fig. 3.30: Total bacterial density of the experimental buckets with respect to time.

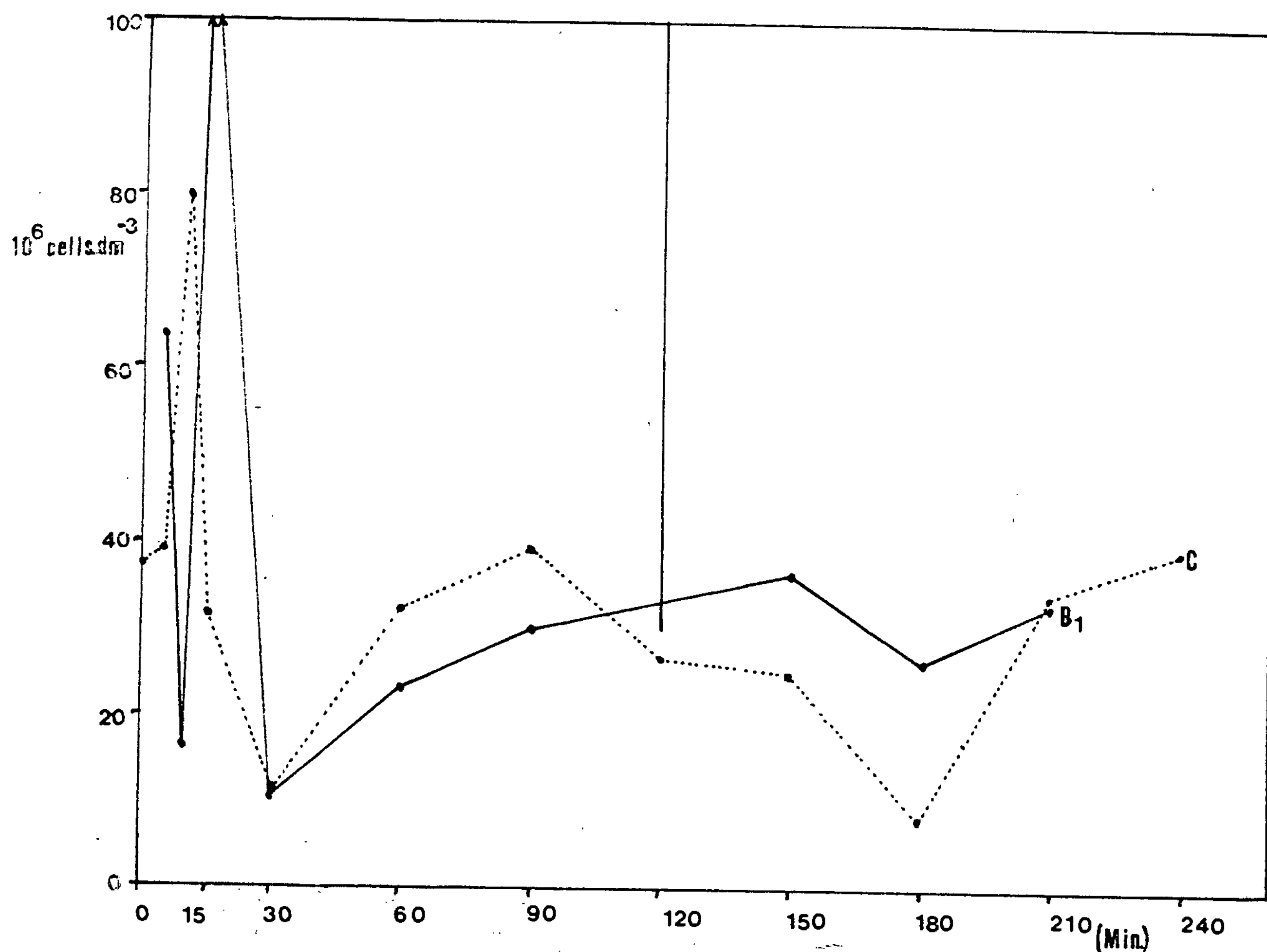


Fig. 3.31: Total bacterial density of the antibiotic and control buckets with respect to time.

In determining the capacity for uptake, the population size and dimensions of the cells must both be considered. For example, the small cocci have the largest numbers, but due to their size the amount taken up by these bacteria would be less than that by the same number of larger bacteria. Therefore, in order to calculate the biomass (weight per organism), we would have to calculate the contribution from each size and shape of bacterial counting group to the nett amount utilized. This can be calculated from the following expression (Doetsch and Cook, 1973, Painting et al., 1986):

$$B = \frac{N \times Sg \times Vc}{10^6}$$

where B = Biomass in $mg.dm^{-3}$.

N = Bacterial density for each counting group ($cells.ml^{-1}$).

Sg = Specific gravity of bacterium (= 1,1).

Vc = cell volumes in μm^3, where the counting groups

SC = 0,009

LC = 0,142

SR; SU; ∿ = 0,198

LR; LU = 0,672

The following graphs of the biomass for the buckets are shown below:

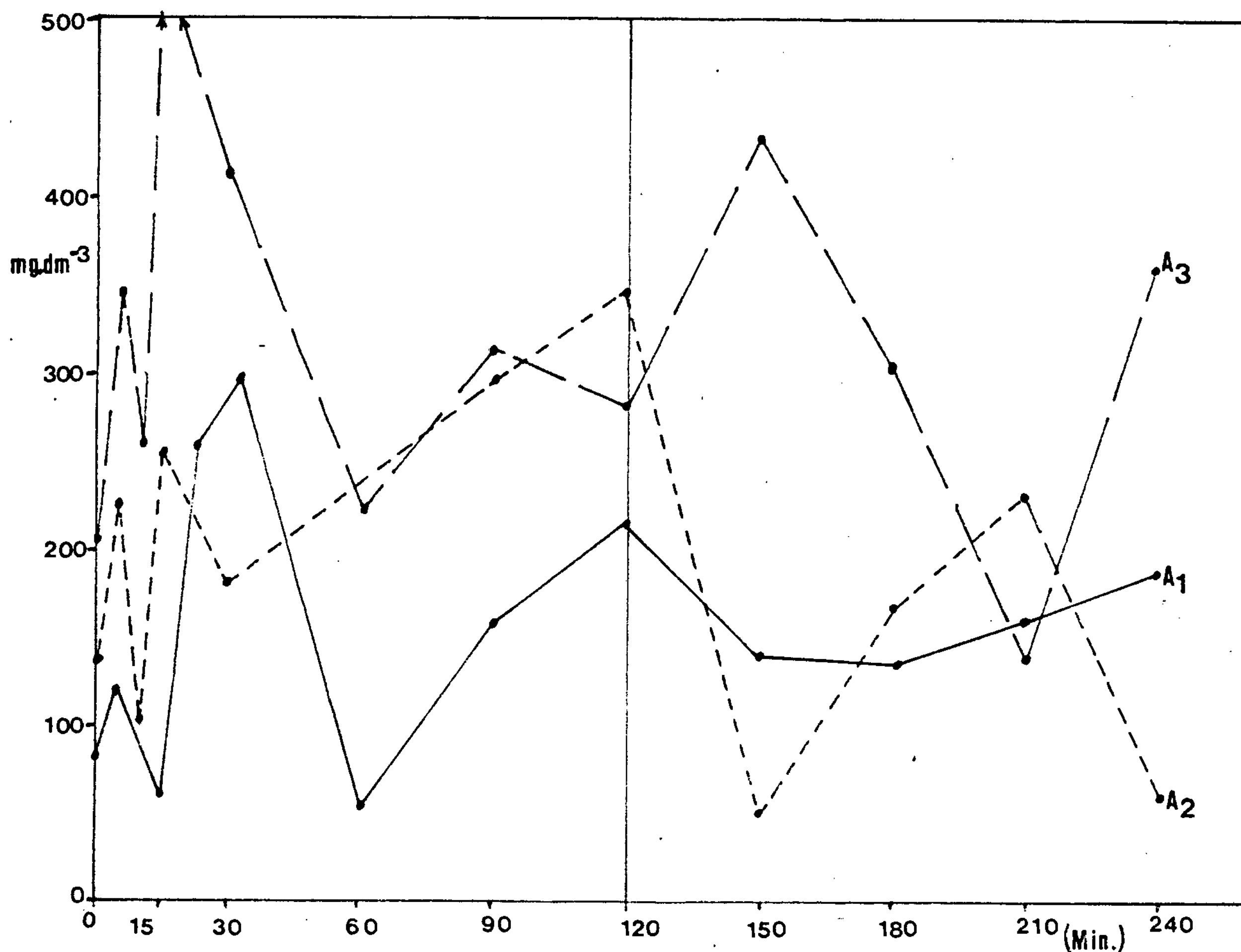


Fig. 3.32: Biomass of the experimental buckets with respect to time.

