# Racemization of Amino Acids in Agricultural Soils

by

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Summary

The broad objective of the thesis is to provide a sound foundation from which to launch more detailed studies of the phenomena of amino acid racemization in agricultural soils and provide a basis for the interpretation of racemization data from paleosols.

The development of the working technique of sample preparation and analysis which has since been adopted by the CSIRO Division of Soils Amino Acid Racemization (AAR) laboratory is described together with realistic expectations of interpretative boundaries based on systematic control experiments. This development includes a description of the technique which provides two hydrolytic fractions from a single soil sample.

AAR analysis of soils, particle size separates, and humic acids from the wheat/fallow and permanent pasture rotations of the Waite agrisequence together with analysis of three soils from the Rothamsted agrisequence indicated racemization data followed trends consistent with established age effects. Hydrolytic fractionation provided the most consistent effect with the D/L ratios of the more resistant fraction generally higher than those of the material released during the first hydrolysis. D/L ratios of these soils/separates were generally low ($D/L < 0.2$).

Extensive analysis of data provided by pyrolysis studies of the pasture soil indicated racemization proceeded largely as predicted by pyrolysis studies of molluscs. Age assessment based on data obtained from this soil indicated the dominance of modern material in the soil hydrolysates. The evidence further

indicated that the hydrolytic fractions had different racemization characteristics and could therefore be defined as kinetic fractions.

Pyrolysis studies of the pasture soil and the wheat/fallow soil indicated that amino acid racemization techniques provide a unique set of data concerning the amino acid status of agricultural soils. Exposure of the soil to the relatively short period of intensive exploitative practices resulted in a marked difference in observed racemization phenomena. The collective evidence was consistent with the thesis that the management of these soils led to breakdown of the mechanisms of protection of amino acids in the wheat/fallow soil and this was measured with the AAR analysis.

I certify that, to the best of my knowledge, this thesis does not incorporate without acknowledgement any material previously accepted for a degree or diploma in any university; and that it does not contain any material previously published or written by another person except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan.

Charles Vincent Griffin

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# CHAPTER 1 AMINO ACID RACEMIZATION IN SOILS : AN INTRODUCTION TO THIS THESIS

## 1.1 INTRODUCTION

Amino acid racemization is the reversible transition between optical isomers of amino acids, most usually D– and L–isomers, as represented by Figure 1.1.

Apparent racemization is racemization as measured. The term apparent is included to account for the complexity of the phenomenon of racemization in natural systems. Apparent racemization changes with time and may be approximated by the model of Wehmiller (1981) illustrated by Figure 1.2.

Complexity of the apparent phenomenon arises because,
(i) amino acids exist in natural systems as a myriad of proteins and peptides of differing molecular weight some of which are shown schematically in Figure 1.3, and,
(ii) racemization is largely a terminal position phenomenon (Kimber and Hare in prep.).

Apparent racemization is the nett result of four processes (i) hydrolysis (ii) racemization (iii) decarboxylation and (iv) deamination. The complexity of the apparent phenomenon is succinctly summarised by the model of Kimber and Griffin (1987) that is presented in Figure 1.4. This model was developed to account for the apparent reversal in racemization of,
(i) most amino acids in the $>1000$ Dalton separate, and,
(ii) aspartic acid in the $<1000$ Dalton separate and the total hydrolysate,
from pyrolysis studies of molluscs. These data are presented in Figure 1.5.

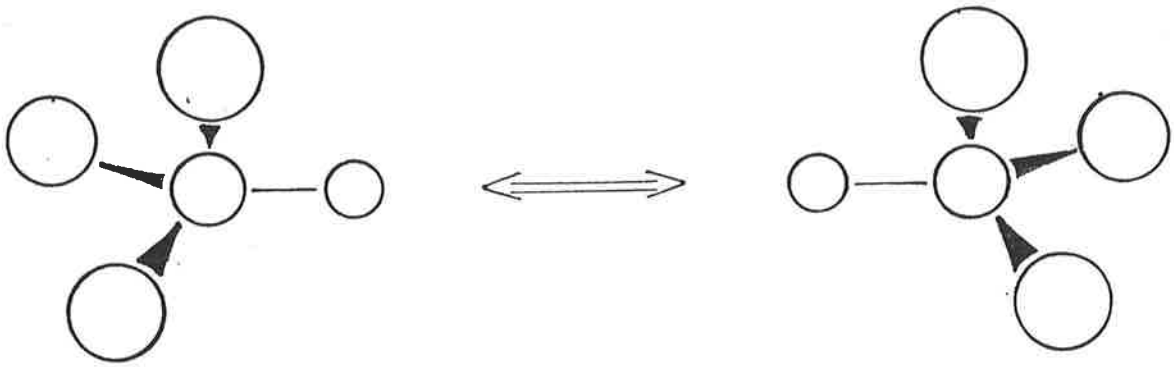



Fig. 1.1 Racemization, the reversible transition between amino acid isomers.

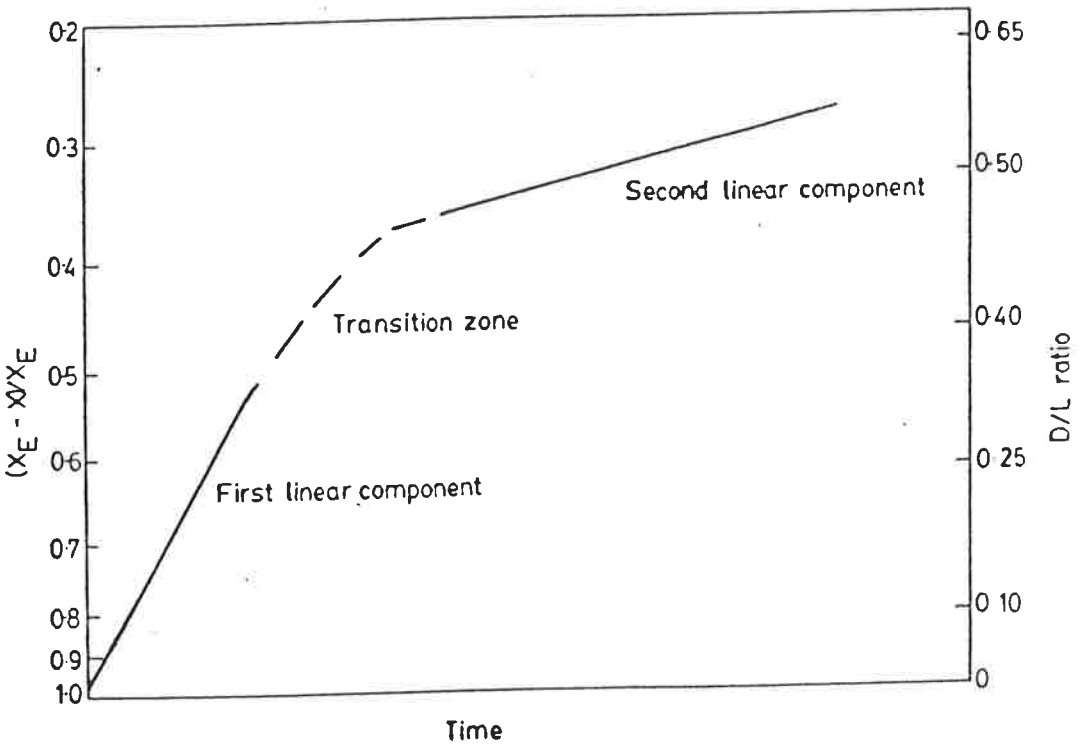
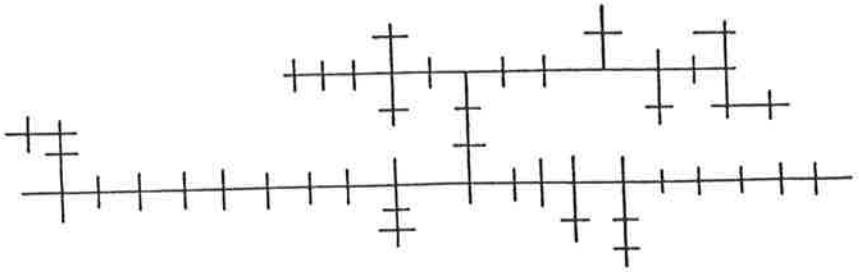
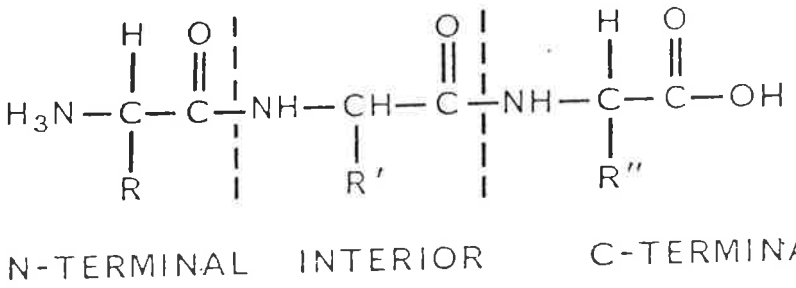


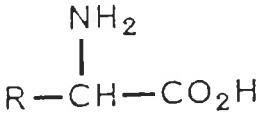

Fig. 1.2 "Non – linear" molluscan racemization kinetic model of Wehmiller (1981, 1982, 1984) with two essentially linear components separated by a transition zone. The second essentially linear component is approximately 10X slower than the first. (After Murray – Wallace 1987).

(i) SCHEMATIC PEPTIDE CHAIN






(ii) TRIPEPTIDE




(iii) FREE AMINO ACID

Fig. 1.3 Schematic diagrams depicting three possible ways for amino acids to combine,
(i) schematic peptide,
(ii) tripeptide showing three main positions (N – terminal, C – terminal and interior), and,
(iii) free amino acids.

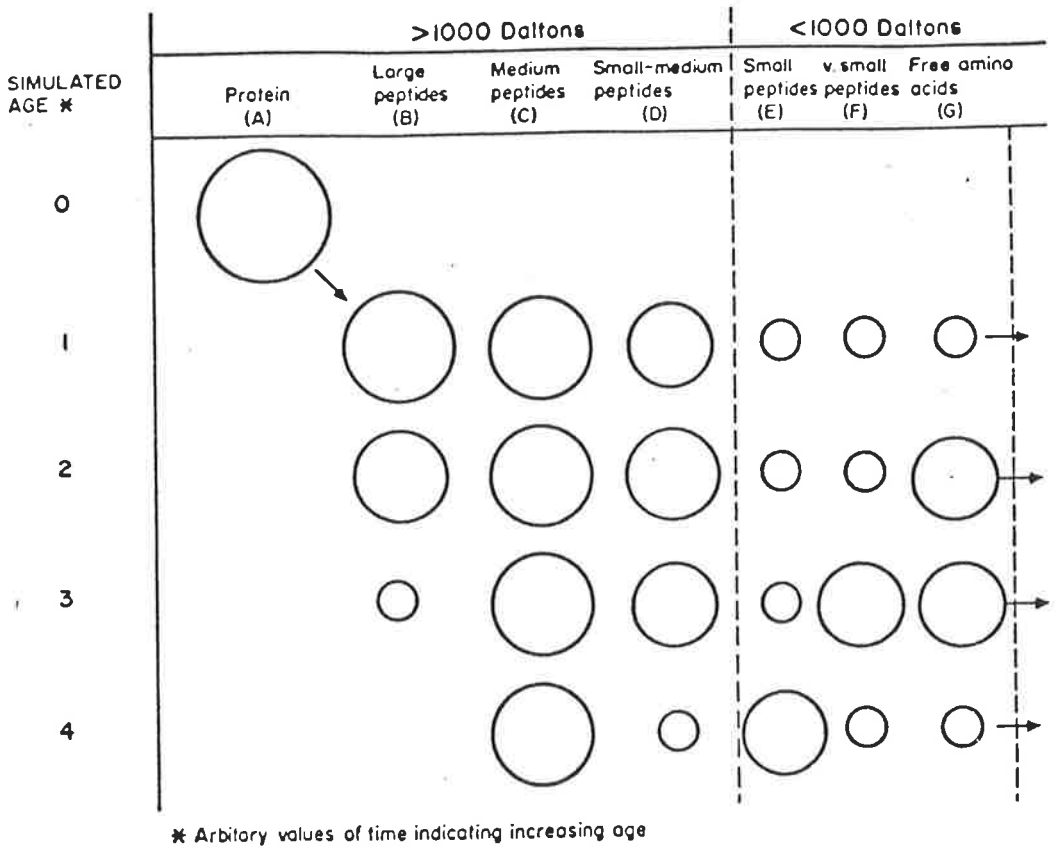



Fig. 1.4 Apparent racemization modelled as the nett result of (i) hydrolysis, (ii) racemization, (iii) decarboxylation and (iv) deamination. The model depicts a possible mode of breakdown during simulated aging of protein via various peptide species (groups A – G). As breakdown progresses with the simulated age increments the original protein is hydrolysed into various molecular weight species which are represented by circles, the areas of which correspond to concentrations. Because racemization is largely a terminal position phenomenon the molecular weight distribution of the contributing peptides determines D/L ratios in that when there is a preferential preservation of larger species (as is depicted here) D/L may reduce as is apparent in Fig 1.5. (After Kimber and Griffin 1987).

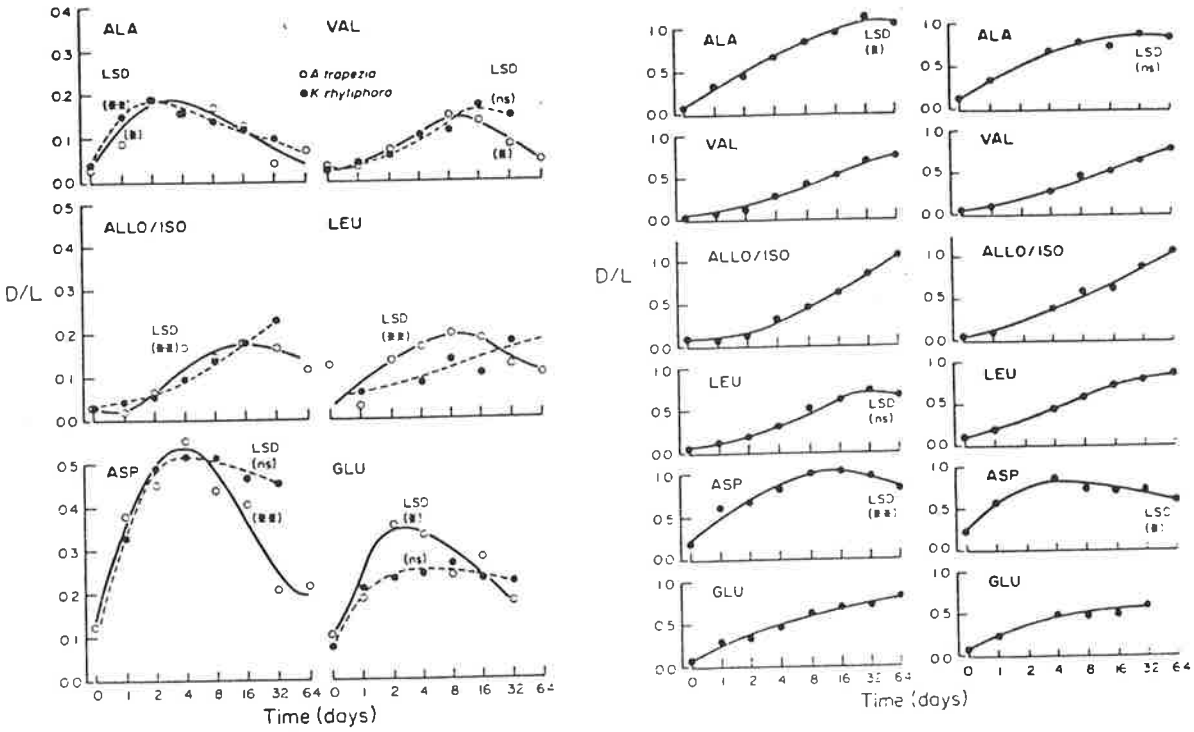



Fig. 1.5A

> 1000 Dalton Separate

Fig.1.5B

< 1000 Dalton Separate

Fig.1.5C

Total Hydrolysate

Fig. 1.5 Evidence for the model described in figure 1.4 arose from pyrolysis studies of molluscs. D/L changes with time are presented for six amino acids from three molecular weight fractions (> 1000 Daltons, < 1000 Daltons and the total hydrolysate) analysed by Kimber and Griffin (1987).

While large molecular weight species (> 1000 Dalton, Fig 1.5A) did not racemize extensively, smaller molecular weight species (< 1000 Daltons, Fig 1.5B) did racemize significantly during pyrolysis.

Generally D/L from the total hydrolysate increases to an equilibrium point close to D/L = 1 indicating a dominance of small molecular weight effects in the total hydrolysate. Apparent racemization of aspartic acid was noted to differ, initially increasing before decreasing (Fig. 1.5C) indicating a dominance of interior aspartic residues in the total.
(Modified after Kimber and Griffin 1987).

## 1.2 AIMS OF THIS THESIS

The broad objective of this thesis is to provide a sound foundation from which to launch more detailed studies of the phenomenon of amino acid racemization in soils.

These investigations are largely restricted to soils which have been subjected to long term agricultural treatments (*ca.* 50 years). The selected treatments represent extremes in management of soil as a nitrogen resource.

There are few publications pertaining to racemization of amino acids in soils. All except those which are a direct result of this work are restricted to the readily hydrolysable portion of soil organic matter.

By restricting the investigations presented in this thesis to particular, representative soils more detailed analysis is possible. The main body of this research is presented in Chapters 4, 5 and 6. Investigations to establish various control parameters are presented in Chapter 3.

Chapter 2 is presented as a review of the literature which is related to the broad objective of this thesis. There are few publications recording the degree of racemization of soil samples. The main body of the literature includes Pollock and Frommhagen (1968), Cheng et al. (1975), Pollock et al. (1977), Pollock and Kvenvolden (1978), Limmer and Wilson (1980), Mahaney et al. (1986), Griffin and Kimber (1988) and Kimber and Griffin (1988). The work of Stevenson and Cheng (1972) is also significant in that it provided amino acid racemization data from a sediment. Excluding those which are a direct result of the research for this thesis the most significant of these publications is that of Limmer and Wilson who proposed the use of a HF fraction in racemization studies of soil.

Known age effects in soils are also discussed in Chapter 2. The concept of age of soil is complicated by the fact that the soil investigated may be considered to be a living organism (Burns 1982, 1983) that has survived for many millennia. Thus quantitative information is really only apparent information and must be interpreted as such.

Extrapolation of the current state of knowledge as presented in Chapter 2 indicates that three main phases of research are required. These phases are,

(i) the development of a working technique to be applied in the laboratory together with realistic expectations of interpretative boundaries based on systematic control experiments,

(ii) to follow the age trends established by radiocarbon techniques by completing amino acid racemization analysis on comparable soils and soil fractions/separates, and,

(iii) to establish a kinetic basis for the interpretation of racemization data from soils.

The first phase of this research is presented as Chapter 3. This chapter includes a detailed discussion of the techniques used for racemization analysis of soil. The investigations designed to establish control parameters such as replication of individual D/L ratios, the effect of the HF treatment, various chromatographic subtleties and elemental and visual analysis of the samples, are reported in the latter half of this chapter.

The second phase of the research is presented as Chapter 4. This chapter details the parallels which arise between known age effects in soils, as detailed in Chapter 2, and the degree of racemization apparent.

The final phase of the research is detailed in Chapters 5 and 6. These pyrolysis experiments provide the first kinetic information concerning racemization in soil.

Chapter 7 is a detailed summary of the validity and relative advantages of amino acid racemization analysis of soils. The need for further research is also examined and possible benefits highlighted.

# CHAPTER 2 AMINO ACID RACEMIZATION : A DATING TECHNIQUE WITH POTENTIAL APPLICATION IN SOIL SCIENCE.

## 2.1 INTRODUCTION

In principle, dating techniques provide a statistical means of differentiating events in a time – framework. Limitations imposed by the type of sample material submitted for age assessment restrict the information a dating technique can be expected to provide. This is very important when the sample material is soil or is derived from soil. These limitations are discussed in Section 2.3 and have been reviewed by Stout et al. (1981), Scharpenseel (1977), Finkl (1984), and Scharpenseel and Neue (1984).

Amino acid racemization is an entropy reaction which has been exploited as a means of chronosequencing fossiliferous material. Recent reviews of traditional applications of the technique include Rutter et al. (1985), Masters (1986) and Murray – Wallace and Kimber (1987).

The overall objective of this thesis is to disclose the results of research on amino acid racemization in agricultural soils. This chapter details the literature from which this investigation arose. Section 2.1 is a brief review of the proven use of the amino acid racemization reaction as a chronological tool. This is followed by a detailed review of the application of the reaction to soils and sediments and the problems that have prevented the routine use of the technique in soil science (Section 2.2).

Subjecting soils to age analysis requires an understanding of specific theoretical limitations. These are detailed in Section 2.3. Radiocarbon dating of soils and soil fractions/separates has provided evidence of age effects in soils. This evidence is detailed in Section 2.4 and forms the basis of the investigations detailed in Chapter 4.

### 2.1.1 Amino acid racemization : Definitions

The twenty commonly occurring alpha – amino acids are carboxylic acids which have an amino group ($-NH_2$), a carboxyl group ($-COOH$), a hydrogen atom and a hydrocarbon group ($-R$) attached to the alpha – carbon atom. This configuration imparts chirality on the alpha – carbon atom. Variation in the hydrocarbon group results in the twenty commonly occurring alpha – amino acids. In glycine, hydrogen is substituted for the hydrocarbon group thus eliminating chirality of the molecule, isoleucine and threonine contain two chiral carbon atoms (alpha and beta) and the remaining seventeen amino acids contain a single chiral carbon atom.

Open chain structures without special symmetry properties have isomer numbers equal to $2^n$ where $n$ is the number of chiral carbon atoms in the structure. Thus for glycine $n$ is zero, one isomer is possible; for the majority $n=1$, two isomers are possible; and for isoleucine and threonine $n=2$ so that four isomers are possible. When $n=1$ the isomers are optical isomers, that is they have identical physical and chemical properties except for their ability to rotate plane polarised light the same amount but in opposite directions. Such isomers are called enantiomers, laevo – or dextro – (L – or D –) rotational enantiomers.

When $n=2$, two sets of enantiomers exist which have some differing physical and chemical properties. These four isomers are called diastereoisomers or epimers. While the enantiomeric pairs are mirror images of their enantiomeric counterparts, the two pairs are not mirror images of their diastereo – counterparts.

Amino acids are present in all living organisms primarily linked together through peptide bonds to form enzymes and structural proteins. These proteins/peptides are essentially composed of L – enantiomers (epimers), a disequilibrium state maintained by various enzymic stereorestrictions. There are few exceptions to the L – configuration. These are mainly restricted to microbial cell wall peptides and are discussed in Section 2.2.3.1. Upon cessation of the stereorestrictions entropy dictates an equilibrium condition and racemization commences (Schroeder and Bada 1976; Wehmiller 1984; Barrett 1985; Rutter et al. 1985; Masters 1986).

Racemization is the reversible transition between L– and D–enantiomers. Epimerization is the equivalent transition when diastereoisomers are involved. In racemization the equilibrium condition involves a racemic mixture *i.e.* equal amounts of L– and D–enantiomers (D/L=1). In epimerization the equilibrium condition may attain a value greater than 1. For isoleucine a range of values between 1.25 and 1.4 has been reported for this constant (Williams and Smith 1977).

As diastereoisomers are chemically distinct molecules they can be resolved with an amino acid analyser, a feature which accounts for the popularity in the use of isoleucine in many amino acid racemization dating laboratories (Bada and Shou 1980; Bada 1985).

There are two basic approaches to separating amino acids using gas–liquid chromatography (GLC). Either volatile enantiomeric derivatives of amino acids are chromatographed on optically active stationary phase or enantiomers are converted to diastereomeric derivatives and chromatographed on optically inactive stationary phase (Kvenvolden et al. 1971). The former method is used in the CSIRO laboratory where these research studies were conducted. The method is detailed in Appendix 1 (1.3 Derivatization, 1.4 Chromatography). This method permits the resolution of most of the amino acid isomers, although in practice about half can be reliably resolved from (i) other amino acid isomers and (ii) chromatographic undesirables (contaminants). Some amino acids degrade during hydrolysis (Hunt 1985) (Section 3.2.2) and are not reliably resolved using any method.

Of the possible diastereoisomers L–isoleucine and D–alloisoleucine are the only ones commonly resolved. The absence of their diastereo–counterparts (D–isoleucine and L–alloisoleucine) has been attributed to the high activation energies required for their formation (Dungworth 1976; Bada et al. 1986).

### 2.1.2 Amino acid racemization : Historical

Abelson (1954) first noted the potential of proteinaceous remnants as age indicators of fossiliferous materials. Apart from the work of Erdman et al. (1956) there was a hiatus in the development of amino acid racemization dating until Hare and coworkers began work in the mid – 1960's. This early work of Hare relied on enzymic degradation to quantify D – enantiomers (Hare and Abelson 1966; Hare and Mitterer 1969; Abelson and Hare 1969). In all probability interest in radiocarbon dating and the need for more routine chromatographic techniques led to this hiatus. Since this work there have been numerous publications pertaining to amino acid racemization techniques.

### 2.1.3 Racemization mechanisms and kinetics

Neuberger (1948) suggested that the initial rate limiting step in the racemization reaction involves the ionization of the alpha – carbon. This forms a planar carbanion molecule and is rapidly followed by non – stereospecific protonation forming either the D – or L – enantiomer. The reaction is base catalysed. Any nucleophilic species may initiate the reaction. Water is the common nucleophile involved (Hare 1974). This mechanism has been supported by evidence from nuclear magnetic resonance studies by Williams and Busch (1965) and Buckingham et al. (1967). Although racemization preferentially occurs at the terminal amino acid (Kriausakul and Mitterer 1980, 1983; Bada 1985; Kimber and Hare in prep.) evidence from Pickering and Li (1964) and Geschwind and Li (1964) shows that racemization of internal amino acids does occur, probably by enolization (Dakin 1912 – 1913; Dakin and Dudley 1913; Dakin and Dale 1919). Reviews of the literature pertaining to mechanisms include Pasini and Cassella (1974), Bada and Schroeder (1975) and Schroeder and Bada (1976).

Currently the "non – linear" kinetic model of Wehmiller (1981, 1982, 1984) is favoured to describe the kinetics of racemization in molluscs. Based on the racemization of leucine in foraminifera from deep sea cores this model describes two essentially linear components joined by a transition zone. The change between the two components arises due to the changing rates of hydrolysis and

racemization. In contrast to this model the "extended linear" model of Kvenvolden et al. (1979, 1981) is based on pyrolysis experiments and requires extrapolation over a much larger temperature range, indeed one order of magnitude larger.

Significant literature favouring the "non – linear" model in accurately describing the racemization of enantiomers in various molluscs includes Mitterer and Kriausakul (1984), Hearty et al. (1986) and Murray – Wallace and Kimber (1987).

No work appears to have been published that describes racemization kinetics in soils.

### 2.1.4 Amino acid racemization age determination

Racemization investigations usually report the degree of racemization of amino acids as unweighted mean D/L ratios of the total hydrolysate. This must be considered as an unweighted mean due to the differential inputs of molecular species of differing degrees of racemization. Quantitative age determination based on D/L ratios can be made using either the calibrated or the uncalibrated method (Bada and Protsch 1973; Schroeder and Bada 1976; Pillans 1982; Rutter et al. 1985).

The calibrated method involves development of racemization rate constants from a sample of known age. Assuming that the known and unknown samples have a comparable diagenic history, values for the racemization rate constants ($K_1$) may be established from the D/L data of the known sample. Application of these constants to D/L data of the unknown sample results in the assignment of a calibrated age. Although this technique overcomes many of the problems associated with the temperature history of the material it is dependent on an alternative dating technique for calibration.

The uncalibrated method requires,
(i) D/L data on the fossil to be dated,
(ii) detailed knowledge of the temperature history of that fossil, and,
(iii) racemization rate constants for that fossil.

Rate constants are derived from pyrolysis experiments on similar fossiliferous material. Difficulties with this method arise in extrapolation from pyrolysis conditions to ambient temperatures and in ascertaining detailed knowledge of the temperature history of the sample.

Using either method the age (t) of the unknown is calculated using Equation 2.1.

$$(1+k')(K_1)t = \ln \frac{[1+(D/L)_x]}{[1-k'(D/L)_x]} - \ln \frac{[1+(D/L)_o]}{[1-k'(D/L)_o]} \qquad 2.1$$

where,
$(D/L)_x$ is the D/L ratio of the unknown,
$(D/L)_o$ is the D/L ratio of a modern sample,
$k' = K_D/K_L = 1/K_{eq}$ and is the reciprocal of the equilibrium constant (unity for enantiomers, 0.8 – 0.7 for isoleucine), and,
$K_1$ is the derived rate of racemization.

$(D/L)_o$ is included to account for racemization induced during sample preparation and any natural abundances of D – enantiomers. This term may become significant for some amino acids in soils due to elevated populations of microorganisms.

## 2.2 AMINO ACID RACEMIZATION AND SOILS

The application of amino acid racemization techniques to soil science is not straight foward. Difficulties of both a technical and an interpretive nature occur. The technical difficulties are divisible into two groups,
(i) release of amino acids into solution, and,
(ii) purification of amino acids for gas – liquid chromatography.

### 2.2.1 Release of amino acids from the soil matrix

The traditional technique of release of amino acids from samples for amino acid racemization analysis (6M HCL at 110°C for 16*h*) does not give quantitative release from soils. Published work on amino acid racemization analysis of soil chronosequences has relied on this readily hydrolysable fraction. Limmer and Wilson (1980) suggest the readily hydrolysable fraction is vulnerable to contamination by groundwater leaching. The evidence presented by Vreeken (1984), Mahaney et al. (1986) and Milnes et al. (1987) supports this with inconsistencies in the depth – D/L ratio (time) pattern and with contradictions to the law of superposition apparent.

To gain a more quantitative release of amino acids from soils Cheng et al. (1975) pretreated samples with hydrofluoric acid (HF). They conclude that such a pretreatment is required for maximum release of organic nitrogen. Limmer and Wilson (1980) suggested that this phenomenon may provide two fractions, a readily hydrolysable fraction and a less readily hydrolysable fraction. They discarded the readily hydrolysable fraction as a contaminant and studied the so called HF fraction. While they were able to make the conclusion that the HF fractions from different aged paleosols were different, based on the general occurrence of amino acids, they were unable to gather any evidence to support any racemization/epimerization effect.

Extrapolation of these ideas forms the basis for much of the work in this thesis and is extensively explored in subsequent chapters. Griffin and Kimber (1988) provided the first report of differences in racemization between the hydrolytic fractions.

It is probable that the supplementary yield of amino acids arises from the release of proteins and peptides intimately associated with the clay matrix and is not merely a feature of peptide bond strength as discussed in Section 3.2.2 (Ensminger and Gieseking 1942; Estermann et al. 1959; Freney 1968; Freney and Miller 1970). The mechanisms controlling supplementary yields discussed by Piper and Posner (1972b) and Paul and van Veen (1978) are based on physical criteria such as the entrapment of organic material within soil aggregates or the

requirement for the removal of some overlying molecule before hydrolysis can proceed. The physico – chemical mechanisms discussed by Greenland (1965, 1971) would not be expected to withstand acid hydrolysis.

Combination of the more plausible theories suggests that organic/inorganic interactions form integral species in the soil (Turchenek and Oades 1976; Stout et al. 1981; Oades et al. 1988). While a traditional hydrolysis largely etches the surfaces of the various soil constituents, the dissolution of the silicon based matrix by treatment with hydrofluoric acid provides access to the interior reaches of the species (Piper and Posner 1972, 1972b; Paul and van Veen 1978; Burns 1982, 1983). Protection of the interior is enhanced by the shrinking of some soil species under conditions of reduced pH.

### 2.2.2 Purification and chromatography of amino acids

The matrix of soil is far more complex than that of other samples routinely analysed. The purification of amino acids from molluscs involves a relatively simple procedure of cation exchange to remove the calcium. Cheng and coworkers have devised a technique to purify amino acids from soils involving the use of both cation and anion exchange (Cheng et al. 1975; Pollock et al. 1977; Pollock and Kvenvolden 1978). In this technique most of the chromatographic contaminants are removed as polyanionic fluoride complexes. The sample thus purified may still contain a significant amount of organic contaminants (lipid type materials).

The technical difficulties which arise in subjecting soils to amino acid racemization analysis are discussed extensively in Chapter 3. Besides purity, losses of amino acids and the potential for co – chromatography are considered in Sections 3.2.3 and 3.2.4.1.

### 2.2.3 Interpretation of D/L ratios

There are both specific and non-specific problems in intrepretation of D/L ratios. The specific problems include natural occurrence of D-amino acids, temperature, clay catalysis and racemases. The non-specific difficulties arise from the fact that the life of a soil occupies an extended portion of time compared to most organisms. The non-specific difficulties are addressed in Section 2.3, specific difficulties are discussed here.

#### 2.2.3.1 D-amino acids

Pollock and Frommhagen (1968) first noted the occurrence of D-amino acids in soil extracts. They concluded that alkali induces significant (*ca.* 20-30%) racemization and suggested that all the D-amino acids in soil were a feature of the procedure used. Bremner (1950) made a similar suggestion. It is now acknowledged that the occurrence of D-amino acids is widespread and that there are specific and non-specific metabolic pathways which involve these isomers (Salton 1964; Aldag and Young 1970; Kjaer and Larsen 1973, 1976, 1977; Porkorny 1974; Robinson 1976; Davies 1977; Metzler 1977; Pollock and Kvenvolden 1978; Larson 1980). The most prolific natural abundance is in cell wall peptides of bacteria. This occurrence appears to be largely restricted to glutamic and aspartic acids and alanine (Salton 1964). The major question pertaining to racemization studies that arises from the occurrence of D-enantiomers in specific peptides is one of kinetics.

A simplified overview depicts racemization as a result of two processes, (i) rates of racemization/hydrolysis, and, (ii) preservation of amino acids. It is plausible that these processes may affect natural D- and L-enantiomers differently. If the rate of racemization is very low and L-amino acids are preferentially preserved, due to different positioning within the peptide chain, decreasing D/L ratio may be apparent without racemization occurring. Conversely preferential preservation of D-enantiomers may lead to an apparent increase in D/L ratios without the involvement of the racemization/time reaction. The greater

the D/L ratio of a modern sample, the greater the likelihood of such deviations from the kinetic models employed to rationalise racemization in molluscs. This problem is overcome by the assumptions that (i) unweighted mean D/L data is used and (ii) the overall equilibrium is a racemic mixture. As no research has pursued this problem one should currently approach age assignation of soils by means of amino acid techniques, based on aspartic and glutamic acids and alanine, with scepticism. As the occurrence of D – enantiomers of other amino acids is much lower, such deviations from proposed models (Kvenvolden et al. 1979, 1981; Wehmiller 1981, 1982, 1984) are probably reduced, and age assignation may be more plausible.

#### 2.2.3.2 Temperature

The material used throughout these investigations was sampled from the 0 – 10cm layer of the soil. The amino acid racemization reaction is sensitive to temperature. This relationship can be crudely described as a $Q_{10}$ relationship *i.e.* rates approximately double for every $10^{o}C$ increase in temperature. The surface soils as analysed were subjected to wide variations in temperature both annually and daily. The development of racemization rate constants has always been contentious. As the comparisons in this thesis are made within individual soils, or between closely related soils, the importance of uncertainties in appropriate effective temperature figures are reduced.

#### 2.2.3.3 Racemases

Racemases are enzymes which enhance racemization. It is known that non – specific racemases do exist (Inagaki et al. 1987). These enzymes offer the potential of racemization without the passage of time. Clearly if a soil contains an organism which produces a non – specific extracellular racemase, the extent of racemization in that soil may not provide any evidence as to the age of the soil.

#### 2.2.3.4 Clay catalysis

Although several workers have invoked clay catalysis to explain accelerated rates of racemization (Pollock et al. 1977; Frenkel and Heller – Kallai 1977) work with lysine by Kimber, Slade and Moen (personal communication) finds no evidence to support this.

## 2.3 THE AGE OF A SOIL

In soil science, the concept of time can be traced to the work of Dokuchaev who used phrases such as "duration of soil formation" and "young or mature" soil in his classic publications. Indeed Jenny (1941) ranks time among the state factors of soil formation.

Soils are distinguished from weathering profiles on the basis of fertility (Burykin 1985). Thus time zero or the onset of pedogenesis corresponds to the first humus formation in the regolith (Scharpenseel 1977). The true age of a soil is therefore the time elapsed since this invasion (Scharpenseel 1971). The first pedogenic organisms are microbial rather than plant (Burykin 1985). After the initiation of pedogenesis, soils continue to develop in a dynamic manner, inorganic constituents continue to weather, organic constituents cycle. This dynamic situation is encapsulated in the definition of soil put forth by Burns (1982, 1983) who described soil as a multicelled organism which responds to or is driven by an almost teleological "thrust" (*sic*).

Obtaining the true age of a soil is not possible unless (i) a relic, contemporary to the initiation of pedogenesis is preserved in the soil and (ii) this relic can be isolated and its age assessed. The constituents of the inorganic part of the solid phase of soil generally predate pedogenesis. Dating techniques directed at inorganic species should give an age older than the soil *e.g.* thermoluminescence dating of loess. Techniques for dating organic species result

in a minimum age for the soil *e.g.* radiocarbon dating (Scharpenseel 1977). Due to continued rejuvenation of the organic phase of the soil the age derived from this phase is generally reported as mean residence time (MRT) (Paul et al. 1964) or more correctly as apparent mean residence time (AMRT). Residence time is defined as the ratio of the total mass of an element in a pool to the annual increment of the element into the pool. MRT is defined as the weighted mean of the different ages of various soil organic matter fractions present in differing quantities. As changes in quantities of the different fractions of soil organic matter during soil development do not follow a strictly linear pattern, the measured age is more correctly termed AMRT (Scharpenseel and Neue 1984) or equivalent age (Jenkinson and Rayner 1977).

Residence time provides information on the rates of removal by the inverse relationship. Thus it provides information by inference on the relative resistance of organic constituents (Stout et al. 1981).

The MRT of a buried soil more closely approximates the true age of the soil than does that of a contemporary soil (Scharpenseel 1977) as burial stops inflow (rejuvenation) but does not stop decomposition (Stout et al. 1981).

A soil fraction which represents a relic, contemporary to the inception of pedogenesis, would be used routinely to assess the age of a soil. Such a fractionation scheme has not been defined. However persistence in the dating of soil fractions will undoubtedly lead to a greater understanding of the significance of processes in soils and in the intensified systems employed in agriculture.

### 2.3.1 Kinetic Fractions

In practical terms mean retention time is a severely restricted concept because of the range of materials and rates within the groups selected for study. Kinetic fractions may provide a more useful conceptual tool (McGill, personal communication). A kinetic fraction is defined and distinguished by rate constants. Information regarding stability is extrapolated from the inverse relationship between specific rate and stability. Lower rates indicate increasing stability. By

restricting the interpretation of the data from kinetic fractions to general conclusions in terms of stability, the inherent limitations of a soil system are better accounted for.

Both schemes, distinctive kinetics and distinctive residence time, describe a mean effect. Kinetic fractions are superior in describing the soil system because a general concept, the concept of stability as indicated by rate, is used to describe the complexity of soil instead of using time.

### 2.3.2 Amino acids in soils

Free amino acids provide a readily assimilable source of both carbon and nitrogen and therefore have short residence times in soil (Schmidt et al. 1960; Sorenson and Paul 1971; Kassim et al. 1981). Free amino acids do exist in soils (Paul and Schmidt 1961; Ivarson and Sowden 1970) and may be extracted using one of a variety of techniques (Schmidt et al. 1960; Paul and Schmidt 1961b; Gilbert and Altman 1966; Monreal and McGill 1985). Longevity of amino acids is increased when they are combined with peptide bonds into larger chains, peptides and proteins. To utilize such chains as a food source for microbes, proteolytic enzymes are required to break the peptide bonds before further assimilation may occur (Burns 1983). As all peptide bonds are not equally vulnerable to proteolytic enzymes (Brisbane et al. 1972) some peptides are inherently more protected. Cytoplasmic materials are more vulnerable than cell wall material (Hurst and Wagner 1969; McGill et al. 1975; Kassim et al. 1981). Longevity may be further increased by association with other inorganic or organic species (Verma et al. 1975). Larger peptides may be strongly absorbed onto clay surfaces (Sorenson 1969; Theng 1982). The literature abounds with suggestions that the primary mechanism of protection of otherwise labile organic constituents is an intimate association of organics and clay (Mattson 1932; Ensminger and Gieseking 1942; Estermann et al. 1959; Sorenson 1963; Stevenson et al. 1967; Freney 1968; Freney and Miller 1970; Legg et al. 1971; Sorenson and Paul 1971; Anderson et al. 1981; Kassim et al. 1981; Scharpenseel and Neue 1984). Alternately longevity may be increased by organic–organic interactions (Parton et al. 1983b) or organic–metallic ion interactions (Campbell et al. 1967). Stout et al. (1981) suggested that

amino acids and proteins may be stabilized by phenolase polymer. Anderson et al. (1981) supported these alternative mechanisms, finding no evidence to support clay protection theories.

Amino acids are ubiquitous in the soil system (Sowden et al. 1977). There is decisive evidence that some portion of the amino acid nitrogen in the soil system is stabilised against short term cycling (Oades and Ladd 1977; Stout et al. 1981; Parton et al. 1983).

Stability is clearly a function of, (i) intrinsic molecular recalcitrance, and, (ii) orientation within the soil matrix (Sorenson 1977; Kassim et al. 1981; Stout et al. 1981; Parton et al. 1983). Although not clearly defined the concepts of physical protection have been incorporated in models describing the turnover of soil organic matter (Hunt 1977; Klein 1977; van Veen et al. 1981; van Veen and Paul 1981; Parton et al. 1983, 1983b).

## 2.4 KNOWN AGE EFFECTS IN SOILS

### 2.4.1 Radiocarbon dating of soils and soil fractions

Radiocarbon dating is a well established dating technique. The problems and prospects of radiocarbon dating are reviewed by Ottaway (1986), Kra (1986) and Waterbolk (1983). Radiocarbon techniques permit the assessment of ages to a maximum of 7.5 half lives (*ca.* 35000 years) with resolution limited to 200 years. Radiocarbon dating is the premier technique for dating soils. There are several thousand radiocarbon dates on soil and soil fractions in the literature. These data have led to many important conclusions regarding soil, carbon cycling and pedogenisis.

Soil carbon is not a homogeneous pool with regard to age. Some authors suggest at least two pools need to be considered (Sauerbeck and Gonzalez 1977). In all probability a continuum of ages exist (Jenkinson and Rayner 1977) ranging

from modern to that of relic material possibly contemporary with the initiation of pedogenesis (Gerasimov 1971). Amino acid nitrogen is not a homogeneous pool with regard to age (Sorenson 1963; Sowden et al. 1977; Limmer and Wilson 1980).

Linear regression of age depth data from soil profiles generally show significant relationships with wide scatter (Scharpenseel and Neue 1984). The relative degree of correlation (expressed as regression coeffficient, *r*) is considered to be indicative of the degree of preservation of organic matter with depth. That alfisols show high coherence apparently confirms the theory that organic matter is protected in argillic horizons.

Such regression information probably results from,
(i) increasing resistance of organic matter to biotic and other types of decay with growing age and depth, and,
(ii) "contamination" of pedogenic relics of old carbon with modern carbon and leaching of modern carbon down profile (Scharpenseel and Neue 1984).

This age information is incompatible with the idea that pedogenesis occurs at the bottom of a profile, at the weathering front at the boundary between the C horizon and parent material. In this case the bottom of the profile is the youngest. Incompatibility arises from the fact that radiocarbon dates an organic fraction of the soil (except in calcrete type horizons) and organic matter invades from the upper reaches of the profile (Dickinson 1974; O'Brien and Stout 1978; O'Brien 1984). Migration of organic matter downward is very low (Munnich 1957; de Vries 1958).

### 2.4.2 Acid hydrolysis

Many authors are in agreement that acid hydrolysis is the most effective method of separating age differing components of soil organic matter (Martel and Paul 1974; Martel and Lasalle 1977; Goh 1978). Goh and Pullar (1977) suggested that the acid hydrolysable fraction from active soil organic matter is dominated by

recent additions, partly humified plant fragments, the active soil biomass *etc.* This results in a modern radiocarbon age assignation (Martel and Paul 1974).

In contrast the non – hydrolysable fraction has a discernible radiocarbon age (Scharpenseel 1977; Goh 1978). Martel and Paul (1974b) show that the non – hydrolysable fraction of soil organic matter constitutes the major portion of resistant soil organic components. Scharpenseel (1977) suggested that an increasing age of fractions results from successive hydrolytic steps.

Despite this there is little evidence to suggest whether the amino acids associated with non – hydrolysable soil organic matter are older than their readily hydrolysable counterparts.

### 2.4.3 Agricultural soils

There are two important features of cultivation that strongly influence radiocarbon dating of arable soils. Cultivation tends to increase decomposition rates of labile carbon substrates and cultivation tends to invert/mix the arable (*Ap*) horizon.

An uncultivated (*Ah*) horizon normally dates younger than an *Ap* horizon (Martel and Paul 1974b). Cultivation may lead to an inversion of the age depth function in the arable layer and result in a sharp increase in age at the boundary of an old arable layer (Gerasimov 1974). Martel and Paul (1974b) show that these effects depend on soil type. Their results indicate that whereas one soil (*Orthic Oxbow*) showed a significant increase in age with cultivation a second soil (*Sceptre*) showed a slight (non – significant) decrease. This conclusion is supported by Stout et al. (1981). Earlier work by Stevenson (1956) demonstrated that cultivation changed the amino acid distribution, increasing the proportion of basic amino acids.

### 2.4.4 Humic acids and radiocarbon

Radiocarbon has been utilized to (i) date classical fractions and (ii) as a tracer to follow humification. Despite this, humification remains the "black box" of soil science. Traditionally, humification was considered to be a largely chemical process mediated by the soil biomass with humic acid being the ultimate product (Ladd and Butler 1966; Kononova 1966; Duchaufour 1976; Coelho et al. 1985). Recently the emphasis has moved to physico – chemical processes. Evidence presented by Scharpenseel (1971, 1972) and Goh and Pullar (1977) indicates that classical fractionation is ineffective in separating age differing components. The authors concluded that the dynamic nature of humification leads to some rejuvenation of all fractions. The method of calculating ages by difference rather than direct measurement may not be accurate (Martel and Paul 1974). The beginning of humus formation is not datable due to rejuvenation (Scharpenseel 1971). Traditional fractionation schemes do not provide a consistently preferential fraction, the date of which approaches the beginning of pedogenesis (Goh et al. 1977). These conclusions are supported by Anderson (1979) and Anderson and Paul (1984). However they contradict the earlier work of Campbell et al. (1967b). The oldest fractions are non – hydrolysable residues from either the humic acid or the humin fractions indicating, (i) support for clay protectionist theories, and, (ii) that hydrolytic fractionation leads to better differentiation of age differing components.

### 2.4.5 Hydrolysis and Humic acids

Amino acids are associated as protein with some but not all humic and fulvic acids (Stevenson and Goh 1971). The concentration and role in nitrogen cycling varies significantly (Lakomiec 1982). The literature pertaining to supplementary yields of amino acids from humic acids is not conclusive.

Kemp and Mudrochova (1973) concluded that traditional hydrolysis of humic acid samples, with and without HF pretreatment, provided similar yields. Over one third of the nitrogen in the sample remained unhydrolysable.

Choudhri and Stevenson (1957), Lowe (1969) and Stevenson and Cheng (1970) suggested that a supplementary yield of amino acids from humic acids does result from treatment of a post – hydrolytic residue with HF and rehydrolysis. Piper and Posner (1972, 1972b) concluded that this treatment provided a significant supplementary yield.

Ladd and Butler (1966) suggested that the amino acid – aromatic ring bond is more stable to acid hydrolysis than a peptide bond. Piper and Posner (1972b) suggested that acid hydrolysis does not release N – terminal amino acids of protein/phenol nor those with free amino groups. Stevenson and Wagner (1970) noted that non – hydrolysable nitrogen is often assumed to occur as part of the structure of humic acids.

McGill and Paul (1976) inversely equate hydrolysability with recalcitrance *i.e.* a fraction of lower hydrolysability is more recalcitrant.

While a humic acid molecule (8 *n*m diameter) is unable to be intercalated, significant inorganic materials are known to be associated with humic acids (Theng and Scharpenseel 1975; Tan and McCreedy 1975; Theng 1982). Therefore a plausible suggestion is that some parts of the humic acid are either intercalated or intimately associated, probably adsorbed to the inorganic structure and such an association imparts some degree of protection on the organic species. This idea and the quest for ash – free humic acids (Goh 1970) are mutually exclusive.

### 2.4.6 Radiocarbon dating of particle size separates

There are few published accounts of the radiocarbon age of various particle size separates. This paucity may relate to difficulties in obtaining material suitable for dating. Work by Scharpenseel (1972) and Anderson and Paul (1984) indicate the oldest separate to be of intermediate size (60 – 1*u*m). This trend is clearly soil specific as the separates from three different soils analysed by Anderson and Paul showed different trends from, (i) all separates dating modern, to, (ii) the coarse silt separate dating oldest, and, (iii) the coarse clay separate dating oldest.

In light of remarks by Burns (1983) suggesting the colloidal surface to be the region of maximum biological activity in a soil, it is not surprising that

enzymes are concentrated in the smaller ($<50u$m) separate (Mateus and Carcedo 1985) as these separates have the highest surface area to volume ratio. It is unfortunate that these authors (Mateus and Carcedo) did not further fractionate this rather large and certainly heterogeneous size separate into its clay and silt components.

Dalal and Mayer (1987) have explored the relationship between density and size separates and the lability of organic matter on cultivation. Both Tiessen and Stewart (1983) and Dalal and Mayer (1987) noted that the greatest decrease in organic carbon as a result of cultivation occurs in the $>50u$m separate. While Tiessen and Stewart suggested that this begins a dynamic cycle of movement of the organic carbon to smaller size fractions with long term losses of *ca.*30%, Dalal and Mayer suggested that the increases in the proportion of organic nitrogen in fine fractions is a result of decreases in the total organic nitrogen (as a result of decreases in the larger fractions). Dalal and Mayer conclude that neither particle size nor density fractionation provides a consistent and clear separation between labile and non – labile organic matter.

## 2.5 CONCLUSIONS

The amino acid racemization reaction has been successfully exploited as a chronological tool. Traditional sample materials include shells (molluscs, foraminifera, egg) and bones. Interpretation of racemization data from other sample materials (coral, shell grit, sands and even some molluscs) is not straightfoward.

There has been little work devoted to elucidating the racemization process in soils and soil fractions/separates.

Soils and soil fractions/separates are unusual materials to subject to age assessment. An apparent age is readily attained using a variety of dating techniques. Within limits, radiocarbon dating remains the premier technique for organic matter studies. Time is generally reported as apparent mean residence time to satisfy restrictions inherent in the system studied.

Radiocarbon dating has provided important evidence of the existence of materials of different ages within a soil. Acid hydrolysis is effective in separating age differing components. Cultivation influences the age of soil by increasing decomposition rates. Classical fractionation may provide an older fraction, the oldest fractions are non-hydrolysable residues from either humic acid or the humin fractions. Particle size separations may provide older and younger separates.

Alternately these same data may indicate procedurally attainable kinetic fractions.

To assess the potential contribution of information derived from application of the amino acid racemization reaction to soils and soil fractions/separates three phases of research are required.

Firstly the requirement for control data must be satisfied. A technique which can be routinely used to assess the degree of racemization of a soil sample must be developed. This technique should minimize chromatographic undesirables as well as minimizing losses of amino acids, especially differential losses of each enantiomer/epimer. The technique should be replicable. The technique should

minimize induced racemization. The technique should be simple, to permit the processing of significant numbers of samples.

The second phase of the research should examine the degree of racemization of samples of known age. Amino acid racemization techniques are unique in that they concentrate exclusively on the amino acid component of the soil. Comparison of amino acid data to other age data is therefore fraught with difficulties. Nonetheless the age effects established by radiocarbon data should be paralleled by racemization data. Caution should be exercised when drawing conclusions from the data provided by this phase of research.

The third phase of the research should examine the kinetics of racemization in soils. Such information provides a vehicle for comparing the racemization process in soils with that of more traditional samples. Further, kinetic information is required to maximise interpretation of racemization data. Kinetic information is derived from pyrolysis experiments. Although there may be some doubt in extrapolating to the situation prior to the establishment of pyrolysis conditions, the results are indicative of the situation which existed at the start of the experiment.

# CHAPTER THREE AMINO ACID RACEMIZATION TECHNIQUES

## 3.1 INTRODUCTION

Standard techniques for processing samples to determine their degree of racemization are well developed and had been routinely applied before this project began (Wehmiller 1984; Kimber and Milnes 1984; Kimber et al. 1986). Processing of the three samples used by Wehmiller for the purposes of inter – laboratory comparison provided evidence that results from this laboratory were within the error margins of the other participating laboratories (Murray – Wallace 1987).

The analytical methods used in the preparation of soils and soil fractions/separates for amino acid racemization analysis are outlined in point form in Appendix 1.

## 3.2 SAMPLE PREPARATION

Sample preparation is readily divisible into four stages, (i) pre – treatment, (ii) hydrolysis, (iii) clean – up, and, (iv) chromatography.

### 3.2.1 Pre – treatment

Pre – treatment includes any modifications to the sample prior to hydrolysis. Pre – treatment of traditional samples includes meticulous removal of external contaminants using dental tools and acid baths and is clearly inappropriate in soil studies. This research investigated the effect of variations in the pre – treatment of soil samples. Pre – treatments used range from no treatment (HCl fractions), preliminary hydrolytic treatment (HF fractions), extraction procedures (humic

acids) and physical treatments (particle size separates) and set combinations of these pre – treatments (*i.e.* HCl and HF fractions from humic acids and size separates).

### 3.2.2 Hydrolysis

Hydrolysis releases amino acids into solution. The traditional hydrolytic conditions imposed during amino acid racemization analysis are 6M HCl @ 110$^{o}$C for 16*h*. These conditions represent a compromise aimed at yielding the best estimates of amino acid composition of "simple" proteinaceous materials (Noltman et al. 1962; Mahoward et al. 1962; Sowden 1969 and Gehrke et al. 1985) while minimizing procedurally induced racemization (Hare 1969). Hydrolysis of simple materials has received liberal attention. Recent reviews include Blackburn (1978), Anderson et al. (1981), Ambler (1981), and Hunt (1985).

Gehrke et al. (1985) have summarised the general conclusions as,
(i) isoleucine and valine are liberated more slowly than other amino acids,
(ii) threonine and serine undergo progressive destruction,
(iii) cystine, cysteine and methionine are destroyed to varying degrees depending on the sample matrix,
(iv) tyrosine can be oxidized by the presence of residual oxidizing agents, and some may be converted to chloroderivatives, and,
(v) tryptophan is generally destroyed, and asparagine and glutamine are quantitatively converted to aspartic and glutamic acids.
The traditional hydrolysis generally yields over 95% recovery for aspartic and glutamic acids, glycine, alanine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine.

These general conclusions are dramatically influenced by the matrix of the sample to be hydrolysed (Hunt 1985). Soil is a very complex matrix of organic and inorganic constituents (Burns 1982, 1983). Traditional hydrolysis of soil samples does not produce quantitative release of amino acids. Limmer and Wilson (1980) suggest this phenomenon may provide two fractions, a readily hydrolysable fraction (HCl fraction) and a less readily hydrolysable fraction (HF fraction). The

readily hydrolysable fraction is gained from a traditional hydrolysis. Treatment of the residue from this hydrolysis with hydrofluoric acid and rehydrolysis provides the less readily hydrolysable fraction. The initial hydrolysis is considered a pretreatment leading to the HF fraction (Fig. 3.1).

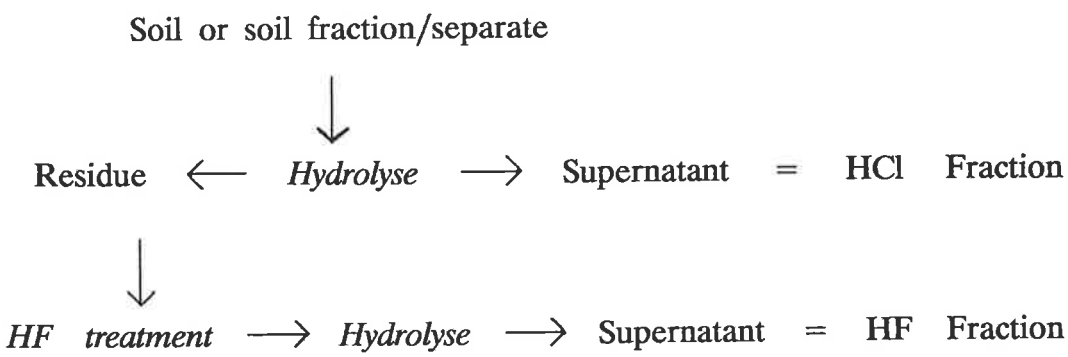



Fig. 3.1 Schematic diagram depicting the relationship of the hydrolytic fractions in a sample of soil or a soil fraction/separate.

### 3.2.3 Clean – up

Clean – up is the procedure which separates amino acids from impurities. Purification of amino acids from soil and soil fractions is more complex than that of the standard technique. The standard technique relies on cation exchange resin to retard amino acids while the impurities are eluted. This is generally sufficient for traditional applications, however inadequate clean – up may occur. Impurities in the final product result from inadequacies in ion exchange. Such inadequacies are of two types, (i) inadequate exchange sites resulting from either low efficiency during rejuvenation of the resin or insufficient resin, and, (ii) inability of the selected resin to distinguish between some impurities and amino acids. In soil there are many compounds which cannot be separated from amino acids by cation exchange.

Clean – up procedures appropriate for soil samples differ from that of the standard technique in that major chemical species present in soil, *i.e.* silicon, iron and aluminium, are removed as polyanionic fluoride complexes. After hydrolysis the dominant anion is chloride, cations reside as chloride complexes. Polyanionic fluoride complexes are synthesised by changing the dominant anion to fluoride. Samples are dried and then resuspended in hydrofluoric acid.

Reduction in the quantities of unwanted cationic species occurs in several stages. Some of the iron and calcium complexes formed are insoluble and are readily concentrated by centrifuging and decanting the fluoride solution.

The majority of the fluoride complexes are removed during anion exchange. On completion of preparation of the anion exchange resin (AG 1 X8, 100 – 200 mesh) fluoride anions are associated with all the active sites. These are readily replaced by the polyanionic fluoride complexes which are subsequently retained by the resin. Retention of amino acids by the anion exchange resin is minor and elution occurs within a short time (Cheng et al. 1975) when hydrofluoric acid (0.5 molar) is used as the eluant. As aspartic acid and glutamic acid are retarded longer than the other amino acids care must be taken to ensure that an adequate amount of the hydrofluoric acid is used. Any error made at this point is readily diagnosed by the absence of peaks corresponding to aspartic and glutamic acids in the final chromatograms.

The standard technique may be further modified when warranted by employing a solvent extraction step (Griffin and Kimber 1988). This step reduces the amounts of undesirable organics, lipid materials, by dissolving such contaminants in dichloromethane. During this step the majority of the amino acids are retained in the aqueous phase (Sowden 1969).

It should be noted that all exchange steps and solvent extractions lead to some loss of amino acids (Thompson et al. 1959; Bremner 1965; Sowden 1969; Pollock and Miyamoto 1971; Cheng et al. 1975). This is not important in racemization studies as such losses will affect each enantiomer equally as these compounds cannot be separated on chemical criteria. In all probability it would not affect epimers (D – allo/L – isoleucine) either. This and the difficulties associated with the volatile nature of the final derivatives make it difficult to derive consistent concentration data using these techniques.

The techniques are similar in that solubility phenomena are used to help remove undesirable materials. Filtration ensures separation of insoluble materials after hydrolysis, before anion exchange and before derivatization.

### 3.2.4 Chromatography

Enantiomers are converted to volatile derivatives and chromatographed on an optically active stationary phase. Derivatization is a two step procedure. Amino acids were esterfied with propan – 2 – ol/3.3 M HCl. Following cooling and evaporation of the solvent the samples were acylated with pentafluoropropionic anhydride (Kimber and Griffin 1987).

Gas – liquid chromatography was performed using a Chirasil – L – Val fused silica capillary column (25m x 0.22mm) in a Hewlett Packard 5890A gas chromatograph equipped with both a nitrogen phosphorus detector and a flame ionisation detector. Chromatographic peaks were automatically integrated by an HP9216 micro – computer running Nelson Analytical *XTRA CHROM data system*

software. The D/L ratios were calculated by using peak area data, as were concentration estimates (Kimber and Griffin 1987).

3.2.4.1 Calibration

Calibration of the chromatographic equipment is essential for peak identification and to determine response factors. The non-protein amino acid L-norleucine was included in known amounts in all samples. L-norleucine was added with the acid in all acid hydrolyses and could therefore be used as an internal standard, providing a reference point for retention times and response factors as well as being a guide to sample recovery. Chromatographic peaks were identified primarily by characteristic retention times. Mixtures of known amounts of known amino acids including L-norleucine were used to determine both retention times and response factors.

There was a marked variation in the characteristic retention times of different columns. Each new column was calibrated for characteristic retention times. Standard mixtures were run regularly to follow minor variations in retention times.

Retention time was found to be characteristic of the particular temperature programme used during chromatography. Figure 3.2 provides a comparison of three different temperature programmes using the same column. The first two programmes (Figs. 3.2a and 3.2b) differ only in the length of the first ramp (period of increasing oven temperature). The shorter ramp (to 85$^{o}$C) resulted in a general slowing of the chromatogram. While this has not affected the relative positions of L-serine and L-leucine, better resolution of D-leucine and D-proline was apparent. Either of these must be considered to be superior to temperature programme 3.2C (Fig. 3.2c) where there was very poor resolution of D-leucine and proline as well as co-chromatography of L-serine and L-leucine.

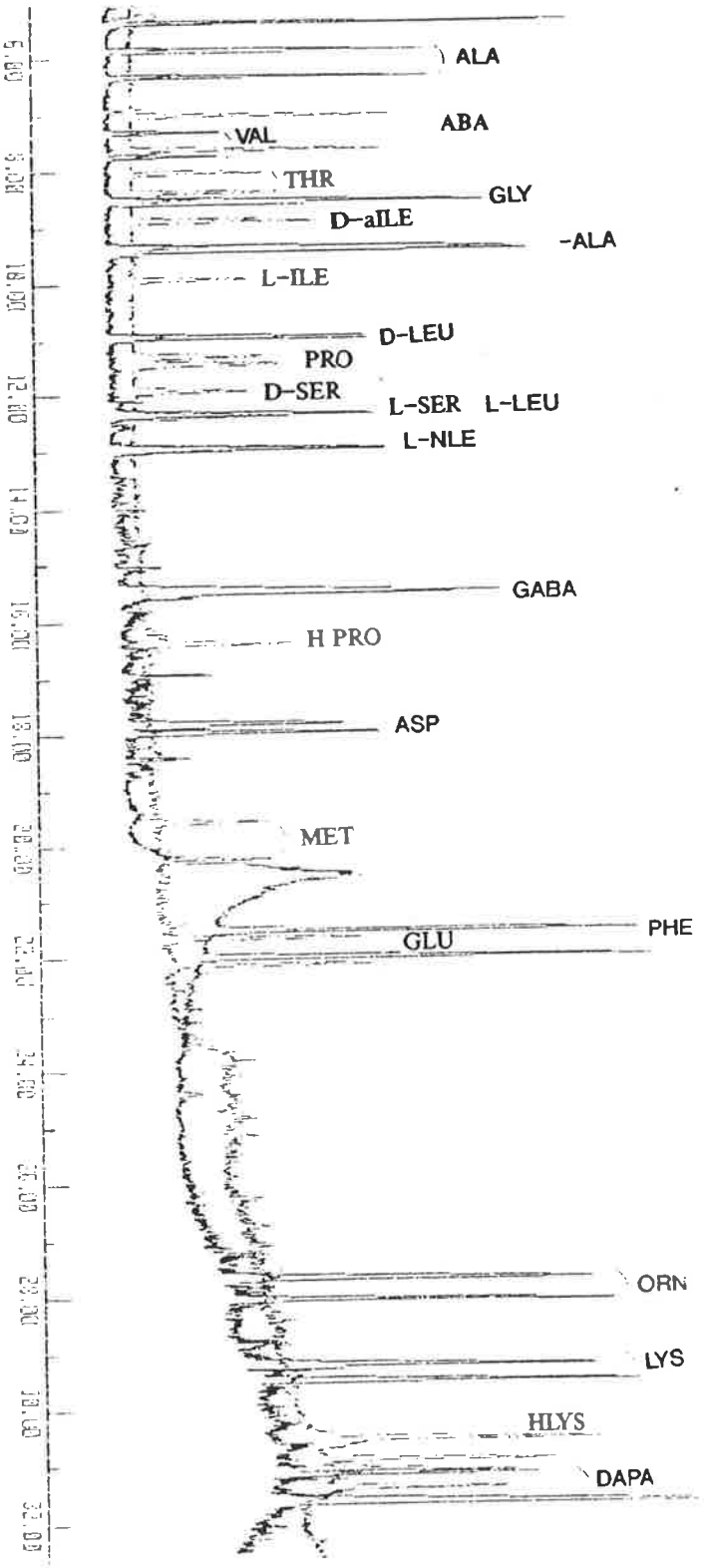



Fig. 3.2a Comparison of three temperature programmes.

Programme 3.2A hold at 45°C for 0.5 min, increase at 20°C/min to 90°C, increase at 2°C/min to 110°C, increase at 5°C/min to 196°C, hold for 5 mins.

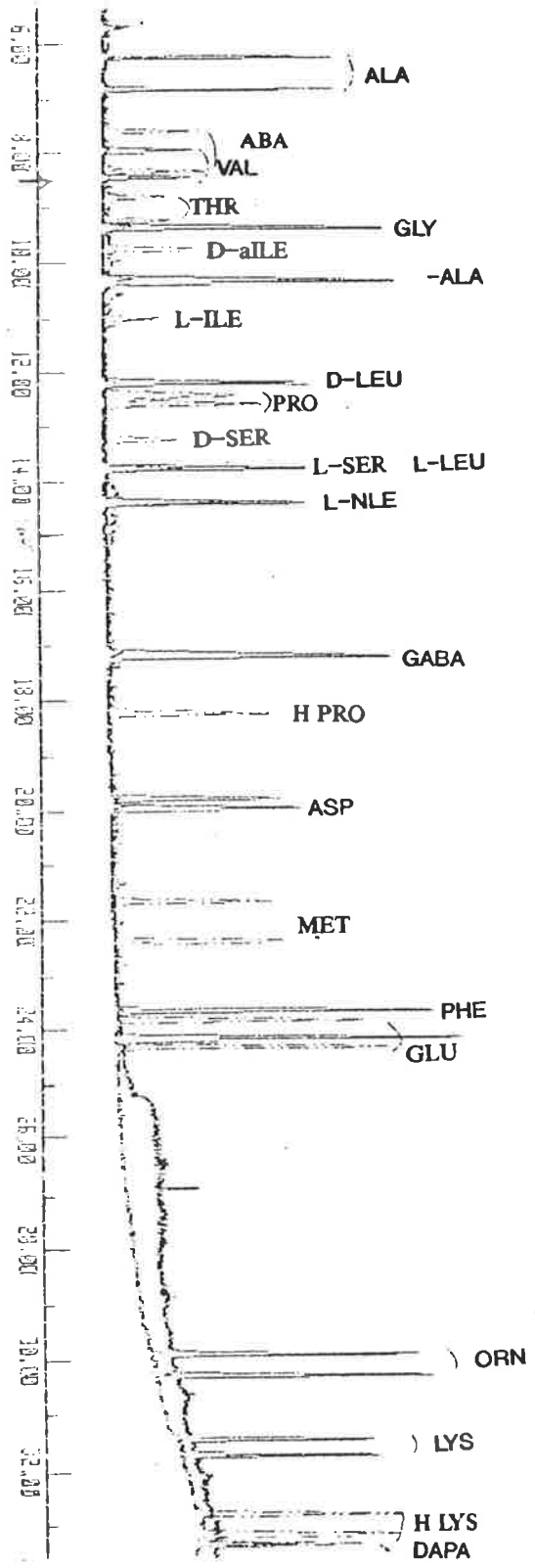



Fig. 3.2b Comparison of three temperature programmes.

Programme 3.2B hold at 45°C for 0.5 min, increase at 20°C/min to 85°C, increase at 2°C/min to 110°C, increase at 5°C/min to 196°C, hold for 5 mins.

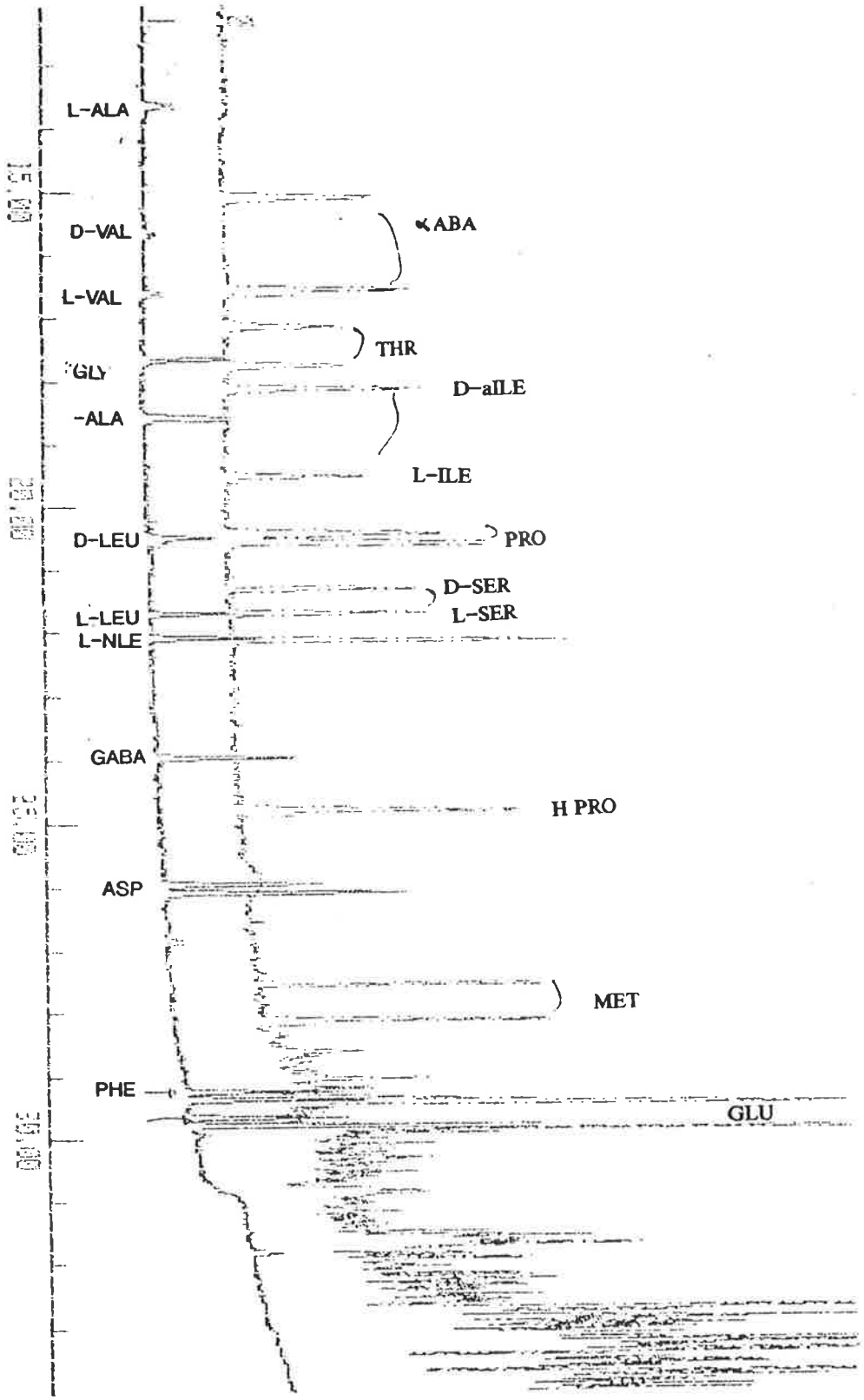



Fig. 3.2c Comparison of three temperature programmes.

Programme 3.2C from 45°C increase at 10°C/min to 70°C, decrease at 5°C/min to 60°C, increase at 5°C/min to 196°C, hold for 5 mins.

Standard mixtures were also used to determine response factors. The response factors were used to determine concentration as described in Section 3.3. The detector was more or less sensitive to different amino acids. This means the same concentrations of different amino acids resulted in peaks of different size (Figs. 3.3a and 3.3b). Response factors (Table 3.1) measured this difference and hence provided a conversion factor for calculation of concentration data in known units. Unfortunately the differential losses expected from clean-up procedures and the volatile nature of the derivative led to large errors in concentration data from this technique. For these reasons concentration data must be considered to be indicative only and the absolute units tend to be irrelevant. Concentration data are presented only in the latter chapters of this thesis and are reported as relative concentration only.

Apart from the twenty commonly occurring amino acids there are several hundred amino carboxylic acids currently characterised (Kjaer and Larsen 1973, 1976, 1977; Robinson 1976; Davies 1977). Few of these compounds will co-chromatograph with the twenty or so routinely examined amino acids. Care had to be taken to ensure the peak purity of unknown chromatograms. Computer facilities permitted the collection, storage and re-examination of chromatograms. Digital processing provided the means whereby chromatograms could be examined on any scale to single sample points. This increased the security in peak identification and peak purity.

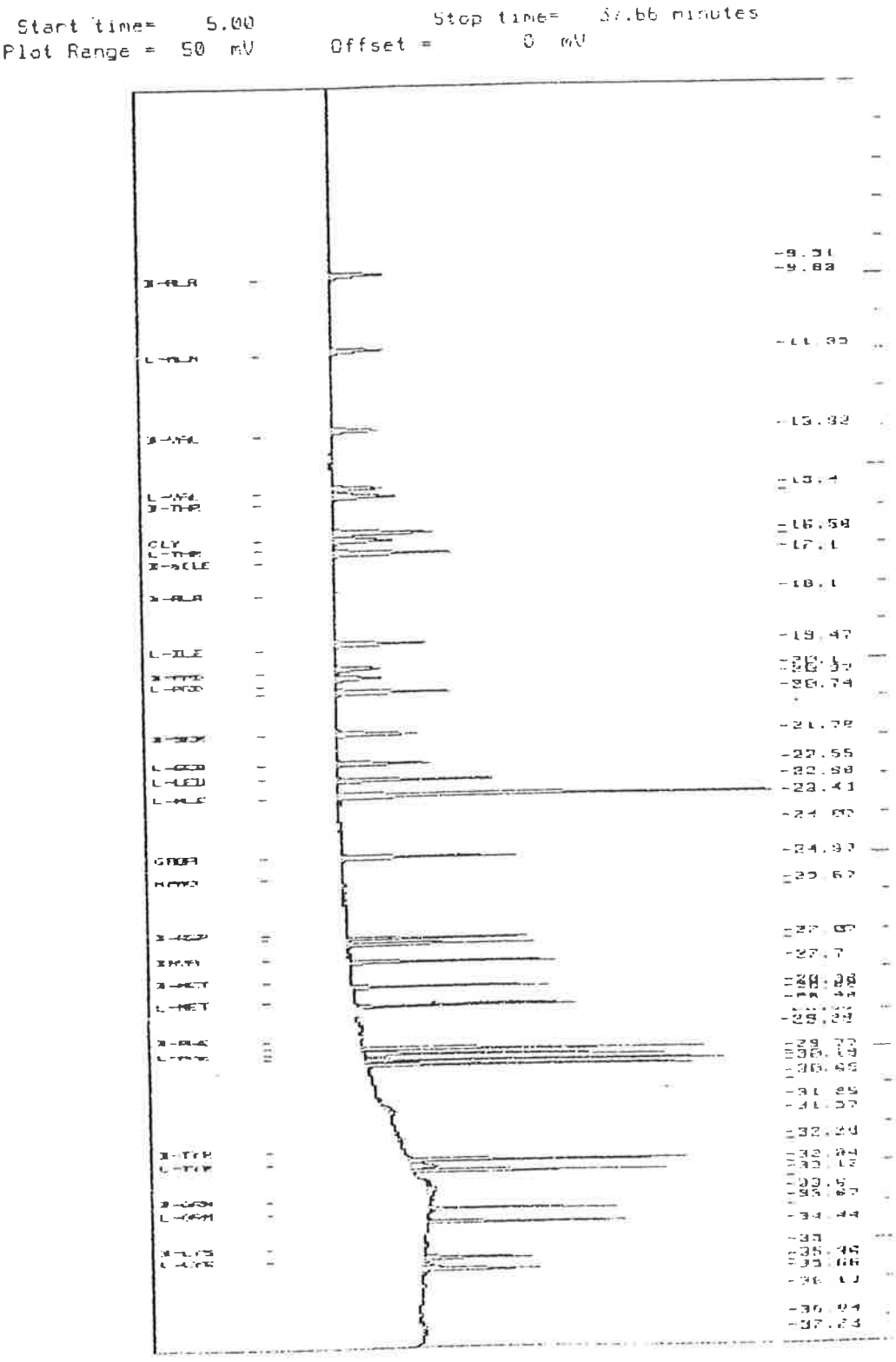



Fig. 3.3a Chromatogram showing differential sensitivity of the detector to different amino acids. Despite the information on the report all the amino acid isomers are in the same concentrations (10*n*mole).

```
**************** INTERNAL STANDARD REPORT **************************
Data From Sample STD                    Collected on 26 Sep 1988 11:43
Delay Time :   5.00                     Run Time :   37.66
Area Reject =   200 uV-sec   Sampling Rate =   5.00 pts/sec
Bunch Factor = 4 pts
Noise Threshold = 20 uV      Area Threshold = 150 uV-sec
Sample Amount = 1 pmoles/g      Injection Vol = 1
Dilution Factor = 100      Multiplier Amount =    1.0000
This Report Uses Normalized Response Ratios
```

| Peak Num | Ret Time | Peak Name | Concentration as pmoles/g | Raw Area | Area Ratio | Cal Range | Peak Type | Ref Peak | Int Std Peak | % Delta Ret Time | Normalized Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 9.51 | UNKNOWN | 2.37769E-11 | 918 | .0000005 | Over | 1 | 3 | 20 | | 19649139.9469 |
| 2 | 9.88 | D-ALA | 1.46839E-02 | 28058 | .0000147 | Over | 1 | 8 | 20 | -1.119 | 1.0000 |
| 3 | 11.85 | L-ALA | 1.46839E-02 | 26853 | .0000147 | Over | 1 | 8 | 20 | -1.161 | 1.0000 |
| 4 | 13.92 | D-ABU | 1.46839E-02 | 26663 | .0000147 | Over | 1 | 8 | 20 | -1.005 | 1.0000 |
| 5 | 15.40 | UNKNOWN L-VAL | 6.98776E-10 | 20579 | .0000137 | Over | 2 | 3 | 20 | | 19649139.9469 |
| 6 | 15.61 | L-ABU D-THR | 1.46839E-02 | 38795 | .0000147 | Over | 2 | 8 | 20 | .52434 | 1.0000 |
| 7 | 16.58 | UNKNOWN GLY | 8.17713E-10 | 58207 | .0000218 | Over | 2 | 8 | 20 | | 26680016.7138 |
| 8 | 16.77 | GLY L-THR | 1.46839E-02 | 39177 | .0000147 | Over | 2 | 8 | 20 | 0 | 1.0000 |
| 9 | 17.16 | SERINE D-allo | 1.46839E-02 | 62764 | .0000147 | Over | 1 | 8 | 20 | .47794 | 1.0000 |
| 10 | 19.10 | B-ALA | 1.46839E-02 | 1338 | .0000147 | Over | 1 | 8 | 20 | -.4084 | 1.0000 |
| 11 | 19.47 | L-ILE | 1.46839E-02 | 45795 | .0000147 | Over | 1 | 8 | 20 | -.7240 | 1.0000 |
| 12 | 20.10 | UNKNOWN D-PRO | 3.42563E-10 | 24384 | .0000091 | Over | 2 | 8 | 20 | | 26680016.7138 |
| 13 | 20.37 | B-PRO L-PRO | 1.46839E-02 | 24266 | .0000147 | Over | 2 | 8 | 20 | .56425 | 1.0000 |
| 14 | 20.74 | D-LEU | 1.46839E-02 | 56294 | .0000147 | Over | 1 | 18 | 20 | -.1351 | 1.0000 |
| 15 | 21.76 | D-SER | 1.46839E-02 | 39695 | .0000147 | Over | 1 | 16 | 20 | -.0890 | 1.0000 |
| 16 | 22.55 | L-SER | 1.46839E-02 | 40086 | .0000147 | Over | 1 | 18 | 20 | -.0360 | 1.0000 |
| 17 | 22.96 | L-LEU | 1.46839E-02 | 56663 | .0000147 | Over | 1 | 18 | 20 | .15865 | 1.0000 |
| 18 | 23.41 | L-MLE | 1.46839E-02 | 199173 | .0000147 | Over | 1 | 18 | 20 | 0 | 1.0000 |
| 19 | 24.07 | UNKNOWN | 2.89568E-13 | 534 | 0.0000000 | Over | 1 | 18 | 20 | | 1.3584177E+08 |
| 20 | 24.97 | GABA | 0.00000E+00 | 68102 | .0000147 | - | 1 | 18 | 15 | .11492 | 1.0000 |
| 21 | 25.67 | NFRO | 2.52301E-05 | 1856 | .0000147 | Over | 1 | 18 | 20 | .58843 | 582.8000 |
| 22 | 25.84 | UNKNOWN | 3.21018E-13 | 592 | 0.0000000 | Over | 1 | 16 | 20 | | 1.3584177E+08 |
| 23 | 27.07 | D-ASP | 1.46839E-02 | 50419 | .0000147 | Over | 1 | 18 | 20 | -.0364 | 1.0000 |
| 24 | 27.22 | UNKNOWN L-ASP | 4.28211E-10 | 50546 | .0000147 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 25 | 27.70 | BABA | 1.46839E-02 | 65276 | .0000147 | Over | 1 | 18 | 20 | .33052 | 1.0000 |
| 26 | 28.36 | B-ASP | 1.46839E-02 | 50524 | .0000147 | Over | 1 | 16 | 20 | .57120 | 1.0000 |
| 27 | 28.68 | L-HCY | 1.46839E-02 | 623 | .0000147 | Over | 1 | 18 | 20 | -.3745 | 1.0000 |
| 28 | 28.96 | UNKNOWN L-ASN | 4.36976E-10 | 51591 | .0000150 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 29 | 29.29 | UNKNOWN | 1.82643E-11 | 1212 | .0000034 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 30 | 29.77 | B-GLU D-PHE | 1.46839E-02 | 86640 | .0000147 | Over | 2 | 16 | 20 | .64245 | 1.0000 |
| 31 | 29.92 | L-PHE D-GLU | 1.46839E-02 | 86923 | .0000147 | Over | 2 | 18 | 20 | -.3011 | 1.0000 |
| 32 | 30.19 | L-GLU L-PHE | 1.46839E-02 | 87160 | .0000147 | Over | 2 | 18 | 20 | .16964 | 1.0000 |
| 33 | 30.31 | UNKNOWN L-GLU | 7.25772E-10 | 85670 | .0000249 | Over | 2 | 23 | 20 | | 34356949.4692 |
| 34 | 30.65 | UNKNOWN | 2.07699E-12 | 340 | .0000001 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 35 | 30.89 | UNKNOWN | 3.88512E-12 | 459 | .0000001 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 36 | 31.25 | UNKNOWN | 4.40020E-12 | 519 | .0000002 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 37 | 31.57 | UNKNOWN | 8.36818E-11 | 9678 | .0000029 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 38 | 32.28 | UNKNOWN | 2.34785E-11 | 2771 | .0000008 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 39 | 32.42 | B-THR | 1.46839E-02 | 429 | .0000147 | Over | 1 | 18 | 20 | -.5108 | 1.0000 |
| 40 | 32.84 | UNKNOWN D-THR | 6.19571E-10 | 73134 | .0000213 | Over | 2 | 23 | 20 | | 34356949.4692 |
| 41 | 33.00 | L-THR | 1.46839E-02 | 16992 | .0000147 | Over | 2 | 17 | 20 | .18362 | 1.0000 |
| 42 | 33.12 | UNKNOWN L-THR | 5.16356E-10 | 60951 | .0000177 | Over | 2 | 23 | 20 | | 34356949.4692 |
| 43 | 33.35 | UNKNOWN | 6.54016E-12 | 772 | .0000002 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 44 | 33.60 | UNKNOWN | 7.87095E-11 | 9291 | .0000027 | Over | 2 | 23 | 20 | | 34356949.4692 |
| 45 | 33.67 | B-GLU | 1.46839E-06 | 1061 | .0000147 | Over | 2 | 16 | 20 | .06781 | 10000.0000 |
| 46 | 34.05 | L-GLU D-ORN | 1.46839E-02 | 46916 | .0000147 | Over | 1 | 18 | 20 | -.2776 | 1.0000 |
| 47 | 34.44 | UNKNOWN L-ORN | 4.27131E-10 | 50418 | .0000147 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 48 | 35.00 | B-LYS | 1.46839E-06 | 855 | .0000147 | Over | 1 | 18 | 20 | -.2662 | 10000.0000 |
| 49 | 35.36 | L-LYS D-LYS | 1.46839E-02 | 38247 | .0000147 | Over | 2 | 16 | 20 | -.1220 | 1.0000 |
| 50 | 35.52 | UNKNOWN | 2.59118E-11 | 3059 | .0000009 | Over | 2 | 23 | 20 | | 34356949.4692 |
| 51 | 35.66 | UNKNOWN L-LYS | 3.70161E-10 | 43654 | .0000127 | Over | 2 | 23 | 20 | | 34356949.4692 |
| 52 | 36.11 | UNKNOWN | 1.00102E-11 | 1182 | .0000003 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 53 | 36.84 | UNKNOWN | 1.15012E-11 | 1358 | .0000004 | Over | 1 | 23 | 20 | | 34356949.4692 |
| 54 | 37.23 | UNKNOWN | 5.43545E-11 | 6416 | .0000019 | Over | 1 | 23 | 20 | | 34356949.4692 |

Fig 3.3b Internal standard report showing differential sensitivity of the detector to different amino acids. Despite the information on the report all the amino acid isomers are in the same concentrations (10*n*mole).

| Amino acid | $\frac{Area_i}{Area_{IS}}$ | R.F |
|---|---|---|
| D – ALA | 0.141 | 0.28 |
| L – ALA | 0.145 | 0.30 |
| D – VAL | 0.134 | 0.27 |
| L – VAL | 0.135 | 0.27 |
| GLY | 0.292 | 0.58 |
| D – aILE | 0.315 | 0.63 |
| L – ILE | 0.229 | 0.46 |
| D – PRO | 0.122 | 0.24 |
| L – PRO | 0.126 | 0.25 |
| D – LEU | 0.282 | 0.56 |
| L – LEU | 0.284 | 0.57 |
| D – SER | 0.199 | 0.40 |
| L – SER | 0.201 | 0.40 |
| D – ASP | 0.253 | 0.51 |
| L – ASP | 0.253 | 0.51 |
| D – PHE | 0.434 | 0.87 |
| L – PHE | 0.436 | 0.87 |
| D – GLU | 0.436 | 0.87 |
| L – GLU | 0.430 | 0.86 |

Table 3.1 RF values calculated from the standard as appears in Figs. 3.3a and 3.3b.

3.2.4.2 Detectors

During the course of this study two types of detectors were used, a nitrogen – phosphorus detector (NPD) and a flame ionisation detector (FID). The NPD is responsive to nitrogen and phosphorus containing compounds. In this way the detector has a diminished response to lipid based chromatographic undesirables which would otherwise obscure peaks. The FID responds to the ionization of column eluent as it burns in a hydrogen flame. The FID provides no protection from non – nitrogenous undesirables.

The response of the NPD and the FID to changing concentration was tested with standard mixtures of amino acids. Mixtures containing amino acids at concentrations of 12.5, 25, 50, 100 and 200 *n*moles were derivatized and chromatographed using the NPD. Mixtures containing amino acids at concentrations of 1, 5, 10, 50, and 100 *n*moles were derivatized and chromatographed using the FID. The data were used in a calibration plotting sub – routine of the *XTRA – CHROM data system*. These plots are presented in Appendix 2. The data are summarized in Table 3.2 as regression coefficients of two models, a first order ($y = ax + b$) and a second order ($y = ax^2$) relationship, (Models 1 and 2 respectively, Table 3.2).

Clearly the superior linearity of the FID outweighs other disadvantages. Only results determined with the FID are presented in this thesis.

| Amino Acid | N – PD | | FID | |
|---|---|---|---|---|
| | Model 1 | Model 2 | Model 1 | Model 2 |
| D – ALA | 0.97 | 0.90 | | |
| L – ALA | 0.97 | 0.90 | | |
| D – VAL | 0.98 | 0.92 | 1.00 | 1.00 |
| L – VAL | 0.97 | 0.90 | 1.00 | 1.00 |
| GLY | 0.97 | 0.94 | 0.99 | 1.00 |
| D – aILE | 0.96 | 0.92 | 1.00 | 1.00 |
| L – ILE | 0.96 | 0.91 | 1.00 | 1.00 |
| D – LEU | 0.95 | 0.93 | 1.00 | 1.00 |
| L – LEU | 0.96 | 0.93 | | |
| L – LEU + L – SER | | | 1.00 | 1.00 |
| D – ASP | 0.92 | 0.93 | 0.98 | 1.00 |
| L – ASP | 0.93 | 0.94 | 0.98 | 1.00 |
| D – PHE | 0.89 | 0.95 | 0.98 | 1.00 |
| L – PHE | 0.89 | 0.95 | 1.00 | 1.00 |
| D – GLU | 0.89 | 0.93 | 0.99 | 1.00 |
| L – GLU | 0.92 | 0.97 | 1.00 | 1.00 |

Table 3.2 Summary of the regression coefficients derived by modelling detector calibration data to first order (Model 1) and second order (Model 2) relationships.

## 3.3 CALCULATION OF D/L RATIOS AND CONCENTRATIONS

D/L ratios were calculated simply as the ratio of the area of the peak identified as the D-enantiomer (epimer) to that of the corresponding L-enantiomer (epimer).

Concentration data were calculated from response factors. The response factor of a component of interest was calculated from Equation 3.1,

$$R.F._i = (CC_i/Area_i)\ (Area_{IS}/CC_{IS}) \qquad 3.1$$

where
$CC_i$ is the concentration of the component of interest used in the calibration sample,
$Area_i$ is the area of the peak of the component of interest,
$Area_{IS}$ is the area of the internal standard peak, and,
$CC_{IS}$ is the concentration of internal standard used in the calibration sample.

Concentration may then be calculated using Equation 3.2,

$$Conc_i = (IS/SA)\ (RF_i \times Area_i/Area_{IS})\ (XF) \qquad 3.2$$

where
$Conc_i$ is the concentration of the component of interest,
IS is the concentration of internal standard added to the unknown,
SA is the amount of unknown sample used for analysis,
$RF_i$ is the response factor of the component of interest,
$Area_i$ is the area of the component of interest in the unknown,
$Area_{IS}$ is the area of the internal standard peak in the unknown, and,
XF is a scaling factor.

As mentioned in Section 3.2.4.1, concentration data is reported as relative concentration and only in the latter chapters of this thesis. Relative concentration is calculated from Equation 3.2 when $R.F._i$ and XF are set to one.

## 3.4 AMINO ACID RACEMIZATION ANALYSIS OF SOILS : SYSTEMATICS

A number of preliminary experiments were conducted to assess the validity of the assumptions,

(i) that a soil has a characteristic degree of racemization,

(ii) that the hydrofluoric treatment does not induce racemization, and,

(iii) that the hydrofluoric treatment is effective in the release of organic matter intimately associated with the surfaces of soil particles.

### 3.4.1 Replication

An experiment was conducted to ascertain the replicability of the technique as modified for soil samples. Three one gram replicate samples of the Urrbrae fine sandy loam from the permanent pasture plot of the rotation experiment at the Waite Agricultural Research Institute, Adelaide, were analysed. Each processed sample was chromatographically analysed three times and the D/L data compared.

The results from this experiment are presented graphically as D/L values from each replicate (Figs. 3.4, 3.5 and 3.6) and as percent standard error (Table 3.3). The error term was generally reduced when sample means were calculated from replicate means rather than from individual results.

Two groups of amino acids were readily distinguished using this data. The first group were technically reliable amino acids. The error associated with replicate data from this group was less than ten percent. These amino acids were alanine, valine, leucine and aspartic acid. Glutamic acid could be included as results from one replicate were easily recognised as outliers. The second group was technically unreliable. The percent standard deviation was consistently large, outliers were not readily distinguished. Phenylalanine and glutamic acid had similar retention times as did a number of unknown peaks. There were up to six peaks in less than one minute in this part of the chromatogram (Fig. 3.7). This led to difficulties in identification and resolution which contributed to the large error associated with these two amino acids. The D – alloisoleucine peak usually appeared as a side peak of glycine. Such fusing of peaks led to inconsistencies in the placement of base lines and the resolution of peak area. As the total area of the D peak was small, variation was significant.

The quality of chromatograms declines progressively with column use. This meant a column had a finite life of approximately six to twelve months depending on use. The quality of chromatograms produced by different columns varied considerably. The categorization of an amino acid as either reliable or unreliable varied during the course of the research. While problems with leucine developed, D – alloisoleucine became clearly resolved. The problems associated with phenylalanine and glutamic acid persisted. The identification of the existence of these problems has provided some hope for their future use.

| | AMINO ACID | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| mean, standard error and percent standard error of raw data | | | | | | | |
| x | 0.098 | 0.017 | 0.040 | 0.050 | 0.085 | 0.033 | 0.140 |
| s | 0.003 | 0.004 | 0.015 | 0.004 | 0.004 | 0.010 | 0.025 |
| %s | 3 | 23 | 38 | 8 | 5 | 30 | 18 |
| Replicate mean and standard error | | | | | | | |
| A (x) | 0.095 | 0.015 | 0.027 | 0.049 | 0.082 | 0.027 | 0.176 |
| (s) | 0.0004 | 0.006 | 0.004 | | 0.0002 | 0.008 | 0.0002 |
| B (x) | 0.101 | 0.017 | 0.035 | 0.051 | 0.090 | 0.030 | 0.122 |
| (s) | 0.002 | 0.004 | 0.004 | 0.007 | 0.004 | 0.008 | 0.017 |
| C (x) | 0.098 | 0.019 | 0.060 | 0.050 | 0.084 | 0.045 | 0.133 |
| (s) | 0.002 | 0.005 | 0.009 | 0.004 | 0.003 | 0.004 | 0.004 |
| mean, standard error and percent standard error of replicate means | | | | | | | |
| x | 0.098 | 0.017 | 0.041 | 0.050 | 0.085 | 0.034 | 0.144 |
| s | 0.003 | 0.002 | 0.017 | 0.001 | 0.004 | 0.009 | 0.029 |
| %s | 3 | 12 | 41 | 2 | 5 | 26 | 20 |

Table 3.3 Manipulation of replication experiment data. This table provides a comparison of the error associated with individual replicates and the errors associated with handling raw data and replicate data.