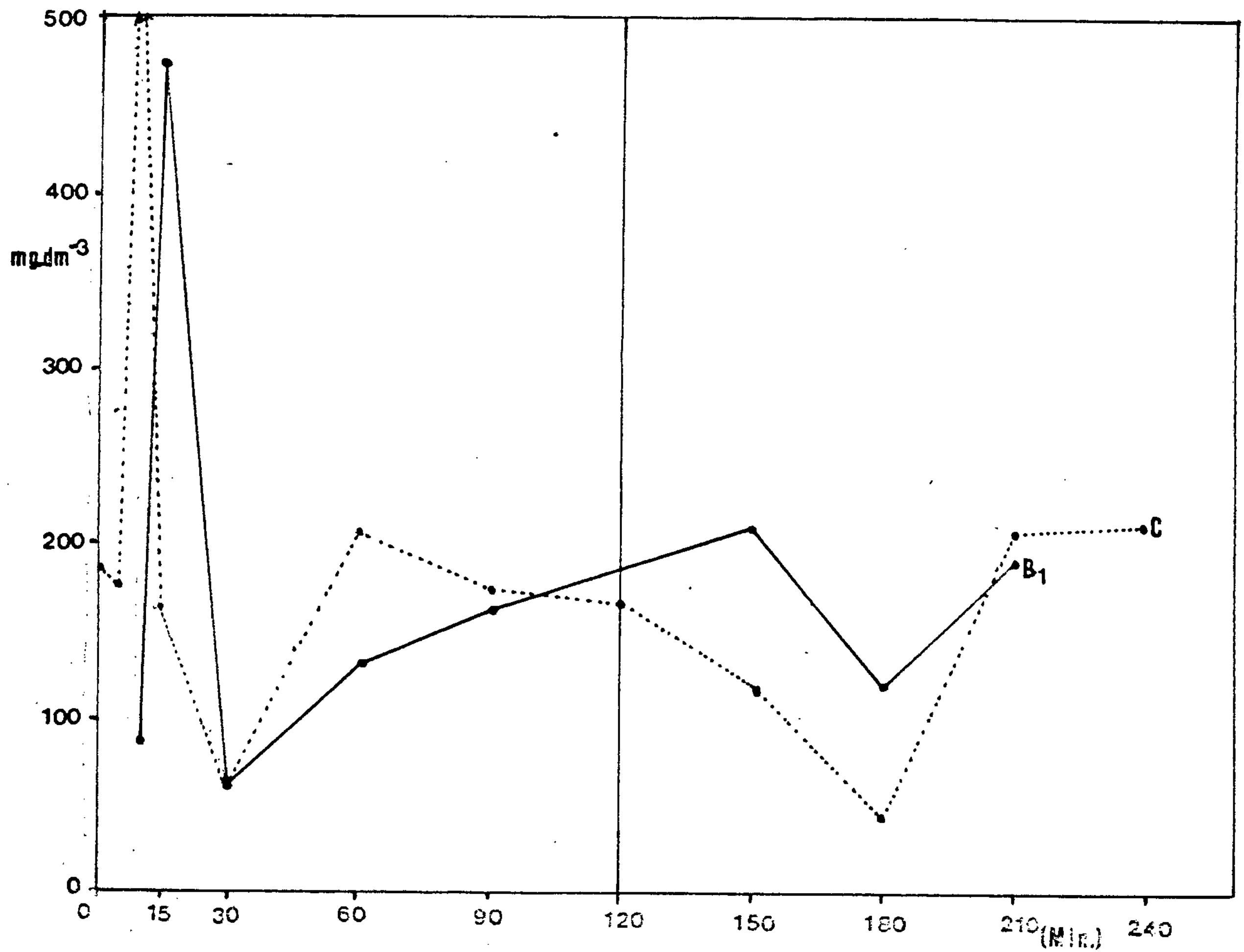


Fig. 3.33: Biomass of the antiobiotic and control buckets with respect to time.

4. DISCUSSION

As noted before (Section 2.2), the results showed that the various chemical and biological processes occurring were extremely complex and, despite the strenuous efforts made to carry out a simple simulation, no straightforward picture emerged. To understand the processes more fully, a much more detailed experiment is needed and therefore such a project is beyond the scope of this thesis. However, the results are instructive in that certain deductions can be made, albeit not quantitatively as was hoped.

4.1 QUALITATIVE ASSESSMENT OF RESULTS

The overall conclusion obtained on the basis of the data gathered from the time series study, was that no large exudation of reduced nitrogen species (amino acids or ammonia) occurred. The reasons are complex and are discussed in a preliminary fashion within the context of changes in bacterial biomass and activity in each microcosm environment.

Microheterotrophic organisms are primary consumers of dissolved organic nitrogen (DON) species, such as amino acids, and because these components are metabolized through oxidative mechanisms, the main products would be dissolved inorganic nitrogen (DIN) species such as nitrate (NO_3), nitrite (NO_2), and ammonia (NH_4) (Ryther and Dunstan, 1971). The relationship between the various nitrogen pools and their linking metabolic pathways is summarized in fig. 4.1

below and includes the pathway linking the DON and DIN pools, which were investigated in this study:

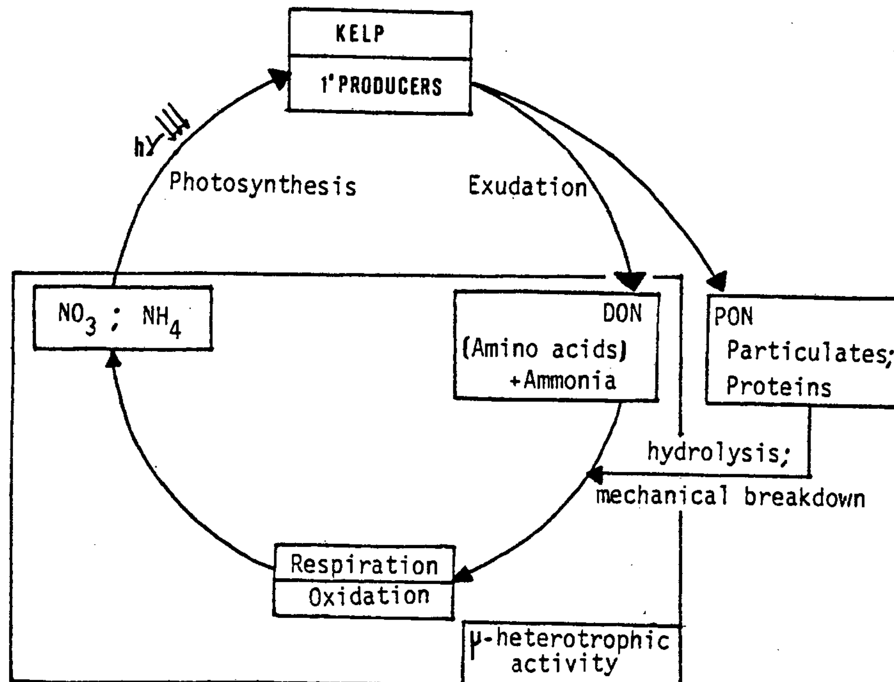


Fig. 4.1: Schematic pathways of the nitrogenous species released from kelp.

The main objective of the experiment was to attempt to quantify the exuded amino acid contribution to the organic nitrogen pool and monitor the response of the system through measurement of bacterial activity and changes in the inorganic nitrogen pool. The effect of input of DON (as measured by amino acid concentrations) through exudation was therefore expected to be seen as:

1. An increase in bacterial biomass and/or activity, as well as inorganic nitrogen species in the experimental buckets, compared with the control bucket.

2. An increase in dissolved amino acid concentration in the antibiotic treated buckets, compared with the control bucket.

4.1.1 Bacterial biomass and activity

The plots of bacterial biomass (in $mg.dm^{-3}$) and activity ($\mu gC.dm^{-3}.d^{-1}$) against time are shown in the Results section (figs. 3.29 - 3.33). It can be seen that there is a sharp decrease (from 25 $\mu gC.dm^{-3}.d^{-1}$ to near zero) in bacterial activity in the control bucket at the 90 - 120 min. sampling interval (fig. 3.29). This significant change contrasts with the plot for experimental bucket A_1, where the activity fluctuates within the range 10 - 22 $\mu gC.dm^{-3}.d^{-1}$. The difference in bacterial activity between the two experimental conditions can perhaps initially be ascribed to a drop in substrate concentration in the control bucket though not necessarily the nitrogen substrate.

The variability of biomass with time for the experimental buckets (fig. 3.32) is also substantial. Part of the explanation for this variability may be in the fact that the fronds used in the experimental buckets were not of similar physiological conditions; however, it is considered that the system is in a state of very low bacterial biomass and activity, and this gives rise to large variations. In support of this, typical activities for in-shore kelp dominated regions are about 8 times higher than the activities determined in this study (Painting, pers. comm.). It can be seen that over the time series, the measured bacterial biomass in the

three buckets varied between 50 and 400mg.dm^{-3} (fig. 3.32), reasonably close to the variability range observed for the control and antibiotic bucket (fig. 3.33) of 100 - 200mg.dm^{-3}.

From the measured biological parameters of activity and biomass, it is evident that bacterial activity is a more sensitive variable, responding readily to substrate concentration level changes.

4.1.2 Chemical variability

On a broad basis, the chemical parameters vary around a mean value similar to that observed with the bacterial parameters.

4.1.2.1 Amino acids

Of all the dissolved free amino acids determined, only alanine showed concentrations substantially above 10nmol.dm^{-3} with all the others falling in the very low range of 2 - 6nmol.dm^{-3}. These values are near the analytical detection limit, resulting in large fluctuations which demonstrate either that the processes involved in amino acid release and uptake are very rapid, or that they are baseline fluctuations attributed to experimental error. Other sources of error could include an inefficient mixing of released matter within the containers, with possible formation of concentration gradients. Adsorption of these species onto the container walls is also possible at these low levels. Finally, the physiological state of the kelp is important in this type of study.

There is little change in a rough mean over the time series common to all the experimental environments, even between the experimental, antibiotic and control buckets. The typical concentration ranges for the amino acids in marine systems are discussed in Section 1.3. Experimental error bars have been given for the alanine profile (fig. 3.7).

4.1.2.2 **Total and inorganic nitrogen**

The total nitrogen concentrations for the antibiotic and control buckets are very nearly constant (fig. 3.16 - 3.21) and low (± 5 $\mu mol.dm^{-3}$) for the duration of the time series study. The experimental buckets (fig. 3.20) on the other hand, show large variability (5 - 50 $\mu mol.dm^{-3}$) but again no significantly increasing or decreasing trend.

The ammonia concentration is nearly constant with respect to time, ranging around about 1 $umol.dm^{-3}$ for the experimental and control buckets (fig. 3.16), but an increasing trend occurs in the antibiotic buckets (fig. 3.17). This increasing trend is likely to be due to the oxidative deamination of dissolved organic nitrogen, as the antibiotic is an unlikely source of this component. Similarly for the nitrate (figs. 3.18 and 3.19), there appears to be no definite trend in the time series.

There are two possible reasons for there being little change in the microcosm used in this study:

1. The flux of exuded amino acid nitrogen was too small or too slow to create any substantial response in the system, possibly because the experimental systems were not mixed continuously.

2. The microheterotrophic population was, for reasons that may or may not be linked with (1), in an abnormally low state of activity, as shown in Section 4.1.1.

If either, or both, of the above possibilities pertained to the study, then the time span used was perhaps too short since it would simply give a small "window" on a system which changes on a time scale of days. This can be demonstrated by using the determined activity levels from the experimental bucket A_1 (fig. 3.2.9). From a mean activity of 15 $\mu gC.dm^{-3}.d^{-1}$, which gives a value of 52,1nmolC.$dm^{-3}.h^{-1}$, and using a 6,5:1 C:N ratio (Reddfield ratio), this would create a calculated nitrogen demand of 8nmol N.$dm^{-3}.h^{-1}$, which is very low, nearly three orders of magnitude smaller than the observed concentration of the inorganic nitrogen and total nitrogen pools.