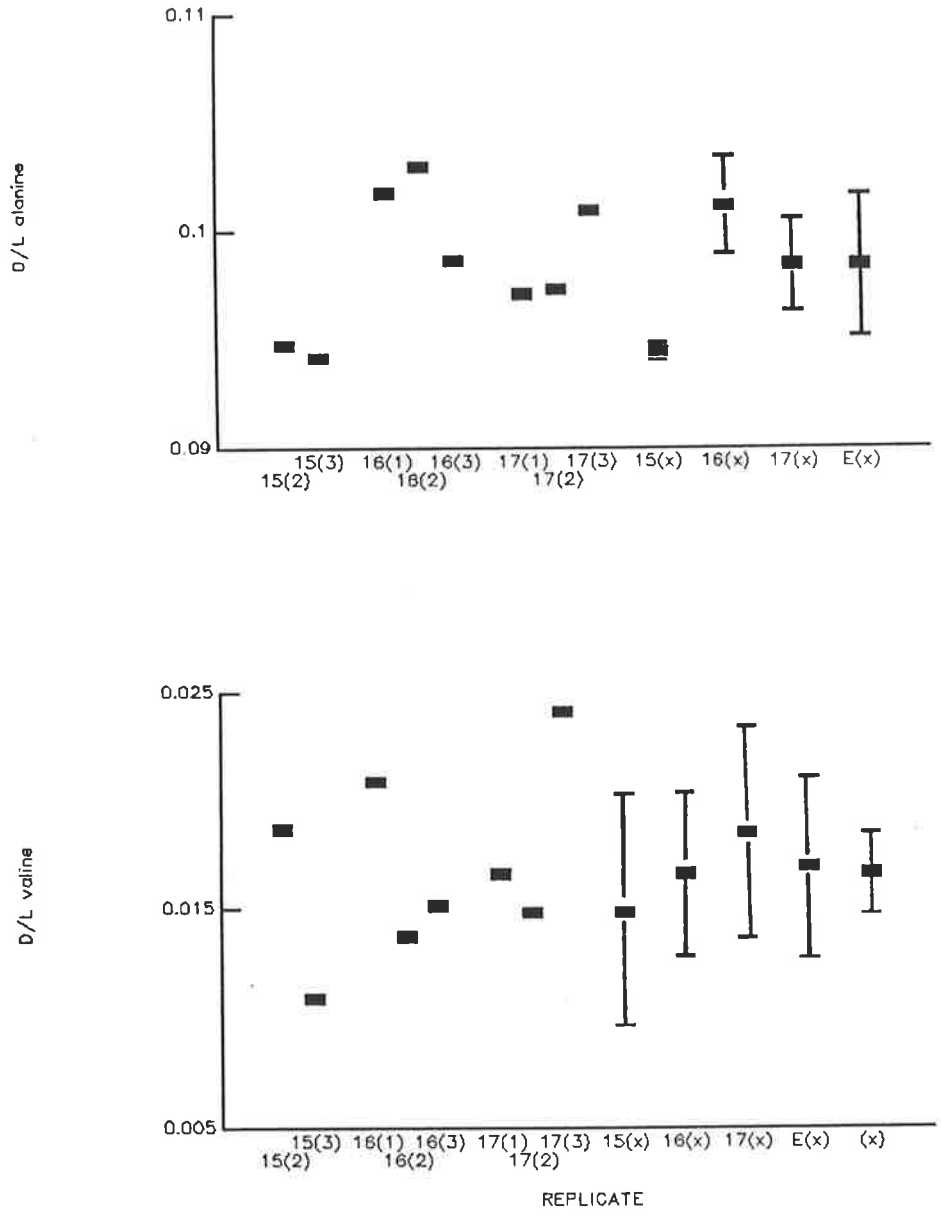



Fig. 3.4 D/L ratios of alanine and valine from replicate injections (1, 2, 3) of replicate subsamples (15, 16, 17) together with the mean and standard deviation as calculated for each subsample (15(x), 16(x), 17(x)), and as calculated for the sample from all the available data (E(x)) and from the subsample means ((x)).

Note the different D/L scale for each amino acid.

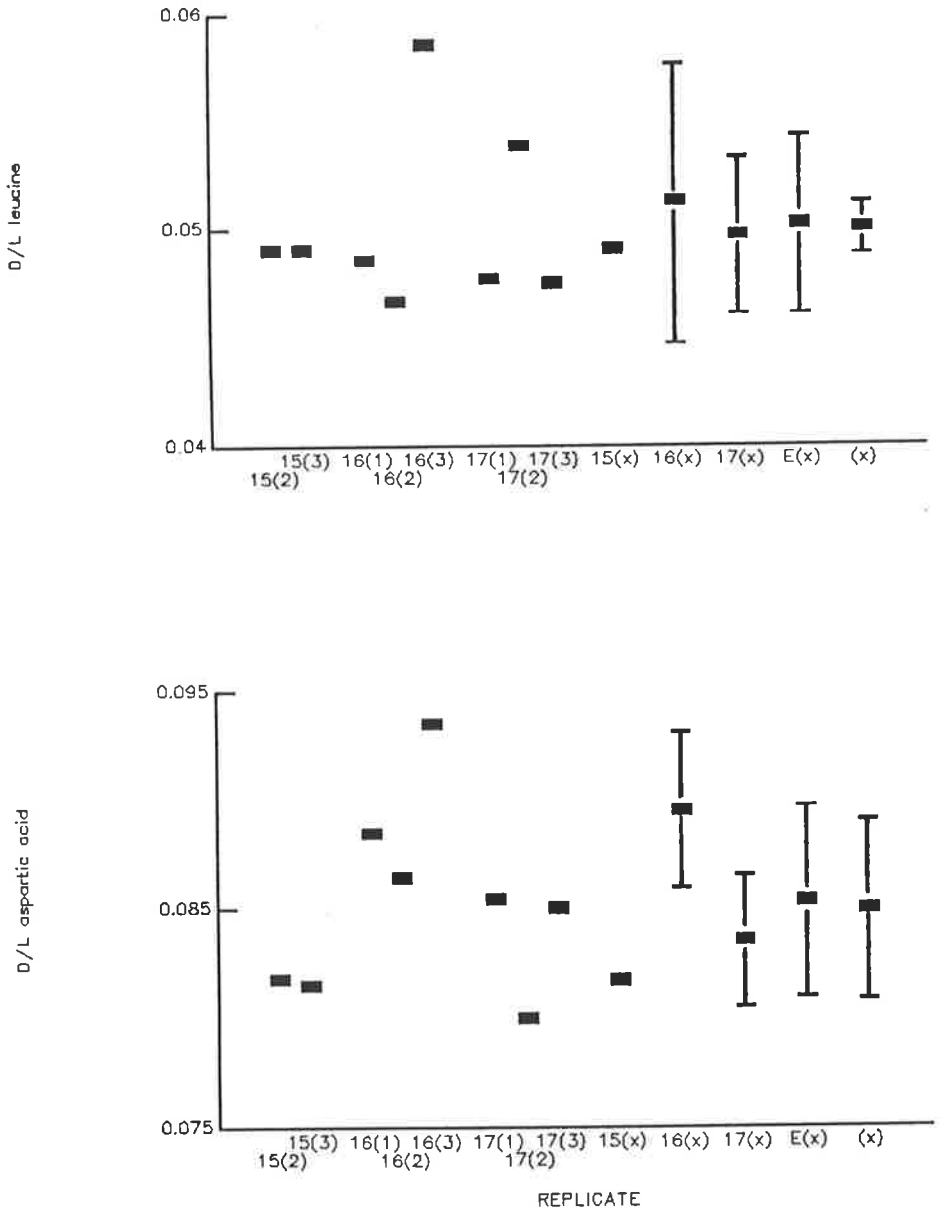



Fig. 3.5 D/L ratios of aspartic acid and leucine from replicate injections (1, 2, 3) of replicate subsamples (15, 16, 17) together with the mean and standard deviation as calculated for each subsample (15(x), 16(x), 17(x)), and as calculated for the sample from all the available data (E(x)) and from the subsample means ((x)).

Note the different D/L scale for each amino acid.

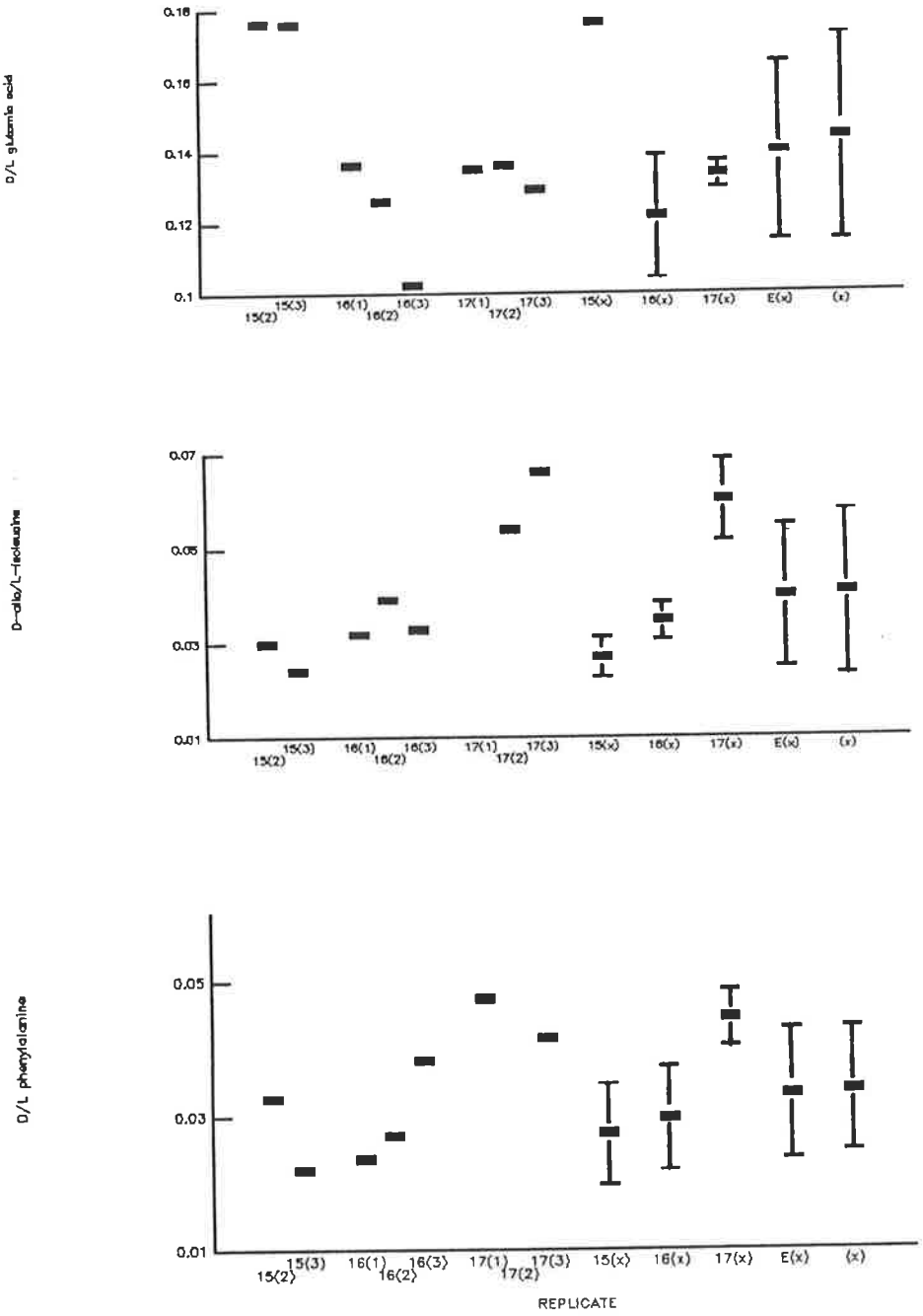



Fig. 3.6 D/L ratios of the three technically unreliable amino acids glutamic acid, isoleucine and phenylalanine from replicate injections (1, 2, 3) of replicate subsamples (15, 16, 17) together with the mean and standard deviation as calculated for each subsample (15(x), 16(x), 17(x)), and as calculated for the sample from all the available data (E(x)) and from the subsample means ((x)).

Note the different D/L scale for each amino acid.

| Chromatogram | Data File | Sample Name | Start Time (min) | Stop Time (min) | Scale Range (mV) | Scale Offset (mV) |
|---|---|---|---|---|---|---|
| 1 | 1 | MIX1 | 29.00 | 30.00 | 50 | 0 |
| 2 | 18 | MIX2 | 29.00 | 30.00 | 50 | 5 |

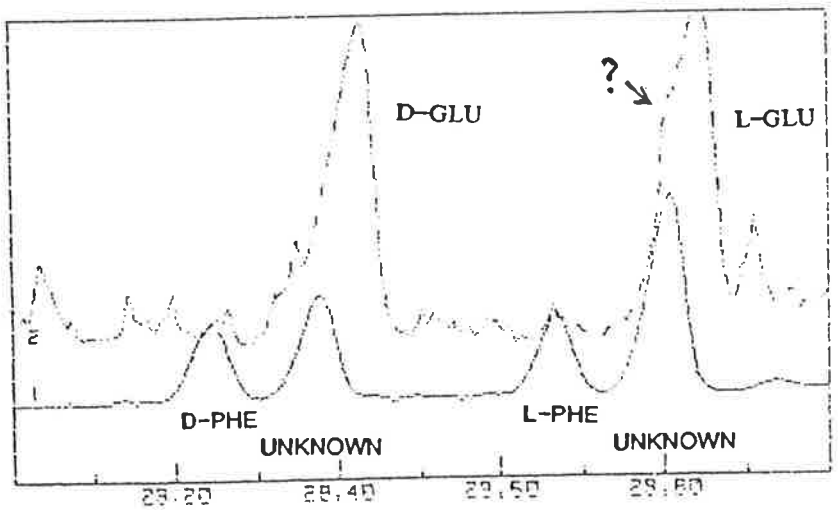



Fig. 3.7 Illustration of the complexity which may arise in the chromatogram in the region of phenylalanine and glutamic acids.

### 3.4.2 HF Induced Racemization

This investigation used three peptides and two proteins; (i) L – prolyl – L – alanine (ii) L – alanyl – L – proline (iii) glycyl – L – prolyl – L – alanine (iv) egg albumen and (v) gelatine. The peptides were Sigma chemicals, the proteins were low grade preparations routinely used in microbiology. Approximate 1% solutions of the samples were made in 0.01% sodium azide. Four replicate 1ml aliquots of these solutions were used, two as a control group and two as treatment.

The treatment consisted of the addition of 3ml of 5.0M HF/0.1M HCl solution to each treatment aliquot and shaking, end – over – end, for 24 hours at 25$^{o}$C. The treatment samples were then evaporated to dryness at 50 – 60$^{o}$C on a rotary film evaporator. All samples, control and treatment, were then subjected to standard acid hydrolysis conditions (6M HCl at 110$^{o}$C for 16*h.*). Hydrolysates were evaporated and desalted on cation exchange resin and derivatised as described by Kimber and Griffin (1987). Gas – liquid chromatography was performed as described by Griffin and Kimber (1988).

The results are presented in Table 3.4.

| Peptide/ Protein | Fraction | D/L ALA | LEU | ASP | PHE | GLU |
|---|---|---|---|---|---|---|
| Pro – Ala | HCl | | | | | |
| | HF | 0.031 | | | | |
| Ala – Pro | HCl | 0.0031 | | | | |
| | HF | 0.0022 | | | | |
| Gly – Pro – Ala | HCl | 0.0064 | | | | |
| | HF | 0.0087 | | | | |
| Albumen | HCl | 0.0064 | 0.0100 | 0.0369 | | 0.0196 |
| | HF | 0.0067 | 0.0214 | 0.0273 | 0.0187 | 0.0154 |
| Gelatin | HCl | 0.0070 | 0.0103 | 0.0400 | 0.0248 | 0.0107 |
| | HF | 0.0080 | | 0.0390 | 0.0160 | 0.0115 |

Table 3.4 D/L ratios from control and HF treated proteins and peptides.

This experiment provided conclusive evidence that hydrofluoric acid does not induce racemization under the conditions routinely used in sample preparation.

The amino acid content of the peptides and proteins was almost exclusively in the L – form. It is possible that the proteins did contain very minor amounts of D – amino acids prior to treatment. Some racemization may occur during refinement of the proteins. It is also possible some racemization has occurred since production as the bottles and contents were of indeterminate age.

Clearly this experiment oversimplifies the situation that exists in soil, the real situation for which control was sought. The amino acid content of soil resides in a plethora of species and associations (Parton et al. 1983b). HF is used to release species not immediately vulnerable to acid hydrolysis. To develop a model system which accurately depicts all the influences leading to the retention of amino acids during acid hydrolysis, the change in vulnerability under HF and subsequent release after further hydrolysis requires *a priori* knowledge of the extent of racemization of this HF fraction.

Evidence from the control experiment indicates that the HF treatment was not responsible for racemization and acid hydrolysis does induce some racemization. The environment of acid hydrolysis, a constant boiling mixture of HCl at elevated temperature, combines the most severe conditions used during processing. HF is a weaker acid (Stevenson and Cheng 1970), the energy (temperature/time) provided to the HF treatment is minimal. Bremner (1950) suggested that all D – amino acids found in soil were formed during acid hydrolysis. More recent evidence suggests that acid hydrolysis is responsible for a smaller proportion of racemization (Larson 1980).

During normal processing amino acids from the HF fraction are subject to macro – environmental acid hydrolysis conditions twice. There is no evidence available to indicate whether the lack of vulnerability to the first hydrolysis equates with a lack of vulnerability to racemization or not. It is generally accepted that there is a link between racemization and hydrolysis. This suggests HF fractions suffer no significant procedurally induced racemization.

### 3.4.3 Elemental analysis

ICP (inductively coupled plasma spectroscopy) analysis provides simultaneous determination of the elements Al, Ca, K, Mn, Na, P, S, B, Co, Cu, Fe, Mo, Zn, and Si (Zarcinas et al. 1987). This technique was employed to quantify the release of these elements during acid hydrolysis and supplementary treatments.

The supernatants from five treatments of the two soils, WF and PP, described in Section 4.2.1, were subjected to ICP spectroscopy. The treatments were,
(i) traditional hydrolysis (6M HCl at 110$^{o}$C for 16*h.*),
(ii) treatment of the residue from (i) with HF (5M HF/0.1M HCl 24*h* end – over – end),
(iii) traditional hydrolysis of the residue from (ii),
(iv) oxidative hydrolysis of the residue from (i) (6M HCl/0.01M $H_2O_2$ at 110$^{o}$C for 16*h),* and,
(v) traditional hydrolysis of the residue from (i).
These treatments are divisible into two groups, (a) the initial treatment, and, (b) supplementary treatments, treatments (ii) and (iii) being parts of a single supplementary procedure (the so – called HF treatment).

Results from the ICP analysis of these samples are presented as Table 3.5. The main proportion of twelve of the fifteen elements was released during the initial treatment (traditional hydrolysis). Silicon, sodium and boron were the exceptions. The results show HF was responsible for the release of silicon into solution. Twelve hundred times as much silicon was released during HF treatment than during traditional hydrolysis. The second hydrolysis released more silicon than the first hydrolysis. Release of sodium followed a similar pattern with twenty times as much sodium released during HF treatment than during the initial treatment. Other supplementary treatments did not release as much sodium as the initial treatment. More boron was released during all of the supplementary treatments than during the initial treatment.

These data support the contention that the procedure leading to the release of amino acids in the HF fraction provided the greatest destruction of the soil matrix. That this would include the destruction of much of the clay was inferred

from the large amounts of silicon released and the concurrent release of aluminium, sodium, iron and other elemental constituents of clays.

ICP results confirmed that the dominant cations requiring attention during clean – up procedures were silicon, iron and aluminium as suggested in Section 3.2.3.

** 05-Aug-89 **

C.Griffin :Acid Hydrolysates & HF treatment solutions.

| Sample No. | Al | Ca | K | Mg | Mn | Na | P | S | B | Co | Cu | Fe | Mo | Zn | Si |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/L | | | | | | | | | | | | | | |
| PP S/N 1 | 23400 | 3160 | 8140 | 3890 | 798 | 252 | 597 | 488 | <1 | 757 | 47 | 57400 | 38 | 248 | 89 |
| PP S/N 2 | 15800 | 349 | 1820 | 228 | 59 | 5090 | 113 | 250 | 198 | 158 | 4.6 | 4310 | 14 | 37 | 110000 |
| PP S/N 3 | 1420 | 550 | 5030 | 89 | 3 | 357 | 10 | 40 | 41 | 10 | 3.4 | 271 | 2 | 49 | 190 |
| PP S/N 4 | 5630 | 219 | 1190 | 649 | 46 | 104 | 76 | 127 | 7.0 | 118 | 6.6 | 3260 | 7 | 37 | 46 |
| PP S/N 5 | 4860 | 2330 | 1040 | 540 | 39 | 59 | 61 | 109 | 2.6 | 90 | 4.0 | 2540 | 6 | 179 | 22 |
| WF S/N 1 | 23400 | 2340 | 6890 | 3720 | 695 | 267 | 374 | 378 | <1 | 766 | 65 | 61200 | 33 | 174 | 150 |
| WF S/N 2 | 23100 | 344 | 2390 | 717 | 67 | 6120 | 107 | 294 | 178 | 184 | 8.2 | 4960 | 24 | 32 | 81000 |
| WF S/N 3 | 1880 | 275 | 7440 | 109 | 4 | 554 | 12 | 46 | 99 | 12 | 3.7 | 318 | 2 | 23 | 990 |
| WF S/N 4 | 4440 | 142 | 938 | 474 | 33 | 113 | 43 | 95 | 7.5 | 83 | 5.0 | 2310 | 5 | 13 | 70 |
| WF S/N 5 | 2740 | 95 | 536 | 280 | 20 | 44 | 23 | 56 | 4.2 | 42 | 4.2 | 1220 | 3 | 16 | 32 |

Table 3.5 ICP spectroscopy results from analysis of dilute solutions of the five supernatants derived from the pasture (PP) and wheat/fallow (WF) soils.

The treatments were,
(i) S/N 1 traditional hydrolysis,
(ii) S/N 2 treatment of the residue from (i) with HF,
(iii) S/N 3 traditional hydrolysis of the residue from (ii),
(iv) S/N 4 oxidative hydrolysis of the residue from (i), and,
(v) S/N 5 traditional hydrolysis of the residue from (i).

### 3.4.4 Electron microscopy

Further evidence of the material contributing to the hydrolytic effect was ascertained by visual examination of the larger particles of the residues from each of the samples submitted for ICP spectroscopy.

Visual examination supported chemical analysis in that treatment with HF resulted in a dramatic change to the residue. The micrographs presented are all magnifications of quartz particles. Untreated soil and residues (i), (iv) and (v) (Plates 3.1, 3.2, 3.3 and 3.4 respectively) were similar in that the quartz particles appear to have grain coatings and adhering particles. In a study of appropriate pretreatments for SEM analysis of weathered grains Cremeens et al. (1987) found acid hydrolysis increased grain coatings and adhering particles. The visual evidence presented here neither supports nor refutes this detail. The evidence shows these treatments did not release all of this material.

Treatment with HF removed the grain coatings and most of the adhering particles, exposing the surfaces of the quartz particles (Plates 3.5 and 3.6).

There are several questions which remain unanswered by this investigation. These include, how much of the resultant surface is an artifact of the treatment? and is there organic matter associated with the material normally residing in these crevices? If the crevices as exposed by HF were real, this may provide the infrastructure for development of microniches, where nutrients are protected from short term cycling within the soil system.

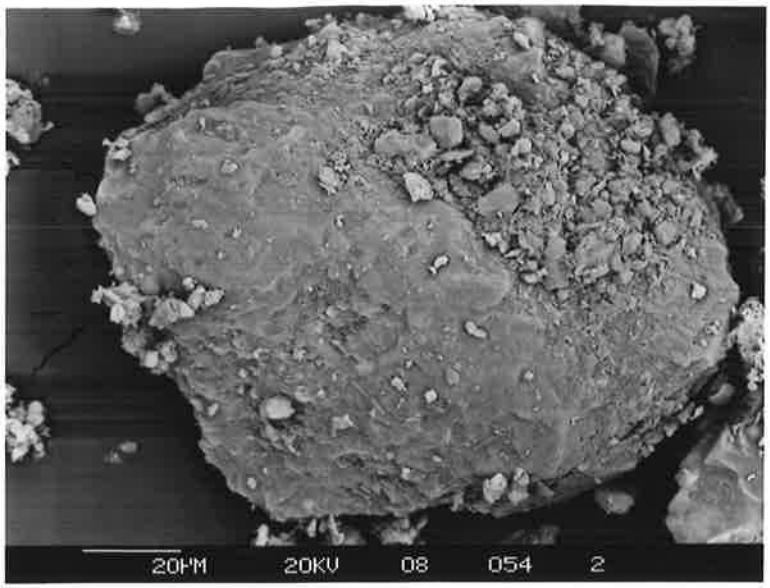



Plate 3.1 PP soil, untreated (0.75kx).
Quartz infrastructure with grain coat and adhering particles.

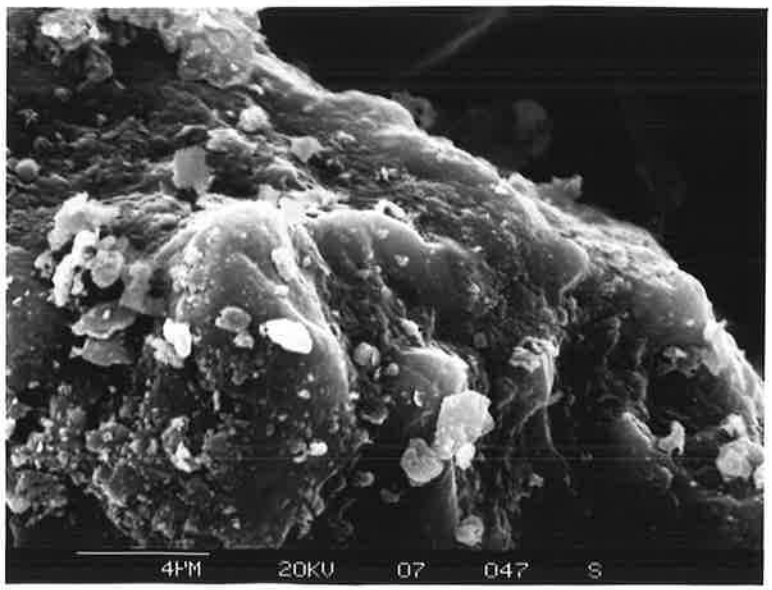



Plate 3.2 PP soil after traditional hydrolysis (5.0kx).
Quartz infrastructure with grain coat and adhering particles.

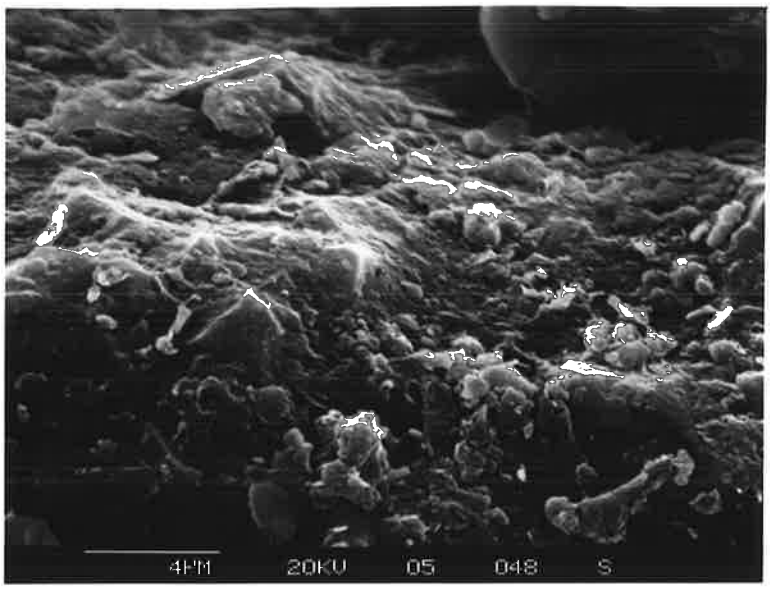



Plate 3.3 PP soil after traditional and oxidative hydrolyses (5.0kx).
Quartz infrastructure with grain coat and adhering particles.

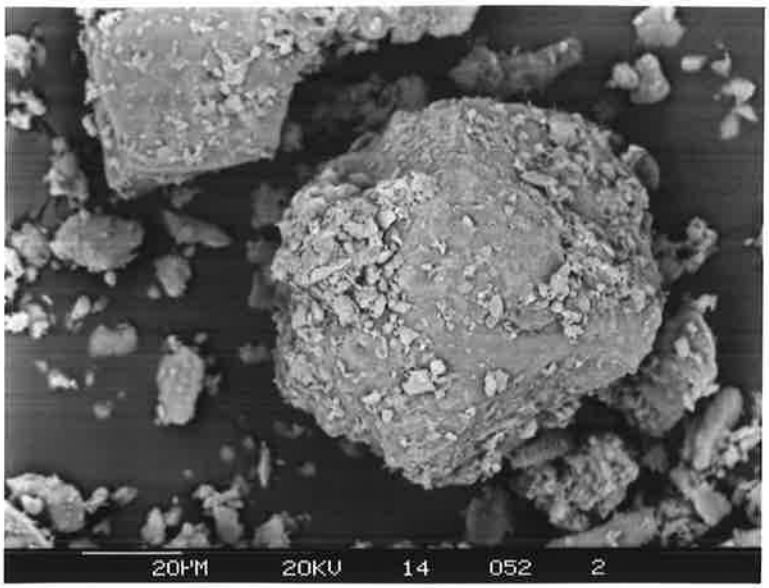



Plate 3.4 PP soil after two traditional hydrolyses (0.75kx).
Quartz infrastructure with grain coat and adhering particles.

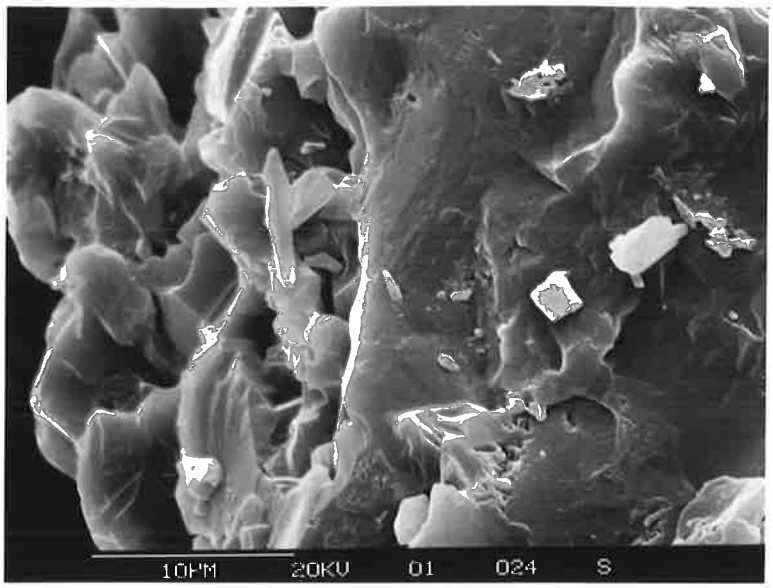



Plate 3.5 PP soil after traditional hydrolysis and HF treatment (2.9kx).
Quartz infrastructure with few adhering particles but devoid of grain – coat.

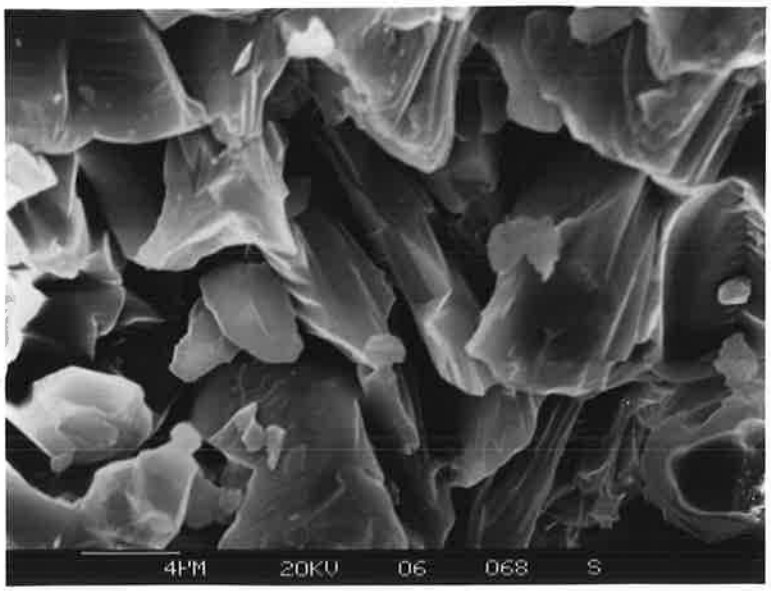



Plate 3.6 PP soil after traditional hydrolysis, HF treatment and a second traditional hydrolysis (5.0kx).
Quartz infrastructure, devoid of grain – coat.

In a second examination of material released in the HF fraction, residue from the initial traditional hydrolysis was treated with HF for varying time periods. After 4.5 hours the separation of the surface coating from the underlying quartz particle was apparent, suggesting the crevices were not all artifact (Plate 3.7).

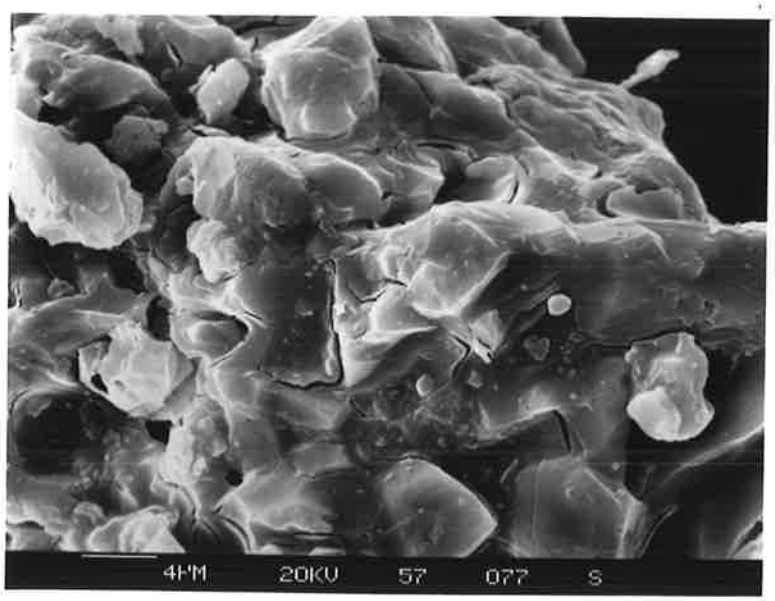



Plate 3.7 PP soil after 4.5h exposure to the HF treatment (2.9kx).
Quartz infrastructure with cracking of grain – coating revealing underlying crevices.

It is interesting to note the types of organisms which may survive hydrolysis. Plate 3.8 shows a diatom, the remains of which have survived the initial hydrolysis. Diatoms have a siliceous exoskeleton which was not vulnerable to acid hydrolysis. Plate 3.9 shows a miospore, probably a pollen grain. The highly sculptured walls of miospores are largely made of sporopollenin a highly resistant hydrocarbon containing a variety of functional groups including hydroxyls, phenols and fatty acids. This specimen has survived the initial hydrolysis, treatment with HF and a second hydrolysis.

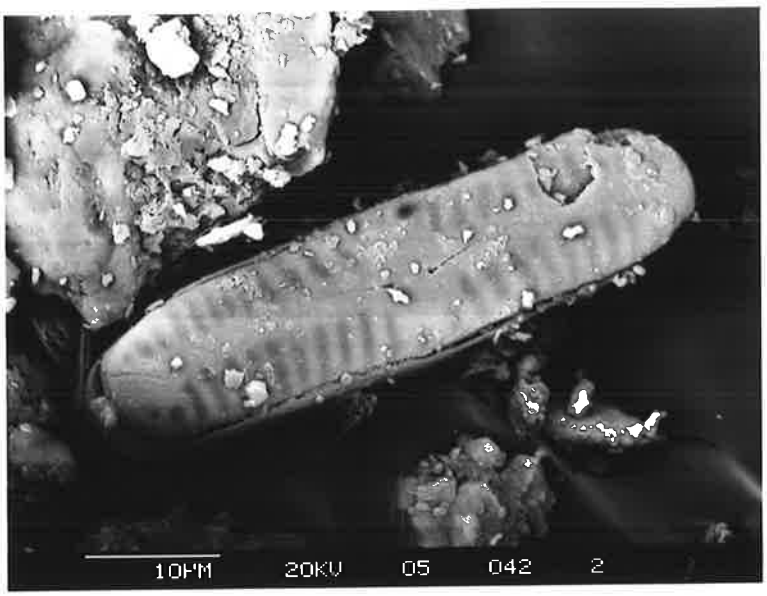



Plate 3.8 PP soil after traditional hydrolysis (1.9kx). Diatom.

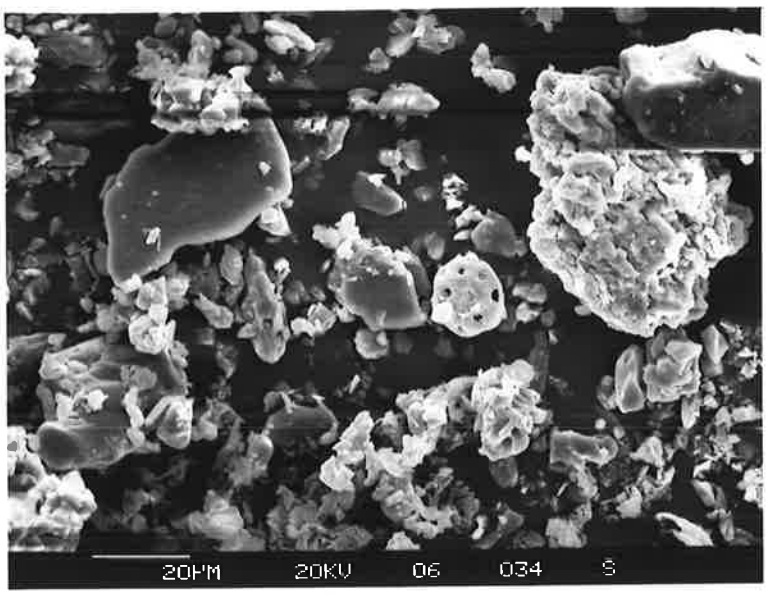



Plate 3.9 PP soil after traditional hydrolysis. HF treatment and a second traditional hydrolysis (1.9kx). Palynomorph.

## 3.5 CONCLUSIONS

Racemization analysis of soil samples is complex relative to that used for the simplest of possible samples (e.g. egg shells) where it is only a matter of hydrolysis and derivatization. Clean – up must include cation and anion exchange steps. Solvent extraction did improve chromatographic legibility but also led to some losses of amino acids. The technique as published (Griffin and Kimber 1988) satisfies the requirements as outlined in Section 2.5.

The technique provides two hydrolytic fractions from a single sample. Examination of both of these fractions is considered important because there is some evidence that such fractions vary considerably and there is a distinct paucity of information regarding the behaviour of amino acids which are part of the non – hydrolysable nitrogen in a soil.

Completion of control experiments has led to the following conclusions:

Elution characteristics (retention times) of the capillary column can be manipulated using different temperature programmes. By using a flame ionisation detector reasonable linearity in response was ensured.

Two broad groups of amino acids were discernible on the basis of errors in replication of D/L ratios. D/L ratios of the technically reliable amino acids (alanine, valine, leucine and aspartic acid) were readily replicated with errors less than ten percent. The other amino acids were categorized as technically unreliable. The inclusion of any amino acid in either group is probably column specific, as different capillary columns have different retention characteristics.

Hydrolysis was used to separate two procedural fractions (the HCl and HF fractions) from a single soil sample. The hydrofluoric acid treatment used to expose the HF fraction for hydrolysis did not induce significant racemization. This treatment released twelve hundred times as much silicon as a traditional hydrolysis and led to wholesale destruction of the soil matrix. Visual evidence indicated that the treatment grossly affected the surfaces of the larger particle, releasing any organic matter intimately associated with these surfaces.

# CHAPTER 4 AMINO ACID RACEMIZATION IN AGRICULTURAL SOILS

## 4.1 INTRODUCTION

Little is known about amino acid racemization in soils. As discussed in Sections 1.2 and 2.2 of this thesis, previous workers have restricted their investigations into racemization of amino acids in soils, to the readily hydrolysable materials in the soil system. The investigations described in this chapter were conducted on a range of agricultural soils and fractions/separates from these soils to ascertain,

(i) the relative importance of readily hydrolysable material in describing racemization in a soil, and,

(ii) whether D/L values parallel age effects as established by radiocarbon techniques.

Such an investigation is required to establish a basis for application of amino acid racemization dating techiques to soils. As described in the previous chapter the technique of extraction, purification and analysis of the degree of racemization of amino acids in soils and soil fractions is both complex and time consuming. This feature places constraints on the extent of any investigation. The broad objective of these preliminary investigations was to outline the complexities of racemization in soils.

As described in Section 2.4 radiocarbon techniques have established four broad age effects, (i) hydrolytic effects, (ii) agricultural management effects, (iii) humification effects, and, (iv) particle size effects. The investigations descibed in this chapter examine racemization in hydrolytic fractions of, (i) untreated soils, (ii) particle size separates, and, (iii) humic fractions (Table 4.1). All the soils are from agrisequences. An agrisequence is a series of soils which are pedogenically identical. Any divergence amongst the series is restricted to the different agricultural treatments imposed during their recent history.

| Agrisequence | Rotation | Symbol | Fraction/separate |
|---|---|---|---|
| Waite | Wheat/Fallow | WF | HCl, HF<br>humic acids<br>sand, silt, clay |
| | Permanent Pasture | PP | HCl, HF<br>humic acids<br>sand, silt, clay |
| | Arboretum | Arb | sand, silt, clay<br>fine clay |
| Rothamsted | Wilderness | BBW | HCl, HF |
| | Permanent Wheat | BB03 | HCl, HF |
| | Permanent Wheat (sampled 1881) | BB1881 | HCl, HF |

Table 4.1 Agrisequences, soils and soil fractions/separates used for these investigations.

## 4.2 SOILS AND SOIL FRACTIONS

All of the soils and prepared soil fractions investigated are described in this section.

### 4.2.1 Waite agrisequence.

Samples of Urrbrae fine sandy loam (Piper 1938) were collected under a wheat – fallow rotation (WF) and permanent pasture (PP) in the rotation experiment at the Waite Agricultural Research Institute, Adelaide in 1985. Twenty randomly placed, 5cm diameter cores of the 0 – 10cm layer from each plot were combined, air dried and ground in a Christy – Norris mill to $< 1.5$mm, to provide a bulk sample for each plot.

Since establishment in 1948, the permanent pasture has not been cultivated. It is a grass – legume pasture and has arrested the decline in soil nitrogen associated with more exploitative rotations. Such an exploitative rotation is exemplified by the wheat – fallow treatment. Each rotation cycle of the wheat – fallow treatment involves at least four cultivations, a monoculture cropping schedule of winter wheat and 15 months fallow. Since establishment in 1928, this rotation has decreased soil nitrogen to *ca.* 0.3 mg $g^{-1}$ compared to 0.8 mg $m^{-1}$ in the pasture (Ladd and Russel 1983).

### 4.2.2 Humic acids

The humic acid samples were provided by the CSIRO Division of Soils and are the same samples described by Bulter, Ladd and co – workers (Butler and Ladd 1969; Brisbane et al. 1972). Source material and extraction of the humic acids was done in the mid – sixties.

### 4.2.3 Particle size separates

Particle size separates were provided by the Department of Soil Science of the Waite Agricultural Research Institute. Source material for the particle size

work was sampled independently also in 1985 and has been used for other investigations (Oades et al. 1987, 1988). A third soil, collected from the arboretum, was analysed during particle size investigations (Section 4.3.4). This soil is accepted as representing the virgin state of the Urrbrae fine sandy loam found in the vicinity of the rotation experiment at the Waite Institute.

### 4.2.4 Rothamsted agrisequence

The three soils provided by Rothamsted form an agrisequence not unlike the Waite agrisequence. These soils are,

(i) Broadbalk plot 03 (<30 mesh), 0 – 23cm, sampled in 1881, (BB1881),

(ii) Broadbalk plot 03 (<6.25mm), 0 – 23cm, sampled in 1987, (BB03), and,

(iii) Broadbalk Wilderness (<6.25mm), 0 – 23cm, sampled in 1987, (BBW).

Russell (1973) described Broadbalk at Rothamsted as "a field that has been in wheat almost continuously since 1843" and plot 3 as having been "unmanured ..... since 1843" (Russell 1973 p33). Later he described the creation of the Wilderness plot as "a third plot ..... carried a wheat crop in 1882 which was never harvested: the plot was allowed to revert to natural vegetation, and became known as Broadbalk Wilderness" (Russell 1973, p349).

The two 1987 samples were sieved and then air dried at room temperature. The 1881 sample was sieved (<6.25mm), air dried and stored in a sealed bottle at room temperature 1881 – 1987. It was ground to pass a 30 mesh sieve in 1982.

BB03 exemplifies a soil after long term exposure to an exploitative rotation and is in this way comparable to the WF soil of the Waite agrisequence. Similarly BB1881 may be comparable to the WF, however there is some doubt about the effect of storage. Water is required for racemization to proceed. This sample was air dried. Air drying does not remove all of the water from a soil. Water that remains is intimately associatied with the matrix. Is this water available for hydrolysis/racemization reactions?

## 4.3 HYDROLYTIC FRACTIONS

### 4.3.1 Introduction

As discussed in Section 2.4.2, many authors agree that acid hydrolysis is the most effective method of separating age differing components of soil organic matter. Limmer and Wilson (1980) proposed the use of hydrolytic fractions in epimerization studies. However they were unable to gather conclusive evidence. This hypothesis was investigated here by analysing the hydrolytic fractions, as described in Section 3.2.2, from the soils and soil separates/fractions described in Section 4.2.

### 4.3.2 Waite Agrisequence

HCl and HF fractions from the two soils (WF and PP) were prepared and analysed in accordance with the procedure outlined in Appendix 1. At least five replicate subsamples were analysed for each soil.

The results indicating the degree of racemization of the four technically reliable amino acids, as described in Section 3.4.1, from each fraction of each soil are presented as means of D/L ratios in Table 4.2. Total hydrolysable amino acid – N is approximately 0.3 mg $g-1$ for the WF and 0.8 mg $g-1$ for the PP (Ladd and Russell 1983). A further 10 – 15% was protected from normal hydrolysis and was released in the HF fraction. The D/L data was transformed logarithmically and then subjected to an analysis of variance. The inferences were based on the assumption that the fraction by amino acid by soil residual was a valid estimate of error; any three – factor interaction resulted in the tests of significance being conservative.

The ANOVA indicated significant differences, (i) between soils ($P<0.01$), (ii) between fractions ($P<0.05$), and, (iii) between amino acids ($P<0.01$) (Table 4.2). The soils were not distinguished on the basis of racemization of amino acids

in the HCl fraction. The degree of racemization of amino acids released in the HF fraction did distinguish the soils. This resulted in the difference between the transferred means of the soils of 0.377 ($-2.817$ for WF and $-2.440$ for PP), indicating that the D/L values from the PP were 1.46 times higher than those from the WF.

The conservative nature of this evidence is highlighted when temperature effects are considered. During the course of a full rotation, the mean effective temperature in the WF is presumed to be higher (albeit slightly) than that of the PP, an effect primarily resulting from the summer months of bare fallow (Radke et al. 1985; Parton and Logan 1981). This assumption implies that the racemization rate constants were higher in the WF than in PP, all other factors being equal. Therefore, if the differences in D/L values are controlled by temperature, the WF values should be higher than those for PP.

The differences between the transferred means of the fractions of 0.279 ($-2.768$ for HCl and $-2.489$ for HF) indicated that D/L values from the HF fractions were 1.32 times those of the HCl fractions (Table 4.3).

| FRACTION | AMINO ACID | | | |
|---|---|---|---|---|
| | Valine | Leucine | Alanine | Aspartic |
| WF HCl | 0.026 (0.010) | 0.048 (0.012) | 0.101 (0.009) | 0.100 (0.026) |
| PP HCl | 0.027 (0.011) | 0.056 (0.012) | 0.110 (0.010) | 0.115 (0.009) |
| WF HF | 0.033 (0.020) | 0.079 (0.062) | 0.057 (0.017) | 0.087 (0.054) |
| PP HF | 0.062 (0.018) | 0.171 (0.046) | 0.126 (0.046) | 0.130 (0.062) |

Table 4.2 D/L ratio means (and standard deviation) of the four technically reliable amino acids from the hydrolytic fractions from two rotations of the Waite agrisequence.

| Amino Acid | Fraction | | Mean |
|---|---|---|---|
| | HCl | HF | |
| Valine | −3.631 | −3.096 | −3.363 |
| Leucine | −2.959 | −2.152 | −2.556 |
| Alanine | −2.250 | −2.468 | −2.359 |
| Aspartic | −2.233 | −2.241 | −2.237 |
| Mean | −2.768 | −2.489 | |

Table 4.3 Mean rotation D/L ratios of four amino acids from the hydrolytic fractions expressed on a logarithmic scale. The *l.s.d.* (1%) for all values is 0.45.

Differences in the degree of racemization between amino acids were quantified in the two-way table (amino acids by fraction) of transferred means and margins (Table 4.3). This table indicates significant differences between fractions for valine and leucine ($P < 0.01$). Racemization of these two amino acids is recognised as providing good potential for age assessment in other chronostudies (Hearty et al. 1986; Kimber and Griffin 1987; Murray-Wallace et al. 1988). Futhermore, it is recognised that racemization of alanine and aspartic acid may be complicated by specific attributes of each amino acid resulting from diagenic processes (Vallentyne 1969; Kimber et al. 1986; Kimber and Griffin 1987) and from natural abundances of D-enantiomers derived from bacterial cell walls (Pollock and Kvenvolden 1978).

Results for other amino acids are presented as mean D/L ratios and standard deviation (Table 4.4). These amino acids are, (i) the technically unreliable amino acids as described in Section 3.4.1 *i.e.* phenylalanine, glutamic acid and isoleucine, and, (ii) amino acids not routinely examined and not supported by control data *i.e.* proline, ornithine and lysine. These data are, as expected, less decisive.

There were a number of difficulties associated with interpreting the D/L values of phenylalanine, glutamic acid and isoleucine. These difficulties were in part predicted by the control experiment described in Section 3.4.1. D-alloisoleucine was present only in very small quantities. This peak was generally not resoved from the glycine peak in these replicates. Complexity in the region of the chromatogram where glutamic acid and phenylalanine were expected led to difficulties in identification and resolution which contributed to the large error associated with these two amino acids. Chromatograms of some of the replicates analysed suggested the presence of a contaminant co-chromatographing with D-phenylalanine.

D/L ratios of proline and lysine supported the general conclusions. An incomplete data group prevented the use of these amino acids as independent evidence. D/L ratios for ornithine are presented. Error terms prevented the resolution of this data.

| Fraction | Amino Acid | | | | | |
|---|---|---|---|---|---|---|
| | Phe | Glu | Ile | Pro | Orn | Lys |
| WF HCl | 0.047<br>0.019) | 0.148<br>(0.018) | 0.025<br>(0.006) | 0.045<br>(0.017) | 0.041<br>(0.014) | 0.043<br>(0.014) |
| PP HCl | 0.132<br>(0.090) | 0.131<br>(0.011) | | 0.081<br>(0.010) | 0.067<br>(0.014) | 0.034<br>(0.018) |
| WF HF | 0.223<br>(0.162) | 0.319<br>(0.163) | | 0.078<br>(0.015) | 0.023<br>(0.004) | |
| PP HF | 0.508<br>(0.292) | 0.196<br>(0.078) | | | 0.056<br>(0.004) | 0.152<br>(0.037) |

Table 4.4 D/L ratio means (and standard deviation) of the technically unreliable amino acids and amino acids not routinely examined from the HCl and HF fractions from two rotations of the Waite agrisequence.

### 4.3.3 Rothamsted Agrisequence

Two replicate samples of the three Rothamsted soils were treated to provide hydrolytic fractions which were subjected to racemization analysis.

The HCl fraction of the two soils sampled in 1987 (BBW and BB03) provided D/L values for nine amino acids. The HCl fraction from BB1881 provided data on fewer amino acids primarily due to doubts about purity of chromatographic peaks. Aspartic acid was the only amino acid consistently resolved in the HF fractions. These data are presented in Table 4.5.

D/L ASP from the six separates is presented graphically in Figure 4.1. This presentation indicates these data were readily divisible into three groups with BB1881 HF being higher, and BBW HCl being lower, than the rest (BBW HF, BB03 HCl, BB03 HF and BB1881 HCl). This analysis indicated differences between the fractions of BB1881 and BBW and no difference between the fractions of BB03.

These data parallel those from the rotation experiment in Adelaide described in Section 4.3.2.2. The exploitative rotation from both agrisequences *i.e.* WF and BB03 resulted in homogeneity of the soil based on degree of racemization while some degree of heterogeneity was evident from less exploitative rotations *i.e.* PP and BBW.

There is clearly some difference between BB1881 and BBW. D/L ASP from both fractions of BB1881 was higher than that of BBW (Fig.4.1). This evidence and the relative paucity of values for BB1881 suggested this soil has changed during storage with some degree of hydrolysis, degradation and racemization continuing. These reactions are initiated by nucleophilic species which suggests the involvement of the water which remains after air drying as discussed in Section 4.2.4.

| Soil/Fraction | Amino Acid | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ala | Val | Ile | Pro | Leu | Asp | Phe | Glu |
| BB03 HCl | 0.081 (.019) | 0.017 (.000) | 0.045 (.013) | 0.037 (.002) | 0.070 (.003) | 0.148 (.002) | 0.062 (.019) | 0.130 (.010) |
| BBW HCl | 0.060 (.014) | 0.010 (.005) | 0.019 (.009) | 0.019 (.007) | 0.051 | 0.100 (.007) | 0.040 (.001) | 0.083 (.001) |
| BB1881 HCl | | 0.040 (.005) | | 0.121 (.011) | 0.057 (.009) | 0.154 (.002) | 0.039 (.004) | 0.142 (.002) |
| BB03 HF | 0.095 | | | 0.070 | | 0.148 (.000) | | 0.131 |
| BBW HF | | 0.050 | | 0.154 | 0.168 | 0.199 (.019) | 0.108 | |
| BB1881 HF | | | | | | 0.224 (.043) | | |

Table 4.5 D/L ratio means (and standard deviation) for nine amino acids from the hydrolytic fractions of the soils from the Rothamsted agrisequence.

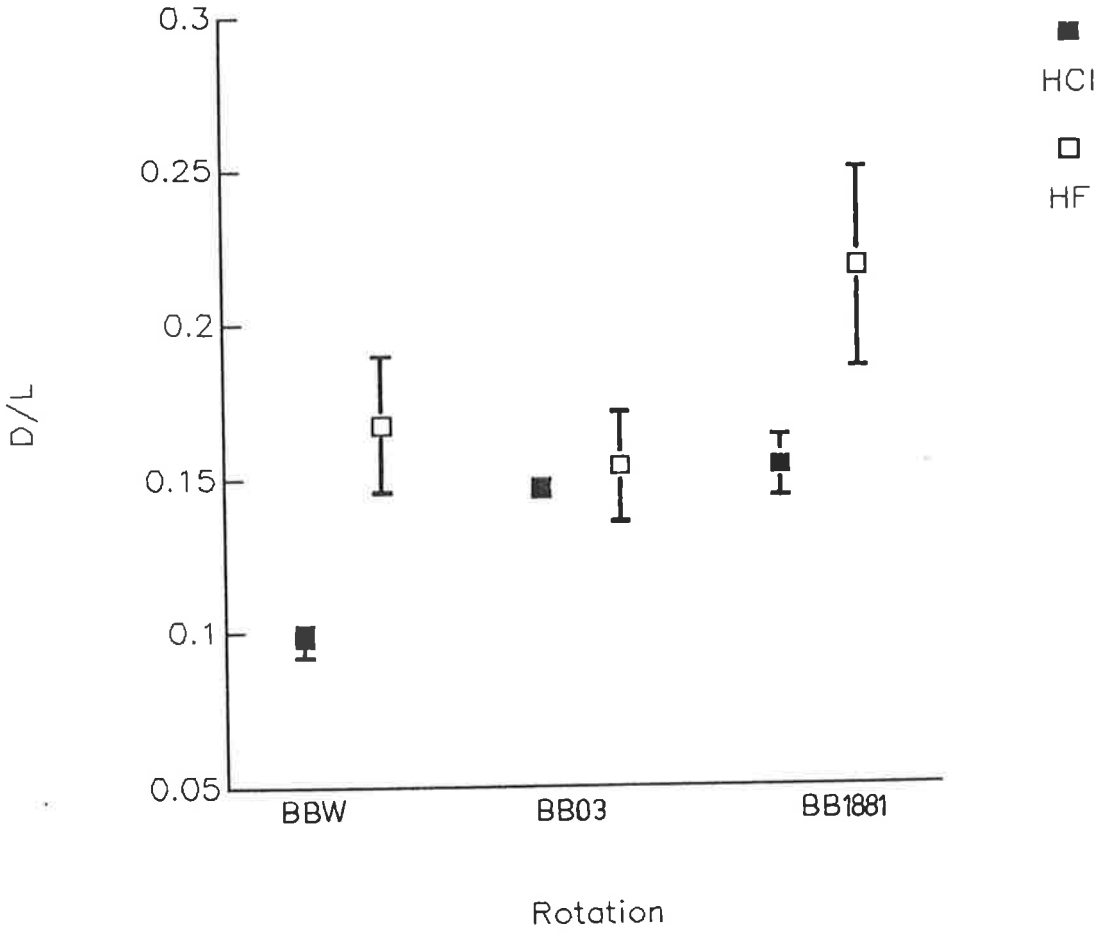



Fig. 4.1 D/L ASP (mean and standard deviation) as measured in the hydrolytic fractions of the three soils from the Rothamsted agrisequence.

The difference between BB1881 and BBW and the interpretation that this difference is a result of storage is strong evidence that D/L values reflect age.

The degree of racemization of the HCl fraction from BBW was lower than that of BB03. The D/L values from the HCl fraction from BBW approached values for procedurally induced racemization with the exceptions of alanine, aspartic acid and glutamic acid (amino acids with high natural abundances of D – enantiomer). This is strong evidence of the rejuvenation (modernization or younging) of the soil from this plot. The dichotomy in the two fractions from BBW supports the long term integrity of the HF fraction.

### 4.3.4 Particle size separates

Particle size separates analysed corresponded to the crude divisions sand, silt, clay and fine clay (53 – 2000 *u*m, 2 – 20 *u*m, 0.2 – 2 *u*m and < 0.2 *u*m respectively). Hydrolytic fractions of the sand, silt and clay separates from the WF and PP soils, described in Section 4.2.1, were analysed together with the four separates of the arboretum soil described in Section 4.2.3.

D/L ratios (mean and standard deviation) for the technically reliable amino acids (alanine, valine, leucine, and aspartic acid) are presented graphically as Figures 4.2a and 4.2b.

These data generally support previous results in that;

(i) Although there were large differences in the mean D/L aspartic acid ratios, generally this was masked by a large error. The silt separate of the arboretum soil was distinctive in that, (a) there was clear differentiation between the hydrolytic fractions, and, (b) this was the highest D/L aspartic acid recorded from this agrisequence.

(ii) Similarly D/L alanine differentiated neither hydrolytic fractions (HCl and HF) nor soils (WF and PP). This was primarily due to the large error terms which arose from the difficulty in resolving the D – enantiomer. Results from the

arboretum soil supported the theory associating differing degrees of racemization with hydrolytic fractions.

(iii) There were strong trends apparent in D/L leucine. While the values from the HCl fractions of the three soils were the same, the values from the HF fractions increased with increasing system stability (WF < PP < ARB).

(iv) There was little distinction made by the hydrolytic procedure as applied to WF separates based on D/L valine. A difference between the hydrolytic fractions of the separates from the PP and Arb soils was apparent.

There was a general trend apparent amongst the particle size separates. Generally silt separates had higher D/L values than did either the larger or smaller separates. There was no consistent trend between the clay and fine clay separates, D/L aspartic acid and D/L valine decreased (clay > fine clay) while D/L alanine and D/L valine increased (clay < fine clay).

The original samples were to be carbon dated and it was hoped that this investigation would provide data whereby the two techniques could be compared (amino acid racemization and carbon dating). Technical difficulties encountered in carbon dating the samples precluded this.

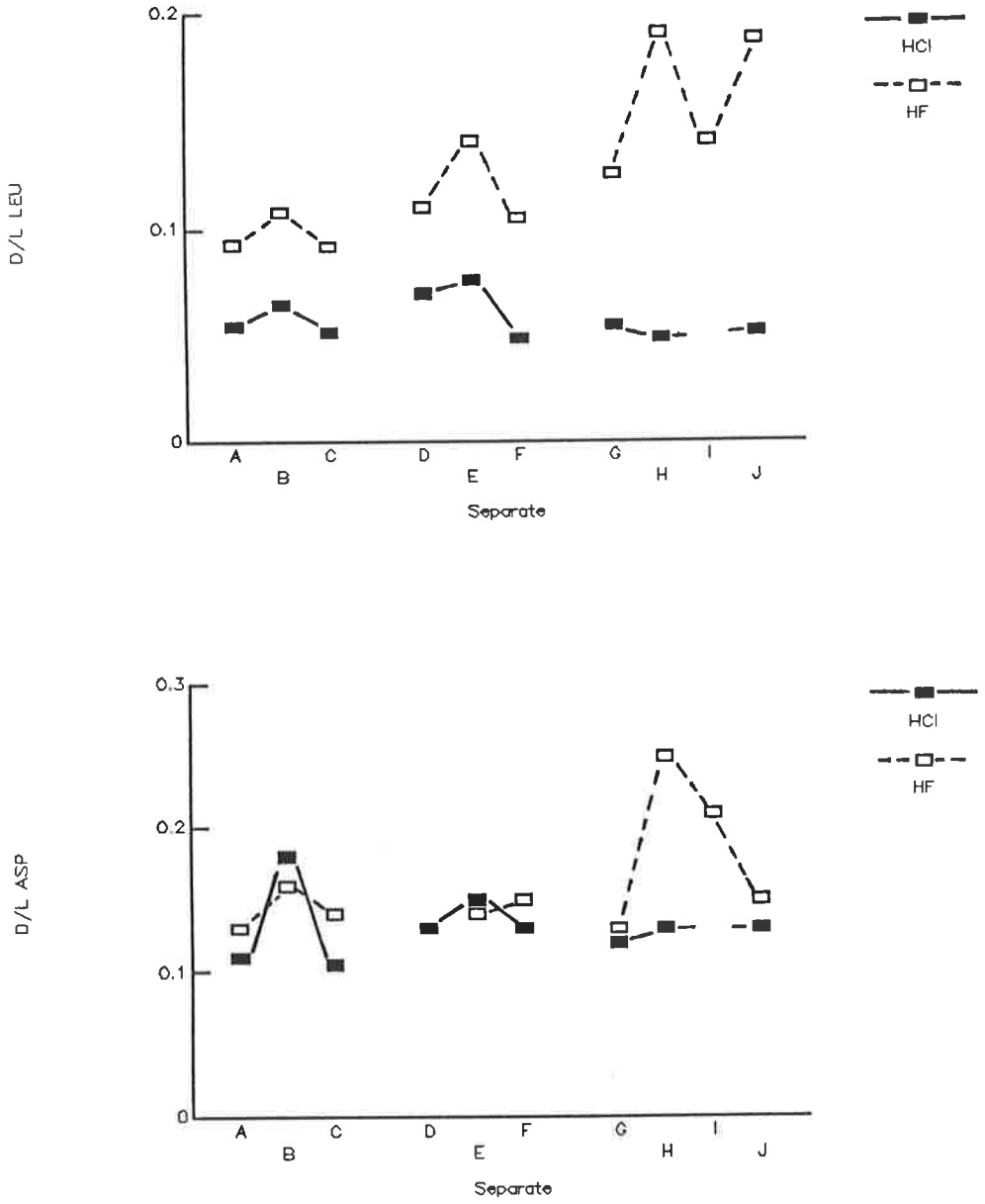



Fig. 4.2a D/L ratios (means) of leucine and aspartic acid from the hydrolytic fractions of particle size separates (sand A,D,G; silt B,E,H; clay C,F,I and fine clay J) of the WF soil (A,B,C), PP soil (D,E,F) and the Arb soil (G,H,I,J) of the Waite agrisequence.

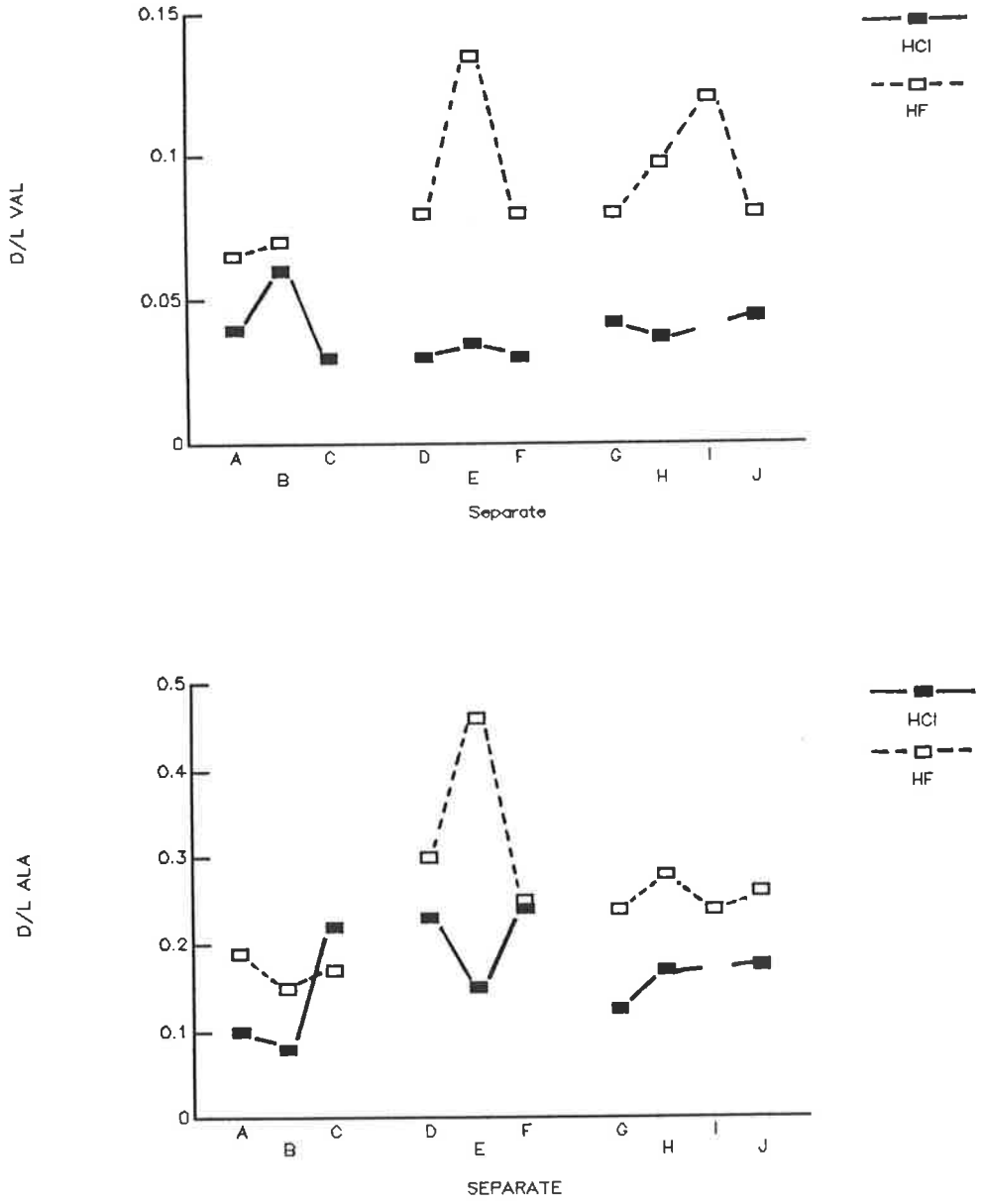



Fig. 4.2b D/L ratios (means) of valine and alanine from the hydrolytic fractions of particle size separates (sand A,D,G; silt B,E,H; clay C,F,I and fine clay J) of the WF soil (A,B,C), PP soil (D,E,F) and the Arb soil (G,H,I,J) of the Waite agrisequence.

### 4.3.5 Classical fractions

#### 4.3.5.1 Introduction

Humic acids are widely accepted as being procedural fractions. The most common extractants used in the preparation of classical fractions are sodium hydroxide and sodium pyrophosphate. It is clear that different extractants do not produce the same fractions. Humic acids precipitated from pyrophosphate contain less acid hydrolysable amino acid nitrogen, have more low molecular weight material, a higher carboxyl content and higher extinction values than the corresponding sodium hydroxide extracts (Butler and Ladd 1969). Tracer work using $^{15}N$ indicated pyrophosphate humic acids were more highly humified (McGill and Paul 1976), leading these authors to conclude pyrophosphate humic acids are more recalcitrant molecules than humic acids derived from other extractants. This conclusion is supported by pyrolysis gas chromatography and rock – eval pyrolysis which indicates pyrophosphate humic acids contain more highly condensed moieties (Kimber and Searle 1970).

#### 4.3.5.2 Influence of Extractant

Humic acids obtained from the two soils (WF and PP) by extraction with pyrophosphate (A), pyrophosphate and then alkali (B) and alkali (C) were subjected to amino acid racemization analysis as an investigation to examine the influence of extractant on resultant D/L ratios.

D/L ratios of the three amino acids which were most consistently resolved are presented in Table 4.6. These data indicate that significant racemization of aspartic acid occurred when alkali was used to extract the humic acid. This effect was less dramatic for valine where there was only a difference between the pyrophosphate humic acids and alkali humic acids. The inclusion of alkali in the extraction procedure had no consistent effect on the D/L ratios of alanine.

Alkali is known to induce significant racemization (Pollock and Frommhagen 1968). The results are consistent with this statement, preventing corroboration of the conclusion of McGill and Paul (1976) regarding recalcitrance.

| Humic Acid | D/L means Alanine | Aspartic | Valine |
|---|---|---|---|
| 1A HCl | 0.100 | 0.111 | 0.024 |
| 1B HCl | 0.098 | 0.265 | 0.028 |
| 1C HCl | 0.108 | 0.294 | 0.100 |
| 2A HCl | 0.115 | 0.131 | 0.019 |
| 2B HCl | 0.098 | 0.268 | |
| 2C HCl | 0.109 | 0.283 | 0.057 |

Table 4.6 D/L ratio means of alanine, aspartic acid and valine from humic acids obtained from soils WF (1) and PP (2) by extraction with pyrophosphate (A), pyrophosphate then alkali (B) and alkali (C).

4.3.5.3 Influence of soil

Pyrophosphate extracted humic acids from the two soils (WF and PP) were subjected to hydrolytic fractionation and the two fractions analysed for degree of racemization.

The results are presented graphically in Figure 4.3. These results are D/L ratio means and standard deviation for alanine, valine and aspartic acid. These data show a conclusive separation of the two fractions from both soils with the degree of racemization of the HF fraction consistently higher than that of the HCl fraction. These data also suggest a consistent trend between the soils. Each fraction of the WF soil had higher D/L ratios than the equivalent fractions from the PP soil. This is in agreement with previous work which suggested that the wheat/fallow was the older (Kimber and Searle 1970).

That this second observation was not statistically significant (means are not separated by more than the sum of the errors) may have been enhanced by the relatively short exposure of the soils to the imposed agricultural management. Humic acids were derived from soils sampled some time before 1965, probably closer to 1960. At this time, the pasture had been in place for only a dozen or so years. That there was a trend to lower D/L values from the pasture humic acids over such a short period demonstrates the vulnerability of humic acids to rejuvenation as previously noted in Section 2.4.4.

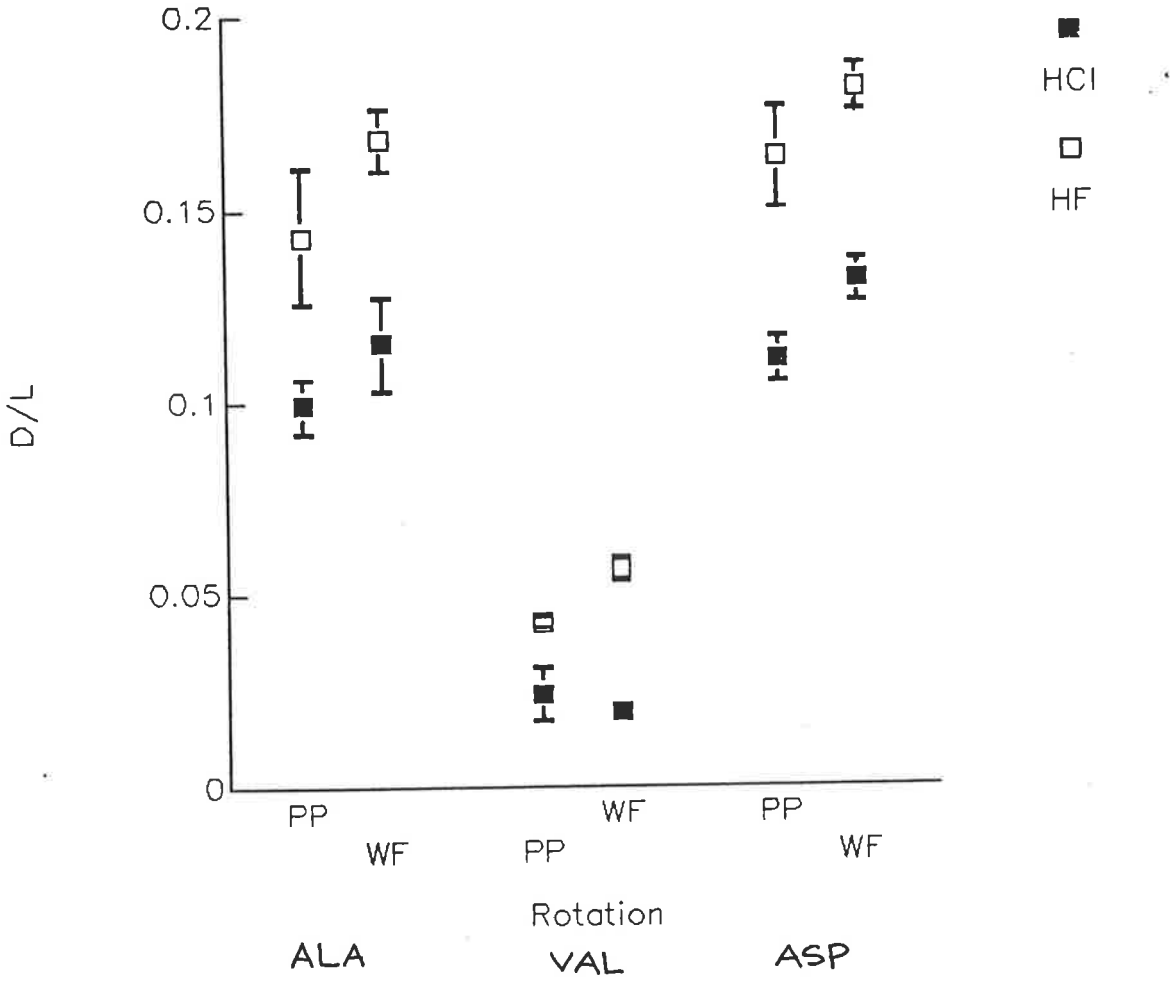



Fig. 4.3 D/L ratios (mean and standard deviation) of three amino acids from the hydrolytic fractions of pyrophosphate humic acids from the WF and PP soils.

## 4.4 CONCLUSIONS

These investigations indicate the degree of racemization of a soil may not be best described solely with reference to a readily hydrolysable fraction. Treatment of the residue from the initial hydrolysis with hydrofluoric acid provided a second fraction for analysis. Although not accurately quantified by this work the relative amount of amino acids released into each fraction was approximately 10:1 (HCl:HF).

Given the complexity of soil and the central role of amino acids in many biochemical pathways, it is expected that individual amino acids will display different racemization characteristics. Not only do rates of racemization differ between amino acids, metabolic pathways differ due to differing biochemical roles. There was however some consistency in the degree of racemization of individual amino acids and known age effects in soils.

This research indicates differences in the degree of racemization of soils and soil fractions/separates parallel age effects as evident from the literature on radiocarbon dating discussed in Section 2.4.

### 4.4.1 Hydrolytic effects

The evidence indicates that the hydrolytic fractions from a single soil/fraction/separate may be expected to have different degrees of racemization with the HF fraction having a higher D/L ratio. This is consistent with trends established by radiocarbon data as discussed in Section 2.4.2.

HCl fractions from both the untreated soils and the pyrophosphate humic acids of the Waite agrisequence (WF and PP) had similar D/L ratios. Generally acid – hydrolysable soil organic matter is considered to be dominated by recent additions, partly humified plant fragments, the active biomass *etc.* (Goh and Pullar 1977), which result in a modern radiocarbon age assignation (Martel and Paul 1974). If, as suggested by some workers, the degree of racemization of the amino acids in soil is some indicator of the age of the soil organic matter (Mahaney et al. 1986), then the D/L values of the HCl fraction must equate with modern

values for this soil. That these values were not zero is partially accounted for by contributions of modern D – isomers from micro – organisms as discussed in Section 2.2.3.1, and, to a minor extent, by racemization induced by experimental procedures as discussed in Section 3.4.2.

### 4.4.2 Agricultural effects

Evidence from both of the agrisequences analysed indicates an inverse relationship between the intensity of management and the heterogeneity of fractions. This was most clearly demonstrated by racemization of leucine and valine in the Waite agrisequence. Not only did hydrolytic fractions of these soils display this relationship (Table 4.2) it was also demonstrated by the particle size analysis (Fig. 4.2). This same relationship was demonstrated in the Rothamsted agrisequence with reference to aspartic acid (Fig. 4.1).

The Rothamsted sequence was distinctive in that the HCl fractions of BBW and BB03 showed the rejuvenation of the organic matter under wilderness and the depletion of this same pool under cultivation as predicted by Martel and Paul (1974b).

The literature lacks evidence to adequately explain the differences noted between the soils of the Waite agrisequence. That the HF fraction of WF soil had lower D/L ratios than the PP HF may be explained by consideration of the exploitative nature of the systems. The more stable system (PP) involves minimal externally initiated pedoturbation, carbon resources parallel nitrogen resources. The physical stability enhances the opportunities of microniche development in which amino acid nitrogen is permitted to age (racemize). This potential is reduced in the exploitative systems by a combination of physical disruption by cultivation and irregular inputs of high C:N material (stubble, roots, etc.). To utilize this carbon flush, nitrogen is used and nitrogen demand increases. Cultivation reorganises the three – dimensional framework of the soil thus exposing old organic matter to renewed activity. It is suggested that these factors increased the vulnerability of the so – called protected amino acid nitrogen to microbial attack, reducing the D/L values as noted.

### 4.4.3 Particle size separates

The relationship noted amongst particle size separates parallels the radiocarbon age effect as indicated by Scharpenseel (1972) and Anderson and Paul (1984) in that silt separates generally had higher D/L ratios than either smaller or larger separates.

### 4.4.4 Classical fractions

Extraction procedures involving only pyrophosphate may be deployed as an appropriate pretreatment before amino acid racemization analysis. Extractions involving alkali should not be used.

The previous suggestion (Kimber and Searle 1970) that the humic acid of the wheat/fallow may be older was supported by racemization data.

Hydrofluoric treatment of pre-hydrolysed humic acids provided a second fraction of amino acids which were then vulnerable to hydrolysis. The degree of racemization of this second fraction was higher than that of the first hydrolysis. This racemization effect is paralleled by evidence from radiocarbon dating as discussed in Section 2.4.4.

# CHAPTER 5 PYROLYSIS STUDIES OF THE RACEMIZATION PROCESS IN THE PASTURE SOIL

## 5.1 INTRODUCTION

The requirement for kinetic information was recognised as one of the aims of this research. The importance of kinetic information is highlighted by the literature review presented in Chapter 2. Kinetic information was considered essential to resolve three important lines of evidence developing in the contemporary literature on amino acid racemization in soils. This evidence is,

(i) analysis of top soils indicates the D/L ratios of the HF fraction are generally higher than those of the HCl fraction (Griffin and Kimber 1988).

(ii) analysis of paleosols, using the same methods, indicates the HCl fraction generally has the higher D/L ratios (Kimber and Griffin 1988).

(iii) published D/L ratios were generally lower than may have been predicted from racemization studies of molluscs. This is particularly evident in the work of Mahaney (Mahaney et al. 1986).

Little is known about racemization in soils. Pyrolysis experiments provide data whereby racemization kinetics can be assessed. Similar experiments have been used to help elucidate the complexity of the racemization process that occurs during the aging of other fossil materials (Vallentyne 1969; Hare and Mitterer 1969; Bada 1972; Turekian and Bada 1972; Williams and Smith 1977; Kriausakul and Mitterer 1980; Kimber et al. 1986; Kimber and Griffin 1987). Indeed pyrolysis studies are essential in age determination as described by Kvenvolden et al. (1979, 1981).

The pyrolysis experiments described in this chapter provide data whereby the racemization processes of the pasture soil and other fossil materials can be directly compared in terms of rate and complexity.

The pyrolysis system is a closed system. The chemical processes are enhanced by heating the system. While there may be some doubt whether the pyrolysis conditions reflect what has happened prior to analysis there is no doubt that the data gathered during the experiment reflect the conditions at the start of the experiment. Pyrolysis experiments provide useful information to elucidate the processes involved and may provide evidence to rationalise previous observations.

Pyrolysis experiments enhance the chemical components of the racemization process and thus may approximate the field situation of a well isolated paleosol only. In this situation biological influences are minimal.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Temperature/fractions and time treatments

Experiments of similar design were performed at three different temperatures (105$^{o}$C, 140$^{o}$C and 160$^{o}$C). These experiments are referred to as H105, H140 and H160 respectively.

One gram samples of the PP soil described in Section 4.2.1, were weighed into Corningware hydrolysis tubes, 5ml of water was added and the tubes sealed with screw caps with teflon inserts. The tubes were then randomised and heated either in an oven (H105) or using an aluminium heating block (H140, H160). Tubes were withdrawn after completion of the time treatment and stored in a cold room at 4$^{o}$C. The time treatments used were 1, 2, 4, 8, 16, 32, 64 days (H105); 1, 2, 4, 8, 16, 32 days (H140) and 1, 2, 4, 8, 16 days (H160). These treatments are referred to as $T_1$ to $T_7$ where $T_1$ is a one day treatment and $T_7$ is a 64 day treatment.

The two hydrolytic fractions were isolated from each temperature/time treatment and are referred to as temperature/fractions. The scheme which led to the separation and clean-up of the hydrolytic fractions has been described

previously and is detailed in Appendix 1. The solvent extraction, Step 2.8, was not performed routinely during experiments H140 and H160.

Standards were run frequently both to confirm retention times and to give some credence to the presented concentration data.

Zero time treatments had been analysed previously. The details of this analysis are presented in Section 4.3.2.

### 5.2.2 D/L data : transformation and test for first order kinetics

D/L ratios were transformed to fit the ordinate of Equation 2.1 *i.e.*

$$y = (1+K')^{-1} \ln \frac{1+D/L}{1-K'(D/L)} \qquad 5.1$$

where
y is the transformed D/L ratio
$K' = K_D/K_L = 1/K_{eq}$ and is the reciprocal of the equlibrium constant (unity for enantiomers, 0.8 – 0.7 for isoleucine) and
D/L is as measured.

Equation 2.1 thus reduces to the form

$$y = kt + c \qquad 5.2$$

where
y is the transformed D/L data,
k the rate constant, is the slope,
t is time (seconds) and,
c is a constant to account for D/L at time zero.

When a significant straight line is indicated the studied reaction is said to be first order and age determination is relatively simple. Although racemization in natural systems is a complex process, any complex process can be approximated

by a simple (first order) process over a short distance. The method used for uncalibrated age determination is described in Section 2.3.1.

Where first order kinetics were found to be inappropriate to describe the racemization processes observed, a cumulative regression analysis was performed. This analysis involved regression of data from $T_0$ to $T_x$ where $T_x$ is each time treatment.

Change in rate was indicated by changes in the slope (m) of Equation 5.3.

$$\log k = m \log t + c \qquad 5.3$$

where
k is the rate calculated by the cumulative regression analysis
t is time
c is some constant
m is obtained by regression of the data collected to each time treatment (cumulative regression).

### 5.2.3 Concentration data

Concentration data was calculated using the peak area obtained from each treatment for each enantiomer/epimer. The data are presented as relative concentration. The relative concentration is defined as,

$$\text{relative concentration} = \text{Area}_i \,/\, \text{Area}_{IS} \qquad 5.4$$

where
$\text{Area}_i$ is the peak area of the component of interest and
$\text{Area}_{IS}$ is the peak area of the internal standard.

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Racemization of amino acids in soils

D/L ratios of seven amino acids from the two hydrolytic fractions for the three temperature experiments are recorded in Appendix 3A. Some of the D/L ratios for zero time treatments have been published (Griffin and Kimber 1988). These data, together with unpublished data for phenylalanine and glutamic acid were transformed to fit Equation 5.1 and also are recorded in Appendix 3A.

The transformed data together with the details of the first regression analysis (the slope, Pearson's correlation coefficient, and the significance of the regression based on this coefficient) are recorded in Appendix 3B.

These data are summarised in Table 5.1 as first order rate constants and the significance of this constant in describing the observed reaction. The analysis did not include zero time values, instead the analysis was used to predict zero time values. This provided a second measure of the accuracy of first order kinetics in describing the racemization process in soils.

Generally D/L values increased with the passage of time, a pattern reflected by the predominance of positive rate constants. This general increase was present in both of the hydrolytic fractions.

| Fraction | Amino acid | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| H105HCl | 0.33*** | 0.15*** | 0.30*** | 0.28*** | 0.23 | 0.39*** | 0.34* |
| H140HCl | 0.31* | 0.41* | 0.28 | | −0.55* | 0.53* | 0.78 |
| H160HCl | 3.81*** | 2.08*** | 1.83*** | 2.88*** | 0.47 | 2.08 | −0.03 |
| H105HF | 0.20*** | 0.08*** | 0.13*** | 0.13** | −0.33*** | 0.15 | 0.12 |
| H140HF | 0.20 | 0.20* | 0.10 | | −0.13 | 0.42* | 0.84* |
| H160HF | 1.12** | 0.52** | −0.06 | 0.51 | −1.53* | 0.50 | 1.93 |

Table 5.1 Racemization rate constants ($x10^{-2}$) (and their significance) of seven amino acids from the hydrolytic fractions analysed from each temperature treatment. Significance was measured as Pearson's correlation coefficient (r) and is noted

( * $P > 0.05$, ** $P > 0.01$, *** $P > 0.001$ ).

Racemization of alanine, valine and leucine and epimerization of isoleucine followed first order kinetics in both the HF and HCl fractions at 105°C. The rates of racemization in the HCl fraction were approximately double those in the HF fraction (1.7, 1.9, 2.2 and 2.3 times respectively).

This pattern was repeated by alanine and valine at 160°C. The difference in rates of racemization of these two amino acids from the two fractions was larger (3.4 and 4.0 times respectively).

These data resolved the superficial dichotomy between the report of Griffin and Kimber (1988), who record D/L HCl < D/L HF in agricultural soils, and the report of Kimber and Griffin (1988), who record D/L HCl > D/L HF in paleosols.

The data from the pyrolysis experiments support the contention that the hydrolytic fractions are kinetic fractions. Each fraction has distinctive rates of racemization. The rate of racemization of the HF fraction was lower than that of the HCl fraction. The D/L ratio of the HCl fraction increased faster than that of the HF fraction. Thus in a modern soil D/L HF > D/L HCl which may be due to an age effect (Griffin and Kimber 1988), racemization is slower in the HF fraction than in the HCl fraction (this work), and thus D/L HCl > D/L HF in paleosols (Kimber and Griffin 1988). Data from all the amino acids analysed support this (Fig. 5.1).

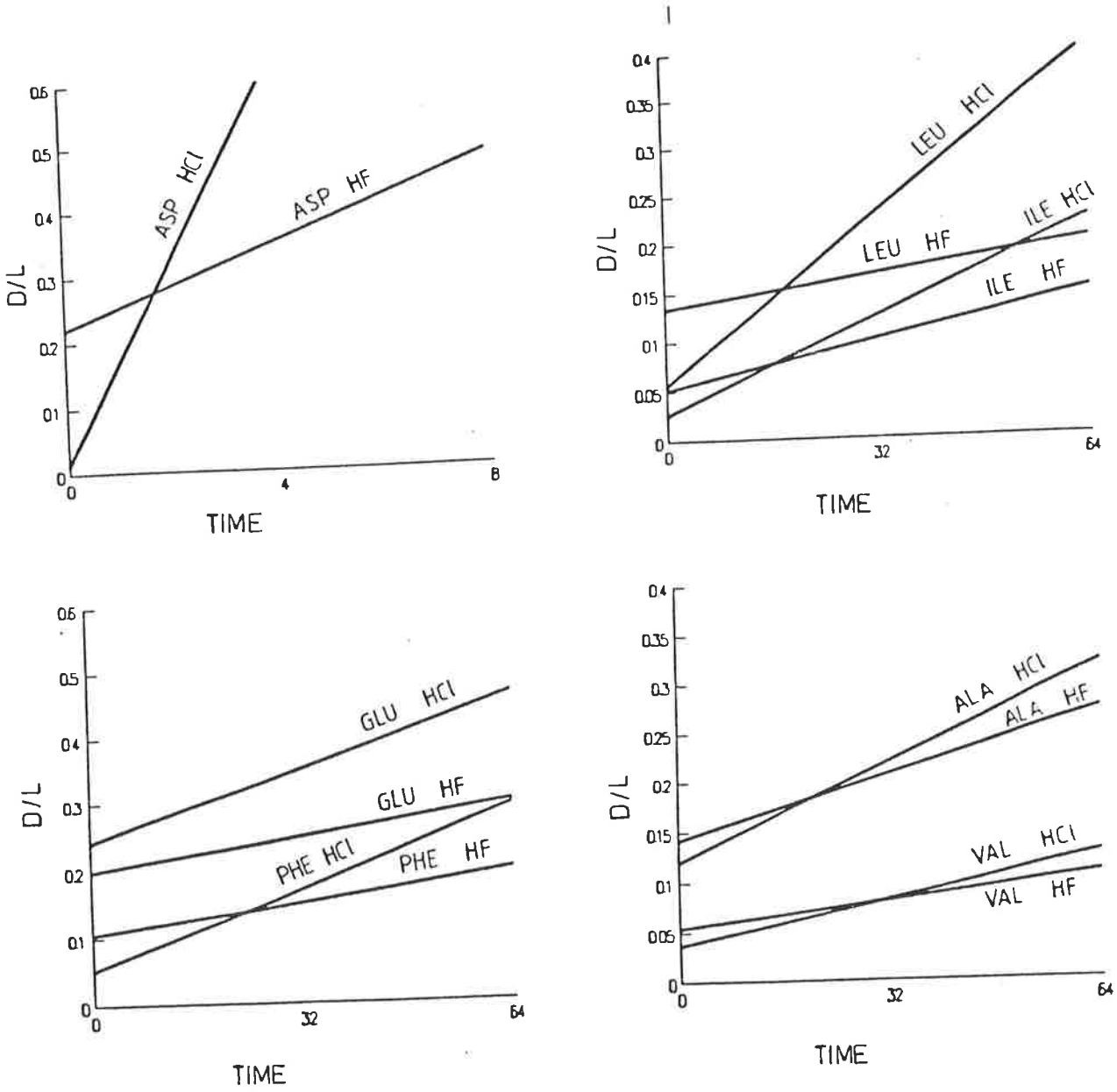



Fig. 5.1 Comparison of the apparent rate of racemization (D/L vs Time) of the hydrolytic fractions after pyrolysis at 105°C.

The rate of racemization was found to be temperature dependent with the higher rates calculated from higher temperature experiments. Racemization of valine provided the most consistent evidence of first order kinetics in both fractions over the three temperatures.

Racemization of aspartic acid did not follow simple kinetics. Not only did the regression coefficients generally lack significance the negative slope indicated decreasing D/L values with time. The regression analysis did not closely predict the observed zero time values (Table 5.2).

Epimerization of isoleucine followed first order kinetics in three of the six treatments.

Racemization of glutamic acid generally did not follow the simple model nor did racemization of phenylalanine in the HF fraction. This was at least partly associated with the general difficulties in resolution of these amino acids (Section 3.4.1). Difficulty increased with the higher temperatures suggesting a diagenetic origin to this problem.

Despite this, racemization of phenylalanine in H105 HCl and H160 HCl did fit first-order kinetics. The temperature dependence was of a similar order to other amino acids *i.e.* valine, isoleucine.

Poor resolution of leucine (D-leucine from L-proline and L-leucine from L-serine) prevented analysis of racemization of leucine in H140 and undoubtedly detracted from the significance of H160.

The observed zero time values are compared with those predicted from the simple linear regression of the transformed data in Table 5.2. Generally the regression analysis predicted a higher degree of racemization than was observed. H105 HF was the only temperature/fraction which consistently predicted lower values for all amino acids except alanine. This supports the existence of a general bias in the data and indicated that first order kinetics only provide a crude approximation of the racemization process. Deviation from first order kinetics are qualified by examination of the plotted D/L means (Figs. 5.2, 5.3, 5.4 and 5.6) and by reference to cumulative regression data of the more significant amino acids (Tables 5.3, 5.4 and 5.5).

| Fraction | Temp. | Amino acid | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| Predicted | HCl | | | | | | | |
| | 105°C | 0.12 | 0.04 | 0.03 | 0.06 | 0.26 | 0.05 | 0.27 |
| | 140°C | 0.20 | 0.07 | 0.13 | | 0.65 | 0.22 | 0.48 |
| | 160°C | 0.16 | 0.04 | 0.13 | −0.01 | 0.47 | 0.04 | 0.53 |
| Observed | HCl | 0.11 | 0.03 | 0.03 | 0.06 | 0.12 | 0.04 | 0.23 |
| Predicted | HF | | | | | | | |
| | 105°C | 0.15 | 0.05 | 0.13 | 0.13 | 0.40 | 0.10 | 0.22 |
| | 140°C | 0.19 | 0.08 | 0.10 | | 0.32 | 0.17 | 0.20 |
| | 160°C | 0.24 | 0.10 | 0.17 | 0.23 | 0.41 | 0.24 | 0.25 |
| Observed | HF | 0.13 | 0.07 | | 0.17 | 0.13 | 0.12 | 0.23 |

Table 5.2 Comparison of zero time D/L ratios as predicted from linear regression of pyrolysis data to the observed zero time values, as recorded in Tables 4.2 and 4.4.

5.3.1.1 Alanine

The regression analysis indicates data for racemization of alanine generally conformed to first order kinetics. A significant linear relationship was evident for both fractions from the two of temperature treatments H105 and H160 (Table 5.1). Results from both fractions of H140 suggested a more complex process may have been responsible (Fig. 5.2).

The results from a cumulative regression of the data are presented in Table 5.3. Data from $T_0$ to $T_x$ (where $T_x$ is the time treatment) were subjected to a simple linear regression analysis which provided cumulative regression coefficients and cumulative rate constants.

Deviation from first order kinetics in H140 is emphasised by the decrease in significance of the regression coefficient after $T_4$ (day 8). This was accompanied by a large difference between the predicted $T_0$ value and that recorded (Table 5.2). Results from H160 HF fit a similar pattern however the deviation occurred earlier (after $T_3$, day 4) and was less severe. This pattern was not repeated in the other temperature/fractions where the regression coefficient remained high (H105 HCl and H160 HCl) or improved as more data was analysed (H105 HF).

The rate constant as calculated varied with time. Generally the rate of racemization of alanine decreased with time. This relationship, when modelled as Equation 5.3, was significant ($P > 0.02$) for each temperature/fraction.