This nitrogen demand is however of the same order of magnitude as the observed amino acid concentrations, thus tentatively explaining the large measured variability, with the exception of alanine which has a higher concentration (1 order of magnitude). This confirms that:

1. The amino acid concentration is clearly very small compared with the inorganic nitrogen pool. This may be expected since the organic nitrogen species were labile.

2. Any future studies to monitor the impact of the organic nitrogen pool on the inorganic nitrogen pool through bacterial activity need to be carried out over an extended period, of the order of days.

Thus, although the exercise may not have been successful from an ecological point of view, it has shown that the methodology of amino acid analyses can be effectively used in the study of the microbiochemistry of the components in the marine system. However, it is quite clear that under the experimental field conditions reported here, there can be very little export of free dissolved amino acids from a kelp bed system to the oceans, since the concentrations are so small.

5. CONCLUSIONS

This study has demonstrated that a simple, precise, sensitive and rapid technique for determining 20 amino acids in seawater using reverse phase high performance liquid chromatography can be established. Also, these methods are suitable for routine use, while the nature of the equipment required and of the instrument itself permits field use, for example, on a research vessel. This opens up many new avenues for research into the contribution of amino acids to the cycling of nitrogen in the oceans. The amino acid concentrations in a given water mass may perhaps be characteristic, enabling the method to be used as a marine tracer, using amino acids for "fingerprinting". Another advantage is that only small volumes of water are needed, permitting analysis of samples such as those from pore waters.

Application of the techniques to a simple study of kelp exudation provided unexpectedly complex results and more detailed studies over a longer time period are required. It is clear, however, that amino acid levels in kelp exudates are low, and a large oceanward transport of amino-nitrogen from kelp beds is unlikely. The amino acid contribution to the nitrogen pool was very low, indicative of the lability of amino acids in the ocean. While in itself not conclusive, the field study demonstrated the successful application of the laboratory methodology to analysis of real samples, indicating the potential value of this technique in probing marine chemical processes.

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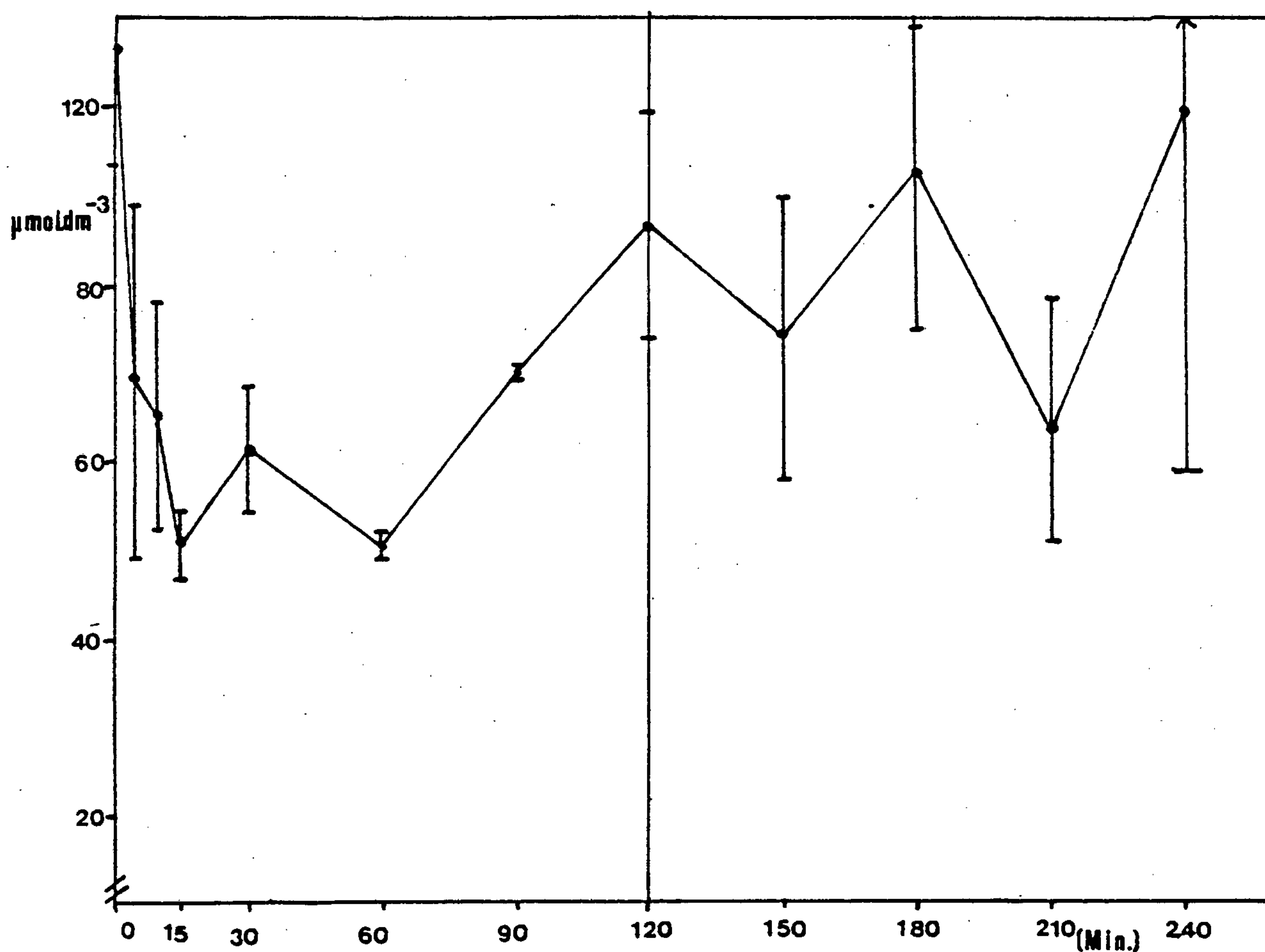
7. APPENDIX

7.1 Source of the chemicals and materials used in this study

9-fluorenylmethyl chloroformate was purchased from Fluka Chemical Corporation. Amino acid standards (catalogue no.AA-S-18, A9781, A2908 and A9906) and amino acid kits (DLAA-24 and LAA-10) were obtained from Sigma Chemical Corporation. Methanol was purchased from Beckman Instruments (Rathburn, HPLC grade) and from Merck (Lichrosolv® grade). Acetonitrile was also purchased from Beckman (Rathburn, HPLC grade S). Milli-Q® water (Millipore) was used throughout.

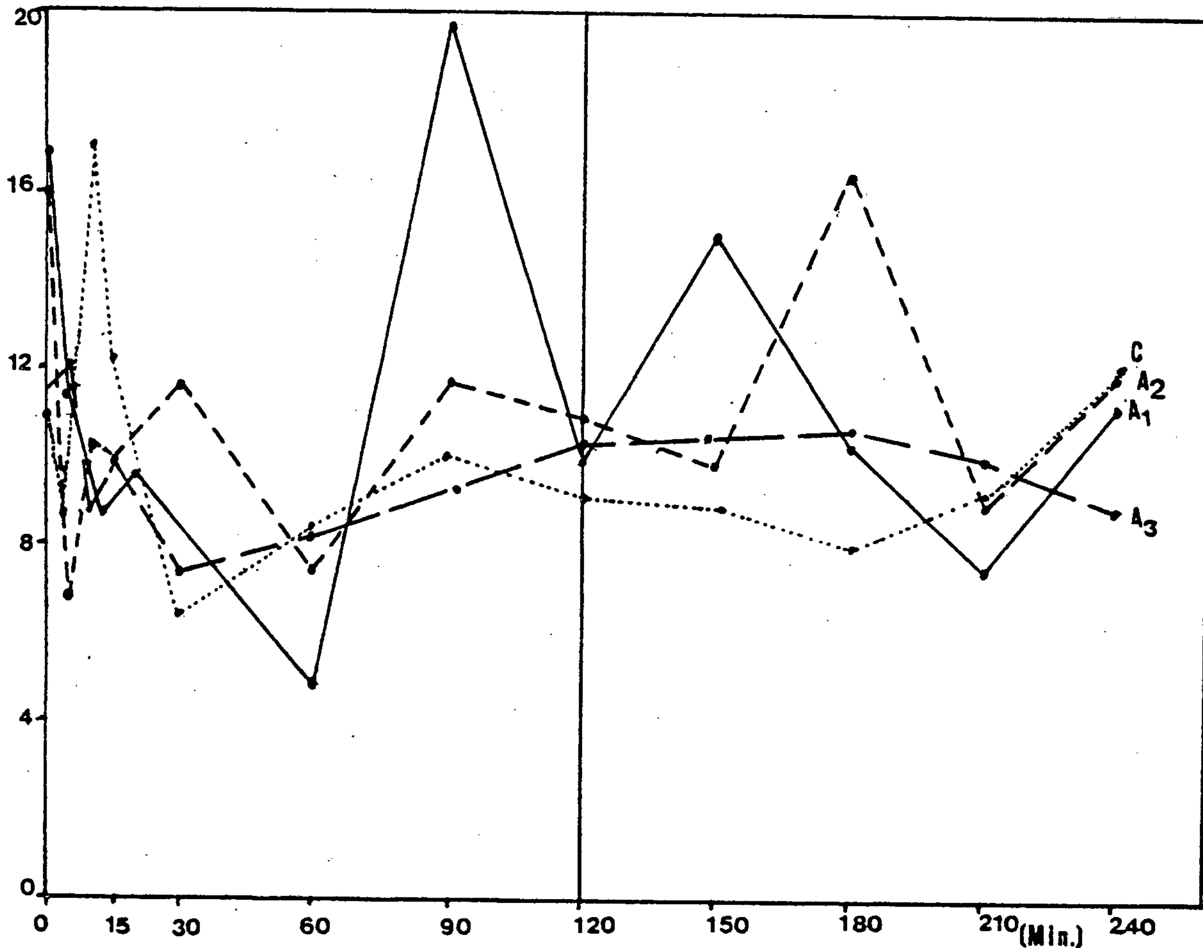
7.2 Statistical distribution of particulate C for the experimental buckets.