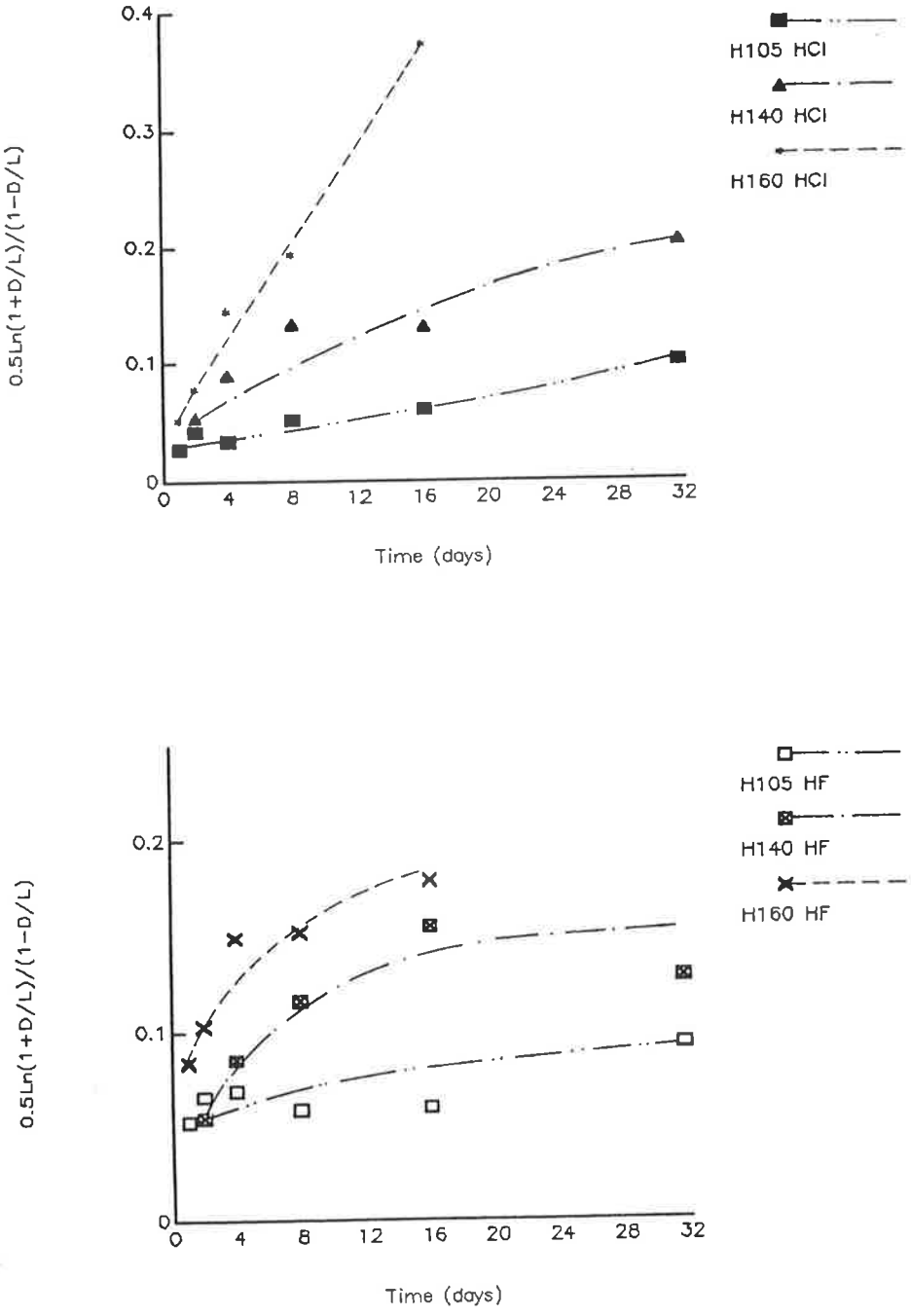



Fig. 5.2 Change in D/L alanine in the hydrolytic fractions (HCl top, HF bottom) of samples from the three temperature treatments.

| Time Treatment | | Temperature/Fraction H105 HCl | H105 HF | H140 HCl | H140 HF | H160 HCl | H160 HF |
|---|---|---|---|---|---|---|---|
| $T_0 - T_2$ | r | 0.73 | 0.37 | | | 0.98 | 0.98 |
| | k | 0.0074 | 0.01 | | | 0.054 | 0.051 |
| | C | 0.106 | 0.126 | | | 0.107 | 0.134 |
| $T_0 - T_3$ | r | 0.90 | 0.56 | 0.99 | 0.89 | 0.99 | 0.97 |
| | k | 0.0069 | 0.0084 | 0.027 | 0.023 | 0.060 | 0.051 |
| | C | 0.107 | 0.128 | 0.104 | 0.110 | 0.102 | 0.134 |
| $T_0 - T_4$ | r | 0.98 | 0.70 | 0.97 | 0.93 | 0.99 | 0.88 |
| | k | 0.0083 | 0.0069 | 0.019 | 0.019 | 0.049 | 0.027 |
| | C | 0.104 | 0.130 | 0.120 | 0.117 | 0.118 | 0.177 |
| $T_0 - T_5$ | r | 0.67 | 0.69 | 0.74 | 0.56 | 0.99 | 0.82 |
| | k | 0.0025 | 0.0034 | 0.0058 | 0.0065 | 0.040 | 0.013 |
| | C | 0.120 | 0.138 | 0.184 | 0.159 | 0.146 | 0.216 |
| $T_0 - T_6$ | r | 0.92 | 0.86 | 0.67 | 0.54 | | |
| | k | 0.0041 | 0.0032 | 0.0031 | 0.0025 | | |
| | C | 0.112 | 0.139 | 0.202 | 0.18 | | |
| $T_0 - T_7$ | r | 0.95 | 0.88 | | | | |
| | k | 0.0033 | 0.0021 | | | | |
| | C | 0.120 | 0.147 | | | | |
| Regression log k vs. $\log_2 t$ (days) | | | | | | | |
| | r | −0.85 | −0.99 | −0.995 | −0.97 | −0.91 | −0.95 |
| | P > | 0.01 | 0.001 | 0.001 | 0.02 | 0.02 | 0.01 |

**Table 5.3** Cumulative regression coefficients (r), rate constants (k) and estimated $T_0$ values (C) for the six temperature/fractions from the transformed D/L data of alanine.

Rate constants were regressed to fit equation 5.3. The significance of this regression is recorded and is a measure of the variation in the rate constant as calculated from cumulative data.

5.3.1.2 Valine

Clearly a first order reaction may be appropriate to describe racemization of valine in the HCl fraction of the two experiments H105 and H160 (Fig 5.3, Table 5.4). The equation describing the change in degree of racemization as calculated from the degree of racemization as measured in each time treatment of the two experiments predicted a time zero value of 0.037 which is within the error margin of the previously recorded value ($0.027 +/- 0.011$, Table 4.2).

Racemization of valine in the HCl fraction of H140 was not linear. The data were biased towards a dual linear form. Restricting the regression to the early data (days 2, 4 and 8) while not improving the measured significance did better predict the zero time value (0.031 compared to 0.062). The rate constant calculated from the restricted data set was 0.013 ($day^{-1}$).

This bias was evident in H105 HCl. The rate constant as calculated varied with time. Generally the rate of racemization decreased with time. This relationship, when modelled as Equation 5.3, was significant ($P > 0.01$).

There was no significant bias apparent in the racemization of valine in H160 HCl. All of the rates calculated in the analysis were within $0.024 +/-$ *ca.*20%.

The equation derived to describe racemization of valine in the HF fraction of H105 closely predicted the zero time value of 0.062 ($+/- 0.018$). Data from the other two temperature treatments were not as closely predicted by the derived equations nor was the zero time value. There is some suggestion of curvature of the early part of the curves describing racemization of valine in the HF fraction of H140 and H160.

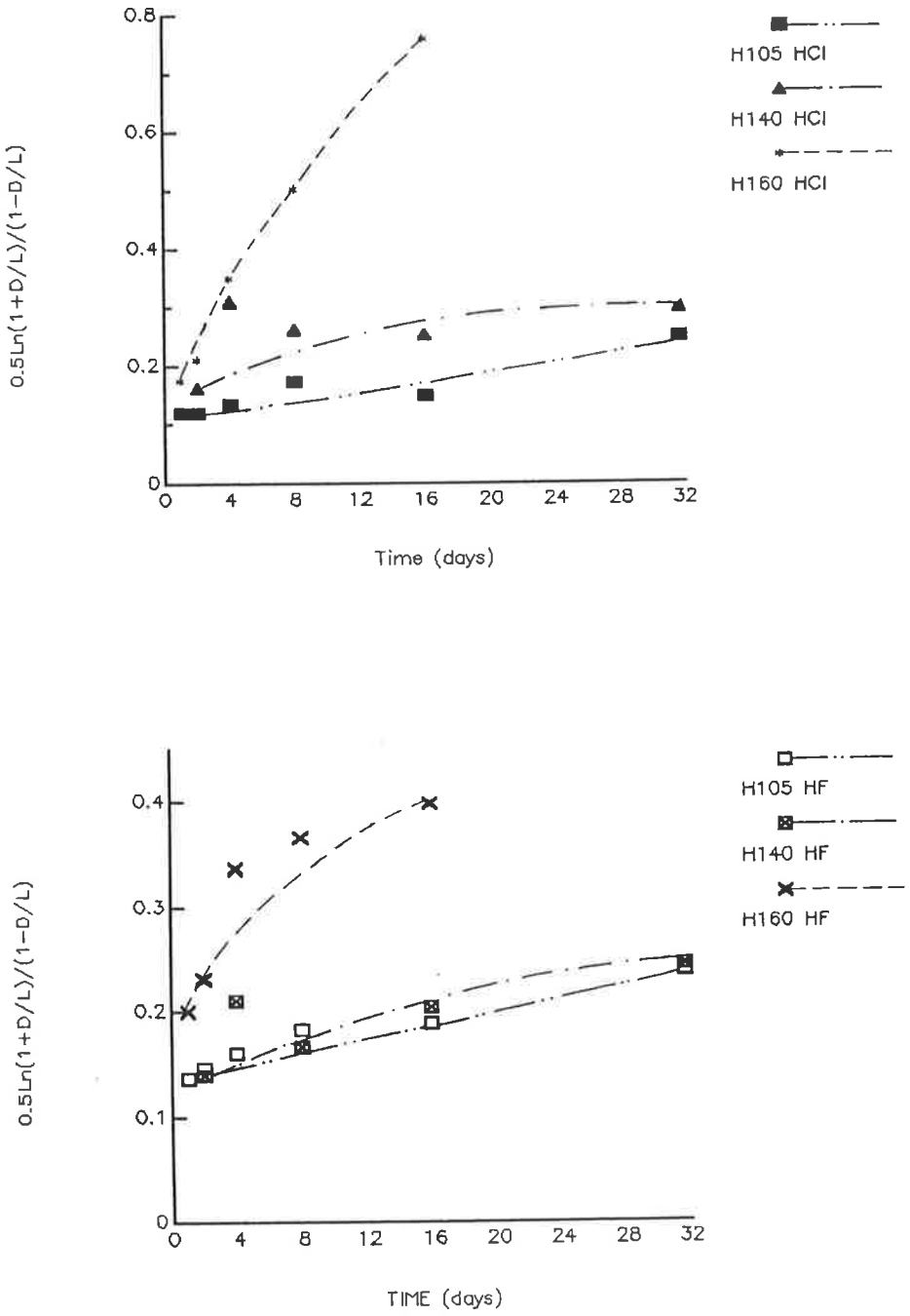



Fig. 5.3 Change in D/L valine in the hydrolytic fractions (HCl top, HF bottom) of samples from the three temperature treatments.

| Time Treatment | | Temperature/Fraction H105 HCl | H105 HF | H140 HCl | H140 HF | H160 HCl | H160 HF |
|---|---|---|---|---|---|---|---|
| $T_0 - T_2$ | r | 0.86 | | | | 1.00 | 0.91 |
| | k | 0.0085 | | | | 0.025 | 0.020 |
| | C | 0.023 | | | | 0.027 | 0.063 |
| $T_0 - T_3$ | r | 0.68 | 0.56 | 1.00 | 0.71 | 1.00 | 0.87 |
| | k | 0.0034 | 0.0049 | 0.016 | 0.0070 | 0.029 | 0.022 |
| | C | 0.028 | 0.049 | 0.025 | 0.052 | 0.023 | 0.061 |
| $T_0 - T_4$ | r | 0.86 | 0.33 | 0.87 | 0.94 | 0.97 | 0.78 |
| | k | 0.0030 | 0.0016 | 0.013 | 0.0083 | 0.020 | 0.010 |
| | C | 0.028 | 0.054 | 0.030 | 0.049 | 0.036 | 0.082 |
| $T_0 - T_5$ | r | 0.81 | 0.18 | 0.75 | 0.96 | 0.99 | 0.79 |
| | k | 0.0019 | 0.0003 | 0.0067 | 0.0067 | 0.021 | 0.006 |
| | C | 0.032 | 0.057 | 0.052 | 0.054 | 0.034 | 0.094 |
| $T_0 - T_6$ | r | 0.88 | 0.73 | 0.83 | 0.73 | | |
| | k | 0.0021 | 0.0010 | 0.0047 | 0.0021 | | |
| | C | 0.031 | 0.053 | 0.062 | 0.076 | | |
| $T_0 - T_7$ | r | 0.89 | 0.84 | | | | |
| | k | 0.0014 | 0.0008 | | | | |
| | C | 0.037 | 0.055 | | | | |
| Regression log k vs. $\log_2 t$ (days) | | | | | | | |
| | r | −0.95 | −0.63 | −0.98 | −0.79 | −0.70 | −0.93 |
| | P > | 0.001 | n.s | 0.01 | n.s. | n.s | 0.05 |

Table 5.4 Cumulative regression coefficients (r), rate constants (k) and estimated $T_0$ values (C) for the six temperature/fractions from the transformed D/L data of valine.

Rate constants were regressed to fit equation 5.3. The significance of this regression is recorded and is a measure of the variation in the rate constant as calculated from cumulative data.

5.3.1.3 Aspartic acid

First order kinetics were not appropriate to describe the behaviour of aspartic acid during the course of these experiments. Racemization of aspartic acid may have approximated first order kinetics only in the very early part of the temperature treatments. During this time the rate of racemization was very high. Regression of zero time data and that from day 1 and day 2 of H105 HCl revealed a rate of 0.127 $(day)^{-1}$. This was two orders of magnitude larger than other rates recorded at this temperature and one order of magnitude larger than rates for racemization of other amino acids at 160°C (Table 5.1).

Examination of the transformed data as plotted against time reveals that the degree of racemization generally peaked and then declined (Fig. 5.4). This pattern is evident in all of the HF fractions and in H140 HCl. An incomplete data set prevented the resolution of the detail in H105 HCl, the data which were recorded do fit the pattern. There is no clear pattern revealed by the data from H160 HCl.

Racemization of aspartic acid as recorded by these experiments was not predicted by either first order kinetics or the more complex models of Wehmiller (1981, 1982, 1984) and Kvenvolden et al. (1979, 1981). The apparent reversal in racemization with time is not uncommon having been noted by racemization/pyrolysis studies of a variety of species of molluscs (Kimber et al. 1986; Kimber and Griffin 1987).

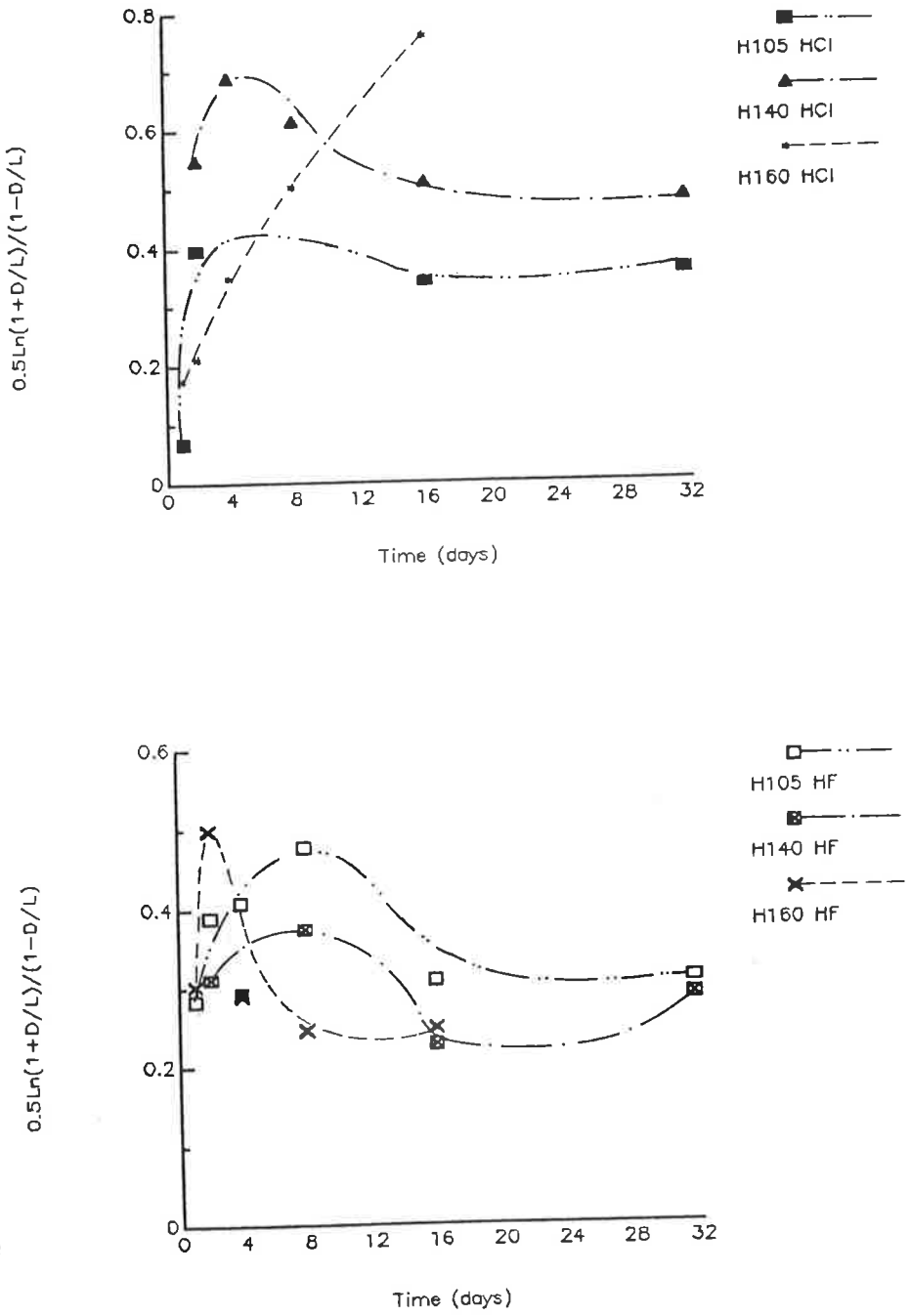



Fig. 5.4 Change in D/L aspartic acid in the hydrolytic fractions (HCl top, HF bottom) of samples from the three temperature treatments.

5.3.1.4 Isoleucine

Isoleucine was described as a technically unreliable amino acid in Section 3.4.1. Nevertheless the data from these pyrolysis investigations warrant further examination. As mentioned in Section 3.4.1.3, capillary columns of the type used vary considerably in their retention characteristics. The pyrolysis studies were performed using a different capillary column (same type) to the replication experiment. Resolution characteristics of these columns were slightly different. D – alloisoleucine still appeared as a peak on the tail of the glycine peak however resolution of the peaks was generally better. Some of the experimental error was a direct result of small differences in the integration of the D – alloisoleucine peak. Although the differences were small in terms of area of the peaks, the peak was generally very small and the errors in terms of percentage difference thus became quite large. This led to large variance in D/L ratios. However there are definite trends established by this amino acid (Fig. 5.5).

The cumulative regression (Table 5.5) indicates that the first order kinetics, initially indicated in Table 5.1 as being appropriate to describe the epimerization process in H105 HCl and HF, were not established until near the end of the experiment. There was no consistent variation in the rates of epimerization within these two temperature/fractions. These features detract from the validity in application of first order kinetics in describing epimerization in these temperature/fractions.

There was no consistent trend in the epimerization measured in H160 HF. All the values fell within the range of 0.14 to 0.18. By the end of the cumulative regression a negative rate constant was indicated. At no time did the model predict the zero time value accurately.

The first approximation describes the epimerization process which occurred in the earlier time treatments of H140 HCl and HF and H160 HCl (to $T_4$, $T_5$ and $T_5$ respectively). During the early phase (to $T_4$ and $T_3$) of the HCl fractions of H140 and H160 the regression accurately predicted the zero time value. After this early phase the rate decreased. The process of epimerization in these temperature/fractions must be interpreted in terms of dual linear or curvi – linear kinetics.

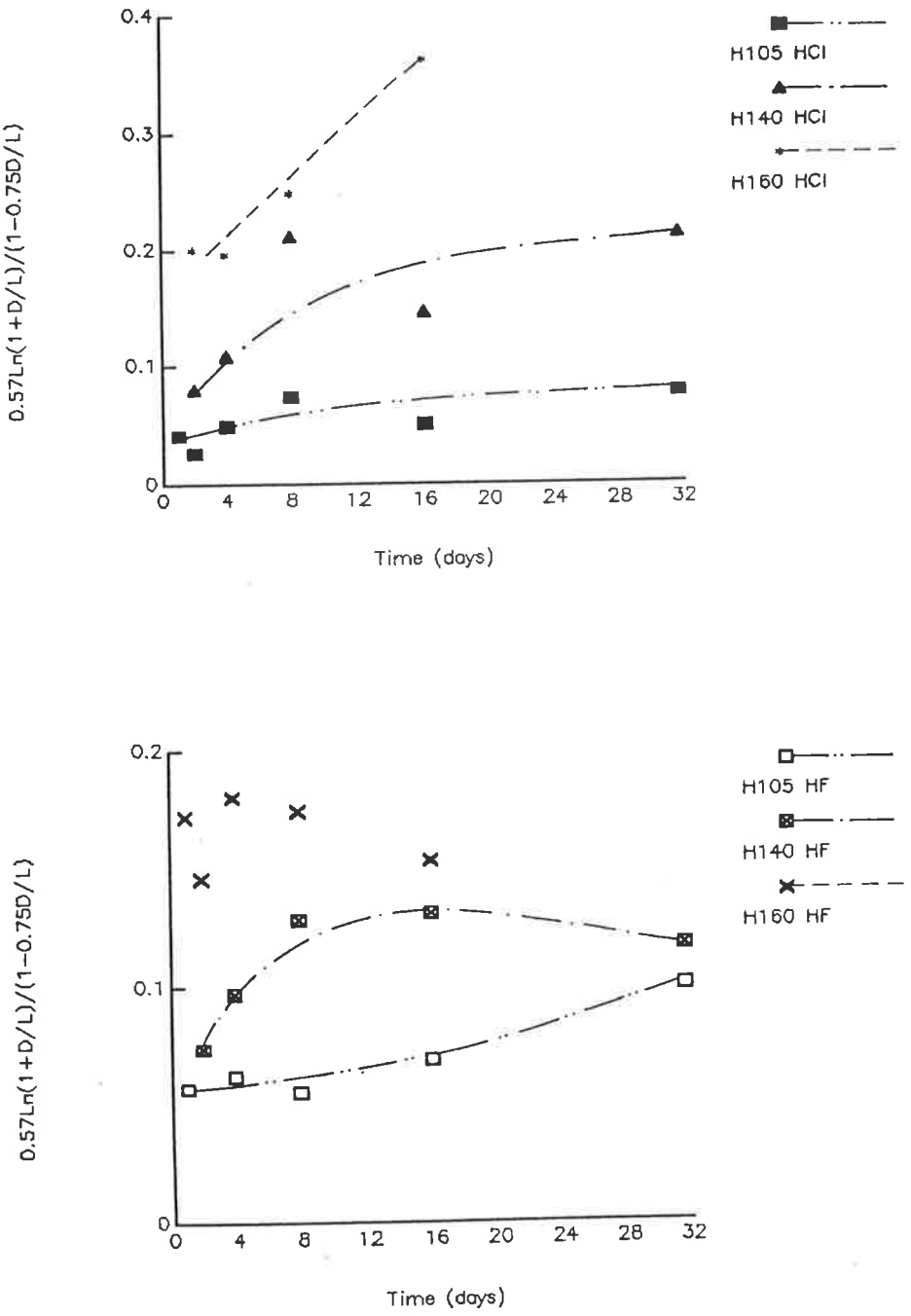



Fig. 5.5 Change in D – allo/L – isoleucine in the hydrolytic fractions (HCl top, HF bottom) of samples from the three temperature treatments.

| Time Treatment | | Temperature/Fraction H105 HCl | H105 HF | H140 HCl | H140 HF | H160 HCl | H160 HF |
|---|---|---|---|---|---|---|---|
| $T_0-T_2$ | r | | | | | | 0.90 |
| | k | | | | | | 0.086 |
| | C | | | | | | 0.0033 |
| $T_0-T_3$ | r | 0.49 | 0.22 | 0.98 | 0.98 | 0.85 | 0.21 |
| | k | 0.0041 | 0.0016 | 0.020 | 0.013 | 0.047 | 0.0042 |
| | C | 0.028 | 0.055 | 0.031 | 0.049 | 0.034 | 0.162 |
| $T_0-T_4$ | r | 0.84 | 0.01 | 0.88 | 0.98 | 0.88 | 0.08 |
| | k | 0.0058 | 0.0000 | 0.023 | 0.0087 | 0.037 | 0.0007 |
| | C | 0.026 | 0.058 | 0.027 | 0.060 | 0.048 | 0.17 |
| $T_0-T_5$ | r | 0.41 | 0.34 | 0.56 | 0.84 | 0.83 | −0.30 |
| | k | 0.0013 | 0.0008 | 0.0082 | 0.0042 | 0.019 | −0.0014 |
| | C | 0.038 | 0.056 | 0.079 | 0.078 | 0.101 | 0.18 |
| $T_0-T_6$ | r | 0.83 | 0.74 | 0.62 | 0.52 | | |
| | k | 0.0024 | 0.0014 | 0.0039 | 0.0010 | | |
| | C | 0.032 | 0.053 | 0.101 | 0.096 | | |
| $T_0-T_7$ | r | 0.95 | 0.88 | | | | |
| | k | 0.0030 | 0.0015 | | | | |
| | C | 0.026 | 0.051 | | | | |
| Regression log k vs. $\log_2 t$ (days) | | | | | | | |
| | r | 0.95 | 0.63 | 0.98 | 0.79 | 0.70 | 0.93 |
| | P> | 0.01 | n.s | 0.01 | n.s. | n.s | n.s |

Table 5.5 Cumulative regression coefficients (r), rate constants (k) and estimated $T_0$ values (C) for the six temperature/fractions from the transformed D/L data of isoleucine.

Rate constants were regressed to fit equation 5.3. The significance of this regression is recorded and is a measure of the variation in the rate constant as calculated from cumulative data.

### 5.3.2 Concentration Data

The relative concentration of the enantiomers of six amino acids from the two fractions of H140 and H160 is presented in Figure 5.6. Concentration data extracted from the racemization studies discussed in the previous chapters of this thesis were generally too crude to use in evidence and were therefore not presented. The data from H140 and H160 were found to be less variable than had been encountered in earlier experiments. This indicates that, by not routinely performing the solvent extraction as mentioned in Section 5.2, losses of amino acids in these two experiments were reduced and concentration data for some amino acids follow predictable trends.

The data from replicate treatments of the heating experiments are very variable. A detailed assessment of RF was therefore considered unnecessary and the data are presented as relative concentration, calculated using Equation 5.4 and should be used only to establish trends. It should be noted that the amount of L – NLE used in the HCl fraction is one order of magnitude greater than that used in the HF fraction. Therefore, a factor of ten must be taken into account when comparing the concentration data of these fractions.

There are three important aspects of these data which have strong implications on D/L ratios:

(i) There was a general decrease in the concentration of L – enantiomers. Changes in concentrations of D – enantiomers were more complex. Concentrations of D – enantiomers of some amino acids remained constant, some increased, some decreased and some initially increased before decreasing.

(ii) Although the concentration data from the HF fraction were more variable, the general decline in the concentration of L – enantiomers of the HF fraction was less than that of the HCl fraction.

(iii) The general decline in concentration of aspartic acid was larger than the other amino acids.

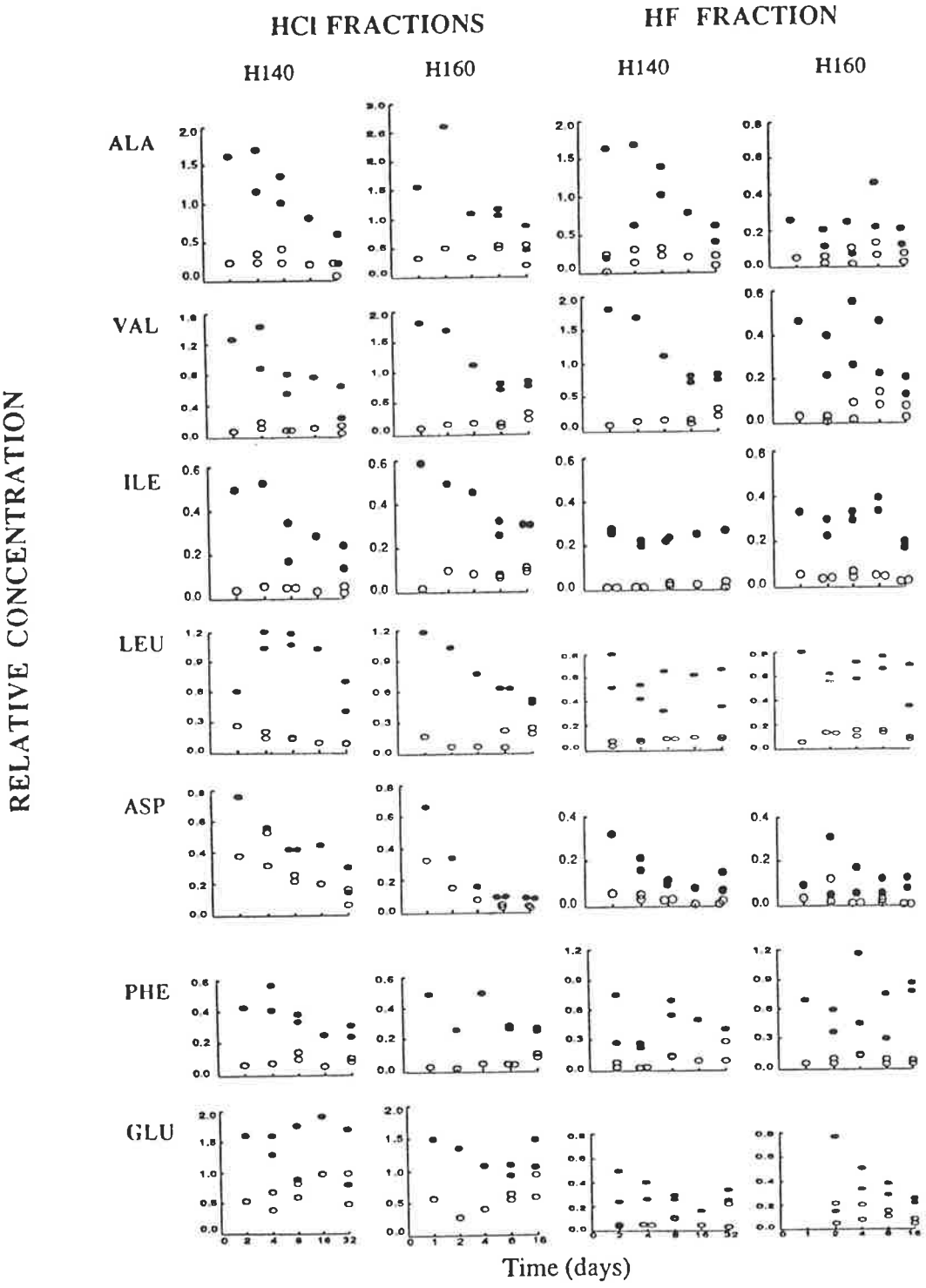



Fig. 5.6 Relative concentration of each enantiomer of seven amino acids (0 D, ● L) from the hydrolytic fractions of samples from two temperature treatments.

5.3.2.1 Mechanisms for change : Racemization, decarboxylation and deamination.

The concentration data highlight how the degree of racemization can change without the involvement of the racemization reaction as depicted in Figure 1.1.

A general decline in concentration of L – enantiomers was expected. The system was closed to input and amino acids were lost via deamination and decarboxylation. Racemization resulted in a further decline in the concentration of L – isomers.

Conversely, racemization resulted in a general increase in the concentration of D – enantiomers. When deamination and decarboxylation of the D – enantiomer were less than the increase due to racemization a nett increase in concentration of D – amino acid was evident. Similarly a nett decrease or an equilibrium situation can be explained.

Usually degradation of amino acids, *e.g.* deamination and decarboxylation, occurs only when the amino acid is in a terminal position on a peptide chain or is a free amino acid. Terminal amino acids are more highly racemized than internal amino acids (Kimber and Hare in prep.). Thus deamination and decarboxylation serve to reduce the degree of racemization as measured by mean D/L, by reducing the impact of a potentially highly racemized pool of amino acids.

The mechanism whereby deamination and decarboxylation influence the degree of racemization prompts the recognition of the fact that the phenomenon observed is apparent racemization and must be qualified as such.

5.3.2.2 Comparison of hydrolytic fractions

Generally there was a ten – fold difference between the fractions. The method developed to isolate the fractions included this factor in determining the

amount of internal standard added to the two fractions. By resolving this difference with the addition of internal standard, the method minimizes errors because comparisons are made with data which fall within a much smaller range. The smaller range applies to both the range of relative concentration as is evident from Figure 5.6 and the detector response range.

Concentration data highlight the relative stability of the two fractions. Although more variable, the concentration of the HF fraction appeared to be more stable *i.e.* declined less than that of the HCl fraction. This is particularly evident from the concentration of isoleucine at both temperatures.

5.3.2.3 Aspartic acid

Unlike most amino acids aspartic acid may decarboxylate within a peptide chain. Aspartic acid has two carboxyl groups. As an interior amino acid one of these carboxyl groups is generally not involved in a peptide bond. This carboxyl group may degrade, releasing carbon dioxide. This process is decarboxylation. Water is not required. Alpha – amino butyric acid is the other product of decarboxylation of aspartic acid. Decarboxylation may be enhanced under pyrolytic conditions.

Decarboxylation of an interior amino acid may enhance the apparent degree of racemization. Interior amino acids form a lowly racemized pool. Thus interior decarboxylation affects predominantly the L – enantiomers of a restricted group of amino acids and thus provides a mechanism of apparent racemization of these amino acids.

The evidence presented in Figure 5.6 indicates a rapid decline in both aspartic acid enantiomers in the HCl fractions from the two temperatures. Concentration in the HCl fraction of H160 decreased asymtopically to approximately 5% of the original. The decline in concentration of the HF fraction was less with some 30% of the original remaining at the end of the time treatments. Most of the decline arose from the decline in concentration of L – aspartic. Decline in concentration of D – aspartic was less dramatic in the HF

fractions and was approximated by the increase due to racemization. The combined effect of these processes led to the relatively constant concentration of D – aspartic acid.

In theory glutamic acid may also decarboxylate within a chain. The concentration data on this amino acid was less precise and provided no evidence to support the theory. Amino acids containing other functional groups *e.g.* a hydroxyl, amino or sulphur group similarly may degrade as an internal amino acid.

## 5.4 COMPARISON WITH RACEMIZATION KINETICS IN MOLLUSCS

### 5.4.1 Introduction

The pyrolysis studies described in this chapter parallel the molluscan pyrolysis studies of Kimber et al. (1986) and Kimber and Griffin (1987). To facilitate a comparison between the racemization processes in soil and molluscs, D/L data from the molluscan work was transformed to fit Equation 5.1. Rate constants were developed from the transformed data using a cumulative regression analysis. Because these data are extensive and much of the detail is not referred to, the transformed data are recorded in Appendix 4A and the result of the analysis is detailed (rate constant and regression coefficient) in Appendix 4B. These data are summarised as Table 5.6.

### 5.4.2 Comparison of D/L Ratios (transformed data)

During the course of the pyrolysis studies of the soil and molluscs, the D/L ratios from the soil were found to increase to a much lower extent than had been found in molluscs.

### 5.4.3 Racemization rate constants

The cumulative regression analysis of the molluscan data indicated that the rate constant calculated for each amino acid was not fixed over the duration of the experiment. As was noted in soil, the rate constant tended to decrease as more data was analysed.

Table 5.6 summarises the calculated rate constants for four amino acids from the soil and molluscan experiments. Rate constants are recorded as the range of values from the first establishment of linear kinetics to the end of the experiment.

Increasing the pyrolysis temperature from 105$^{o}$C to 160$^{o}$C increased the calculated racemization rates in this soil almost one order of magnitude. The increasing age of the shells studied decreased the calculated rates almost one order of magnitude. Rate constants from the higher temperature experiments on soil approached those from the oldest shells. A realistic comparison of the relative rates of racemization in this soil and the molluscs indicates that the differences approach two orders of magnitude. Even if pyrolysis studies are artificial this difference should be maintained at ambient temperatures. This conclusion supports the low D/L values which have been recorded from soils (Mahaney et al. 1986).

| Sample | Temperature | Rate $k$ ($\times 10^{-2}$ day$^{-1}$) | | | |
|---|---|---|---|---|---|
| | | ALA | VAL | ILE | LEU |
| Soil | | | | | |
| HCl fraction | 105°C | 0.7 – 0.3 | 0.8 – 0.1 | 0.3 | 0.3 |
| | 140°C | 2.7 – 0.3 | 1.6 – 0.5 | | |
| | 160°C | 5.4 – 4.0 | 2.5 – 2.1 | 1.8 | 2.9 |
| HF fraction | 105°C | 1.0 – 0.2 | 0.5 – 0.1 | 0.1 | 0.1 |
| | 140°C | 2.3 – 0.3 | 0.7 – 0.2 | | |
| | 160°C | 5.1 – 1.3 | 2.0 – 0.6 | | |
| Molluscs | | | | | |
| *Katylesia rhytiphora* (Modern) | 110°C | 20.9 – 1.2 | 6.5 – 1.8 | 9.8 – 1.6 | 10.1 – 2.9 |
| *Anadara trapezia* (Modern) | 110°C | 13.0 – 1.8 | 5.3 – | 4.7 – 2.0 | 6.3 – |
| *Anadara trapezia* (Holocene) | 110°C | 7.2 – 1.2 | 2.3 – 1.1 | 4.2 – 1.7 | 3.8 – 1.3 |
| *Anadara trapezia* (Pleistocene) | 110°C | 4.1 – | 1.7 – 1.0 | 3.6 – 1.5 | 5.4 – 0.9 |

Table 5.6 The range of rate constants calculated from pyrolysis studies of a number of molluscs and the hydrolytic fractions of the pasture soil. The first rate is the apparent rate of racemization after first order kinetics is established (*i.e.* a statistical straight line is evident). The second rate is calculated from the complete data set. Data for molluscs after Kimber and Griffin (1987).

### 5.4.4 Change in rate constants

When tested the D/L data of alanine, valine, isoleucine and leucine from pyrolysis studies of molluscs were found to be described by Equation 5.3 (Appendix 3B).

The slope of this equation ($m$) describes the amount of change in the rate. When $m=0$ there is no change in rate and first order kinetics are appropriate to describe the reaction. When $m$ is larger the change in rate against time is significant. The complexity of this relationship (*i.e.* log format) indicates that the change in rate is not a simple relationship.

Table 5.7 contains values of $m$ for alanine and valine from a variety of molluscs and soil pyrolysis studies. It is interesting to note that the values from the two sources (soil and molluscs) are similar (mean values of 0.20 and 0.20 for ALA and 0.18 and 0.13 for VAL respectively).

| Sample | Temperature | Amino acid | |
|---|---|---|---|
| | | ALA | VAL |
| Soil | | | |
| HCl fraction | 105°C | 0.12*** | 0.15* |
| | 140°C | 0.34*** | 0.19** |
| | 160°C | 0.21*** | |
| HF fraction | 105°C | 0.06** | |
| | 140°C | 0.32** | |
| | 160°C | 0.12** | 0.19* |
| Molluscs | | | |
| *Anadara trapezia* (Modern) | 110°C | 0.18*** | 0.12** |
| *Anadara trapezia* (Pleistocene) | 110°C | 0.17*** | 0.11** |
| *Katylesia rhytiphora* (Modern) | 110°C | 0.26*** | 0.17*** |

Table 5.7 Comparison of the change in the apparent rate of racemization of alanine and valine in soil and molluscs. The value recorded is the slope from regression to fit Equation 5.3. Significance of the relationship is noted

( * $P > 0.05$, ** $P > 0.01$, *** $P > 0.001$ ).

### 5.4.5 Comparison to the >1000 Dalton separate of molluscs

The above comparison of racemization in the pasture soil with racemization in molluscs is based on the premise of equivalence of the total hydrolysate. The total hydrolysate of molluscs represents the mean of a wide range of molecular weight peptides which arise from hydrolysis of the initial proteins. Further examination of the data of Kimber and Griffin (1987) indicated that the racemization process in this soil approximated that of the larger molecular separates of molluscs. This comparison is made in Figure 5.7.

Racemization proceeds much slower in the larger (peptide) molecular weight separates because of the predominance of lowly racemized internal amino acids (Kimber and Griffin, 1987).

Racemization of valine (Fig. 5.7a) in both hydrolytic fractions of the soil approximated that of the large molecular weight separate of the molluscs (>1000 Daltons). Racemization in the HCl fraction of the soil started and finished at similar levels to the >1000 Dalton separate of the molluscs. While the degree of racemization in the HF fraction at the end of the experiment was similar to that of the mollusc separates, the D/L ratio of the HF fraction at the start was higher.

Epimerization of isoleucine proceeded in a similar manner (Fig. 5.7b). While epimerization in the HCl fraction and the mollusc separates was similar, epimerization in the HF fraction was lower than that in the separates of molluscs.

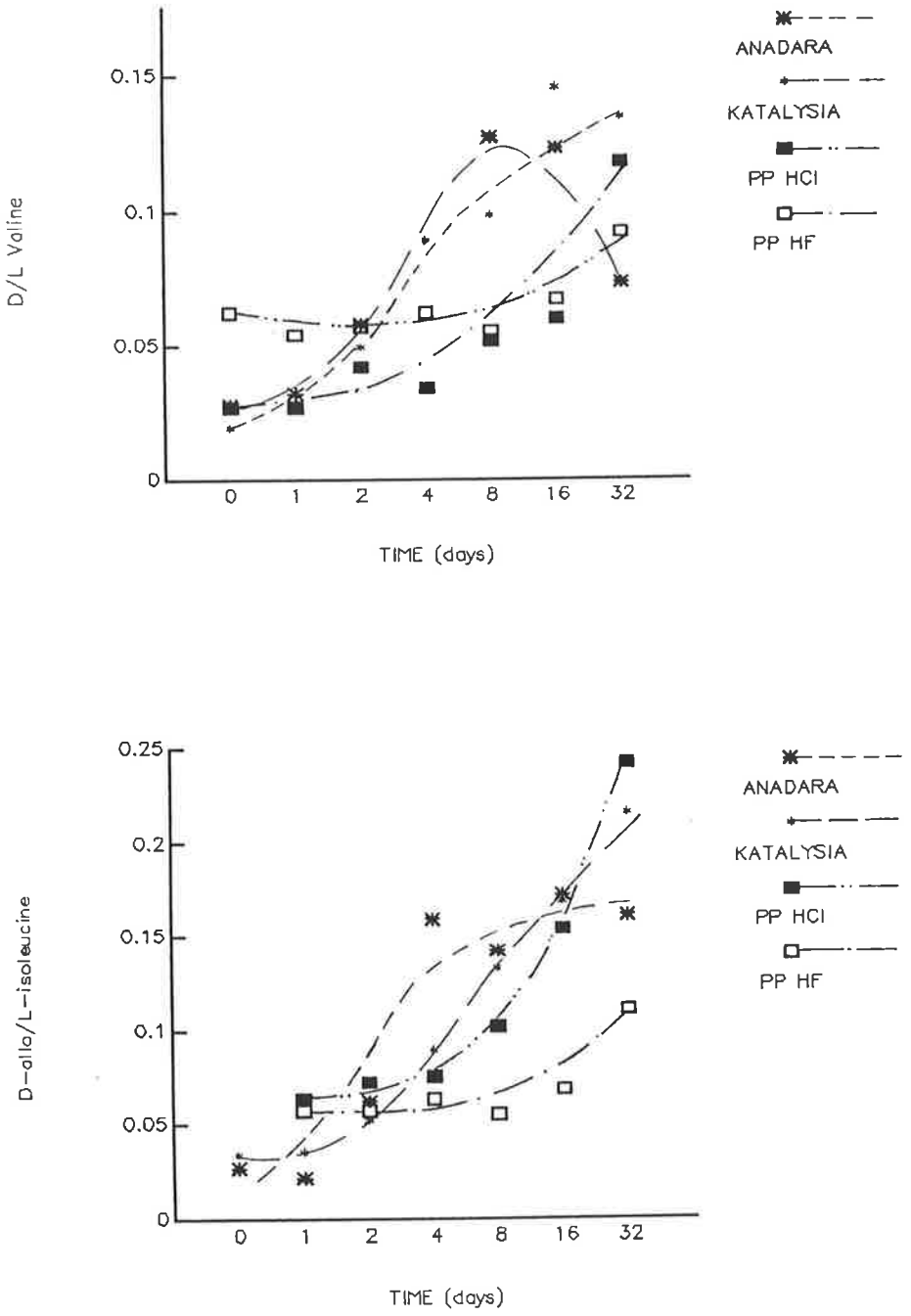



Fig. 5.7 Comparison of
(a) apparent racemization of valine (top) and
(b) apparent epimerization of isoleucine (bottom)
in the >1000 Dalton (molecular weight) separate of two molluscs (after Kimber and Griffin 1987) with data obtained from the hydrolytic fractions of the pasture soil.

## 5.5 AGE ASSESSMENT OF THE HYDROLYTIC FRACTIONS OF THE PASTURE SOIL BASED ON RACEMIZATION OF VALINE

Application of the uncalibrated method of age determination is based on the extrapolation of rate constants determined by pyrolysis to ambient temperatures. There are several assumptions involved in application of the method.

The first assumption is that although racemization is complex, any complex process is approximated by a simple process over a short distance. Changes in the D/L ratio of valine during pyrolysis were relatively minor and did not approach the transition zone recognized by Wehmiller (1981). This aspect of racemization in this soil supports the validity of this assumption.

The second assumption involves the application of Arrhenius parameters. The Arrhenius parameters are, the activation energy, *Ea*, and the pre – exponential factor, *A*. An Arrhenius plot relates the rate of a reaction to the temperature via a straight line correlation of the log of the rate and 1000 times the inverse of the temperature in *Kelvin*.

Because this relationship is a straight line, extrapolation is a valid exercise. Clearly the extrapolation is increasingly accurate if taken over short distances and with several pyrolysis temperatures.

Pyrolysis studies of the hydrolytic fractions of this soil indicated that the complex nature of racemization of valine was not apparent until considerable data had been gathered, as is evident from Figure 5.3 and Table 5.4. The transformed data was subjected to a second, more accurate cumulative regression analysis presented as Tables 5.8 and 5.9. This analysis provided the rate of racemization in each temperature/fraction at the first establishment of linearity. These selected rates are presented in Figure 5.8. Selection of this rate to use in the construction of the Arrhenius plot for each hydrolytic fraction supported the validity of the assumption of simplicity further.

The Arrhenius parameters indicated that the activation energy characteristic of the HF fraction was higher than that of the HCl fraction. The

pre – exponential factor characteristic of each fraction was the same. This evidence is presented in Tables 5.10 and 5.11.

The pre – exponential factor describes the degree of ordering required by each reaction. This factor was derived as the y – intercept of the Arrhenius plot (from *Ln k* when *1/Tx1000* = zero). The similarity in the pre – exponetial factor calculated for each fraction indicates racemization is basically the same reaction in each fraction. The activation energy was derived as the slope of the regression. Though not significant the trend supports the contention that the hydrolytic fractions are kinetic fractions. More data may statistically separate these fractions on the basis of activation energy.

Extrapolation of the plot to ambient temperatures provides the rate of racemization for uncalibrated age assessment. This extrapolation is shown in Figure 5.9. These data indicate there was a difference in the rate of racemization of the two fractions of one order of magnitude at ambient temperature *i.e.* 16$^{o}$C.