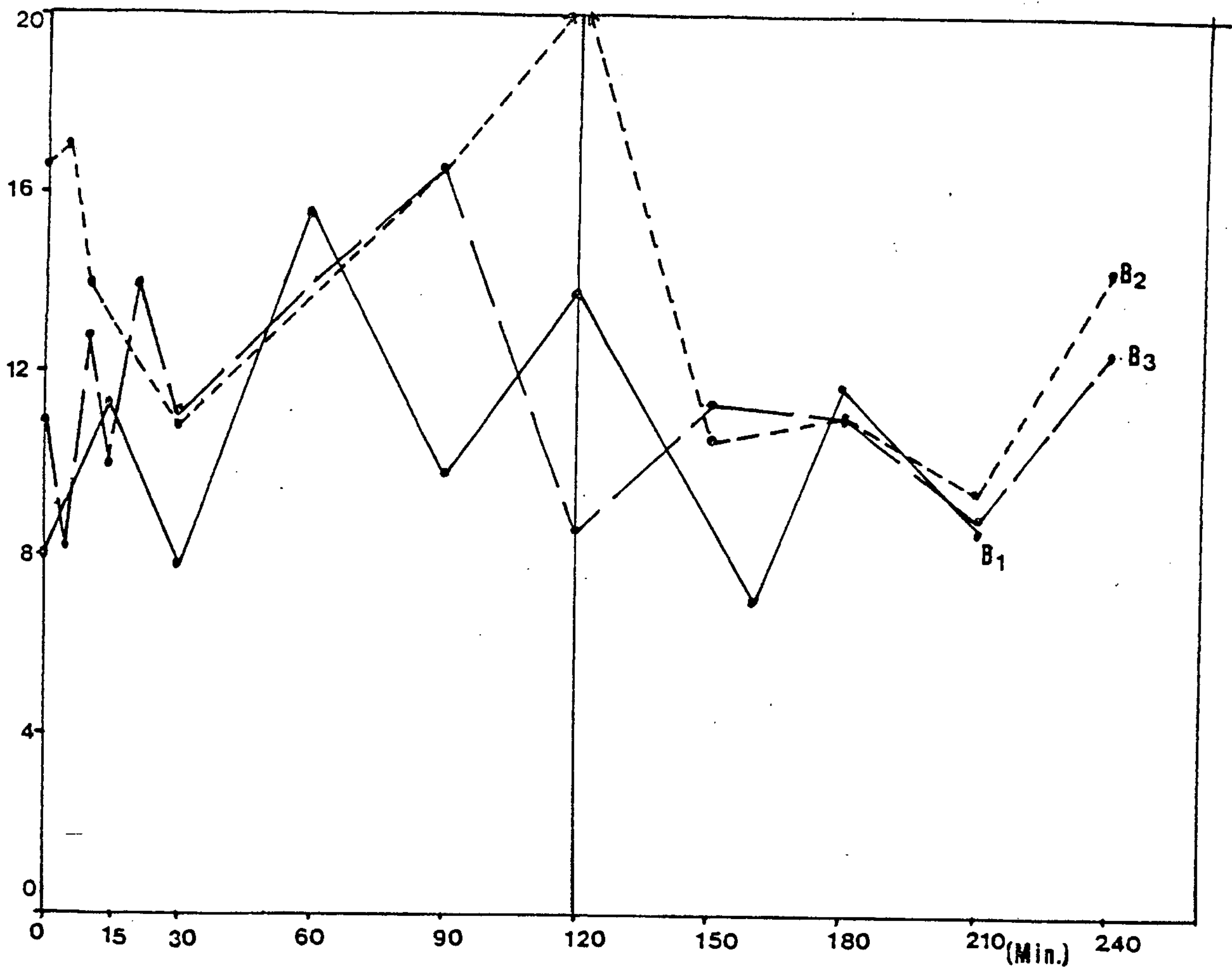
This gives both a large error at (time 240 minutes and an acceptable error at time 90 minutes. Variations for the other profiles (e.g. amino acids) are larger than this example given. Values are shown overleaf:

SAMPLING TIME	MEAN	STANDARD DEVIATION
A 0	106,48	12,23
A 5	69,44	20,69
A 10	65,28	13,37
A 15	50,46	4,58
A 30	61,81	7,64
A 60	50,46	1,73
A 90	70,14	0,70
A 120	87,04	13,63
A 150	74,31	15,98
A 180	93,06	17,82
A 210	63,89	15,88
A 240	99,07	41,01

7.3 C:N ratios of the buckets used in the field study



C:N ratio of the experimental ~~and control~~ buckets.



C:N values for the antibiotic buckets.

AMINO ACID CONCENTRATION VALUES

The following amino acid tables list the concentration values plotted in figs 3.6 - 3.15 and serve only as a clarification of these plotted points.

The error of these graphs is the normal experimental error calculated on page 109. These have been demonstrated on the alanine profiles in figs 3.6 and 3.7. This small error range is roughly representative of the other amino acids.

Sample: A,0				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,39	0,788	3,6
glutamic acid	5	1,10	0,624	3,4
glycine	7	–	4,833	5,4
arginine	9	0,65	0,369	1,5
alanine	10	–	2,387	9,6
tyrosine	11	0,70	0,397	0,9

Sample: A,5				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,11	0,629	2,8
glutamic acid	5	0,75	0,425	2,3
glycine	7	2,42	1,372	1,5
arginine	9	0,60	0,340	1,4
alanine	10	2,20	1,247	5,0
tyrosine	11	0,50	0,284	0,7

Sample: A,12				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,75	0,992	4,5
glutamic acid	5	0,71	0,403	2,2
glycine	7	–	1,315	1,4
arginine	9	0,50	0,284	1,2
alanine	10	–	1,075	4,3
tyrosine	11	0,50	0,284	0,7

Sample: A,23				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	2,15	1,219	5,6
glutamic acid	5	1,19	0,675	3,6
glycine	7	–	13,373	15,
arginine	9	1,30	0,737	3,
alanine	10	–	3,13	12,
tyrosine	11	1,30	0,737	1,8

Sample: A,30				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,74	0,987	4,5
glutamic acid	5	0,90	0,510	2,7
glycine	7	–	1,286	1,4
arginine	9	0,90	0,510	2,1
alanine	10	–	1,792	7,2
tyrosine	11	0,50	0,284	0,7

Sample:	A,60			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,15	0,652	3,0
glutamic acid	5	0,70	0,397	2,1
glycine	7	-	1,025	1,1
arginine	9	0,50	0,284	1,2
alanine	10	1,74	0,987	3,9
tyrosine	11	0,50	0,284	0,7

Sample:	A,90			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,52	0,862	3,9
glutamic acid	5	0,75	0,425	2,3
glycine	7	2,90	1,644	1,8
arginine	9	0,75	0,340	1,4
alanine	10	-	1,357	5,4
tyrosine	11	0,50	0,284	0,7

Sample:	A, 120			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,40	0,794	3,6
glutamic acid	5	1,40	0,794	4,3
glycine	7	-	2,355	2,6
arginine	9	0,80	0,454	1,9
alanine	10	—	8,022	32,2
tyrosine	11	0,50	0,284	0,7

Sample:	A,150			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,90	1,077	4,9
glutamic acid	5	1,05	0,595	3,2
glycine	7	—	2,062	2,3
arginine	9	1,10	0,624	2,6
alanine	10	—	4,941	19,9
tyrosine	11	0,90	0,510	1,2

Sample:	A,180			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	
aspartic acid	3	1,70	0,964	4,4
glutamic acid	5	0,95	0,539	2,9
glycine	7	1,00	0,567	0,6
arginine	9	0,5	0,284	1,2
alanine	10	1,85	1,049	4,2
tyrosine	11	—	—	—

Sample: A_1 210				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	2,41	1,366	6,2
glutamic acid	5	1,75	0,992	5,4
glycine	7	—	3,920	4,4
arginine	9	0,90	0,510	2,1
alanine	10	—	4,633	18,6
tyrosine	11	0,80	0,454	1,1

Sample: A_1 240				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,80	1,021	4,6
glutamic acid	5	0,70	0,397	2,1
glycine	7	2,00	1,134	1,2
arginine	9	0,70	0,397	1,7
alanine	10	—	2,217	8,9
tyrosine	11	—	—	—

Sample: A_2 0				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	0,95	0,539	2,4
glutamic acid	5	0,80	0,454	2,4
glycine	7	—	2,056	2,3
arginine	9	0,5	0,286	1,2
alanine	10	—	2,662	10,7
tyrosine	11	0,83	0,471	1,1

Sample: A_2 5				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	0,83	0,471	2,1
glutamic acid	5	1,31	0,743	4,[illegible]
glycine	7	—	2,403	2,7
arginine	9	0,60	0,340	1,4
alanine	10	—	35,431	142,6
tyrosine	11	0,50	0,284	0,7

Sample: A_2 10				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,5	0,284	0,8
aspartic acid	3	1,34	0,760	3,4
glutamic acid	5	0,69	0,391	2,1
glycine	7	—	1,955	2,2
arginine	9	0,5	0,284	1,2
alanine	10	—	1,016	4,0
tyrosine	11	0,5	0,284	0,7

Sample: $A_2 15$				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	–	1,556	7,1
glutamic acid	5	0,98	0,556	3,0
glycine	7	–	2,193	2,4
arginine	9	0,50	0,284	1,2
alanine	10	–	1,699	6,8
tyrosine	11	0,80	0,454	1,1

Sample: $A_2 30$				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	0,50	0,284	1,3
glutamic acid	5	1,96	1,111	6,0
glycine	7	–	4,323	4,8
arginine	9	1,68	0,953	4,1
alanine	10	–	42,754	172,1
tyrosine	11	1,6	0,907	2,2

Sample: $A_2 60$				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,00	0,567	2,6
glutamic acid	5	1,00	0,567	3,0
glycine	7	–	3,406	3,6
arginine	9	0,80	0,454	1,9
alanine	10	–	3,099	12,4
tyrosine	11	0,5	0,284	0,7

Sample: $A_2 90$				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,81	1,026	4,7
glutamic acid	5	1,45	0,822	4,4
glycine	7	2,91	1,650	1,8
arginine	9	0,5	0,284	1,2
alanine	10	–	3,309	13,
tyrosine	11	–	–	–

Sample: $A_2 120$				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,55	0,879	4,0
glutamic acid	5	1,19	0,634	3,4
glycine	7	–	2,777	3,1
arginine	9	0,9	0,510	2,1
alanine	10	–	4,907	19,7
tyrosine	11	0,7	0,397	0,9

Sample:	A2150			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,48	0,839	3,8
glutamic acid	5	0,90	0,649	2,7
glycine	7	–	3,648	4,1
arginine	9	0,70	0,397	1,7
alanine	10	–	2,112	8,5
tyrosine	11	0,5	0,284	0,7

Sample:	A2180			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	2,04	1,157	5,3
glutamic acid	5	2,49	1,412	7,7
glycine	7	–	3,560	4,0
arginine	9	0,92	0,522	2,2
alanine	10	–	20,044	80,7
tyrosine	11	0,90	0,510	1,2

Sample:	A2210			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,85	1,049	4,8
glutamic acid	5	1,00	0,567	3,0
glycine	7	–	3,791	4,2
arginine	9	0,90	0,510	2,1
alanine	10	–	2,025	8,1
tyrosine	11	0,50	0,284	0,7

Sample:	A2240			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,17	0,663	3,0
glutamic acid	5	0,91	0,516	2,8
glycine	7	–	3,103	3,5
arginine	9	0,80	0,454	1,9
alanine	10	–	1,780	7,1
tyrosine	11	0,50	0,284	0,7

Sample:	A30			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,25	0,709	3,2
glutamic acid	5	1,25	0,709	3,8
glycine	7	–	3,299	3,7
arginine	9	0,80	0,454	1,9
alanine	10	–	3,618	14,5
tyrosine	11	0,50	0,284	0,7

Sample: A3 5

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	0,5	0,284	0,8
aspartic acid	3	2,26	1,281	5,8
glutamic acid	5	1,84	1,043	5,6
glycine	7	–	4,712	5,3
arginine	9	1,00	0,567	2,4
alanine	10	–	9,490	38,2
tyrosine	11	0,50	0,284	0,7

Sample: A3 10

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	–	–	–
aspartic acid	3	1,01	0,573	2,6
glutamic acid	5	0,68	0,386	2,1
glycine	7	–	1,148	1,2
arginine	9	0,60	0,340	1,4
alanine	10	–	0,738	2,9
tyrosine	11	0,70	0,397	0,9

Sample: A3 15

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,20	0,680	3,1
glutamic acid	5	0,88	0,499	2,7
glycine	7	–	1,988	2,2
arginine	9	0,80	0,454	1,9
alanine	10	–	1,391	5,6
tyrosine	11	0,70	0,397	0,9

Sample: A3 30

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,92	1,089	5,0
glutamic acid	5	1,30	0,737	4,0
glycine	7	–	6,726	7,5
arginine	9	1,00	0,567	2,4
alanine	10	–	4,279	17,2
tyrosine	11	1,00	0,567	1,4

Sample: A3 60

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	0,60	0,340	1,0
aspartic acid	3	1,15	0,652	3,0
glutamic acid	5	2,22	1,252	6,8
glycine	7	–	5,227	5,8
arginine	9	1,44	0,816	3,5
alanine	10	–	24,718	99,5
tyrosine	11	0,9	0,510	1,2

Sample:	A_3 90			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,71	0,403	1,2
aspartic acid	3	0,51	0,856	3,9
glutamic acid	5	1,00	0,567	3,0
glycine	7	–	2,612	2,9
arginine	9	1,89	1,072	4,6
alanine	10	–	2,167	8,7
tyrosine	11	0,71	0,403	1,0

Sample:	A_3 120			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	1,90	1,077	3,3
aspartic acid	3	–	1,507	6,9
glutamic acid	5	0,90	0,510	2,7
glycine	7	–	6,532	7,3
arginine	9	–	0,875	3,7
alanine	10	–	2,354	9,4
tyrosine	11	0,5	0,284	0,7

Sample:	A_3 150			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	1,50	0,851	2,6
aspartic acid	3	–	1,901	8,7
glutamic acid	5	1,90	1,077	5,8
glycine	7	–	7,519	8,4
arginine	9	–	3,253	14,0
alanine	10	–	4,570	18,3
tyrosine	11	1,10	0,624	1,5

Sample:	A_3 180			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,85	0,482	0,8
aspartic acid	3	–	1,645	7,5
glutamic acid	5	0,9	0,510	2,7
glycine	7	–	9,859	11,1
arginine	9	–	2,191	9,4
alanine	10	–	2,498	10,0
tyrosine	11	0,6	0,340	0,8

Sample:	A_3 210			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	2,15	1,219	3,7
aspartic acid	3	–	2,497	11,4
glutamic acid	5	1,10	0,624	3,4
glycine	7	–	9,792	11,0
arginine	9	–	3,038	13,1
alanine	10	–	6,830	27,4
tyrosine	11	0,5	0,284	0,7

Sample: A3 240				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	1,53	0,868	2,6
aspartic acid	3	–	1,400	6,4
glutamic acid	5	0,84	0,476	2,5
glycine	7	–	11,000	12,4
arginine	9	–	2,684	11,5
alanine	10	–	3,518	14,1
tyrosine	11	0,50	0,284	0,7

Sample: B, 0				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,55	0,879	4,0
glutamic acid	5	0,97	0,550	3,0
glycine	7	1,03	0,584	0,6
arginine	9	0,50	0,284	1,2
alanine	10	1,12	0,635	2,5
tyrosine	11	0,57	0,323	0,8

Sample: B, 5				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	–	1,594	7,3
glutamic acid	5	1,54	0,873	4,7
glycine	7	1,68	0,953	1,0
arginine	9	0,50	0,284	1,2
alanine	10	–	4,211	16,9
tyrosine	11	0,50	0,284	0,7

Sample: B, 10				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,5	0,284	0,8
aspartic acid	3	–	0,874	4,0
glutamic acid	5	1,72	0,975	5,3
glycine	7	0,95	0,539	0,6
arginine	9	0,50	0,284	1,2
alanine	10	1,58	0,896	3,6
tyrosine	11	–	–	–

Sample: B, 15				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,10	0,625	2,8
glutamic acid	5	1,10	0,625	3,4
glycine	7	1,10	0,625	0,7
arginine	9	0,50	0,284	1,2
alanine	10	–	2,558	10,2
tyrosine	11	–	–	–

Sample: B, 33				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	1,28	0,726	2,2
aspartic acid	3	0,75	0,425	1,9
glutamic acid	5	0,5	0,284	1,5
glycine	7	1,02	0,578	0,6
arginine	9	0,5	0,284	1,2
alanine	10	1,52	0,862	3,4
tyrosine	11	0,5	0,284	0,7

Sample: B, 60				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	—	—	—
aspartic acid	3	2,12	1,202	5,5
glutamic acid	5	1,90	1,077	5,8
glycine	7	—	1,273	1,4
arginine	9	1,60	0,907	4,9
alanine	10	—	27,538	110,8
tyrosine	11	1,50	0,851	2,1

Sample: B, 90				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	2,40	1,361	6,2
glutamic acid	5	—	0,761	4,1
glycine	7	3,32	1,882	2,1
arginine	9	2,15	1,219	5,2
alanine	10	—	13,135	52,8
tyrosine	11	0,50	0,284	0,7

Sample: B, 120				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,39	0,788	3,6
glutamic acid	5	1,92	1,089	5,9
glycine	7	—	11,305	12,7
arginine	9	—	2,040	8,7
alanine	10	—	4,625	18,6
tyrosine	11	0,70	0,397	0,9

Sample: B, 150				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm-3)
asparagine	2	0,5	0,284	0,8
aspartic acid	3	2,30	1,304	5,9
glutamic acid	5	—	0,930	5,0
glycine	7	—	18,526	20,8
arginine	9	—	2,959	12,7
alanine	10	—	7,042	28,3
tyrosine	11	1,20	0,680	1,7

Sample:	B_1 180			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	2,00	1,134	5,2
glutamic acid	5	–	1,003	5,4
glycine	7	–	1,256	1,4
arginine	9	1,25	0,709	3,0
alanine	10	–	3,427	13,7
tyrosine	11	1,20	0,680	1,7

Sample:	B_1 210			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,51	0,856	3,9
glutamic acid	5	–	1,803	9,8
glycine	7	–	15,582	17,5
arginine	9	0,50	0,284	1,2
alanine	10	–	4,000	16,1
tyrosine	11	0,50	0,284	0,7

Sample:	B_1 240			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	–	–	–
aspartic acid	3	1,65	0,936	4,3
glutamic acid	5	1,00	0,567	3,0
glycine	7	–	14,084	15,8
arginine	9	1,13	0,641	2,7
alanine	10	–	4,257	17,1
tyrosine	11	1,47	0,833	2,0

Sample:	B_2 0			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	–	2,482	11,4
glutamic acid	5	2,01	0,856	4,6
glycine	7	–	2,259	2,5
arginine	9	0,70	0,397	1,7
alanine	10	–	12,067	48,5
tyrosine	11	0,80	0,454	1,1

Sample:	B_2 5			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,80	1,021	4,6
glutamic acid	5	0,72	0,408	2,2
glycine	7	–	1,736	1,9
arginine	9	0,70	0,397	1,7
alanine	10	–	0,765	3,0
tyrosine	11	0,60	0,340	0,8

Sample: $B_2$10				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,41	0,799	3,6
glutamic acid	5	1,00	0,567	3,0
glycine	7	1,16	0,658	0,7
arginine	9	0,5	0,284	1,2
alanine	10	—	0,987	3,9
tyrosine	11	—	—	—

Sample: $B_2$15				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,72	0,975	4,4
glutamic acid	5	1,30	0,737	4,0
glycine	7	2,71	1,537	1,7
arginine	9	1,81	1,026	4,4
alanine	10	—	1,520	6,1
tyrosine	11	0,50	0,284	0,7

Sample: $B_2$30				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	1,90	1,077	4,9
glutamic acid	5	—	2,00	10,9
glycine	7	—	11,165	12,5
arginine	9	1,65	0,936	3,7
alanine	10	—	12,005	48,3
tyrosine	11	1,85	1,049	2,6

Sample: $B_2$60				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	0—	0,765	3,5
glutamic acid	5	0,95	0,539	2,9
glycine	7	1,12	0,635	0,7
arginine	9	0,50	0,284	1,2
alanine	10	—	0,987	3,9
tyrosine	11	1,97	1,117	2,8

Sample: $B_2$90				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	1,38	0,782	3,5
glutamic acid	5	1,05	0,595	3,2
glycine	7	1,49	0,845	0,9
arginine	9	0,78	0,442	1,9
alanine	10	—	2,949	11,8
tyrosine	11	1,10	0,624	1,5