Substituting, (i) these rates of racemization, and, (ii) the difference between (a) the D/L ratio of the hydrolytic fraction and (b) some reasonable figure to account for racemization which may have occurred as a result of sample processing, into Equation 2.1 provides the age of the two fractions. Assuming a mean annual temperature of 16$^{o}$C the ages thus calculated are 5 years and 50 years for the HCl and HF fractions respectively. While these actual figures do not approach the true age of the soil this analysis does provide conclusive evidence that the amino acids contributing to the HF fraction of this soil are older than those contributing to the HCl fraction.

The details of the preceding analysis are presented in the following tables and figures.

| Regression Output: | | | |
|---|---|---|---|
| Constant | 0.055084 | | |
| Std Err of Y Est | 0.011759 | | |
| R Squared | 0.710232 | | |
| No. of Observations | 15 | | |
| Degrees of Freedom | 13 | | |
| X Coefficient(s) | 0.000819 | | |
| Std Err of Coef. | 0.000145 | | |
| Regression Output: | | | |
| Constant | 0.05343 | 0.075792 | |
| Std Err of Y Est | 0.011464 | 0.02546 | |
| R Squared | 0.531351 | 0.528935 | |
| No. of Observations | 13 | 10 | |
| Degrees of Freedom | 11 | 8 | |
| X Coefficient(s) | 0.001026 | 0.002112 | |
| Std Err of Coef. | 0.00029 | 0.000705 | |
| Regression Output: | | | |
| Constant | 0.056898 | 0.054341 | 0.084764 |
| Std Err of Y Est | 0.01108 | 0.010456 | 0.019852 |
| R Squared | 0.032643 | 0.926363 | 0.822105 |
| No. of Observations | 11 | 8 | 9 |
| Degrees of Freedom | 9 | 6 | 7 |
| X Coefficient(s) | 0.000292 | 0.006734 | 0.006476 |
| Std Err of Coef. | 0.000529 | 0.000775 | 0.001139 |
| Regression Output: | | | |
| Constant | 0.054042 | 0.049286 | 0.07493 |
| Std Err of Y Est | 0.012939 | 0.009695 | 0.011723 |
| R Squared | 0.109127 | 0.89024 | 0.901572 |
| No. of Observations | 8 | 7 | 7 |
| Degrees of Freedom | 6 | 5 | 5 |
| X Coefficient(s) | 0.001603 | 0.00825 | 0.009939 |
| Std Err of Coef. | 0.00187 | 0.001295 | 0.001469 |
| Regression Output: | | | |
| Constant | 0.049125 | 0.051714 | 0.068636 |
| Std Err of Y Est | 0.012462 | 0.012231 | 0.011056 |
| R Squared | 0.310873 | 0.552646 | 0.826524 |
| No. of Observations | 7 | 5 | 5 |
| Degrees of Freedom | 5 | 3 | 3 |
| X Coefficient(s) | 0.004856 | 0.007036 | 0.014091 |
| Std Err of Coef. | 0.003233 | 0.003655 | 0.003727 |
| Regression Output: | | | |
| Constant | 0.0516 | 0.062 | 0.062636 |
| Std Err of Y Est | 0.01498 | 0.007778 | 0.010553 |
| R Squared | 0.011744 | 0.317669 | 0.832861 |
| No. of Observations | 5 | 3 | 4 |
| Degrees of Freedom | 3 | 1 | 2 |
| X Coefficient(s) | 0.002 | -0.00325 | 0.020091 |
| Std Err of Coef. | 0.010592 | 0.004763 | 0.006364 |

Table 5.8 Cumulative regression analysis of the transformed D/L valine data derived from the three temperature treatments for the HCl fraction.

N.B. Data for Table 5.8 appear on p124.

| Regression Output: | | | | |
|---|---|---|---|---|
| Constant | 0.03602 | | | |
| Std Err of Y Est | 0.016422 | | | |
| R Squared | 0.775429 | | | |
| No. of Observations | 17 | | | |
| Degrees of Freedom | 15 | | | |
| X Coefficient(s) | 0.001429 | | | |
| Std Err of Coef. | 0.000199 | | | |

| Regression Output: | | | |
|---|---|---|---|
| Constant | 0.02958 | 0.062536 | |
| Std Err of Y Est | 0.013866 | 0.042878 | |
| R Squared | 0.784849 | 0.685151 | |
| No. of Observations | 15 | 8 | |
| Degrees of Freedom | 13 | 6 | |
| X Coefficient(s) | 0.00214 | 0.004566 | |
| Std Err of Coef. | 0.000311 | 0.001264 | |

| Regression Output: | | | |
|---|---|---|---|
| Constant | 0.030439 | 0.051531 | 0.036657 |
| Std Err of Y Est | 0.009219 | 0.037395 | 0.021929 |
| R Squared | 0.663311 | 0.563789 | 0.974947 |
| No. of Observations | 12 | 6 | 8 |
| Degrees of Freedom | 10 | 4 | 6 |
| X Coefficient(s) | 0.001971 | 0.006653 | 0.019923 |
| Std Err of Coef. | 0.000444 | 0.002926 | 0.001304 |

| Regression Output: | | | |
|---|---|---|---|
| Constant | 0.028016 | 0.029234 | 0.036153 |
| Std Err of Y Est | 0.006267 | 0.029421 | 0.018131 |
| R Squared | 0.675925 | 0.768952 | 0.949167 |
| No. of Observations | 9 | 5 | 6 |
| Degrees of Freedom | 7 | 3 | 4 |
| X Coefficient(s) | 0.002862 | 0.012992 | 0.02009 |
| Std Err of Coef. | 0.000749 | 0.004112 | 0.002325 |

| Regression Output: | | | |
|---|---|---|---|
| Constant | 0.029571 | 0.025333 | 0.0234 |
| Std Err of Y Est | 0.006747 | 0.004082 | 0.004817 |
| R Squared | 0.186159 | 0.991403 | 0.993819 |
| No. of Observations | 7 | 3 | 4 |
| Degrees of Freedom | 5 | 1 | 2 |
| X Coefficient(s) | 0.001929 | 0.0155 | 0.0292 |
| Std Err of Coef. | 0.001803 | 0.001443 | 0.001628 |

| Regression Output: | | | |
|---|---|---|---|
| Constant | 0.023 | | 0.026667 |
| Std Err of Y Est | 0.004778 | | 0.000816 |
| R Squared | 0.747046 | | 0.999467 |
| No. of Observations | 5 | | 3 |
| Degrees of Freedom | 3 | | 1 |
| X Coefficient(s) | 0.0085 | | 0.025 |
| Std Err of Coef. | 0.002856 | | 0.000577 |

Table 5.9 As for table 5.8. HF fraction.

N.B. Data for Table 5.9 appear on p123.

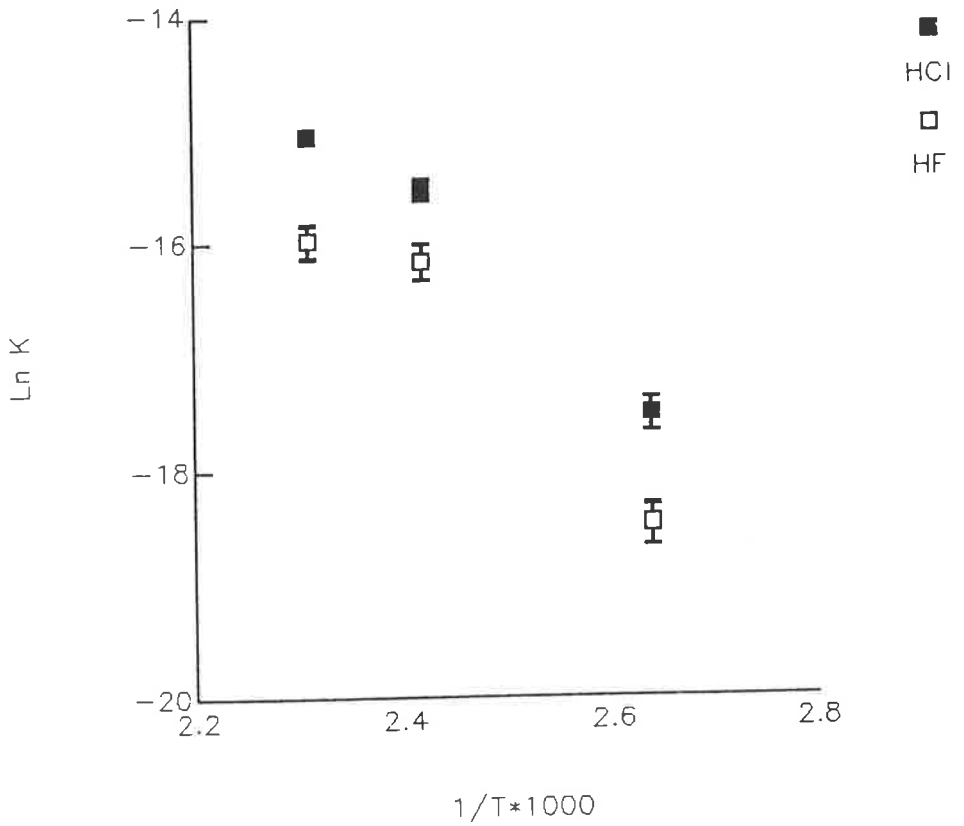



Fig. 5.8 Rates of racemization of valine (mean and standard deviation), in the hydrolytic fractions, at three temperatures. These rates were selected as being most appropriate for use in development of the Arrhenius plots for each fraction. Selection is based on the establishment of linearity. The error terms indicate the fractions differ significantly. This evidence supports the hypothesis that hydrolytic fractionation provides kinetic fractions.

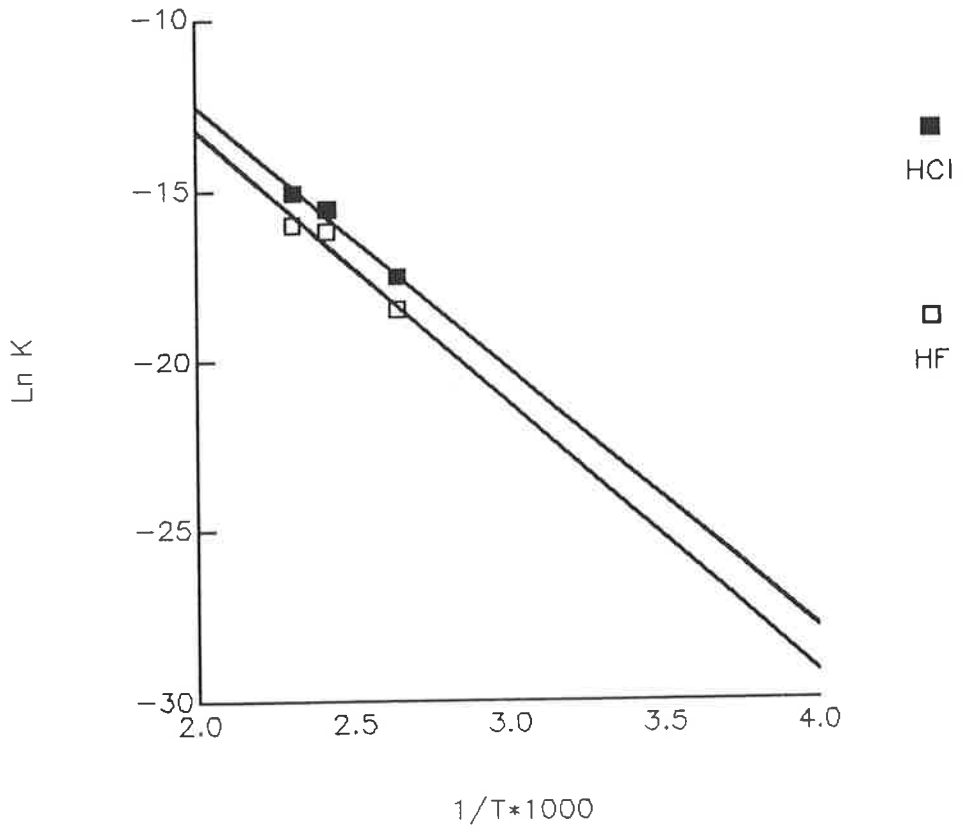



Fig. 5.9 Arrhenius plots of racemization of valine in the two hydrolytic fractions from the pasture soil.

| | | | | | | |
|---|---|---|---|---|---|---|
| SELECTED RATES | | | | | | |
| MEAN | PER DAY | 0.025 | 0.0155 | 0.00214 | | |
| | PER SEC | 2.89E-07 | 1.79E-07 | 2.48E-08 | | |
| ERROR | PER DAY | 0.000577 | 0.001443 | 0.000311 | | |
| | PER SEC | 6.68E-09 | 1.67E-08 | 3.6E-09 | | |
| | TEMP | 160 | 140 | 105 | 16 | |
| RATE | | 2.89E-07 | 1.79E-07 | 2.48E-08 | | |
| RATE + ERROR | | 2.96E-07 | 1.96E-07 | 2.84E-08 | | |
| RATE - ERROR | | 2.83E-07 | 1.63E-07 | 2.12E-08 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| ARRHENIUS PARAMETERS | | | | | | |
| TEMP | | 433 | 413 | 378 | 289 | 250 |
| 1/T*1000 | 0 | 2.309469 | 2.421308 | 2.645503 | 3.460208 | 4 |
| HF | | | | | | |
| HCl | | -15.978 | -16.1643 | -18.4743 | | |
| Ln K | | -15.0556 | -15.5337 | -17.5138 | | |
| Ln (K+ERROR) | | -15.0328 | -15.4446 | -17.3782 | | |
| Ln (K-ERROR) | | -15.079 | -15.6314 | -17.6707 | | |

| Regression Output: | |
|---|---|
| Constant | 2.810248 |
| Std Err of Y Est | 0.273705 |
| R Squared | 0.97795 |
| No. of Observations | 3 |
| Degrees of Freedom | 1 |
| X Coefficient(s) | -7.67081 |
| Std Err of Coef. | 1.151824 |

| PREDICTED ARRHENIUS PARAMETERS | | |
|---|---|---|
| 8.31 Ea (KJ) | MEAN | 0.923082 |
| | +ERROR | 1.061689 |
| | -ERROR | 0.784475 |
| A | | 16.61404 |
| | | 21.84458 |
| | | 12.63592 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HCl | | | 160 | 140 | 105 | 16 | |
| | | | 433 | 413 | 378 | 289 | 250 |
| | | 0 | 2.309469 | 2.421308 | 2.645503 | 3.460208 | 4 |
| | | -7.67081 | -7.67081 | -7.67081 | -7.67081 | -7.67081 | -7.67081 |
| | | 2.810248 | 2.810248 | 2.810248 | 2.810248 | 2.810248 | 2.810248 |
| PREDICTED LINES | | 2.810248 | -14.9052 | -15.7631 | -17.4829 | -23.7323 | -27.873 |
| (D/L)x-(D/L)o | | 0.017 | | | | | |
| Ln K | | -23.7323 | | | AMRT | | 1994.034 |
| K PER SEC | | 4.93E-11 | | | DAYS | | |
| K PER DAY | | 4.26E-06 | | | YEARS | | 5.46 |

Table 5.10 Selected rates, parameters used to develop the Arrhenius plot (Ln k and 1/Tx1000), regression of these parameters, the predicted Arrhenius parameters (Ea and A), extrapolation of the Arrhenius plot to provide the rate of racemization at ambient temperature and the apparent mean residence time of the amino acid nitrogen based on racemization dating. HCl fraction.

| | | | | | | |
|---|---|---|---|---|---|---|
| | TEMP | 160 | 140 | 105 | 16 | |
| SELECTED RATE | | | | | | |
| MEAN | PER DAY | 0.009939 | 0.00825 | 0.000819 | | |
| | PER SEC | 1.15E-07 | 9.55E-08 | 9.48E-09 | | |
| ERROR | | 0.001469 | 0.001295 | 0.000145 | | |
| | PER SEC. | 1.7E-08 | 1.5E-08 | 1.68E-09 | | |
| RATE | | 1.15E-07 | 9.55E-08 | 9.48E-09 | | |
| RATE + ERROR | | 1.32E-07 | 1.1E-07 | 1.12E-08 | | |
| RATE - ERROR | | 9.8E-08 | 8.05E-08 | 7.8E-09 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| ARRHENIUS PARAMETERS | | | | | | |
| TEMP | | 433 | 413 | 378 | 289 | 250 |
| 1/T*1000 | 0 | 2.309469 | 2.421308 | 2.645503 | 3.460208 | 4 |
| Ln K | | -15.978 | -16.1643 | -18.4743 | | |
| Ln (K+ERROR) | | -15.8402 | -16.0184 | -18.3112 | | |
| Ln (K-ERROR) | | -16.1379 | -16.3351 | -18.6693 | | |

| | |
|---|---|
| Regression Output: | |
| Constant | 2.741414 |
| Std Err of Y Est | 0.517786 |
| R Squared | 0.930676 |
| No. of Observations | 3 |
| Degrees of Freedom | 1 |
| X Coefficient(s) | -7.98382 |
| Std Err of Coef. | 2.178986 |

| | | |
|---|---|---|
| PREDICTED ARRHENIUS PARAMETERS | | |
| 8.31 Ea (KJ) | MEAN | 0.960749 |
| | +ERROR | 1.222961 |
| | -ERROR | 0.698536 |
| A | | 15.50891 |
| | | 26.02872 |
| | | 9.240798 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HF | | | 160 | 140 | 105 | 16 | |
| | temp | | 433 | 413 | 378 | 289 | 250 |
| | 1/t*1000 | 0 | 2.309469 | 2.421308 | 2.645503 | 3.460208 | 4 |
| | Ea | -7.98382 | -7.98382 | -7.98382 | -7.98382 | -7.98382 | -7.98382 |
| | Ln A | 2.741414 | 2.741414 | 2.741414 | 2.741414 | 2.741414 | 2.741414 |
| PREDICTED LINE | | 2.741414 | -15.697 | -16.5899 | -18.3798 | -24.8843 | -29.1939 |
| | | | | | | 86400 | |
| (D/L)x-(D/L)o | | 0.052 | | | | | |
| Ln K | | -24.8843 | | | AMRT | | 19300.18 |
| K PER SEC | | 1.56E-11 | | | DAYS | | |
| K PER DAY | | 1.35E-06 | | | YEARS | | 52.88 |

Table 5.11 As for table 5.10. HF fraction.

## 5.6 CONCLUSIONS

The pyrolysis studies described in this chapter were useful in resolving the conflict between the relative degree of racemization of the hydrolytic fractions of agricultural soils, where D/L HCl < D/L HF, and paleosols, where D/L HCl > D/L HF. The pyrolysis studies highlight the dichotomy in the hydrolytic fractions. The apparent rates of racemization at ambient temperature are approximately one order of magnitude lower in the HF fraction than in the HCl fraction. The HCl fraction is more prone to degradation than the HF fraction.

This information supports the concept of protection of the HF fraction. Protection against racemization, hydrolysis and degradation may be facilitated by the exclusion of water. This phenomenon exists with a soil system at the micro–niche level.

Racemization in this soil is much slower than in molluscs. The rate of racemization declined during the course of these experiments. The change in rate may be described by a log log relationship. Data from molluscan studies also fit this relationship. Changes in the apparent rate of racemization were similar in this soil and in molluscs.

Racemization of aspartic acid did not follow either of the kinetic models widely accepted as best describing the process of racemization (Kvenvolden et al. 1979, 1981; Wehmiller 1981, 1982, 1984). Racemization of aspartic acid is divisible into two phases, a sharp increase (phase I) and an equilibrium zone during which there was some suggestion of declining D/L ratio (phase II). The sharp decline in concentration of aspartic indicates amino acids contributing to phase I and phase II are distinct. The two phase nature of racemization of aspartic acid that was apparent in the pyrolysis studies of this soil is not uncommon. This phenomenon has been noted in a variety of molluscs. Parallel investigations in the CSIRO laboratory indicate racemization of aspartic acid in New Zealand chronosequences followed a pattern with striking similarities to these pyrolysis studies (Kimber, Kennedy and Milnes in prep).

Although the consistencies in racemization phenomena of this soil and molluscs are encouraging, ages calculated from the pyrolysis data did not approach

the true age of this soil. This was an expected limitation which is inherent in the organic fractions of soils. The calculated age of the HF fraction is one order of magnitude greater than the calculated age of the HCl fraction.

These data support the contention that amino acids are stabilised in the pasture soil as long chain peptides. Because peptides themselves provide a readily attainable nitrogen source, the peptides must be protected by the soil for this stability to be maintained. Protection in this context is against hydrolysis. Hydrolysis is the first step in assimilation of amino acid nitrogen and is enhanced by proteolytic enzymes. Protection is measured in these experiments as protection against hydrolysis, racemization and degradation. This protection is better developed in the HF fraction suggesting a mineralogical origin. There may be some involvement of hydrophobic components. Most of the protection probably arises from well developed physico – chemical mechanisms.

Evidence supporting the importance of physicochemical mechanisms in the protection of proteins and peptides in soil is widespread in the literature. Hayes et al. (1989) indicate that the current model for genesis of stable organic matter in soil is a heterogeneous process giving rise to a random assemblage of components, an understanding of the detail is uncertain. The following interactions result in some degree of protection.

The significant chemical interactions enhancing the stability of proteins and peptides in soils are,
(i) covalent bonds (Hayes et al. 1989, Rowell et al. 1973)
    (a) hydrogen bonds (Rowell et al. 1973) or multiple hydrogen bonds (Fusi et al. 1989),
    (b) ion dipole bonds (Fusi et al. 1989), and,
(ii) strong ligand to metal bonds arising from the juxtapositioning of amino acid functional groups and inorganic constituents (Hayes et al. 1989),
(iii) electrostatic bonding (Mayaudon 1968),
(iv) irreversible physical adsorption (Mayoudon 1968, Fusi et al. 1989).

Physical criteria contributing to protein/peptide stability in soil systems result in physical isolation of enzymes and substrates (Ladd pers.comm). This isolation is a result of the random complexity of humic material described by Hayes et al. (1989). Isolation may result from the entrapment of peptides/proteins within macromolecules that prevent the intrusion of proteolytic enzymes while permitting the intrusion of substrates (Burns et al. 1972). Isolation may be enhanced by hydrophobic moieties such as long chain hydrocarbons and fatty acids (Hayes et al. 1989). Gianfreda et al. (1991, 1992) and Nannipieri et al. (1978) demonstrate enzyme-clay complexes alter the thermal and proteolytic stability of the enzyme as well as the kinetic parameters describing its activity.

This evidence suggest that even whole proteins and large peptides may be afforded some protection by soil. One might therefore expect that intrinsically less vulnerable peptide sequences may be provided almost complete protection and not participate in the active soil cycles. The evidence presented in this chapter supports the contention that the mechanisms of protection are better developed in the HF fraction.

# CHAPTER 6 PYROLYSIS STUDY OF THE WHEAT/FALLOW SOIL

## 6.1 INTRODUCTION

Racemization of amino acids in the pasture soil was found to be suppressed relative to racemization of amino acids from two modern molluscs. The pattern of racemization in the soil was strongly reminiscent of racemization in the >1000 Dalton separate of the molluscs. The two most plausible mechanisms to explain this observation are,
(i) the contribution of small molecular weight peptides to the mean effect as measured in the hydrolytic fractions is small, or,
(ii) water is excluded from the microenvironment of the peptides and thus hydrolysis and racemization cannot proceed.

The literature as reviewed in Chapter 2 and the data from the pyrolysis studies of the pasture soil support the following model of protection of amino acid nitrogen in the soil.

Amino acids provide a readily assimilable source of nitrogen. Unprotected amino acids have a half life measured in hours. Amino acids are protected when in combination as chains, peptides and proteins (Fig. 1.3). Interior amino acids are protected at the N- and C-terminal positions by peptide bonds to other amino acids. Further protection may arise from the secondary and tertiary structure of the protein/peptide, disulphide bonds, hydrogen bonds and hydrophobic moieties. Proteins and peptides are protected from hydrolysis and racemization by combination with inorganic and organic constituents of the soil. The suggested mechanism is one of multi-hydrogen bonding on to the surface of clay particles. These particles interact with each other and with other constituents in a wide variety of scenarios which ultimately contribute to the fertility of the soil.

The most important feature of the pasture soil is that it is a fertile, non-degraded soil. The pyrolysis studies described in the previous chapter indicated that the mechanisms of protection of the amino acids are well developed in the pasture soil.

The main aim of the pyrolysis experiments described in this chapter is to provide data to compare racemization in the non – degraded pasture soil and the degraded wheat/fallow soil.

## 6.2 MATERIALS AND METHODS

Sub – samples of the wheat/fallow soil, described in Section 4.2.1, were heated at 140$^{o}$C in an experiment which paralleled H140 of the pasture soil, described in Section 5.2. The details of the procedures used in the wheat/fallow pyrolysis are the same as H140 except that the wheat/fallow soil was used instead of the pasture soil. Hydrolytic fractions were separated and analysed as described in Appendix 1. Solvent extraction was not performed. Time treatments used were day 1, 2, 4, 8, 16 and 32. The samples were heated in an oven at 140$^{o}$C. The capillary column used provided good resolution of most of the amino acids routinely examined including D – alloisoleucine and in the glutamic/phenylalanine region. Resolution of D – leucine and proline was not good.

## 6.3 RESULTS

Racemization of alanine, valine, and aspartic acid was recorded together with epimerization of isoleucine as the change in D/L ratio with time in the hydrolytic fractions. The data obtained from this experiment were compared with that from H140 of the pasture soil. This comparison is presented in Figures 6.1, 6.2, 6.3 and 6.4.

The data describing change in relative concentration of aspartic acid in the four soil/fractions are presented as Figures 6.5 and 6.6.

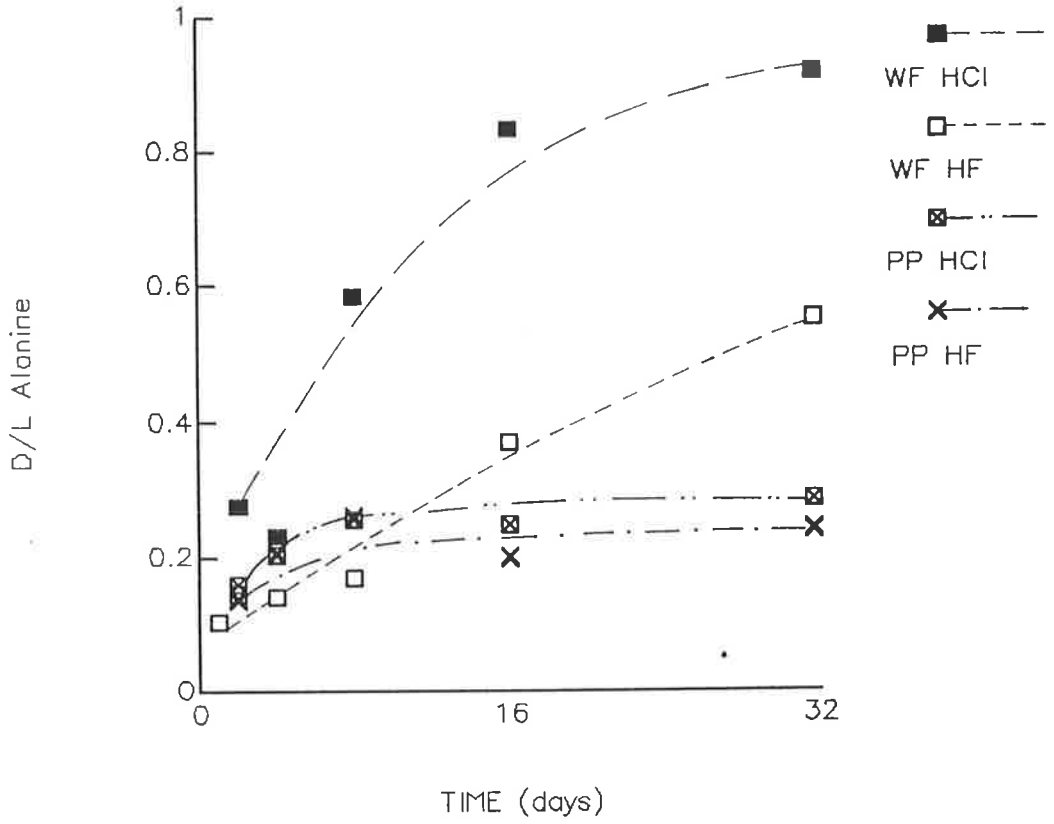



Fig. 6.1 Apparent racemization of alanine at 140°C in the hydrolytic fractions of two soils (WF and PP).

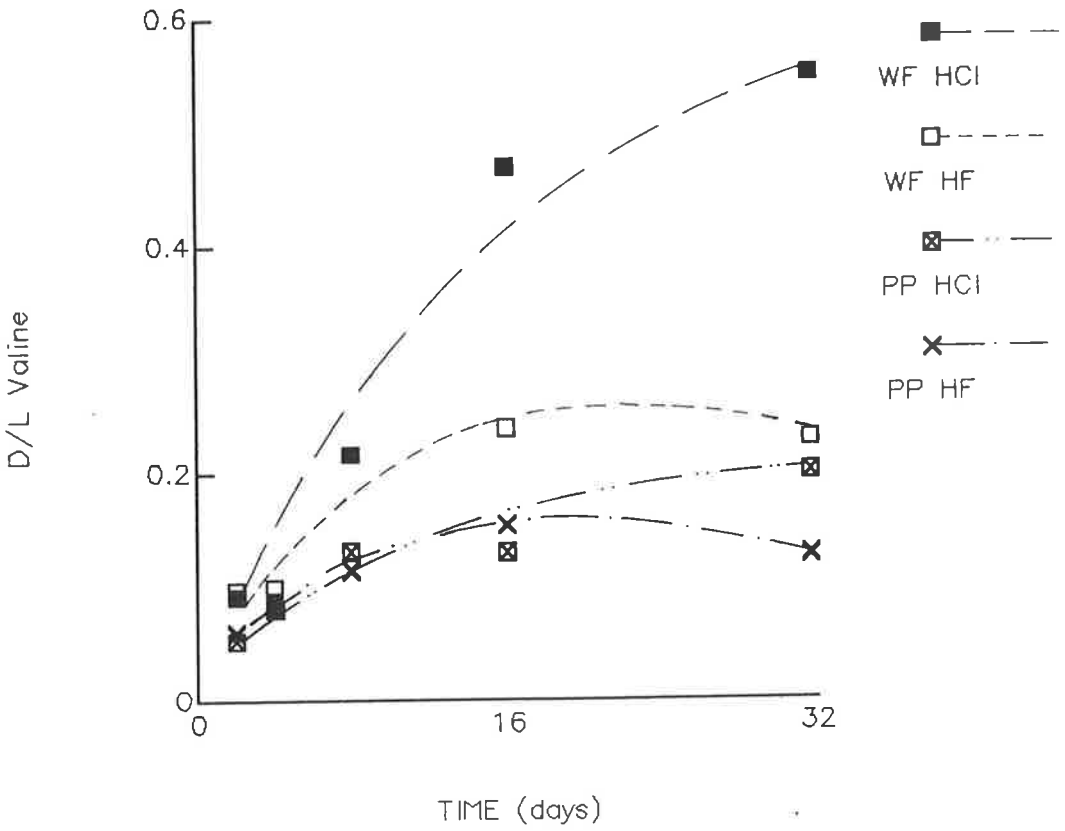



Fig. 6.2 Apparent racemization of valine at 140°C in the hydrolytic fractions of two soils (WF and PP).

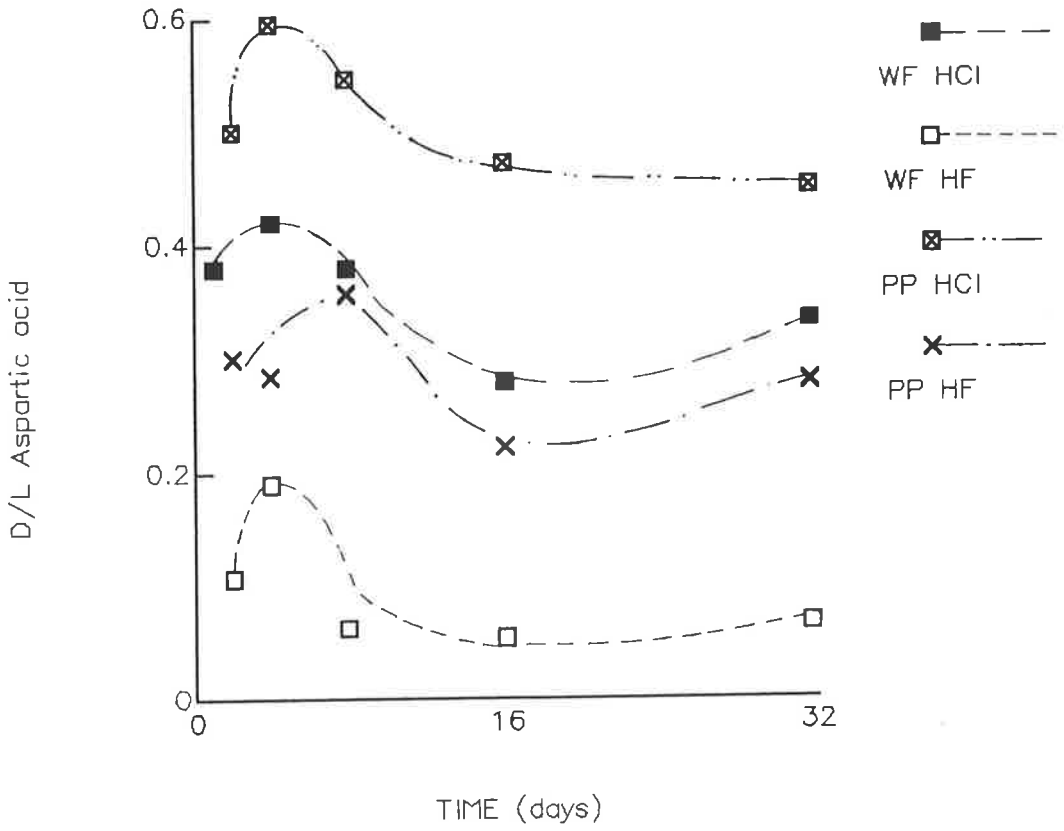



Fig. 6.3 Apparent racemization of aspartic acid at 140°C in the hydrolytic fractions of two soils (WF and PP).

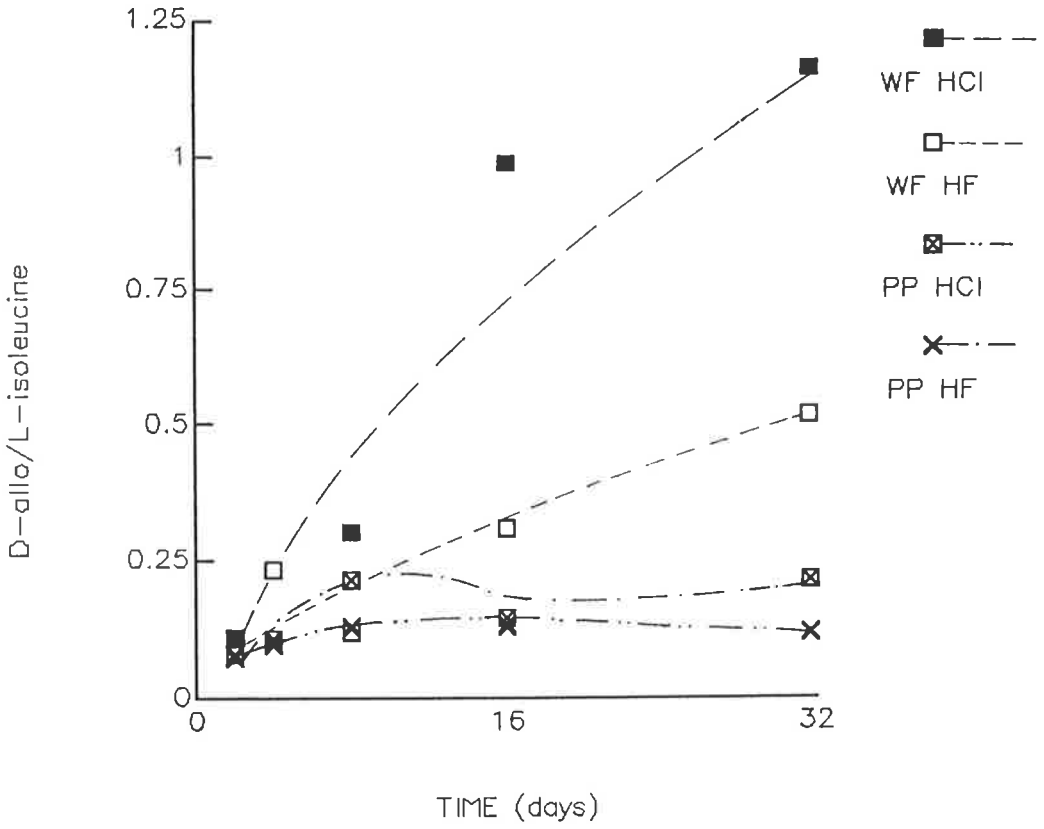



Fig. 6.4 Apparent epimerization of isoleucine at 140°C in the hydrolytic fractions of two soils (WF and PP).

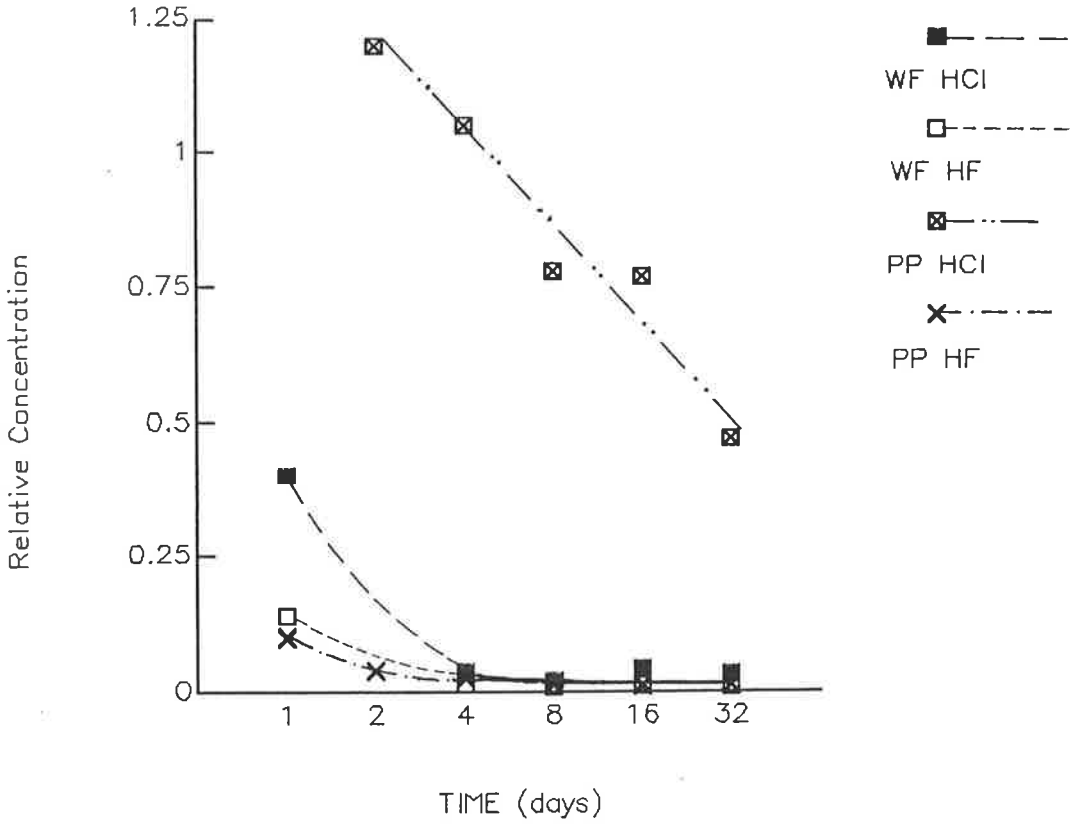



Fig. 6.5 Relative concentration of aspartic acid in the hydrolytic fractions of two soils (WF and PP) after conversion to account for the ten fold difference introduced with the internal standard.

## 6.4 DISCUSSION

The pattern of racemization established by alanine and valine and epimerization of isoleucine was not mimicked by aspartic acid.

Racemization of alanine and epimerization of isoleucine in the HCl fraction of the wheat/fallow soil attained a racemic condition during the experiment (Figs. 6.1 and 6.4). Racemization of valine in this soil/fraction reached D/L = 0.6 (Fig. 6.2). These data indicate that the rates of racemization in this soil/fraction were much higher than those from the pasture soil. Further the rates of racemization in the WF soil were similar to the rates of racemization in the total hydrolysate of modern molluscs (Fig 1.5).

Racemization in the WF HF was slower than in the WF HCl. The rates of racemization of alanine and valine and the rate of epimerization of isoleucine were similar in the WF HF and the PP HCl.

The two phase pattern established by racemization of aspartic acid in the hydrolytic fractions of the pasture soil was repeated with the wheat/fallow soil. The two components of the first phase, duration and extent, were different for each of the four soil/fractions. The extent (change in D/L) of the reaction in WF HCl was half that of PP HCl. The duration (time to commencement of phase II) of phase I in WF HCl was shorter than that of PP HCl although the precise detail of this was not clearly established. This same pattern was repeated in the HF fractions where both the extent and duration of phase I were lower in the wheat/fallow soil. The extent of phase I reaction in the WF HCL and the PP HF was similar. The duration of phase I in WF HCl was much shorter than in PP HF. There was very little racemization of aspartic acid apparent in WF HF.

The extent and duration of phase I racemization may equate with the amount of phase I aspartic acids in the sample and the distribution of mechanisms hindering the phase I process respectively. Where the range of protective mechanisms includes those leading to greatest stability of the moiety the duration of phase I is increased. When this diversity is narrowed, protection is lowered, equivalence in susceptibility is enhanced and the duration of phase I is shortened.

The correlation between the extent of phase I and the amount of phase I amino acids is supported by the concentration data presented in Figure 6.5. The PP HCl contains the most aspartic acid. The concentration of this fraction decreased consistently during the course of the pyrolysis. The concentration in WF HCl and PP HF was the same at the end of the pyrolysis. This is demonstrated by the comparison of the relative concentration from these soil/fractions in Figure 6.6. The concentration in the WF HCl started at a higher level than that in PP HF. In comparison to the other soil/fractions there is very little aspartic acid in WF HF.

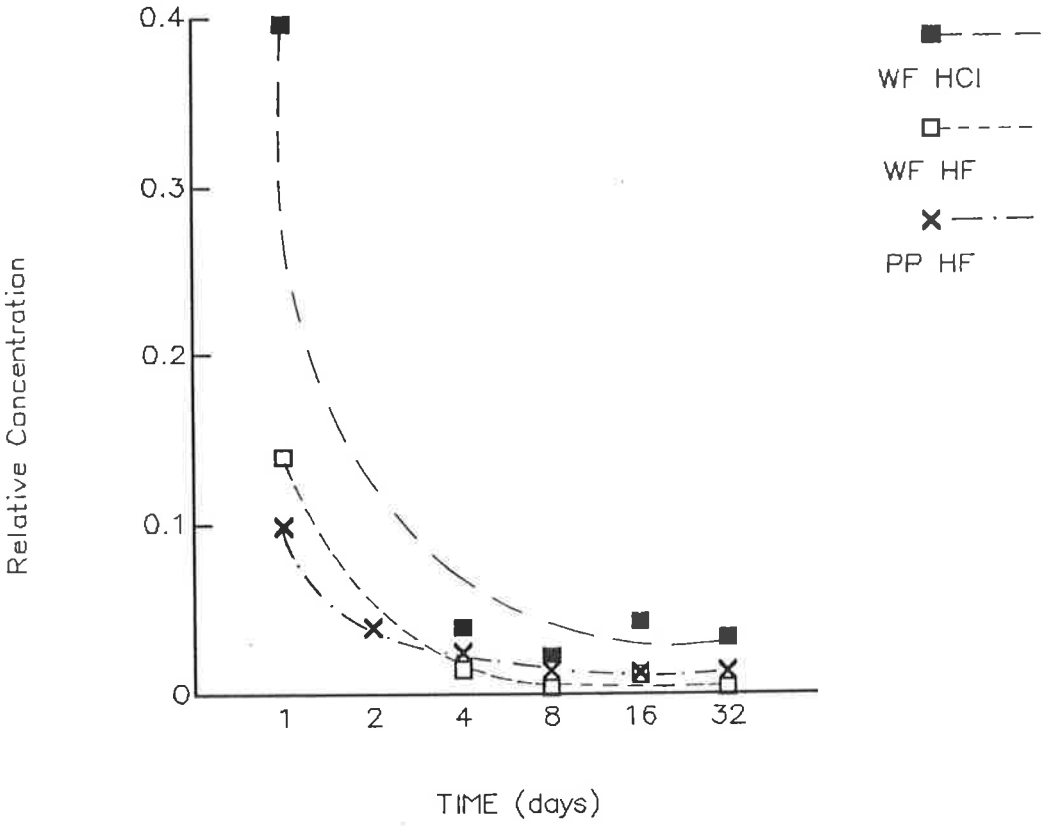



Fig. 6.6 Relative concentration of aspartic acid in WF HCl and HF and PP HCl after conversion to account for the ten factor introduced with the internal standard.

## 6.5 IMPLICATIONS

The differences between the WF and PP soil/fractions noted in Chapter 4, Section 4.3.2, are minor in comparison to the differences under pyrolytic conditions. Data from the pyrolysis experiments described in Chapters 5 and 6 permit a more detailed interpretation of the previously derived data and instigate the need for reassessment of the conclusions presented in Griffin and Kimber (1988).

The pyrolysis experiments have provided conclusive evidence that amino acid nitrogen in soil is not homogeneous with regard to its residence time. Hydrolytic fractionation provides procedurally attainable fractions with different characteristic kinetics and different apparent mean residence times.

Where the hydrolytic fractions of the pasture soil can be described as a fast (HCl) fraction and a slow (HF) fraction the equivalent hydrolytic fractions from the WF soil are very fast (HCl) and fast (HF). This interpretation is different from the conclusion of Griffin and Kimber (1988) and illustrates the difficulty in applying the concept of residence time.

Traditional interpretation of the data from the WF soil may lead to the conclusion that the organic matter in this soil is younger than that in the PP soil. Although the static D/L ratios from the hydrolytic fractions recorded in Tables 4.2, 4.3, and 4.4 are similar, the rate of racemization is higher in the equivalent hydrolytic fractions of the WF. More pyrolysis data is required before the apparent age of the hydrolytic fractions from the WF soil can be calculated. Given (i) similar D/L (ii) similar temperature and (iii) higher rate, the age calculated by Equation 2.1 would be less than that previously calculated for the equivalent fractions of the PP soil.

Previous authors have suggested that the organic matter in the WF soil should be older. This is based on the degraded nature of the organic matter and radiocarbon evidence. The difference between this and the racemization evidence arises because of the inappropriateness of the application of the equation used to determine age from amino acid racemization data *i.e.* Equation 2.1 and from the inappropriate nature of residence time in describing organic matter turnover.

Application of Equation 2.1 involves several assumptions. In the case of these soils these assumptions are not well validated.

Age assessment of the pasture soil, as discussed in Section 5.5, indicated that the age calculated does not approach the true age of the soil. The soil system serves to suppress the apparent racemization relative to the true age. Suppression arises from the dilution of old amino acids with modern material and the continued cycling of amino acid nitrogen in soil/plant sytems. This suppression leads to similarities in the apparent racemization of these soils.

Another assumption in age assessment which may not be valid in soil is that the rate is selected as being representative of the rate experience by the sample *i.e.* the sample experiences some degree of steady state. The WF management is exploitative, the soil becomes increasingly degraded and is not in a steady state. Thus the rate of racemization calculated from pyrolysis studies does reflect the situation at the start of the pyrolysis treatment but does not reflect what happened prior to the establishment of pyrolysis conditions.

That the apparent racemization in the WF soil approximated that of the total hydrolysate of molluscs and the apparent racemization in the pasture soil approximated that of the large molecular weight separate of molluscs is most interesting. Given a general similarity in the chemical nature of the organic matter in the two soils (Oades et al. 1988) one may expect the role of hydrophobic moieties in the protection of amino acids to be similar. This suggests that the difference in rates noted between these soils arises from different molecular weight distribution of the peptides which protect amino acids from racemization.