Sample: $B_2$120				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	—	2,353	10,8
glutamic acid	5	1,60	0,851	4,6
glycine	7	1,80	1,021	1,1
arginine	9	0,84	0,476	2,0
alanine	10	—	5,188	20,8
tyrosine	11	1,60	0,907	2,2

Sample: $B_2$150				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	1,80	1,021	4,6
glutamic acid	5	1,15	0,652	3,5
glycine	7	—	1,104	1,2
arginine	9	0,50	0,284	1,2
alanine	10	—	2,622	10,5
tyrosine	11	0,50	0,284	0,7

Sample: $B_2$180				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	—	1,686	7,7
glutamic acid	5	1,60	0,907	4,9
glycine	7	2,00	1,134	1,2
arginine	9	0,90	0,510	2,1
alanine	10	—	8,890	35,7
tyrosine	11	0,80	0,454	1,1

Sample: $B_2$210				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,72	0,975	4,4
glutamic acid	5	1,40	0,794	4,3
glycine	7	1,63	0,924	1,0
arginine	9	0,93	0,527	2,2
alanine	10	—	2,229	8,9
tyrosine	11	0,80	0,454	1,1

Sample: $B_2$240				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,50	0,851	3,9
glutamic acid	5	—	0,817	4,4
glycine	7	—	2,944	3,3
arginine	9	0,91	0,516	2,2
alanine	10	—	19,964	78,3
tyrosine	11	0,98	0,556	1,3

Sample: $B_3 0$				
compound	peak	measured height	intgr./calc. area	concentration ($nmol.dm^{-3}$)
asparagine	2	—	—	—
aspartic acid	3	1,30	0,737	3,3
glutamic acid	5	1,06	0,601	3,2
glycine	7	1,34	0,760	2,2
arginine	9	0,50	0,284	1,2
alanine	10	1,71	0,969	3,8
tyrosine	11	0,70	0,397	0,9

Sample: $B_3 5$				
compound	peak	measured height	intgr./calc. area	concentration ($nmol.dm^{-3}$)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,40	0,794	3,6
glutamic acid	5	1,41	0,799	4,3
glycine	7	—	0,862	0,9
arginine	9	1,50	0,851	3,6
alanine	10	—	1,626	6,5
tyrosine	11	0,80	0,454	1,1

Sample: $B_3 15$				
compound	peak	measured height	intgr./calc. area	concentration ($nmol.dm^{-3}$)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,42	0,805	3,7
glutamic acid	5	0,80	0,454	2,4
glycine	7	1,05	0,595	0,6
arginine	9	0,50	0,284	1,2
alanine	10	0,95	0,539	3,8
tyrosine	11	—	—	—

Sample: $B_3 30$				
compound	peak	measured height	intgr./calc. area	concentration ($nmol.dm^{-3}$)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,20	0,680	3,1
glutamic acid	5	1,31	0,743	4,0
glycine	7	2,70	1,531	1,7
arginine	9	0,91	0,516	2,2
alanine	10	—	1,465	5,8
tyrosine	11	0,80	0,454	1,1

Sample: $B_3 60$				
compound	peak	measured height	intgr./calc. area	concentration ($nmol.dm^{-3}$)
asparagine	2	—	—	—
aspartic acid	3	1,97	1,117	5,1
glutamic acid	5	0,89	0,505	2,7
glycine	7	1,00	0,567	0,6
arginine	9	—	—	—
alanine	10	1,87	1,060	4,5
tyrosine	11	0,65	0,369	0,9

Sample:	B_3 90			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,81	1,026	4,7
glutamic acid	5	2,08	1,179	6,4
glycine	7	2,05	1,162	1,3
arginine	9	0,80	0,454	2,4
alanine	10	2,85	1,616	6,5
tyrosine	11	0,50	0,284	0,7

Sample:	B_3 10			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,30	0,737	3,3
glutamic acid	5	1,35	0,765	4,1
glycine	7	1,35	0,765	0,8
arginine	9	0,50	0,284	1,2
alanine	10	—	2,558	10,2
tyrosine	11	—	—	—

Sample:	B_3 120			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	2,05	1,162	5,3
glutamic acid	5	0,78	0,442	2,4
glycine	7	1,80	1,021	1,1
arginine	9	0,55	0,312	1,7
alanine	10	—	1,753	7,0
tyrosine	11	—	—	—

Sample:	B_3 150			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	—	1,209	5,5
glutamic acid	5	1,20	0,680	3,7
glycine	7	2,20	1,247	1,4
arginine	9	0,70	0,397	1,7
alanine	10	—	5,507	22,1
tyrosine	11	—	—	—

Sample:	B_3 180			
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,80	1,021	4,6
glutamic acid	5	1,05	0,595	3,2
glycine	7	1,70	0,964	1,0
arginine	9	0,88	0,499	2,1
alanine	10	—	1,293	5,2
tyrosine	11	0,50	0,284	0,7

Sample: B$_3$210				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	2,05	1,162	5,3
glutamic acid	5	0,85	0,482	2,6
glycine	7	1,89	1,072	1,2
arginine	9	0,75	0,425	1,8
alanine	10	1,91	1,083	4,3
tyrosine	11	0,90	0,510	1,2

Sample: B$_3$240				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,80	1,021	4,6
glutamic acid	5	1,54	0,873	4,7
glycine	7	–	0,872	0,9
arginine	9	1,08	0,612	2,6
alanine	10	–	4,534	18,2
tyrosine	11	0,5	0,284	0,7

Sample: C0				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,25	0,612	2,8
glutamic acid	5	0.80	0,454	2,4
glycine	7	–	1,740	1,9
arginine	9	0,50	0,284	1,2
alanine	10	2,42	1,372	5,5
tyrosine	11	0,50	0,284	0,7

Sample: C5				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,46	0,828	3,8
glutamic acid	5	1,57	0,890	4,8
glycine	7	–	2,086	2,3
arginine	9	0,50	0,284	1,2
alanine	10	–	24,393	98,2
tyrosine	11	0,95	0,539	1,3

Sample: C10				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,61	0,913	4,2
glutamic acid	5	1,00	0,567	3,0
glycine	7	–	2,560	2,8
arginine	9	0,70	0,397	1,7
alanine	10	–	1,297	5,2
tyrosine	11	0,60	0,340	0,8

Sample: C15				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,95	1,106	5,0
glutamic acid	5	1,05	0,595	3,2
glycine	7	—	1,779	2,0
arginine	9	0,50	0,284	1,2
alanine	10	—	2,243	9,0
tyrosine	11	0,70	0,397	0,9

Sample: C 30				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,50	0,850	3,9
glutamic acid	5	1,50	0,850	4,6
glycine	7	—	11,263	12,7
arginine	9	1,12	0,635	2,7
alanine	10	—	5,018	20,2
tyrosine	11	1,49	0,845	2,1

Sample: C 60				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	1,85	1,049	4,8
glutamic acid	5	—	0,826	4,5
glycine	7	—	4,071	4,5
arginine	9	1,20	0,680	2,9
alanine	10	—	10,484	42,2
tyrosine	11	0,50	0,284	0,7

Sample: C90				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	0,50	0,284	0,8
aspartic acid	3	1,81	1,026	4,7
glutamic acid	5	1,62	0,919	5,0
glycine	7	—	4,951	5,5
arginine	9	0,80	0,454	1,9
alanine	10	—	5,055	20,3
tyrosine	11	1,25	0,709	1,7

Sample: C120				
compound	peak	measured height	intgr./calc. area	concentration (nmol.dm^{-3})
asparagine	2	—	—	—
aspartic acid	3	1,20	0,680	3,1
glutamic acid	5	1,39	0,788	4,3
glycine	7	—	10,994	12,4
arginine	9	1,50	0,851	3,6
alanine	10	—	4,146	16,6
tyrosine	11	1,20	0,680	1,7

Sample: C 150

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,20	0,680	3,1
glutamic acid	5	0,85	0,482	2,6
glycine	7	—	1,916	2,1
arginine	9	0,75	0,425	1,8
alanine	10	—	1,397	5,6
tyrosine	11	0,70	0,397	0,9

Sample: C 180

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,70	0,964	4,4
glutamic acid	5	1,30	0,737	4,0
glycine	7	—	2,724	3,0
arginine	9	0,80	0,454	1,9
alanine	10	—	3,234	13,0
tyrosine	11	0,96	0,544	1,3

Sample: C210

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	1,41	0,799	3,6
glutamic acid	5	1,62	0,896	4,3
glycine	7	—	5,247	5,9
arginine	9	0,80	0,454	1,9
alanine	10	—	3,230	13,0
tyrosine	11	0,80	0,454	1,1

Sample: C240

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	2,33	1,321	6,0
glutamic acid	5	1,80	1,021	5,5
glycine	7	—	42,541	47,9
arginine	9	2,70	1,531	6,6
alanine	10	—	19,550	78,7
tyrosine	11	—	3,723	9,3

Sample: Blank

compound	peak	measured height	intgr./calc. area	concentration (nmol.dm⁻³)
asparagine	2	—	—	—
aspartic acid	3	0,80	0,454	2,0
glutamic acid	5	0,60	0,340	1,8
glycine	7	0,60	0,340	0,3
arginine	9	—	—	—
alanine	10	2,32	1,315	5,2
tyrosine	11	—	—	—

Origin of samples : J. BRAUER UCT 7 NOV. 1985

Date analysed: 7 -11 -1985

No of Blanks: 0 No of Standards: 7

Blank peak ht: 0 Standard peak ht: 30.4809

Standard concentration: 15 ug at/l

STD CAL Setting: 5

SAMPLE No		PEAK Ht	uG N/L	$\mu mol \cdot dm^{-3}$
Sample Series:				
1	$B_3$240	7.4973	51.6847	3,6917
2	$B_3$120	7.4822	51.5447	3,6817
3	$A_1$240	20.9406	144.409	10,3149
4	$A_2$240	23.0171	158.696	11,3354
5	$B_3$210	13.8294	95.3856	6,8132
6	C240	21.9294	151.132	10,7951
7	$B_1$180	6.0042	41.3198	2,9514
8	C210	38.0313	262.205	18,7289
9	$B_3$180	8.475	58.4079	4,1719
10	$A_1$210	20.1043	138.526	9,8947
11	$A_3$210	89.1181	614.334	43,8810
12	$B_2$210	1.00000E-04	60.0887	4,2905
13	$A_2$240	86.543	596.545	42,6103
14	$A_2$120	93.4743	(644.308)	46,0220
15	$B_2$150	16.5004	113.734	8,1238
16	$B_1$240	19.3721	133.484	9,5345
17	C150	19.7424	136.145	9,7246
18	$A_3$120	80.2984	553.545	39,5389
19	C60	21.9987	151.693	10,8352
20	$A_1$150	103.356	(712.381)	50,8843
21	$B_1$210	7.4904	51.6847	3,6917
22	$B_2$180	11.0556	76.1964	5,4426
23	C90	20.5241	141.468	10,1048
24	B,120	6.7014	46.2221	3,3015
25	$B_3$150	10.8516	74.7958	5,3425
26	B,155	5.5669	38.3784	2,7413
27	$A_1$180	24.964	172.142	12,2958
28	$A_2$210	102.636	(707.479)	50,5291
29	C180	99.6823	(687.029)	49,0685
30	$A_2$180	105.459	(726.948)	51,9196
31	$A_3$240	100.369	(691.791)	49,4086
32	$A_3$150	31.5002	217.104	15,5058
33	$B_2$10	14.3423	98.8873	7,0626
34	$A_1$90	23.8741	164.579	11,7544
35	$B_2$60	14.1308	97.3466	6,9526
36	$A_1$60	105.454	(726.808)	51,9096
37	$A_3$5	23.8443	164.299	11,7344
38	$B_3$0	5.9885	41.3198	2,9511
39	$B_2$0	6.2	42.7204	3,0511
40	$A_1$0	27.2414	187.83	13,4150
41	$A_2$90	22.1	152.393	10,8841
42	$A_3$10	59.9414	413.198	29,5111

Total N continued.

SAMPLE No		PEAK Ht	uG N/L	$\mu mol \cdot dm^{-3}$
Sample Series:				
43	B_2 5	11.2435	93.5648	6,6825
44	A_3 30	15.9895	133.064	9,5036
45	A_1 120	82.2181	684.227	48,8684
46	B_2 90	8.0014	66.5318	4,7517
47	A_1 30	17.9899	149.732	10,6940
48	B_2 15	8.2225	68.4928	4,8948
49	A_1 12	87.7788	730.59	52,1797
50	A_2 30	21.5951	179.706	12,8348
51	A_2 0	68.8635	573.154	40,9354
52	B_3 60	9.0152	75.0759	5,3620
53	B_1 33	5.9073	49.1635	3,5113
54	C 0	17.9127	149.031	10,6439
55	A_2 15	21.9635	182.787	13,0548
56	A_3 15	23.3992	194.693	13,9052
57	B_1 30	4.8898	40.7595	2,9110
58	A_2 60	20.5902	171.302	12,2346
59	B_3 10	14.8204	123.399	8,8133
60	B_2 30	6.9966	58.2679	4,1615
61	A_3 60	86.4786	719.804	51,4093
62	A_2 10	81.4709	678.064	48,4282
63	?	76.8641	639.686	45,6872
64	B_3 30	1.00000E-04	222.566	15,8959
65	C 5	3.0144	25.072	1,7906
66	B_1 0	4.4825	37.2578	2,6610
67	B_1 10	5.0701	42.1602	3,0111
68	MQ	11.9301	99.3075	7,0926
69	B_1 90	9.8554	82.0793	5,8622
70	B_3 5	6.0827	50.5642	3,6114
71	A_3 90	18.1643	151.132	10,7940
72	C 30	17.5579	146.09	10,4339
73	B_2 120	9.6322	80.1183	5,7222
74	A_1 23	17.0414	141.888	10,1338
75	A_2 5	21.291	177.185	12,6548
76	A_2 150	24.1028	200.576	14,3250
77	B_3 21	7.4373	61.9096	4,4217
78	C 15	18.4552	153.654	10,9741
79	B_1 5	6.6341	55.1864	3,9415
80	B_1 15	9.8404	81.9392	5,8522
81	A_3 0	19.5055	162.338	11,5893
82	A_1 5	19.9292	165.839	11,8392
83	Tp.	15.1742	126.34	9,0194
84	A_3 180	52.5	~~663.7~~ 214	15,2774
85	C 120	58.0695	155.614	11,1092

DETERMINATION OF AMMONIUM-N IN SEA WATER

Origin of samples :J.BRAUER UCT 5 NOV. 1985

Date analysed: 7 -11 -1985

No of Blanks: 5 No of Standards: 5

Blank peak ht: 4.87022 Standard peak ht: 13.3317

Standard concentration: 2.5 ug at/l

STD CAL Setting: 4

SAMPLE No		PEAK Ht	uG N/L
Sample Series:			
1	A_2 15	3.3845	24.9319
2	A_2 30	.7263	5.32255
3	A_1 60	2.9457	21.7104
4	A_2 0	2.0474	14.9872
5	A_2 60	1.3533	9.94476
6	B_3 21	6.606	48.4632
7	B_2 10	6.0382	44.4012
8	B_3 5	7.1547	52.5251
9	B_3 0	10.7066	78.7177
10	A_1 10	1.9882	14.567
11	A_2 5	2.8932	21.2902
12	A_1 0	3.9249	28.8538
13	B_3 5	6.0645	44.5413
14	A_3 5	2.6519	19.4693
15	A_1 90	2.506	18.3488
16	C 60	7.26004E-02	.560268
17	B_3 30	4.9689	36.5575
18	B_1 90	5.4592	40.0592
19	B_2 30	5.2291	38.3784
20	A_2 10	1.4643	10.7852
21	A_1 23	1.6091	11.7656
22	B_1 120	5.7331	42.1602
23	C 120	3.5247	25.9124
24	B_3 150	7.9745	58.548
25	A_3 15	1.235	9.10435
26	A_2 90	2.5556	18.769
27	B_2 90	7.0193	51.5447
28	B_2 15	5.7967	42.5804
29	B_1 210	6.9025	50.7043
30	A_2 240	2.278	16.668
31	C 150	2.1789	15.9676
32	B_1 180	5.7628	42.3002
33	A_1 240	.9965	7.28348
34	A_3 180	1.5782	11.6256
35	A_1 150	2.0572	15.1272
36	A_3 240	2.2072	16.2478
37	B_2 180	8.1702	60.0887
38	B_3 210	9.9442	73.115
39	A_2 180	3.1666	23.2511
40	A_2 210	2.0436	14.9872
41	A_3 0	2.4207	17.7885
42	B_1 240	8.4776	62.3298
43	A_3 210	1.7349	12.7461
44	A_2 150	.925	6.86328
45	A_1 210	2.6264	19.3292
46	C 180	1.2486	9.10435

DETERMINATION OF AMMONIUM-N IN SEA WATER

Origin of samples :J.BRAUER UCT

Date analysed: 5 -11 -1985

SAMPLE No		PEAK Ht	uG N/L
Sample Series:			
47	B_3 240	10.5306	77.317
48	B_1 150	7.0652	51.9649
49	B_2 240	7.8165	57.4275
50	B_2 210	6.8058	50.0039
51	B_2 150	12.0286	88.3823
52	C 210	2.1648	15.9676
53	C 240	1.7032	12.466
1	B_3 10	7.7688	32.2154
2	B_1 60	9.5924	39.639
3	C 15	1.856	7.70368
4	A_3 60	2.5058	10.365
5	A_3 150	1.92	7.98382
6	B_2 60	9.8169	40.6194
7	B_3 120	9.3988	38.9386
8	A_1 12	4.476	18.4888
9	C0	2.5314	10.505
10	B_2 0	16.1978	67.0921
11	A_3 30	1.3696	5.60268
12	B_1 15	6.91	28.5737
13	A_1 120	1.3978	5.74275
14	A_1 5	2.2828	9.38449
15	C 30	2.0217	8.40402
16	B_1 0	17.1978	71.154
17	B_1 10	2.2978	9.52456
18	A_3 90	2.1736	8.96429
19	C90	1.8854	7.84375
20	B_1 5	8.6978	35.9972
21	B_3 60	8.9505	36.9777
22	B_2 120	7.8282	32.3555
23	B_1 155	7.9509	32.9157
24	A_1 30	7.1996	29.8343
25	B_1 33	.9609	3.92188
26	A_2 120	10.0663	41.5999
27	C5	5.1863.	21.4303

Particulate C values from CHN - analysis.

Sample	amount filtered (g C)	concentration ($mol.dm^{-3}$)
$A_1$0	72	100,00
$A_1$5	59	81,94
$A_1$10	37	51,39
$A_1$20	41	56,94
$A_1$60	36	50,00
$A_1$90	51	70,83
$A_1$120	51	70,83
$A_1$130	65	90,28
$A_1$180	53	73,61
$A_1$210	32	44,44
$A_1$240	48	66,67
$A_2$0	69	95,83
$A_2$5	29	40,28
$A_2$10	44	61,11
$A_2$15	34	47,22
$A_2$30	50	69,44
$A_2$60	38	52,78
$A_2$90	50	69,44
$A_2$120	75	104,17
$A_2$150	42	58,33
$A_2$180	84	116,67
$A_2$210	46	63,89
$A_2$240	113	156,94
$A_3$0	89	123,61
$A_3$10	60	83,33
$A_3$5	62	86,11
$A_3$15	34	47,22
$A_3$30	39	54,17
$A_3$60	35	48,61
$A_3$120	62	86,11
$A_3$180	64	88,89
$A_3$210	60	83,33
$A_3$240	53	73,61
$B_1$0	71	84,72
$B_1$15	33	54,17
$B_1$33	39	45,83
$B_1$60	50	93,06
$B_1$90	59	69,44
$B_1$120	67	98,61

Sample	amount filtered (g C)	concentration (mol.dm^{-3})
$B_1$160	35	48,61
$B_1$1[illegible]0	50	69,44
$B_1$210	61	81,94
$B_2$0	300	416,67
$B_2$5	132	183,33
$B_2$10	60	83,33
$B_2$30	65	90,28
$B_2$90	113	156,94
$B_2$120	148	205,56
$B_2$150	54	75,00
$B_2$180	105	145,83
$B_2$210	48	66,67
$B_2$240	86	119,44
$B_3$0	159	220,83
$B_3$5	42	58,33
$B_3$10	165	229,17
$B_3$15	60	83,33
$B_3$21	73	101,39
$B_3$30	104	144,44
$B_3$90	85	118,06
$B_3$120	58	80,56
$B_3$150	41	56,94
$B_3$180	47	65,28
$B_3$210	37	51,39
$B_3$240	43	59,72
C 0	59	81,94
C 5	51	70,83
C 5	137	190,28
C 10	73	101,39
C 15	63	87,5o
C 30	33	45,83
C 60	36	50,00
C 90	69	95,83
C 120	47	65,27
C 150	38	52,78
C 180	34	47,22
C 210	47	65,28
C 240	52	72,22

DPM values and subsampling times for the experimental bucket's sampling times.