The observation of high D/L ratios of alanine, valine and allo – /isoleucine suggests the peptides from the WF soil during the later parts of the pyrolysis experiment are of low molecular weight. If racemization is largely an end group phenomenon racemic conditions arise when a majority of the amino acids are in terminal positions and there are few internal amino acids.

Pyrolysis of the pasture soil did not create this situation. This suggests that the protection of amino acids in peptides was better developed in the pasture.

The complicating factors referred to by Griffin and Kimber (1988) are evident in the process of racemization of aspartic acid. The rates of racemization of aspartic acid in the soils may lead to ages which support the previous inference that the organic matter from the WF soil is older (Kimber and Searle 1970). The pyrolysis experiments indicate that the rate of racemization (phase I) of aspartic acid decreased in the order

PP HCl > WF HCl > PP HF > WF HF.

Therefore substituting (i) similar D/L ratios (ii) similar temperatures and (iii) these differences in rates into Equation 2.1 would lead to the apparent age increasing

PP HCl < WF HCl < PP HF < WF HF

as has been suggested.

The final conclusion of Griffin and Kimber (1988) that acid hydrolysis releases a predominantly modern fraction is correct. This analysis indicates that the D/L ratios from the hydrolytic fractions are elevated beyond the amount expected from both natural contributions (Section 2.2.3.1) and procedurally induced racemization. This elevation indicates that while predominantly modern, the HCl fraction is not entirely modern. This leads to a discernable apparent age for this fraction as calculated in Section 5.5.

## 6.6 CONCLUSIONS

The differences in the static D/L ratios as recorded in Table 4.2 and as reported by Griffin and Kimber (1988) are minor in comparison to the differences under pyrolytic conditions.

The collective evidence is consistent with the hypothesis that degradation of a soil leads to the degradation of the mechanisms of protection of amino acids in the soil system and this can be measured with amino acid racemization analysis. The degradation is reflected in both hydrolytic fractions. The difference between the hydrolytic fractions is still unclear but probably does relate to the more intimate association of the organic and inorganic constituents as discussed in Section 2.3.1.

The data obtained from the pyrolysis experiments indicate that the dominant mechanism involved in the process of racemization in these soils originates in the molecular weight distribution of the amino acids. The evidence indicates that,
(i) the process of racemization and epimerization is dominated by large molecular weight effects in the pasture soil, and,
(ii) the contribution of low molecular weight peptides to the nett effect is much higher in the wheat fallow.

Despite the more intimate association of inorganic/organic constituents in the HF fraction and the severity of the treatment required to release the amino acids of the HF fraction, these amino acids are not immune to the effect of soil management. Whereas in the pasture soil the HF fraction is of considerable quantity and displays well developed protection mechanisms, the HF fraction of the wheat fallow is depleted and the mechanisms of protection are less well developed. This evidence suggests that complexes which resist HCl hydrolysis are not stabilized indefinitely as suggested by Oades et al. (1988).

# CHAPTER 7 THE SIGNIFICANCE OF RACEMIZATION OF AMINO ACIDS IN AGRICULTURAL SOILS, FUTURE PROSPECTS

## 7.1 INTRODUCTION

Racemization analysis of soil provides a unique set of data. Amino acids contain approximately one third of the soil nitrogen yet little is known of the behaviour of amino acids in soils. The first evidence of protection of amino acids in peptide chains in soils was recorded twenty years ago by Brisbane et al. (1972). It is of historical significance that this work with proteolytic enzymes was conducted in the same laboratory as the racemization analyses.

Amino acid nitrogen is generally modelled as part of the more labile pools of the nitrogen cycle. Free amino acids in the soil solution form a readily assimilable source of nitrogen and have a half life measured in hours. Amino acids are stabilised in peptides which in turn are stabilised by association with the other components of the soil system. In this way the amino acid nitrogen is increasingly protected from the active cycles of soil.

The amino acid status of a soil can be measured *en masse* using ninhydrin techniques or individually using an amino acid analyser. Alternately proteolytic enzymes may be deployed. These techniques provide a crude overview of soil amino acids and have led to the above conclusions and inference.

## 7.2 THE SIGNIFICANCE OF RACEMIZATION OF AMINO ACIDS IN AGRICULTURAL SOILS.

The research presented in this thesis, while raising many unanswered questions, develops this knowledge base significantly and eliminates some of the inference.

The research examines two procedural components of the soil amino acids. The HCl fraction is the main portion of soil amino acids. Concentration of amino acids in the HF fraction is approximately one order of magnitude lower than in the HCl fraction. D/L ratios can be assigned to each of these fractions with reasonable accuracy. The errors associated with the HCl fraction are generally smaller than those of the HF fraction. The HF treatment dissolves a large portion of silica removing most of the grain coat and particles adhering to the larger quartz grains.

Chapter 4 is devoted to the gathering of static D/L ratios. This research demonstrates the difficulties in interpreting static D/L ratios. Interpretation is difficult because the analysis examines mean effects. Unless there is some understanding of the various aspects contributing to the mean there can be little understanding of the observation.

There are however definite trends established by these static D/L ratios. The HF fraction has a higher D/L ratio. The pasture soil has a higher D/L ratio than the wheat/fallow. Intensive management involving repeated cultivations leads to smaller D/L differences between the hydrolytic fractions. The intermediate sized soil separates *i.e.* silt, had higher D/L ratios than either larger or smaller size separates. Convergence of the hydrolytic fractions under intensive management is demonstrated in a variety of clay, silt and sand separates. A HF fraction can be obtained from humic acids. The HF fraction of pyrophosphate humic acids has a higher D/L ratio than the HCl fraction.

The D/L ratios of these soil and soil fractions/separates were generally low ($<0.25$). D/L ratios recorded from soils by other workers (Mahaney et al. 1986, Kimber, Kennedy and Milnes in prep.) are generally lower than may have been expected from racemization data of molluscs.

The observation of generally low D/L ratios from the red-brown earth which originated tens of thousands of years ago indicates that the biological component of the soil system suppresses racemization. This is not unexpected. Amino acids are ubiquitous in biological systems. The biomass in soil dominates the amino acid pool, amino acids provide readily assimilable nitrogen.

The pyrolysis experiments immensely improve interpretation of racemization data. The pyrolysis conditions mimic a low intensity hydrolysis. In this way the physico – chemical components of the racemization process are enhanced and isolated from the biological component.

The pyrolysis experiments demonstrate that the hydrolytic fractions are kinetic fractions. The racemization kinetics of the HF fraction are slower than those of the HCl fraction. Although the D/L ratio of the HF fraction of an active soil may be higher than that of the HCl fraction, racemization proceeds at a slower rate. Thus it is expected that D/L HCl > D/L HF in a paleosol.

The inference that the HF fraction is older was made early in the research and is demonstrated after extensive pyrolysis investigations. The actual figures may be irrelevant, the arguments as to the real meaning of any derived figure have only just begun.

The most significant observation of this research is the difference between pyrolysis of the pasture and wheat/fallow soils. In the pasture the mechanisms of protection of the amino acids are well developed. The mechanisms of protection of the amino acids in the wheat/fallow system are not well developed. The different management of the soil influences the HF fraction, a fraction which should have very well developed protective mechanisms. Management of these soil resources has resulted in the situation where the four soil/fractions fall into three groups with distinctive kinetics. The WF HCl is the fastest, the WF HF and the PP HCl have some equivalence and the PP HF is the slowest.

The two soils were chosen as being extremes in management of natural fertility in soil. The pasture soil is fertile having no major nutritional deficiencies and being relatively well structured. The wheat/fallow soil is degraded, nutritionally and structurally.

The evidence presented in this thesis supports the contention that the degradation of the soil is reflected in racemization data. The racemization data indicates that the primary mechanism of protection of amino acids in soils, the peptide chain, is not well developed in a degraded soil. Amino acids reside in smaller peptides in the degraded soil.

## 7.3 IMPLICATIONS FOR AMINO ACID RACEMIZATION DATING OF PALEOSOLS

The age calculated in Chapter 5, Section 5.5, indicated that the soil suppresses apparent racemization relative to that which may approach a relic indicative of the actual age of the soil. Thus racemization age assessment of a paleosol should approximate the time of burial of that paleosol.

The pyrolysis work highlights the need for kinetic information when interpreting static D/L ratios. If the assumption that pyrolysis mimicks a well isolated paleosol is correct and the soils, WF and PP, were buried and exhumed many years later and then subjected to amino acid racemization analysis the two soils would provide different degrees of racemization. The naive interpretation of such data would suggest different ages. Pyrolysis studies would highlight the differences in molecular weight distribution of the peptides and hence the need for different rate constants. While other workers (Pillans 1982; Murray – Wallace 1987) have highlighted the importance of temperature in age assessment, this work highlights the importance of hydrolysis or the molecular weight distribution of the constituent peptides and the appropriateness of the chosen rate constant in accounting for differences in this distribution. By choosing well preserved, minimally contaminated monogeneric samples for amino acid racemization analysis traditional applications of the reaction avoid many of the problems with different peptide/molecular weight distributions. Problems which do arise with certain samples traditionally processed may stem from peptide/molecular weight differences.

Given that organic matter enters a profile from the top and migration downward is slow, one may expect smaller molecular weight peptides to be more mobile than larger ones. This would lead to differences in interpreting static D/L ratios within a paleosol as different rates of racemization may be characteristic of the different samples from different vertical positions within the paleosol. Without some kinetic information interpretation is less accurate.

## 7.4 CONCLUSION

Pyrolysis experiments on a wide range of selected soils may lead to a clearer understanding of the processes contributing to the phenomenon of racemization of amino acids in modern soils and in paleosols. Such experiments may provide a unique opportunity to study amino acid turnover in soils and a better understanding of the apparent age which can be calculated.

# APPENDIX 1 AMINO ACID RACEMIZATION ANALYSIS: ANALYTICAL METHODS

Analytical methods used during the course of this research are outlined below in point form. This outline is in four sub – appendices (1.1, 1.2, 1.3 and 1.4).

Appendix 1.1 includes all the steps involved in the isolation of the hydrolytic fractions (the HCl and HF fractions). This method is an adaptation of the observation that a hydrofluoric acid pretreatment is required for maximum release of soil organic matter (Cheng et al. 1975) and the idea (Limmer and Wilson 1980) that this phenomenon may provide two fractions for analysis. The detail of this separation was developed specifically for this research and has since been used routinely for analysis of soil samples in the CSIRO amino acid racemization laboratory.

Appendix 1.2 is a description of the ion exchange techniques used for the purification of the amino acids from the hydrolysates. The initial adaptation of the methods of Cheng et al. (1975) has been published by Griffin and Kimber (1988). This was modified by Kimber (Kimber, Kennedy and Milnes *in prep.*) to use 0.5 M HF in the anion exchange steps (steps 2.4 and 2.5) instead of 5.0 M HF. The advantages of the use of 0.5 M HF include expense and safety. Appendix 1.2B and 1.2C set out the procedure used to rejuvenate exchange resins AG1 and AG50W respectively. The initial adaptation of the technique of Cheng et al. (1975) to the CSIRO laboratory was completed as an integral part of the research for this thesis.

Appendices 1.3 and 1.4 set out the standard procedures of derivatization and chromatography respectively.

1.1.0 Preparation of HF and HCl fractions.

(Steps 1.1.1 to 1.1.9)

1.1 Approximately 1 g of soil or 10 – 50 mg of humic acid sample is weighed into a 10 ml Kimble tube.

1.2 1ml of 0.2 *u*mole L – norleucine spiked 8M HCl is added. A further 4ml of 8M HCl is added. The tube sealed with a cap containing a teflon seal and placed in an oven at 110$^{o}$C for sixteen hours.

1.3 After cooling the contents of the hydrolysis the tube is transferred to a disposable, capped, 50ml polypropylene centrifuge tube using repeated rinses with double – distilled water to ensure complete transfer.

1.4 The insoluble residue is sedimented by centrifugation (14500g for 15 min). The supernatant is filtered through Whatman No.2 filter paper into a 50 ml pear – shaped flask. The insoluble residue is resuspended in 5 ml double – distilled water and centrifuged as above. The supernantant is filtered into the same pear – shaped flask. The washing procedure is repeated twice more. The combined filtrate is the HCl fraction (and is ready for ION EXCHANGE 2.0)

1.6 5.0 ml of 5.0M HF/0.1 M HCl is added to the tube containing the insoluble residue, the tube is sealed and shaken (end – over – end) at 25$^{o}$C for 24 hours.

1.7 The tube and contents are frozen at a slope and evaporated to dryness on a freeze dryer. Alternately the tubes may be evaporated on a rotary film evaporator. However this was found to be less efficient.

1.8 1 ml of 0.02 *u*mole L – norleucine spiked 8M HCl is added to the dry tube and the contents transferred to a hydrolysis tube using 8M HCl. The tube is sealed and hydrolysed as described in step 1.2.

1.9 After cooling the contents of the hydrolysis tube are filtered through Whatman No.2 filter paper into a 50 ml pear – shaped flask. This filtrate is the HF fraction (and is ready for ION EXCHANGE 2.0).

## 1.2.0 ION EXCHANGE

(Steps 1.2.1 to 1.2.10)

2.1 The filtrate is evaporated to dryness on a rotary film evaporator.

2.2 The sample is transferred as described in step 1.3

2.3 5.0 ml of 5.0M HF is added to the tube, the tube frozen on a slope and evaporated using a freeze dryer.

2.4 Dried sample is suspended in 2 ml 0.5 M HF and transferred to anion exchange columns (Biorad *Econo – column* polypropylene column containing 8 – 10 ml, AG1 X8, 100 – 200 mesh) with repeated washings with 0.5 M HF (2x1 ml). Rejuvenation of the anion exchange media is outlined in Appendix 2B

2.5 Columns are eluted using 15 ml of 0.5 N HF. All the eluent is collected, frozen and freeze dried.

2.6 Sample is transferred to 25 ml pear – shaped flask using minimal amount of double distilled water (3x2 ml). 5 ml of 1.0 M HCl is added. The sample is then evaporated to near dryness using a rotary film evaporator, resuspended in 0.01 M HCl and re – evaporated.

2.7 The sample is transferred to a cation exchange column (10 – 15 ml of Biorad AG50W – X8). The loaded column is washed with 20 ml double distilled water before being eluted using 20 ml 2M ammonium hydroxide. The first 3 – 4 ml of the ammonium hydroxide eluent is allowed to run to waste, the remainder is collected in a 50 ml round bottom flask. Cation exchange columns are rejuvenated immediately prior to use using the method outlined in 1.2C.

2.8 Solution is transferred to 50 ml drop – funnels by pouring and washing with minimal quantities of distilled water (3x2 ml). Approximately 5 ml of dichloromethane is added to the funnel, solvent and solute efficiently mixed and left to separate. The solvent is drained (dichloromethane has a density greater than 1.0 and therefore forms the lower layer) and discarded. This extraction is repeated twice more.

2.9 The solute (aqueous layer) is transferred to 50 ml round – bottom flasks, shell frozen and freeze dried. The resulting powder should be a reasonably pure mixture of amino acids.

2.10 Further clean – up may be achieved during and after derivatization by dissolving the amino acid derivative in dichloromethane (anhydrous) and transferring it away from the insoluble impurities.

## 1.2B.0 REJUVENATION OF ANION EXCHANGE RESIN AG1 $F^-$ FORM

(Steps 1.2B.1 to 1.2B.8)

2B.1 Used resin is combined in a polypropylene bottle.

2B.2 1.0M NaOH is added, approximately twice the volumn of resin which is to be rejuvenated. This reaction is exothermic and is accompanied by a distinct darkening in the colour of the mixture.

2B.3 The bottle is centrifuged at low speed (500 RPM for 5 min) to enhance the separation of resin and liquor. The liquor is decanted and discarded.

2B.4 Steps 2.B.2 and 2.B.3 are repeated using double distilled water instead of NaOH.

2B.5 Step 2.B.4 is repeated using 5.0M HF instead of double – distilled water.

2B.6 Step 2.B.4 is repeated until the pH of the liquor starts to increase.

2B.7 The columns are poured and washed with double distilled water until the pH rises to 4 – 5.

2B.8 The column is washed with 10 ml 0.5M HF and is then ready for the addition of the sample.

## 1.2C.0 REJUVENATION OF CATION EXCHANGE RESIN AG50W $NH_4^+$ FORM

(Steps 1.2C.1 to 1.2C.2)

2C.1 Used columns are washed with 20 ml of strong acid (4 – 8M HCl). The addition of ammonium hydroxide to the columns generally highlights the location of some of the compounds retained by the resin. Acid must be added until all of this material has been mobilized. While 4M HCl is generally effective, acid strength may be increased if warranted.

2C.2 The columns are washed with water (< 100 ml) until there is no chloride in the eluent as tested with silver nitrate solution.

## 1.3.0 DERIVATIZATION

(Steps 1.3.1 to 1.3.8)

3.1 Sample is transferred into 3 ml *reactivials* by dissolving in minimum amount (< 1ml) of double – distilled water and filtering through Whatman No.2 filter paper. The round bottom flask is rinsed twice more.

3.2 The sample is dried at 50°C under a stream of industrial dry nitrogen. This nitrogen is delivered through a moisture trap packed with molecular sieve to ensure dryness. Temperature control and nitrogen distribution is facilitated by a Pierce *Reacti – therm* heating module.

3.3 The sample is removed from the heating module when minimum volume is achieved. Care must be exercised to prevent overheating of the sample. The sample is placed in a vacuum desiccator for three hours to ensure complete dryness.

3.4 The sample is removed from the desiccator and 1 ml of 3.3M Propan – 2 – ol/HCl is added. The vial is flushed with nitrogen, capped, mixed and placed in an oven at 110°C for 1 hour.

3.5 *Reativials* are allowed to cool to room temperature.

3.6 Sample is dried at room temperature using heating module.

3.7 50 *u*l dichloromethane/pentafluoropropionic anhydride mixture (1:4) is added to each *reactivial*, the vial flushed with nitrogen and capped. Vials are heated in an oven at 110°C for 10 minutes.

3.8 *Reactivials* are cooled to – 10°C using an aluminium block kept in a deep freeze. Vials are opened and dried under nitrogen using this cold block.

## 1.4.0 CHROMATOGRAPHY

(Steps 1.4.1 to 1.4.4)

4.1 Sample is diluted with anhydrous dichloromethane (50 – 1000 *u*l). The extent of this dilution depends on a number of aspects including the amount of sample initially used, the recovery of amino acids during processing, the particular fraction being investigated and the split ratio used during chromatography. The precise dilution used therefore varies with the sample.

4.2 An appropriate amount of sample (0.5 – 15 *u*l) is tranferred to a SGE spiral – tipped solids injector and the solvent allowed to evaporate.

4.3 Gas – liquid chromatography was performed by using a *Chirasil – L – Val* fused silica capillary column (25 m x 0.22 mm) in a Hewlett Packard 5890A gas chromatograph equipped with both a flame ionization detector and a nitrogen phosphorus detector. Chromatographic peaks were automatically integrated by an HP9216 micro – computer running Nelson Analytical *XTRA CHROM data system* software.

4.4 The D/L ratios were calculated using peak area data, as was relative concentration.

# APPENDIX 2 COMPARISON OF THE RESPONSE OF A FLAME IONISATION DETECTOR AND A NITROGEN PHOSPHORUS DETECTOR.

The response of the nitrogen phosphorous and the flame ionization detectors (NPD and FID respectively) to changing concentration was tested with standard mixtures of amino acids. Mixtures containing amino acids at concentrations of 12.5, 25, 50, 100 and 200 *n*moles were derivatized and chromatographed using the NPD. Mixtures containing amino acids at concentrations of 1, 5, 10, 50 and 100 *n*moles were derivatized and chromatographed using the FID. The data was used in a calibration plotting programme (a sub – routine of the *XTRA – CHROM data system*) which compares the given concentration of the amino acid to the ratio of the area of each amino acid to that of the internal standard. In essence this programme (CALPLOT) plots the respose factor (Equation 3.1) against concentration.

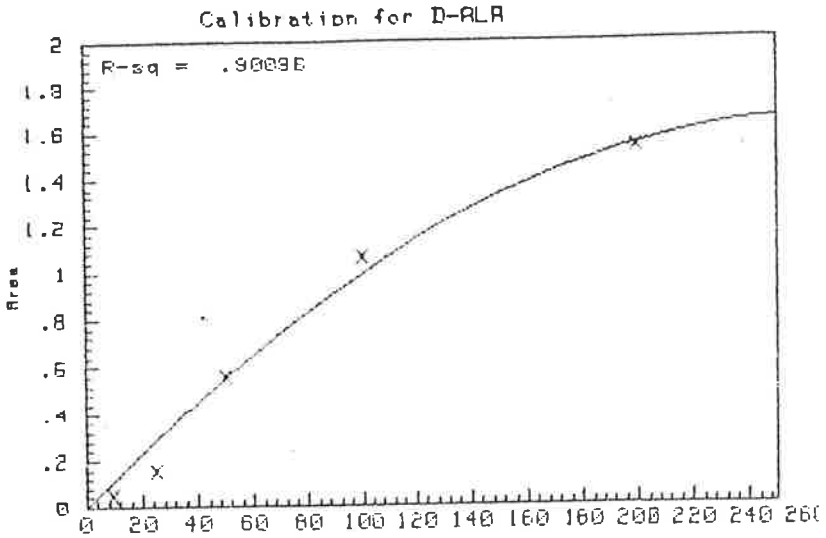
The computer generated results from this comparison are presented in the following pages. The calibration of the NPD was tested as a second order relationship. The regression coefficient reported with each plot is indicative of the fit to a second order relationship.

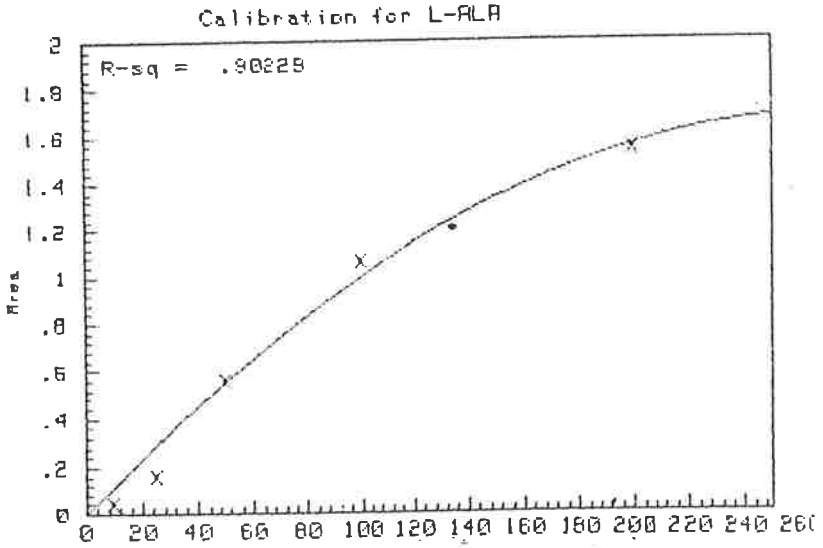
The calibration of the FID was more complex because of the increased linearity of the response. The CALPLOT sub – routine tested for both first order and second order relationships.

These results indicate the FID displays superior linerarity. It should be noted that the NPD was tested over a larger range of concentrations and this could explain in part the differences noted.

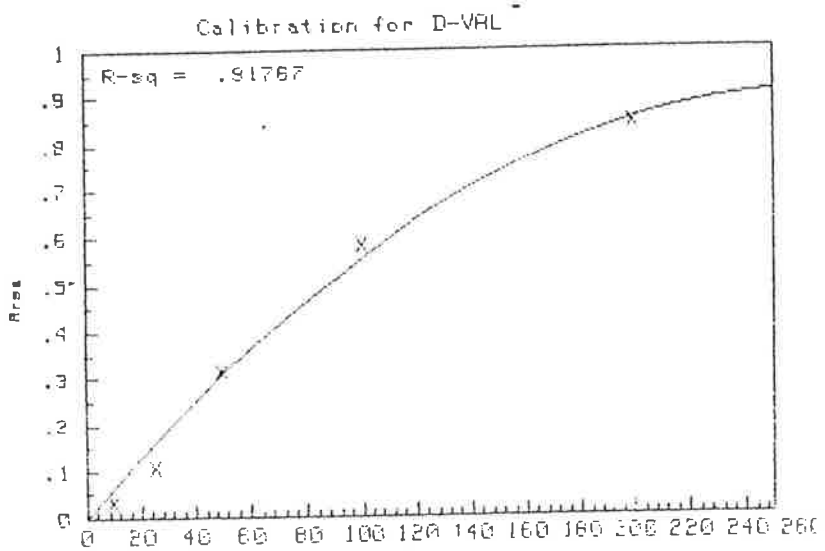
Contents of Appendix 2

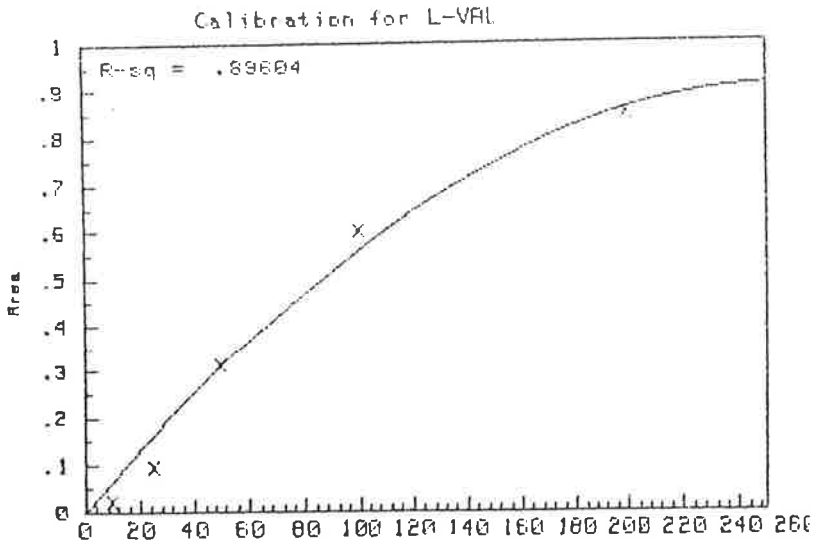
Appendix 2A.1 Alanine (D− and L−)



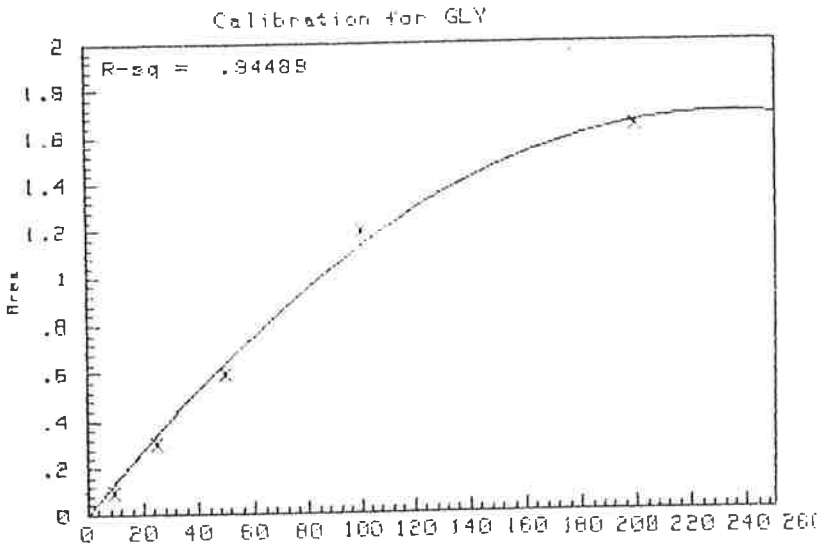



Appendix 2A.2 Valine (D− and L−)

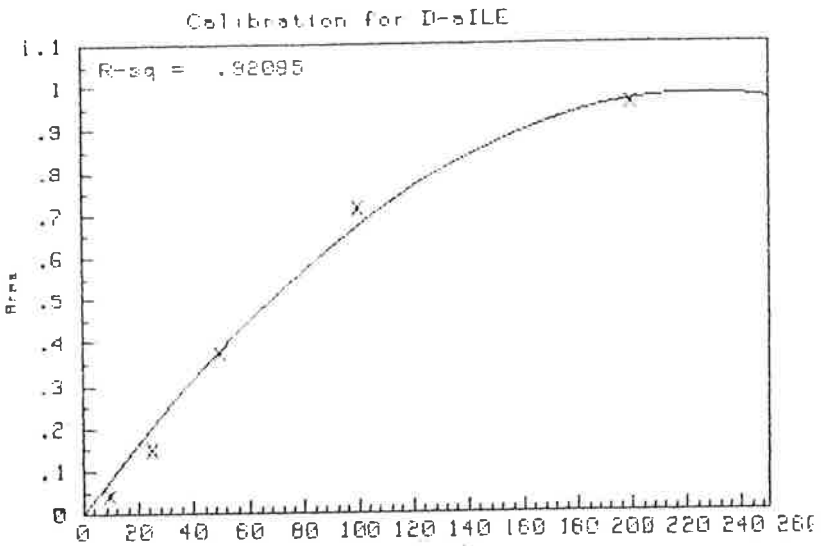


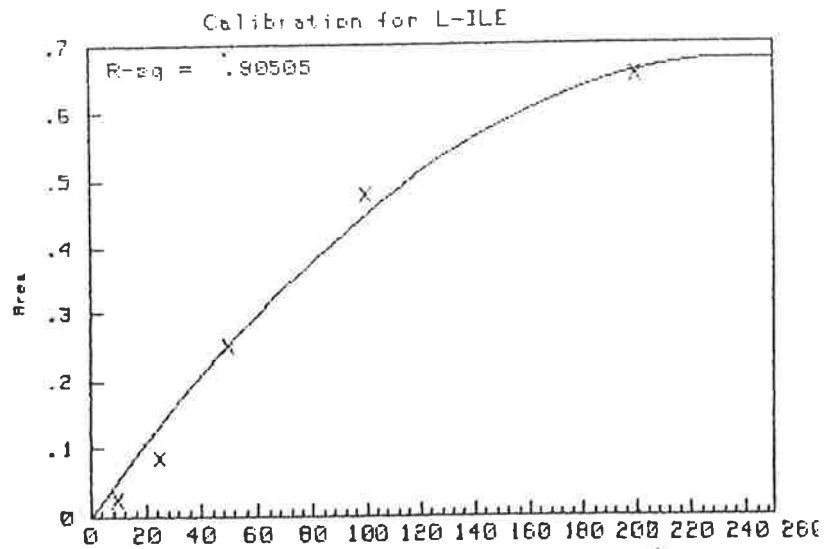



Appendix 2A.3 Glycine

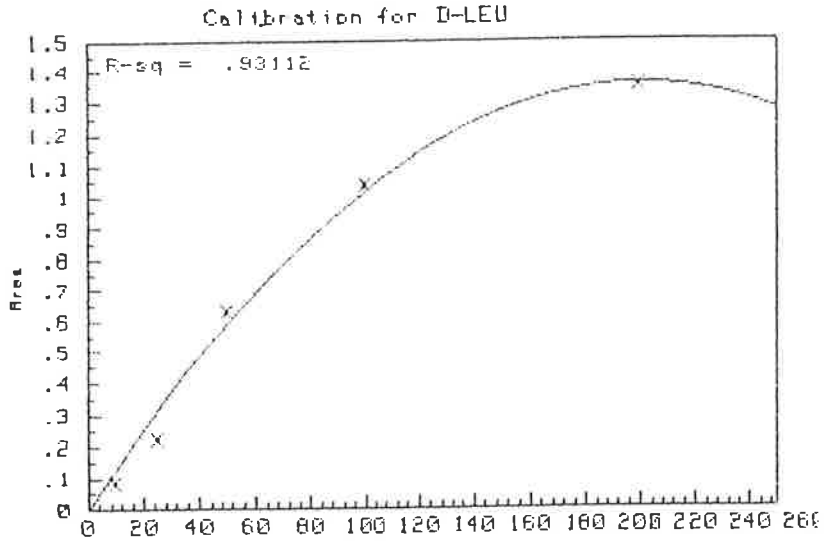


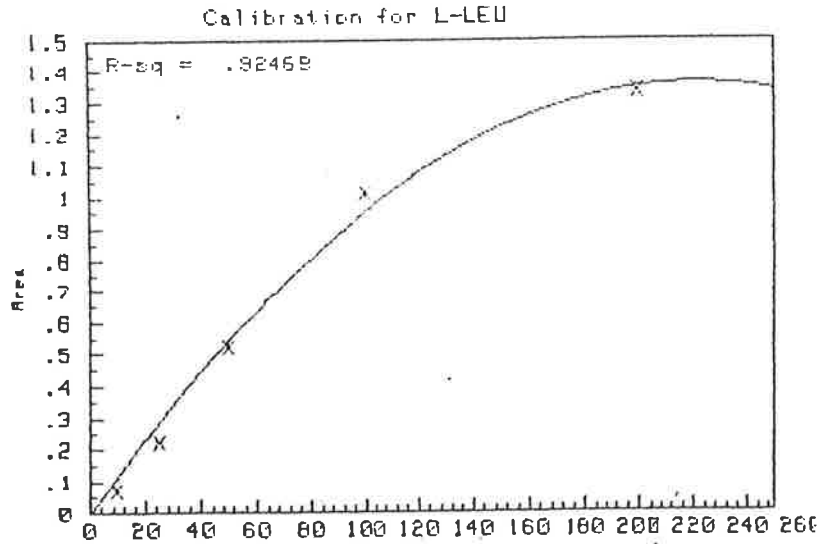
Appendix 2A.4 D – alloisoleucine and L – isoleucine



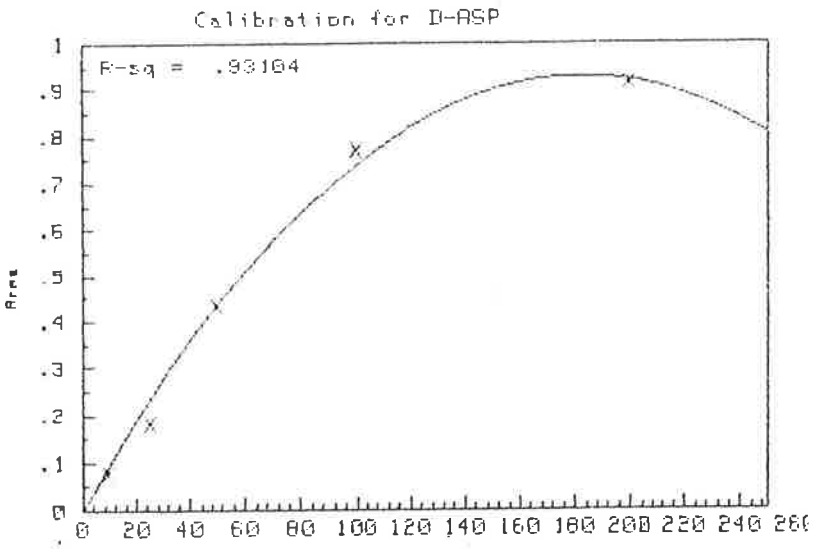



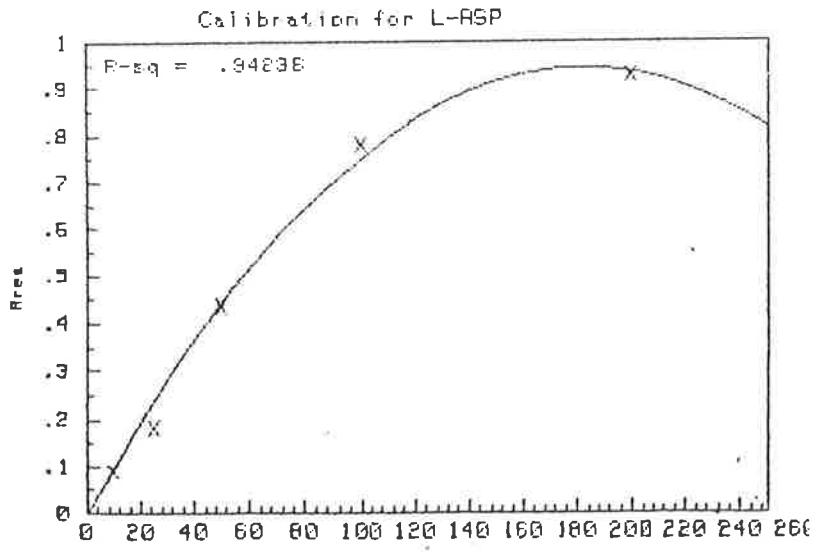
Appendix 2A.5 Leucine (D− and L−)



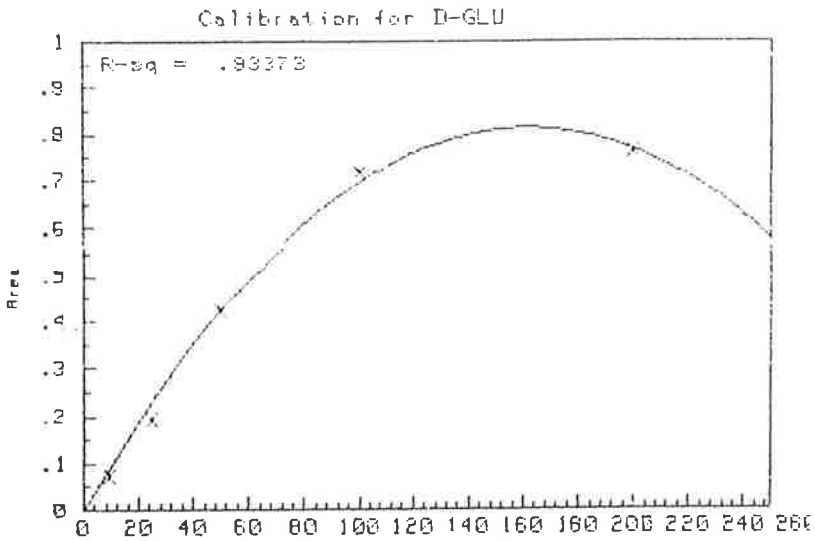



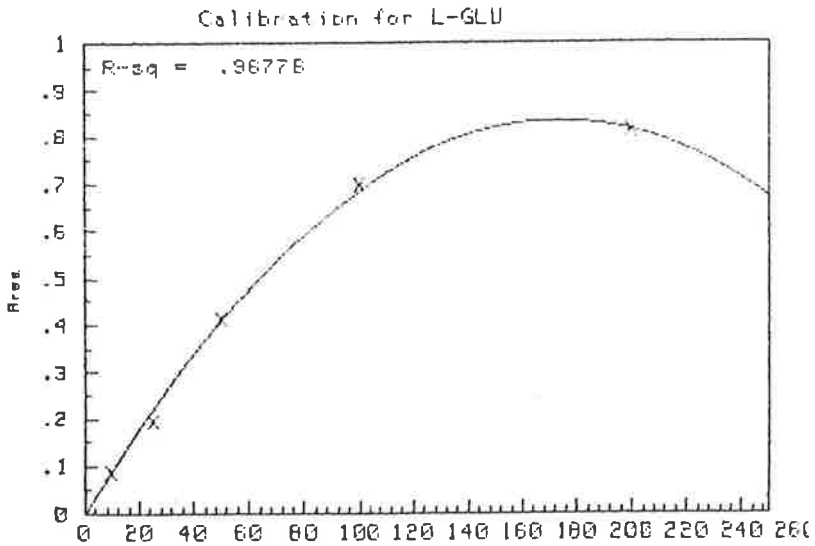
Appendix 2A.6 Aspartic acid (D – and L – )



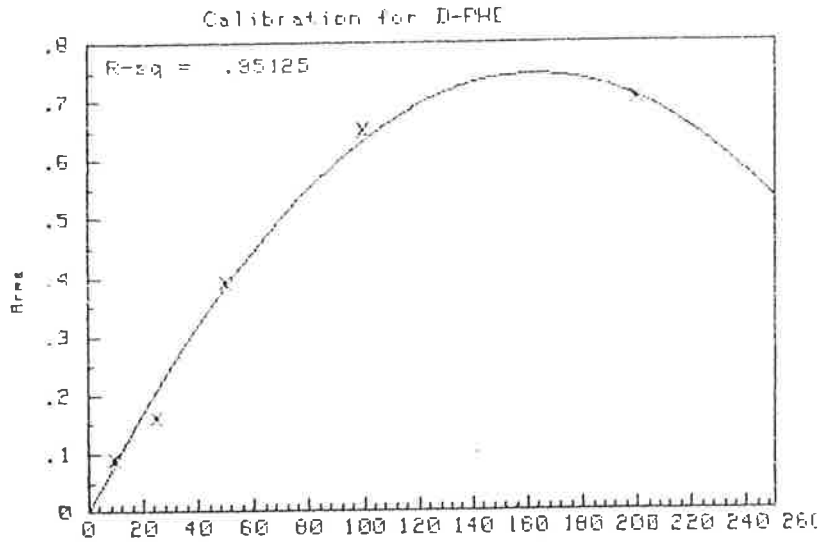



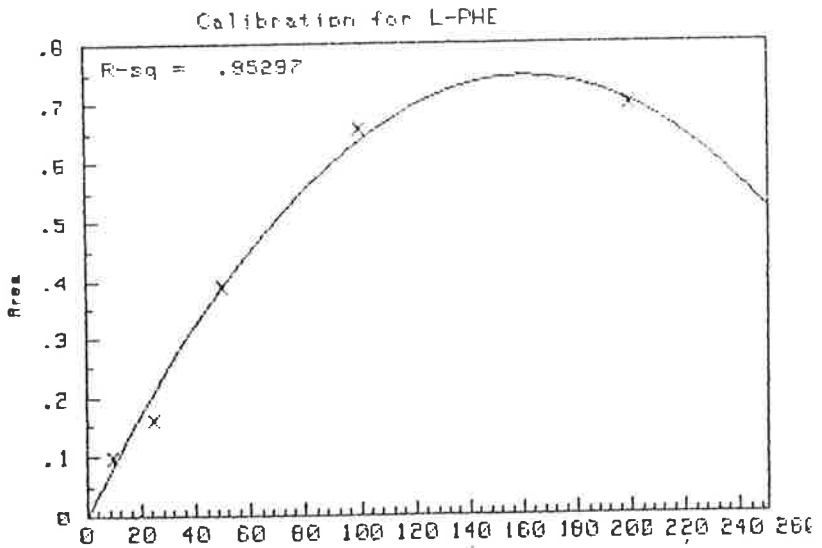
Appendix 2A.7 Glutamic acid (D− and L−)



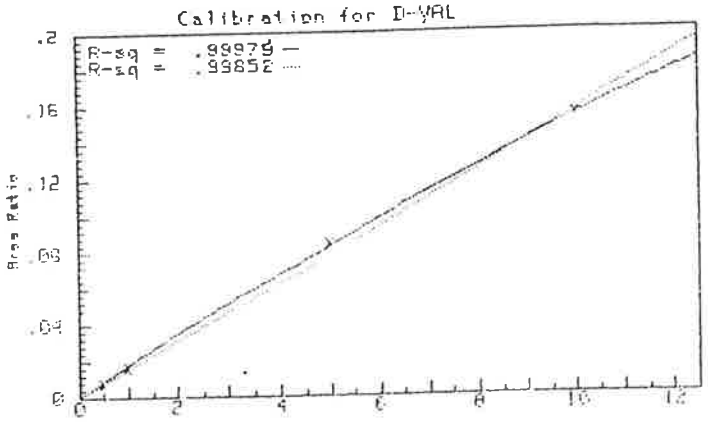



Appendix 2A.8 Phenylalanine (D – and L – )



## Appendix 2B.1 Valine (D− and L−)




Plot # 1 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
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The Index of Determination (r-square) is .99979

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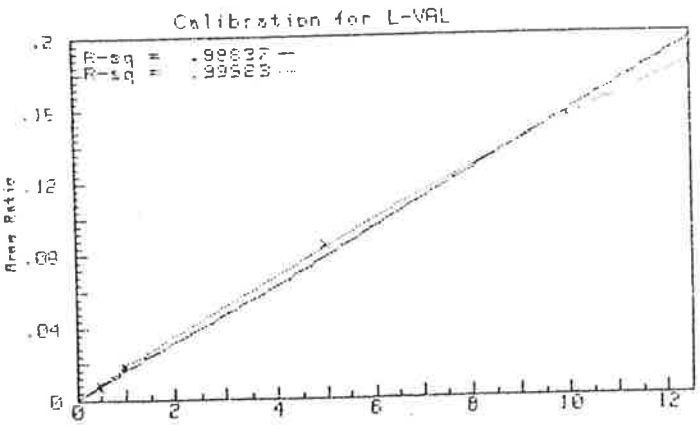
The Index of Determination (r-square) is .99852




Plot # 1 is calibrated by a 1st order fit constrained through 0
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The Index of Determination (r-square) is .99837

Plot # 2 is calibrated by a 2nd order fit constrained through 0
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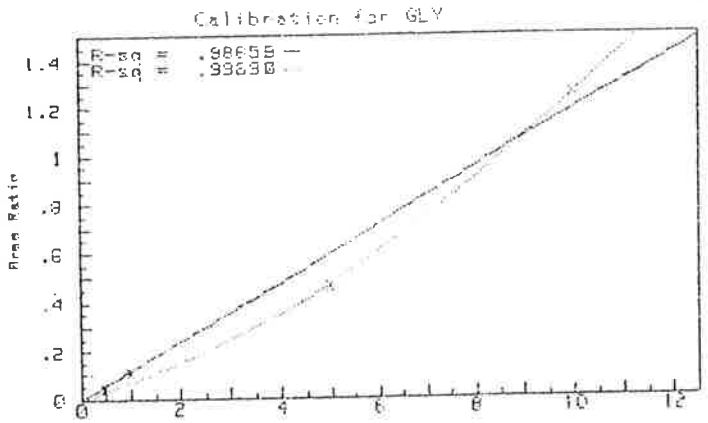
The Index of Determination (r-square) is .99983

## Appendix 2B.2 Glycine

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The Index of Determination (r-square) is .98659

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
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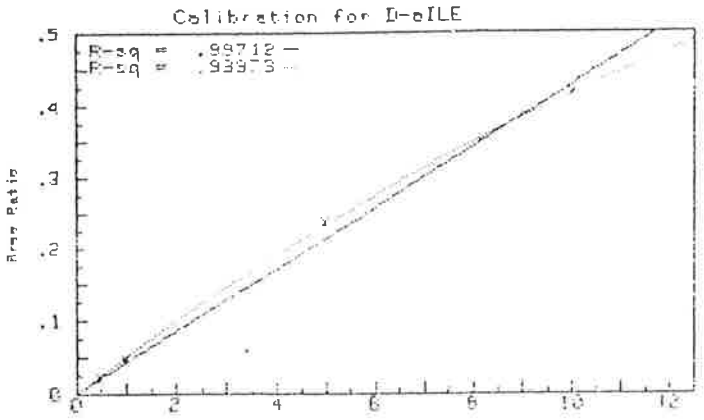
## Appendix 2B.3 D – alloisoleucine and L – isoleucine

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The Index of Determination (r-square) is .99712

Plot # 2 is calibrated by a 2nd order fit constrained through 0
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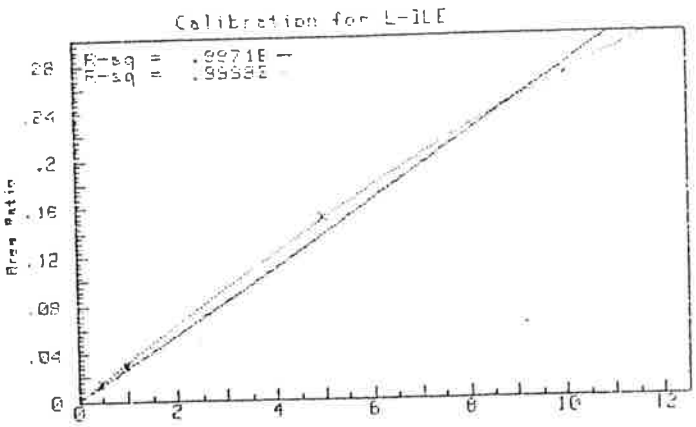
The Index of Determination (r-square) is .99973




Plot # 1 is calibrated by a 1st order fit constrained through 0
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C0 = 0 C1 = .0275