Time (min)
Count (DPM)

	Times		a	b	c	d	e	f
1.	0	Time	0	48	119	179	239	303
		Count	2043,37	2136,36	2545,59	2674,75	2934,13	3891,37
2.	5	Time	5	65	128	185	248	308
		Count	655,06	2304,04	2694,42	321086	3902,45	468303
3.	10	Time						
		Count						
4.	15	Time	11	71	139	199	258	318
		Count	2189,43	2092,49	2919,06	3503,11		3931,58
5.	30	Time	29	89	152	239	273	333
		Count	1028,04	2539,44	2837,02	3168,26	5088,60	6021,66
6.	60	Time	59	119	179	239	303	363
		Count	786,64	2921,54	2591,71	3644,26		4661,82
7.	90	Time	89	152	239	273	333	393
		Count	958,41	1538,77	2503,56	3356,11	4011,14	5112,61
8.	120	Time	119	179	239	303	363	423
		Count	1027,54	1300,17	1729,43	3698,23	2917,78	3095,6
9.	150	Time	152	239	273	333	393	453
		Count	1135,61	2386,19	2294,33	3969,80	3287,38	385954
10.		Time						
		Count						
11.	180	Time	179	239	303	363	423	
		Count	605,88	1078,80	1966,25	2723,74	3303,10	—
12.	210	Time	214	273	333	393	453	
		Count	1181,36	1250,16	1838,00	2428,49	2581,39	—
13.	240	Time	239	303	393	423	453	
		Count	1451,42	5225,52	2330,77	2551,53	2835,54	—

DPM values and subsampling times for the control bucket's sampling times.

Time (min)
Count (DPM)

	Times	a.	b	c	d	e	f
1.	0	0	48	119	179	239	303
		911,01	1559,94	2438,40	4016,07	3991,31	6769,74
2.	5	5	65	128	185	248	308
		1500,75	1589,97	3349,91	3649,59	5232,28	5705,30
3.	10						
4.	15	11	71	139	199	258	318
		1505,84	1577,86	2595,99	3665,11		4992,19
5.	30	29	89	152	239	273	333
		1402,04	2388,69	3820,67	3485,30	5950,58	6756,56
6.	60	59	119	179	239	303	363
		575,43	3085,89	3011,74	4276,72		6322,09
7.	90	89	152	239	273	333	393
		1218,96	2961,34	3545,72	4644,75	9372,12	6953,65
8.	120	119	179	239	303	363	423
		2065,16	1544,72	1188,16	1396,55	1010,75	1272,06
9.	150	152	239	273	333	393	453
		940,56	827,69	806,28	1281,45	1314,86	1257,56
10.							
11.	180	179	239	303	363	423	–
		1166,79	699,07	786,58	739,64	584,89	–
12.	210	214	273	333	393	453	–
		1011,80	735,34	800,82	931,71	832,38	–
13.	240	239	303	393	423	453	–
		550,85	4894,80	1192,79	2215,80	961,70	–

Carbon production values - activities.

A - Experimental bucket.

Sample	Sample time	Carbon production range (10^{-3})	activity mean
1	0	2,155 - 14,008	8,082
2	5	4,702 - 30,563	17,633
4	11	2,547 - 16,555	9,551
5	29	5,803 - 37,720	21,762
6	59	4,443 - 28,882	16,663
7	89	5,372 - 34,918	20,145
8	119	3,162 - 20,553	11,858
9	152	3,503 - 22,769	13,136
11	179	4,510 - 29,315	16,913
12	214	2,609 - 16,962	9,786
13	239	2,449 - 15,918	9,184

C - Control bucket.

Sample	Sample time	Carbon production range (10^{-3})	activity mean
1	0	6,979 - 45,360	26,170
2	5	6,042 - 39,270	22,656
4	11	5,670 - 35,280	20,475
5	29	6,516 - 42,360	24,438
6	59	6,775 - 44,040	25,408
7	89	7,034 - 45,720	26,377
8	119	-0,462 - - 3,005	-1,734
9	152	0,623 - 4,050	2,337
11	179	-0,717 - -4,661	- 2,689
12	214	0,097 - 0,630	0,364
13	239	0,568 - 3,693	2,131

Total bacterial density (N) of the samples and individual biomass values (B) for the different bacterial species.

Sample	N(10^6)	B SC	B LC	B SR	B LR	B SU	B	Biomass(10^{-7}) $mg.dm^{-3}$
Blank 1	5,620	1,09	0,141	2,178	-	4,356	-	7,7
$A_3$10	33,378	7,722	76,538	124,150	29,568	17,420	2,178	257,5
$B_1$10	16,273	4,950	34,360	37,026	-	13,068	-	89,4
$A_1$5	17,397	5,148	45,298	52,272	7,392	8,712	4,356	123,1
C 5	39,438	15,050	71,852	58,806	7,392	21,780	2,178	177,0
$A_2$5	37,288	11,580	81,224	76,230	29,568	28,314	2,178	229,0
$A_3$5	57,080	19,500	90,596	167,706	36,960	28,314	4,356	347,4
C 0	37,483	14,160	60,918	80,586	14,784	15,246	-	185,6
$A_1$0	11,728	3,762	23,430	37,026	7,392	2,178	-	73,7
$A_2$0	25,901	9,108	56,232	45,738	-	8,712	2,178	121,9
$A_3$0	36,164	10,593	110,902	69,696	-	13,068	-	204,2
$A_3$30	64,020	12,276	187,440	213,440	51,744	50,094	2,178	412,6
$A_2$30	25,754	6,930	43,736	108,900	14,784	4,356	2,178	180,8
$A_1$23	54,246	20,196	104,654	93,654	14,784	23,958	2,178	259,4
C 15	31,912	11,286	59,356	76,230	7,392	8,712	-	162,9
$A_3$15	169,824	44,847	221,804	163,350	66,528	13,068	4,356	1360,1
$B_1$15	123,394	26,631	176,506	174,240	29,568	52,272	13,068	472,2
$A_2$15	67,538	16,830	87,472	126,320	-	26,136	-	256,7
Blank 2	3,176	0,198	9,372	10,890	-	-	-	40,0
$A_1$15	9,041	2,772	28,116	30,490	-	2,178	-	63,5
C 10	80,391	10,494	104,654	187,300	147,840	69,696	15,246	535,2
$A_2$10	17,251	6,138	34,364	34,850	22,176	4,356	-	101,8
$A_2$150	9,627	3,465	15,620	19,602	-	10,890	-	49,5
$A_2$120	61,430	18,513	114,026	158,994	7,392	45,738	4,356	349,0
$B_1$30	10,898	3,663	15,620	30,492	-	10,890	2,178	62,8
$B_1$90	30,152	10,296	49,984	82,764	7,392	13,068	-	163,5
C 60	32,743	9,009	71,852	95,830	7,392	19,602	2,178	205,8
$B_1$60	23,604	7,227	57,794	56,630	-	10,890	-	132,5
$A_3$60	29,957	7,029	59,356	84,940	14,784	52,270	6,534	224,9
$A_1$33	44,814	12,670	82,786	141,570	22,176	34,848	2,178	296,2
C 30	11,142	4,455	14,058	32,670	7,392	4,356	-	62,9
$A_1$150	25,901	8,514	56,232	56,628	14,784	4,356	-	140,5
$A_3$150	73,696	14,949	139,018	145,926	51,744	54,450	26,136	432,2
$A_1$120	29,664	8,613	62,480	67,518	66,528	8,712	2,178	216,0
$A_3$120	40,415	10,593	85,910	124,146	14,784	41,382	4,356	281,1
C 120	26,878	8,217	49,984	80,586	14,784	15,246	-	168,8
$A_2$90	79,169	17,127	93,720	156,816	-	30,492	-	298,1
$A_1$90	26,536	8,613	48,420	67,518	22,176	10,890	2,178	159,7
C 90	39,438	15,741	54,670	74,052	14,784	15,246	-	174,4
$A_3$90	45,250	11,682	137,456	98,010	14,784	45,738	6,534	314,2

Total bacterial density (N) of the samples and individual biomass values (B) for the different bacterial species continued...

Sample	$N(10^6)$	B SC	B LC	B SR	B LR	B SU	B	Biomass(10^{-7}) $mg.dm^{-3}$
A_1 60	11,875	4,554	23,430	26,136	-	-	-	54,1
B_1 160	36,652	11,286	62,480	80,586	22,176	30,492	2,178	209,1
B_1 180	26,732	10,098	40,612	52,272	-	17,424	-	120,4
A_1 180	22,138	6,534	53,108	43,560	7,392	26,136	-	136,7
A_1 240	36,506	12,276	68,728	78,408	-	30,492	-	189,9
A_1 210	24,288	4,950	81,224	56,628	-	17,424	-	160,2
C 210	34,840	10,989	60,918	100,188	14,784	21,780	-	208,6
A_2 180	37,288	14,652	57,794	67,518	14,784	10,890	2,178	167,8
C 180	8,454	2,970	15,620	21,780	-	4,356	-	44,7
A_3 180	53,122	17,325	99,968	117,612	22,176	34,848	13,068	304,9
C 150	25,070	9,009	43,736	52,272	-	15,246	-	120,2
A_2 240	9,089	3,366	20,306	28,314	-	10,890	-	62,8
C 240	39,438	13,662	76,538	84,942	14,784	21,780	-	211,7
A_3 240	77,948	12,870	117,150	130,680	22,176	71,874	10,890	365,6
A_2 210	36,652	10,593	71,852	108,900	14,784	21,780	4,356	232,2
B_1 210	33,085	10,296	81,224	71,874	14,784	10,890	2,178	191,2
A_3 210	24,581	8,514	45,298	41,382	14,784	28,314	-	138,2