The Index of Determination (r-square) is .99716

Plot # 2 is calibrated by a 2nd order fit constrained through 0
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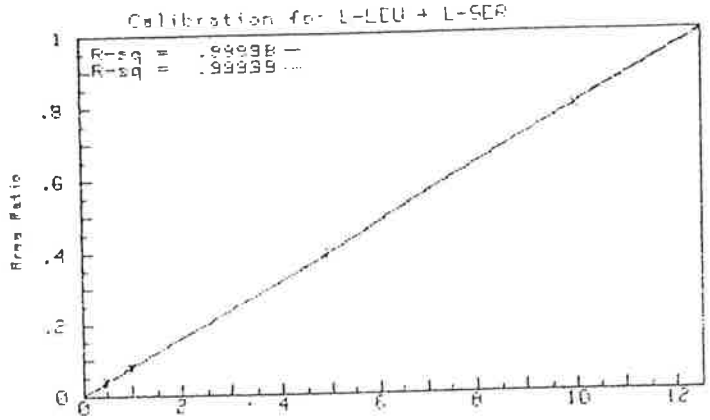
The Index of Determination (r-square) is .99982

## Appendix 2B.4 Leucine (D− and L−)

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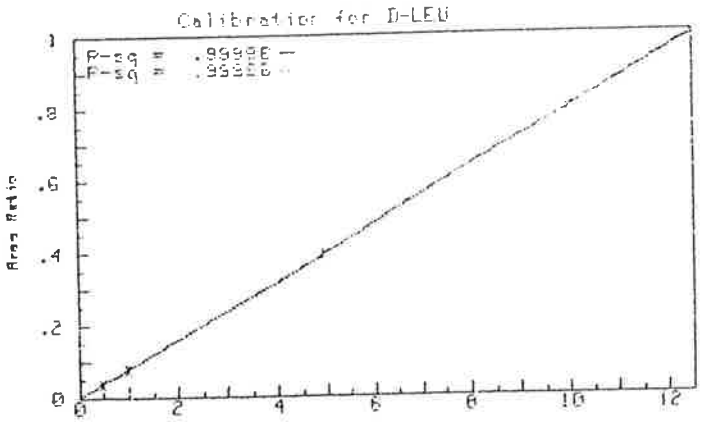
The Index of Determination (r-square) is .99999

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The Index of Determination (r-square) is .99996

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
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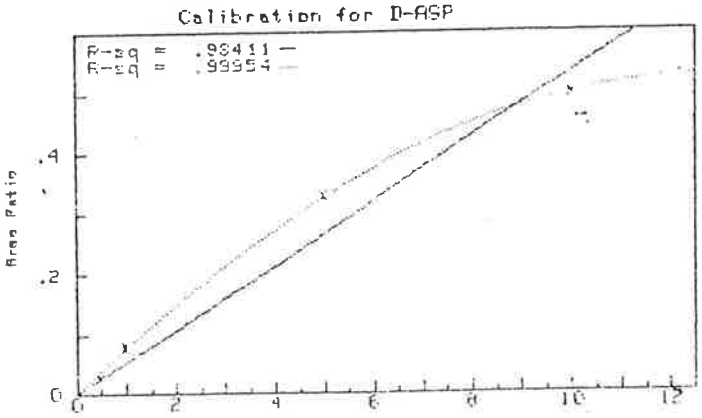
The Index of Determination (r-square) is .99998

## Appendix 2B.5 Aspartic acid (D− and L−)

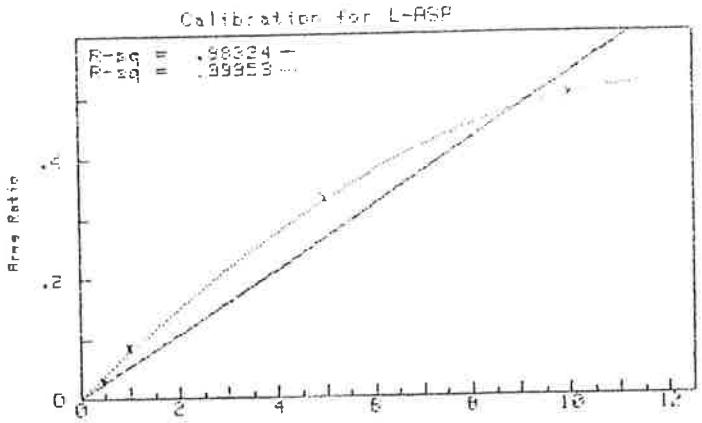



Plot # 1 is calibrated by a 1st order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .0532

The Index of Determination (r-square) is .98411

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .081 C2 = -.00311

The Index of Determination (r-square) is .99954




Plot # 1 is calibrated by a 1st order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .0535

The Index of Determination (r-square) is .98324

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .0821 C2 = -.00321

The Index of Determination (r-square) is .99953

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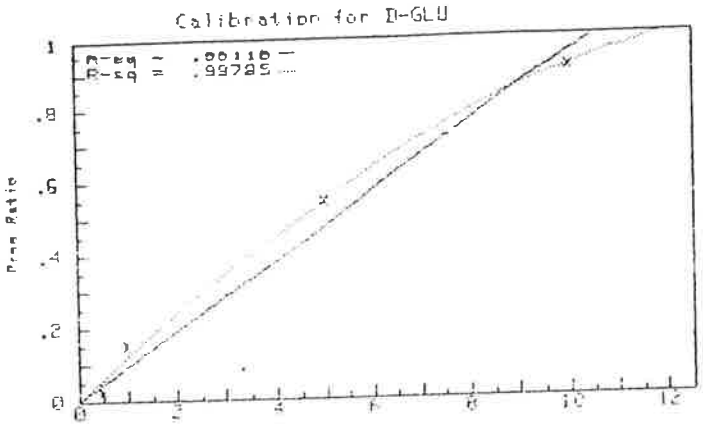
OF 0 1

## Appendix 2B.6 Glutamic acid (D− and L−)

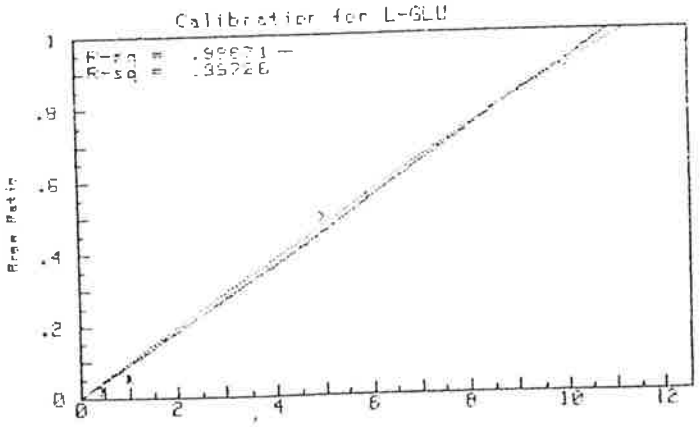



Plot # 1 is calibrated by a 1st order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .095

The Index of Determination (r-square) is .99118

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .127 C2 = -.00363

The Index of Determination (r-square) is .99785



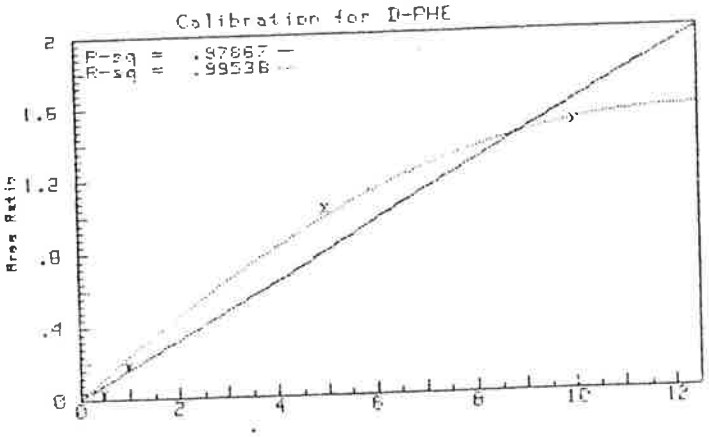

Plot # 1 is calibrated by a 1st order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .0918

The Index of Determination (r-square) is .99671

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .101 C2 = -.001

The Index of Determination (r-square) is .99726

## Appendix 2B.7 Phenylalanine (D− and L−)

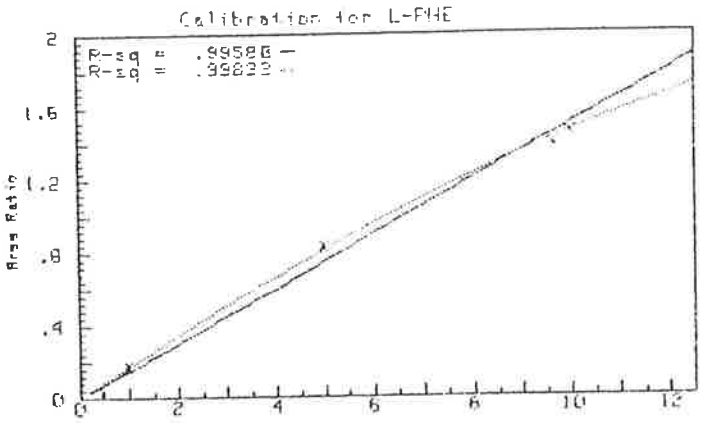



Plot # 1 is calibrated by a 1st order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .159

The Index of Determination (r-square) is .97867

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .245 C2 = -.00971

The Index of Determination (r-square) is .99536




Plot # 1 is calibrated by a 1st order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .15

The Index of Determination (r-square) is .99580

Plot # 2 is calibrated by a 2nd order fit constrained through 0
The Fit Coefficients are:
C0 = 0 C1 = .182 C2 = -.00353

# APPENDIX 3 PYROLYSIS STUDIES: PERMANENT PASTURE SOIL

D/L data from the pyrolysis studies of soil (Chapter 5) are presented as Appendix 4A.

The data were trasnsformed to fit Equation 5.1. These transformed data are presented in Appendix 3B together with the details of the first regression analysis (the slope or racemization rate constant, *m*, the Pearson's correlation coefficient, *r*, and the significance of the model based on this coefficient).

Contents of Appendix 3

Appendix 3A.1 H105 HCl

| SAMPLE CODE | D/L | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| H105D1HCl/1 | 0.122 | 0.030 | 0.054 | 0.068 | 0.066 | | |
| H105D1HCl/2 | 0.115 | 0.025 | 0.028 | 0.058 | 0.069 | 0.046 | 0.209 |
| H105D2HCl/1 | 0.118 | 0.042 | 0.026 | 0.073 | 0.382 | 0.085 | 0.227 |
| H105D2HCl/2 | 0.117 | 0.042 | 0.025 | 0.070 | 0.373 | 0.071 | 0.224 |
| H105D4HCl/1 | 0.137 | 0.032 | 0.049 | 0.074 | | 0.049 | 0.229 |
| H105D4HCl/2 | 0.130 | 0.036 | 0.048 | 0.075 | | 0.061 | |
| H105D8HCL/1 | 0.174 | 0.056 | 0.084 | 0.108 | | 0.111 | |
| H105D8HCL/2 | 0.168 | 0.048 | 0.066 | 0.095 | | 0.104 | 0.277 |
| H105D16HCL/1 | 0.135 | 0.056 | 0.033 | 0.097 | 0.328 | 0.067 | 0.237 |
| H105D16HCL/2 | 0.150 | 0.047 | 0.049 | 0.083 | 0.287 | 0.061 | 0.305 |
| H105D16HCl/3 | 0.162 | 0.078 | 0.068 | 0.099 | 0.370 | 0.097 | 0.521 |
| H105D32HCl/1 | 0.217 | 0.076 | 0.089 | 0.125 | 0.309 | 0.136 | 0.375 |
| H105D32HCl/2 | 0.256 | 0.090 | 0.133 | 0.155 | 0.310 | 0.137 | 0.388 |
| H105D32HCl/3 | 0.269 | 0.131 | 0.131 | 0.181 | 0.410 | 0.262 | 0.448 |
| H105D64HCl/1 | 0.315 | 0.122 | 0.225 | 0.237 | 0.344 | 0.319 | 0.455 |
| H105D64HCl/2 | 0.297 | 0.106 | 0.249 | 0.246 | 0.368 | 0.272 | 0.359 |

## Appendix 3A.2 H105 HF

| SAMPLE CODE | D/L | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| H105D1HF/1 | 0.162 | 0.047 | 0.066 | 0.095 | | 0.071 | |
| H105D1HF/2 | 0.114 | 0.034 | 0.037 | 0.070 | | 0.047 | |
| H105D1HF/3 | 0.130 | 0.059 | 0.069 | 0.161 | 0.276 | | 0.235 |
| H105D2HF/1 | 0.163 | | | 0.181 | 0.428 | 0.178 | 0.270 |
| H105D2HF/2 | 0.128 | 0.066 | 0.057 | 0.161 | 0.310 | 0.115 | 0.170 |
| H105D4HF/1 | 0.174 | 0.063 | 0.063 | 0.142 | 0.378 | 0.121 | |
| H105D4HF/2 | 0.144 | 0.076 | 0.062 | 0.109 | 0.396 | 0.093 | |
| H105D8HF/1 | 0.180 | 0.059 | 0.055 | 0.101 | 0.441 | 0.063 | |
| H105D8HF/2 | | | | | 0.445 | | |
| H105D16HF/1 | 0.226 | 0.062 | 0.048 | 0.154 | 0.289 | 0.143 | 0.277 |
| H105D16HF/2 | 0.172 | 0.060 | 0.065 | 0.127 | 0.334 | 0.090 | 0.251 |
| H105D16HF/3 | 0.160 | 0.058 | 0.096 | 0.159 | 0.283 | 0.255 | 0.254 |
| H105D32HF/1 | 0.239 | 0.090 | 0.093 | 0.184 | 0.250 | 0.102 | 0.113 |
| H105D32HF/2 | 0.228 | 0.095 | 0.110 | 0.174 | 0.349 | 0.123 | 0.223 |
| H105D64HF/1 | 0.248 | 0.115 | 0.184 | 0.242 | 0.209 | 0.213 | 0.349 |
| H105D64HF/2 | 0.269 | 0.093 | 0.122 | 0.159 | 0.159 | | |

## Appendix 3A.3 H140 HCl

| SAMPLE CODE | D/L | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| H140D2HC1/1 | | | | | | | |
| H140D2HC1/2 | 0.161 | 0.053 | 0.081 | | 0.500 | 0.144 | 0.358 |
| H140D4HC1/1 | 0.210 | | | 0.123 | 0.631 | | |
| H140D4HC1/2 | 0.201 | 0.089 | 0.109 | 0.199 | 0.561 | 0.198 | 0.412 |
| H140D8HCL/1 | 0.259 | 0.167 | 0.279 | 0.171 | 0.497 | 0.373 | 0.685 |
| H140D8HCL/2 | 0.251 | 0.096 | 0.150 | | 0.594 | 0.309 | 0.471 |
| H140D16HCL/1 | 0.248 | 0.130 | 0.147 | | 0.471 | 0.246 | 0.528 |
| H140D16HCL/2 | | | | | | | |
| H140D32HC1/1 | 0.340 | 0.250 | 0.223 | | 0.448 | 0.351 | 0.588 |
| H140D32HC1/2 | 0.227 | 0.154 | 0.209 | | 0.451 | 0.379 | 0.639 |

## Appendix 3A.4 H140 HF

| SAMPLE CODE | D/L | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| H140D2HF/1 | 0.155 | 0.058 | 0.074 | 0.051 | | 0.134 | 0.204 |
| H140D2HF/2 | 0.121 | 0.061 | 0.076 | 0.142 | 0.301 | 0.088 | 0.087 |
| H140D4HF/1 | 0.201 | 0.082 | 0.097 | 0.168 | 0.275 | 0.153 | 0.146 |
| H140D4HF/2 | 0.212 | 0.088 | 0.103 | 0.195 | 0.295 | 0.226 | 0.287 |
| H140D8HF/1 | 0.286 | 0.115 | 0.131 | | 0.355 | 0.275 | 0.324 |
| H140D8HF/2 | 0.234 | 0.116 | 0.128 | | 0.359 | 0.265 | 0.415 |
| H140D16HF/1 | 0.201 | 0.154 | 0.133 | | 0.223 | 0.205 | 0.298 |
| H140D16HF/2 | | | | | | | |
| H140D32HF/1 | 0.245 | 0.129 | 0.121 | | 0.270 | 0.305 | 0.353 |
| H140D32HF/2 | 0.234 | 0.124 | 0.117 | | 0.290 | 0.262 | 0.495 |

## Appendix 3A.5 H160 HCl

| SAMPLE CODE | D/L | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| H160D1HCl/1 | 0.172 | 0.051 | 0.042 | 0.077 | 0.494 | 0.082 | 0.403 |
| H160D1HCl/2 | | | | | | | |
| H160D2HCl/1 | 0.206 | 0.077 | 0.202 | 0.072 | 0.411 | 0.075 | 0.313 |
| H160D2HCl/2 | | | | | | | |
| H160D4HCl/1 | | | | | | | |
| H160D4HCl/2 | 0.335 | 0.143 | 0.198 | 0.077 | 0.491 | 0.135 | 0.423 |
| H160D8HCL/1 | 0.440 | 0.176 | 0.425 | | 0.378 | 0.191 | 0.437 |
| H160D8HCL/2 | 0.487 | 0.205 | 0.252 | 0.113 | 0.475 | 0.178 | 0.571 |
| H160D16HCL/1 | 0.649 | 0.383 | 0.362 | 0.490 | 0.497 | 0.400 | 0.607 |
| H160D16HCL/2 | 0.630 | 0.327 | 0.371 | 0.403 | 0.534 | 0.329 | 0.677 |

## Appendix 3A.6 H160 HF

| SAMPLE CODE | D/L | | | | | | |
|---|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | LEU | ASP | PHE | GLU |
| H160D1HF/1 | 0.197 | 0.084 | 0.175 | 0.135 | 0.293 | | |
| H160D1HF/2 | | | | | | | |
| H160D2HF/1 | 0.227 | 0.113 | 0.165 | 0.253 | 0.511 | 0.239 | |
| H160D2HF/2 | 0.227 | 0.092 | 0.132 | 0.231 | 0.409 | 0.179 | 0.267 |
| H160D4HF/1 | 0.297 | 0.118 | 0.146 | 0.156 | 0.361 | 0.142 | 0.237 |
| H160D4HF/2 | 0.351 | 0.177 | 0.221 | 0.293 | 0.199 | 0.300 | 0.395 |
| H160D8HF/1 | 0.370 | 0.159 | 0.195 | 0.187 | 0.230 | 0.164 | 0.328 |
| H160D8HF/2 | 0.327 | 0.142 | 0.159 | 0.246 | 0.251 | 0.252 | 0.453 |
| H160D16HF/1 | 0.381 | 0.155 | 0.130 | 0.120 | 0.287 | | |
| H160D16HF/2 | 0.375 | 0.200 | 0.180 | 0.152 | 0.207 | 0.142 | 0.318 |

# Appendix 3B.1 H105 HCl

$$0.5 \ln \frac{1+D/L}{1-D/L}$$

| SAMPLE CODE | ALA | VAL | LEU | ASP | PHE | GLU |
|---|---|---|---|---|---|---|
| H105D1HCl/1 | 0.123 | 0.030 | 0.068 | 0.066 | | |
| H105D1HCl/2 | 0.116 | 0.025 | 0.058 | 0.069 | 0.046 | 0.212 |
| H105D2HCl/1 | 0.119 | 0.042 | 0.073 | 0.403 | 0.085 | 0.231 |
| H105D2HCl/2 | 0.118 | 0.042 | 0.070 | 0.392 | 0.071 | 0.228 |
| H105D4HCl/1 | 0.138 | 0.032 | 0.074 | | 0.049 | 0.233 |
| H105D4HCl/2 | 0.131 | 0.036 | 0.075 | | 0.061 | |
| H105D8HCL/1 | 0.176 | 0.056 | 0.109 | | 0.112 | |
| H105D8HCL/2 | 0.170 | 0.048 | 0.096 | | 0.105 | 0.285 |
| H105D16HCL/1 | 0.136 | 0.056 | 0.098 | 0.341 | 0.067 | 0.242 |
| H105D16HCL/2 | 0.151 | 0.047 | 0.083 | 0.296 | 0.061 | 0.315 |
| H105D16HCl/3 | 0.164 | 0.078 | 0.099 | 0.389 | 0.098 | 0.578 |
| H105D32HCl/1 | 0.221 | 0.077 | 0.126 | 0.320 | 0.137 | 0.394 |
| H105D32HCl/2 | 0.262 | 0.090 | 0.157 | 0.321 | 0.138 | 0.410 |
| H105D32HCl/3 | 0.276 | 0.132 | 0.183 | 0.436 | 0.269 | 0.482 |
| H105D64HCl/1 | 0.326 | 0.128 | 0.242 | 0.359 | 0.331 | 0.491 |
| H105D64HCl/2 | 0.306 | 0.107 | 0.251 | 0.386 | 0.279 | 0.376 |

REGRESSION DATA

| LINEAR MODEL | | | | | | |
|---|---|---|---|---|---|---|
| m | 0.0693 | 0.0015 | 0.0028 | 0.0023 | 0.0039 | 0.0034 |
| r | 0.946 | 0.881 | 0.970 | 0.420 | 0.907 | 0.623 |
| P> | 0.001 | 0.001 | 0.001 | n.s. | 0.001 | 0.025 |

# Appendix 3B.2 H105 HF

$$0.5 \ln \frac{1+D/L}{1-D/L}$$

| SAMPLE CODE | ALA | VAL | LEU | ASP | PHE | GLU |
|---|---|---|---|---|---|---|
| H105D1HF/1 | 0.163 | 0.047 | 0.095 | | 0.071 | |
| H105D1HF/2 | 0.114 | 0.034 | 0.070 | | 0.047 | |
| H105D1HF/3 | 0.131 | 0.059 | 0.162 | 0.283 | | 0.239 |
| H105D2HF/1 | 0.164 | | 0.183 | 0.457 | 0.180 | 0.277 |
| H105D2HF/2 | 0.129 | 0.066 | 0.162 | 0.321 | 0.116 | 0.172 |
| H105D4HF/1 | 0.176 | 0.063 | 0.143 | 0.398 | 0.122 | |
| H105D4HF/2 | 0.145 | 0.076 | 0.109 | 0.419 | 0.093 | |
| H105D8HF/1 | 0.182 | 0.059 | 0.110 | 0.473 | 0.063 | |
| H105D8HF/2 | | | | 0.478 | | |
| H105D16HF/1 | 0.230 | 0.062 | 0.155 | 0.297 | 0.144 | 0.284 |
| H105D16HF/2 | 0.174 | 0.060 | 0.128 | 0.347 | 0.090 | 0.256 |
| H105D16HF/3 | 0.161 | 0.058 | 0.160 | 0.291 | 0.261 | 0.260 |
| H105D32HF/1 | 0.244 | 0.090 | 0.186 | 0.255 | 0.102 | 0.113 |
| H105D32HF/2 | 0.232 | 0.095 | 0.176 | 0.364 | 0.124 | 0.227 |
| H105D64HF/1 | 0.253 | 0.116 | 0.247 | 0.212 | 0.216 | 0.364 |
| H105D64HF/2 | 0.276 | 0.093 | 0.160 | 0.160 | | |

REGRESSION DATA

| LINEAR MODEL | | | | | | |
|---|---|---|---|---|---|---|
| m | 0.0020 | 0.0008 | 0.0013 | -0.0033 | 0.0015 | 0.0012 |
| r | 0.876 | 0.846 | 0.631 | -0.734 | 0.460 | 0.351 |
| P> | 0.001 | 0.001 | 0.010 | 0.001 | n.s | n.s. |

## Appendix 3B.3 H140 HCl

| SAMPLE CODE | 0.5 ln (1+D/L)/(1-D/L) | | | | | |
|---|---|---|---|---|---|---|
| | ALA | VAL | LEU | ASP | PHE | GLU |
| H140D2HC1/1 | 0.162 | 0.053 | | 0.549 | 0.145 | 0.375 |
| H140D2HC1/2 | | | | | | |
| H140D4HC1/1 | 0.213 | | 0.124 | 0.743 | | |
| H140D4HC1/2 | 0.204 | 0.089 | 0.202 | 0.634 | 0.201 | 0.438 |
| H140D8HCL/1 | 0.265 | 0.169 | 0.173 | 0.545 | 0.392 | 0.838 |
| H140D8HCL/2 | 0.256 | 0.096 | | 0.684 | 0.319 | 0.511 |
| H140D16HCL/1 | 0.253 | 0.131 | | 0.511 | 0.251 | 0.587 |
| H140D16HCL/2 | | | | | | |
| H140D32HC1/1 | 0.354 | 0.255 | | 0.482 | 0.367 | 0.675 |
| H140D32HC1/2 | 0.231 | 0.155 | | 0.486 | 0.399 | 0.756 |

REGRESSION DATA

| LINEAR MODEL | | | | | |
|---|---|---|---|---|---|
| m | 0.0031 | 0.0041 | -0.0055 | 0.0053 | 0.0078 |
| r | 0.669 | 0.790 | -0.695 | 0.673 | 0.588 |
| P> | 0.050 | 0.025 | 0.050 | 0.050 | n.s. |

## Appendix 3B.4 H140 HF

$$0.5 \ln \frac{1+D/L}{1-D/L}$$

| SAMPLE CODE | ALA | VAL | LEU | ASP | PHE | GLU |
|---|---|---|---|---|---|---|
| H140D2HF/1 | 0.156 | 0.050 | 0.051 | | 0.135 | 0.207 |
| H140D2HF/2 | 0.122 | 0.061 | 0.143 | 0.311 | 0.088 | 0.087 |
| H140D4HF/1 | 0.204 | 0.082 | 0.170 | 0.282 | 0.154 | 0.147 |
| H140D4HF/2 | 0.215 | 0.088 | 0.198 | 0.304 | 0.230 | 0.295 |
| H140D8HF/1 | 0.294 | 0.116 | | 0.371 | 0.282 | 0.336 |
| H140D8HF/2 | 0.238 | 0.117 | | 0.376 | 0.271 | 0.442 |
| H140D16HF/1 | 0.204 | 0.155 | | 0.227 | 0.208 | 0.307 |
| H140D16HF/2 | | | | | | |
| H140D32HF/1 | 0.250 | 0.130 | | 0.277 | 0.315 | 0.369 |
| H140D32HF/2 | 0.238 | 0.125 | | 0.289 | 0.268 | 0.543 |

REGRESSION DATA

| LINEAR MODEL | | | | | |
|---|---|---|---|---|---|
| m | 0.0020 | 0.0020 | -0.0013 | 0.0042 | 0.0084 |
| r | 0.467 | 0.695 | -0.335 | 0.670 | 0.714 |
| P> | n.s. | 0.025 | n.s. | 0.025 | 0.025 |

## Appendix 3B.5 H160 HCl

| SAMPLE CODE | 0.5 ln (1+D/L)/(1-D/L) | | | | | |
|---|---|---|---|---|---|---|
| | ALA | VAL | LEU | ASP | PHE | GLU |
| H160D1HCl/1 | 0.174 | 0.051 | 0.077 | 0.541 | 0.082 | 0.427 |
| H160D1HCl/2 | | | | | | |
| H160D2HCl/1 | 0.209 | 0.077 | 0.072 | 0.437 | 0.075 | 0.324 |
| H160D2HCl/2 | | | | | | |
| H160D4HCl/1 | | | | | | |
| H160D4HCl/2 | 0.348 | 0.144 | 0.077 | 0.537 | 0.136 | 0.451 |
| H160D8HCL/1 | 0.472 | 0.178 | 0.113 | 0.398 | 0.193 | 0.469 |
| H160D8HCL/2 | 0.532 | 0.208 | | 0.517 | 0.180 | 0.649 |
| H160D16HCL/1 | 0.774 | 0.404 | 0.536 | 0.545 | 0.424 | 0.704 |
| H160D16HCL/2 | 0.741 | 0.339 | 0.427 | 0.596 | 0.342 | 0.824 |

REGRESSION DATA

| LINEAR MODEL | | | | | | |
|---|---|---|---|---|---|---|
| m | 0.0381 | 0.0208 | 0.0288 | 0.0047 | 0.0208 | -0.0003 |
| r | 0.988 | 0.983 | 0.944 | 0.425 | 0.975 | -0.006 |
| P> | 0.001 | 0.001 | 0.001 | n.s. | 0.001 | n.s |

## Appendix 3B.6 H160 HF

$$0.5 \ln \frac{1+D/L}{1-D/L}$$

| SAMPLE CODE | ALA | VAL | LEU | ASP | PHE | GLU |
|---|---|---|---|---|---|---|
| H160D1HF/1 | 0.200 | 0.084 | 0.136 | 0.302 | | |
| H160D1HF/2 | | | | | | |
| H160D2HF/1 | 0.231 | 0.113 | 0.259 | 0.564 | 0.244 | |
| H160D2HF/2 | 0.231 | 0.092 | 0.235 | 0.434 | 0.181 | 0.295 |
| H160D4HF/1 | 0.306 | 0.119 | 0.157 | 0.378 | 0.143 | 0.242 |
| H160D4HF/2 | 0.367 | 0.179 | 0.302 | 0.202 | 0.310 | 0.418 |
| H160D8HF/1 | 0.388 | 0.160 | 0.189 | 0.234 | 0.165 | 0.341 |
| H160D8HF/2 | 0.339 | 0.143 | 0.251 | 0.256 | 0.258 | 0.488 |
| H160D16HF/1 | 0.401 | 0.156 | 0.121 | 0.295 | | |
| H160D16HF/2 | 0.394 | 0.203 | 0.153 | 0.210 | 0.143 | 0.329 |

REGRESSION DATA

| LINEAR MODEL | | | | | | |
|---|---|---|---|---|---|---|
| m | 0.0112 | 0.0052 | 0.0051 | -0.0153 | -0.0050 | 0.0193 |
| r | 0.820 | 0.752 | -0.462 | -0.592 | -0.385 | 0.531 |
| P> | 0.010 | 0.010 | n.s. | 0.050 | n.s. | n.s. |

Appendix 3B.7 Isoleucine all temperature/fractions

$$0.57 \ln \frac{1+D/L}{1-0.75D/L}$$

| TIME TREATMENT | HCl | | | HF | | |
|---|---|---|---|---|---|---|
| | H105 | H140 | H160 | H105 | H140 | H160 |
| D1/1 | 0.054 | | 0.042 | 0.065 | | 0.172 |
| D1/2 | 0.028 | | | 0.037 | | |
| D1/3 | | | | 0.068 | | |
| D2/1 | 0.026 | | 0.199 | | 0.073 | 0.162 |
| D2/2 | 0.025 | 0.080 | | | 0.075 | 0.130 |
| D4/1 | 0.049 | | | 0.062 | 0.096 | 0.144 |
| D4/2 | 0.048 | 0.108 | 0.195 | 0.061 | 0.102 | 0.217 |
| D8/1 | 0.083 | 0.274 | | 0.055 | 0.129 | 0.192 |
| D8/2 | 0.065 | 0.148 | 0.248 | | 0.126 | 0.156 |
| D16/1 | 0.033 | 0.145 | 0.357 | 0.048 | 0.131 | 0.128 |
| D16/2 | 0.049 | | 0.366 | 0.064 | | 0.177 |
| D16/3 | 0.067 | | | 0.095 | | |
| D32/1 | 0.088 | 0.219 | | 0.092 | 0.119 | |
| D32/2 | 0.013 | 0.205 | | 0.109 | 0.115 | |
| D32/3 | 0.129 | | | | | |
| D64/1 | 0.221 | | | 0.181 | | |
| D64/2 | 0.245 | | | 0.120 | | |

REGRESSION DATA

| LINEAR MODEL | | | | | | |
|---|---|---|---|---|---|---|
| m | 0.0030 | 0.0046 | 0.0183 | 0.0015 | 0.0006 | 0.0006 |
| r | 0.95 | 0.92 | 0.93 | 0.05 | 0.52 | 0.11 |
| P< | 0.001 | 0.001 | 0.010 | n.s. | n.s. | n.s. |

# APPENDIX 4 PYROLYSIS STUDIES : TRANSFORMED MOLLUSC DATA.

The results from previous pyrolysis studies of molluscs, published by Kimber and Griffin (1987) were transformed to fit Equation 5.1. The results of this transformation are presented as Appendix 4A.

The results of cumulative regression analysis of these data are presented as Appendix 4B.

The results of modelling the change in rate as Equation 5.3 are presented as Appendix 4C.

Contents of Appendix 4

## 4A.1 *Anadara trapezia* (Modern)

| Time / Treatment | 0.5 Ln {(1+D/L)/(1-D/L)} ALA | VAL | LEU |
|---|---|---|---|
| $T_0/1$ | 0.061 | 0.041 | 0.048 |
| $T_0/2$ | 0.063 | 0.023 | 0.048 |
| $T_1/1$ | 0.273 | 0.091 | 0.109 |
| $T_1/2$ | 0.288 | 0.063 | 0.142 |
| $T_2/1$ | 0.382 | 0.120 | 0.192 |
| $T_2/2$ | 0.363 | 0.103 | 0.187 |
| $T_3/1$ | 0.594 | 0.237 | 0.367 |
| $T_3/2$ | 0.618 | 0.250 | 0.377 |
| $T_4/1$ | 0.900 | 0.361 | 0.549 |
| $T_4/2$ | 0.818 | 0.420 | 0.544 |
| $T_5/1$ | 1.000 | 0.482 | 0.678 |
| $T_5/2$ | 1.007 | 0.538 | 0.683 |
| $T_6/1$ | 1.478 | 0.711 | 0.915 |
| $T_6/2$ | 1.571 | 0.679 | 0.843 |
| $T_7/1$ | 1.322 | 0.822 | 0.948 |
| $T_7/2$ | 1.322 | 0.745 | 1.670 |

4A.2 *Katalysia rhytiphora* (Modern)

| Time / Treatment | 0.5 Ln {(1+D/L)/(1-D/L)} ALA | VAL | LEU |
|---|---|---|---|
| $T^0/1$ | 0.059 | 0.011 | 0.052 |
| $T_0/2$ | 0.068 | 0.021 | |
| $T_1/1$ | 0.296 | 0.110 | |
| $T_1/2$ | 0.427 | 0.091 | 0.267 |
| $T_2/1$ | 0.492 | 0.157 | 0.235 |
| $T_2/2$ | 0.472 | 0.134 | 0.181 |
| $T_3/1$ | 0.972 | 0.318 | 0.479 |
| $T_3/2$ | 0.755 | 0.276 | 0.479 |
| $T_4/1$ | 1.085 | 0.456 | 0.659 |
| $T_4/2$ | 1.110 | 0.440 | 0.624 |
| $T_5/1$ | 1.186 | 0.569 | 0.672 |
| $T_5/2$ | 0.798 | 0.416 | 0.603 |
| $T_6/1$ | 1.331 | 0.642 | 0.693 |
| $T_6/2$ | 1.317 | 0.639 | 0.755 |
| $T_7/1$ | 0.934 | 0.752 | 0.721 |
| $T_7/2$ | 1.210 | 0.853 | 0.943 |

4A.3 *Anadara trapezia* (Holocene)

| Time | 0.5 Ln {(1+D/L)/(1-D/L)} | | |
|---|---|---|---|
| Treatment | ALA | VAL | LEU |
| $T_0/1$ | 0.205 | 0.130 | 0.093 |
| $T_0/2$ | 0.127 | 0.065 | 0.089 |
| $T_1/1$ | 0.641 | 0.093 | 0.190 |
| $T_1/2$ | 0.343 | 0.088 | 0.176 |
| $T_2/1$ | | 0.256 | 0.126 |
| $T_2/2$ | 0.293 | 0.147 | 0.204 |
| $T_3/1$ | 0.534 | 0.186 | 0.320 |
| $T_3/2$ | 0.446 | 0.144 | 0.273 |
| $T_4/1$ | 0.761 | 0.311 | 0.446 |
| $T_4/2$ | 0.545 | 0.263 | 0.333 |
| $T_5/1$ | 0.727 | 0.427 | 0.516 |
| $T_5/2$ | 0.904 | 0.441 | 0.643 |
| $T_6/1$ | 0.851 | 0.609 | 0.765 |
| $T_6/2$ | 0.753 | 0.460 | 0.632 |
| $T_7/1$ | 1.062 | 0.831 | 1.096 |
| $T_7/2$ | 1.065 | 0.822 | 0.862 |

## 4A.4 *Anadara trapezia* (Pleistocene)

| Time | 0.5 Ln {(1+D/L)/(1-D/L)} | | |
|---|---|---|---|
| Treatment | ALA | VAL | LEU |
| $T_0/1$ | 0.837 | 0.336 | 0.397 |
| $T_0/2$ | 0.853 | 0.314 | 0.448 |
| $T_1/1$ | 0.875 | 0.318 | 0.476 |
| $T_1/2$ | 0.910 | 0.307 | 0.481 |
| $T_2/1$ | 0.970 | 0.347 | 0.516 |
| $T_2/2$ | 0.748 | 0.321 | 0.544 |
| $T_3/1$ | 0.989 | 0.385 | 0.572 |
| $T_3/2$ | 1.032 | 0.389 | 0.586 |
| $T_4/1$ | 0.636 | 0.400 | 0.547 |
| $T_4/2$ | 0.911 | 0.464 | 0.593 |
| $T_5/1$ | 1.167 | 0.608 | 0.749 |
| $T_5/2$ | 1.195 | 0.613 | 0.774 |
| $T_6/1$ | 1.004 | 0.759 | 0.936 |
| $T_6/2$ | 1.004 | 0.782 | 0.926 |
| $T_7/1$ | 1.574 | 0.949 | 1.033 |
| $T_7/2$ | 1.282 | 0.930 | 0.980 |

## 4A.5 Isoleucine

| Time / Treatment | 0.57 Ln { (1+D/L) / (1 - 0.75D/L) } *A.trapezia* Modern | *A.trapezia* Holocene | *A.trapezia* Pleistocene | *K.rhytiphora* Modern |
|---|---|---|---|---|
| $T_0/1$ | 0.061 | 0.103 | 0.428 | 0.021 |
| $T_0/2$ | 0.141 | 0.061 | 0.428 | |
| $T_1/1$ | 0.056 | 0.128 | 0.433 | 0.154 |
| $T_1/2$ | 0.076 | 0.113 | 0.417 | 0.145 |
| $T_2/1$ | 0.124 | 0.180 | 0.485 | 0.258 |
| $T_2/2$ | 0.115 | 0.148 | 0.460 | 0.200 |
| $T_3/1$ | 0.271 | 0.292 | 0.558 | 0.514 |
| $T_3/2$ | 0.315 | 0.198 | 0.562 | 1.069 |
| $T_4/1$ | 0.423 | 0.437 | 0.513 | 0.751 |
| $T_4/2$ | 0.448 | 0.321 | 0.591 | 0.725 |
| $T_5/1$ | 0.545 | 0.558 | 0.757 | 1.163 |
| $T_5/2$ | 0.614 | 0.568 | 0.810 | 0.660 |
| $T_6/1$ | 0.879 | 0.833 | 1.031 | 1.358 |
| $T_6/2$ | 0.852 | 0.729 | 1.066 | 0.897 |
| $T_7/1$ | 1.025 | 1.254 | 1.390 | 1.059 |
| $T_7/2$ | 1.700 | 1.084 | 1.350 | 1.014 |

4B.1 *Anadara trapezia* (Modern)

| Time Treatment | coefficient* | Amino acid ALA | VAL | ILE | LEU |
|---|---|---|---|---|---|
| to $T_2$ | k | 0.142 | 0.040 | 0.010 | 0.071 |
| | r | 0.95 | 0.95 | 0.23 | 0.99 |
| to $T_3$ | k | 0.129 | 0.053 | 0.053 | 0.081 |
| | r | 0.98 | 0.98 | 0.86 | 0.99 |
| to $T_4$ | k | 0.095 | 0.046 | 0.048 | 0.063 |
| | r | 0.97 | 0.99 | 0.95 | 0.99 |
| to $T_5$ | k | 0.055 | 0.030 | 0.033 | 0.039 |
| | r | 0.92 | 0.96 | 0.95 | 0.95 |
| to $T_6$ | k | 0.041 | 0.020 | 0.025 | 0.025 |
| | r | 0.95 | 0.95 | 0.96 | 0.92 |
| to $T_7$ | k | 0.018 | 0.012 | 0.021 | 0.011 |
| | r | 0.77 | 0.89 | 0.94 | 0.76 |

* Where,
k is the rate of racemization/epimerization, and,
r is the regression coefficient.

4B.2 Katalysia rhytiphora (Modern)

| Time Treatment | coefficient* | Amino acid ALA | VAL | ILE | LEU |
|---|---|---|---|---|---|
| to $T_2$ | k | 0.209 | 0.065 | 0.101 | |
| | r | 0.95 | 0.97 | 0.96 | 0.66 |
| to $T_3$ | k | 0.192 | 0.069 | 0.202 | 0.098 |
| | r | 0.97 | 0.99 | 0.86 | 0.93 |
| to $T_4$ | k | 0.124 | 0.054 | 0.094 | 0.069 |
| | r | 0.95 | 0.98 | 0.78 | 0.95 |
| to $T_5$ | k | 0.052 | 0.029 | 0.050 | 0.031 |
| | r | 0.76 | 0.90 | 0.74 | 0.82 |
| to $T_6$ | k | 0.031 | 0.018 | 0.029 | 0.016 |
| | r | 0.79 | 0.89 | 0.75 | 0.77 |
| to $T_7$ | k | 0.012 | 0.011 | 0.011 | 0.009 |
| | r | 0.59 | 0.88 | 0.56 | 0.76 |

* Where,
k is the rate of racemization/epimerization, and,
r is the regression coefficient.

## 4B.3 *Anadara trapezia* (Holocene)

| Time Treatment | coefficient* | Amino acid ALA | VAL | ILE | LEU |
|---|---|---|---|---|---|
| to $T_2$ | k | 0.007 | | 0.022 | 0.054 |
| | r | 0.85 | 0.27 | 0.77 | 0.93 |
| to $T_3$ | k | 0.039 | 0.017 | 0.036 | 0.039 |
| | r | 0.66 | 0.87 | 0.95 | 0.95 |
| to $T_4$ | k | -0.008 | 0.015 | 0.018 | 0.017 |
| | r | -0.19 | 0.91 | 0.82 | 0.78 |
| to $T_5$ | k | 0.017 | 0.018 | 0.022 | 0.018 |
| | r | 0.60 | 0.98 | 0.96 | 0.94 |
| to $T_6$ | k | 0.006 | 0.015 | 0.020 | 0.015 |
| | r | 0.44 | 0.98 | 0.99 | 0.97 |
| to $T_7$ | k | 0.008 | 0.010 | 0.015 | 0.009 |
| | r | 0.79 | 0.96 | 0.98 | 0.92 |

* Where,
k is the rate of racemization/epimerization, and,
r is the regression coefficient.

4B.4 *Anadara trapezia* (Pleistocene)

| Time Treatment | coefficient* | Amino acid ALA | VAL | ILE | LEU |
|---|---|---|---|---|---|
| to $T_2$ | k | 0.079 | 0.052 | 0.042 | 0.037 |
| | r | 0.71 | 0.68 | 0.90 | 0.66 |
| to $T_3$ | k | 0.072 | 0.021 | 0.041 | 0.038 |
| | r | 0.90 | 0.54 | 0.91 | 0.80 |
| to $T_4$ | k | 0.056 | 0.023 | 0.037 | 0.035 |
| | r | 0.91 | 0.83 | 0.95 | 0.91 |
| to $T_5$ | k | 0.038 | 0.021 | 0.030 | 0.029 |
| | r | 0.91 | 0.94 | 0.97 | 0.96 |
| to $T_6$ | k | 0.018 | 0.014 | 0.022 | 0.019 |
| | r | 0.80 | 0.92 | 0.97 | 0.93 |
| to $T_7$ | k | 0.012 | 0.011 | 0.017 | 0.013 |
| | r | 0.84 | 0.96 | 0.97 | 0.93 |

* Where,
k is the rate of racemization/epimerization, and,
r is the regression coefficient.

APPENDIX 4C Molluscan data : Change in rate constants

Change in rate constant is modelled as Equation 5.3. The data reported here is the slope of rate of change. The significance of the model is as indicated, ( $^{*}P<0.1$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{n.s.}$ $P>0.1$).

| Mollusc species (age) | Amino acid ALA | VAL | ILE | LEU |
|---|---|---|---|---|
| *Anadara trapezia* | | | | |
| (Modern) | $-0.18^{***}$ | $-0.12^{**}$ | $-0.037^{n.s.}$ | $-0.17^{**}$ |
| (Holocene) | $-0.052^{n.s.}$ | $-0.046^{n.s.}$ | $-0.042^{n.s.}$ | $-0.14^{***}$ |
| (Pleistocene) | $-0.17^{***}$ | $-0.11^{**}$ | $-0.081^{***}$ | $-0.094^{**}$ |
| *Katalysia rhytiphora* | | | | |
| (Modern) | $-0.26^{***}$ | $-0.17^{***}$ | $-0.27^{***}$ | $-0.22^{**}$ |

# APPENDIX 5 PYROLYSIS STUDIES: WHEAT/FALLOW

## Appendix 5A Pyrolysis studies: Wheat/fallow D/L data HCl fraction.

| WHEAT/FALLOW SAMPLE CODE | D/L | | | | | |
|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | ASP | PHE | GLU |
| D1HCl/1 | 0.246 | 0.787 | | 0.448 | 0.137 | 0.322 |
| D1HCl/2 | 0.226 | 0.661 | 0.464 | 0.331 | | 0.285 |
| D2HCl/1 | 0.271 | 0.092 | 0.105 | | 0.191 | 0.334 |
| D2HCl/2 | 0.278 | 0.093 | 0.119 | | 0.183 | 0.256 |
| D4HCl/1 | 0.464 | 0.160 | | 0.467 | 0.341 | 0.555 |
| D4HCl/2 | | | | 0.426 | | |
| D8HCL/1 | 0.585 | 0.216 | 0.302 | | 0.162 | |
| D16HCL/1 | 0.865 | 0.500 | 0.985 | 0.272 | 0.124 | 0.569 |
| D16HCL/2 | 0.801 | 0.430 | 1.062 | 0.357 | 0.518 | 0.705 |
| D16HCl/3 | 0.834 | 0.469 | 0.988 | 0.280 | 0.202 | 0.620 |
| D32HCl/1 | 0.949 | 0.566 | 1.234 | 0.316 | 0.386 | 0.759 |
| D32HCl/2 | 0.875 | 0.544 | 1.109 | 0.349 | 0.528 | 0.730 |
| D32HCl/3 | 0.719 | 0.550 | 1.161 | 0.338 | 0.457 | 0.744 |

Appendix 5B Pyrolysis studies: Wheat/fallow D/L data HF fraction.

| WHEAT/FALLOW SAMPLE CODE | D/L | | | | | |
|---|---|---|---|---|---|---|
| | ALA | VAL | ILE | ASP | PHE | GLU |
| D1HF/1 | 0.132 | 0.111 | | 0.203 | 0.048 | 0.077 |
| D1HF/2 | 0.099 | 0.057 | 0.080 | | | |
| D2HF/1 | 0.146 | 0.106 | 0.059 | 0.136 | 0.158 | |
| D2HF/2 | 0.148 | 0.088 | 0.107 | 0.079 | 0.243 | |
| D4HF/1 | 0.142 | 0.100 | 0.234 | 0.068 | 0.188 | |
| D4HF/2 | 0.111 | | | 0.178 | 0.176 | 0.334 |
| D8HF/1 | 0.122 | 0.132 | 0.120 | 0.083 | | |
| D8HF/2 | 0.178 | 0.101 | 0.409 | 0.067 | 0.193 | |
| D16HF/1 | 0.472 | 0.239 | 0.307 | 0.054 | 0.213 | 0.169 |
| D16HF/2 | 0.280 | 0.198 | 0.360 | | 0.077 | 0.022 |
| D16HF/3 | | | | | 0.145 | 0.096 |
| D32HF/1 | 0.673 | 0.247 | 0.410 | | 0.426 | 0.055 |
| D32HF/2 | 0.425 | 0.259 | 0.641 | 0.069 | 0.771 | 0.063 |
| D32HF/3 | 0.551 | 0.229 | 0.516 | 0.067 | 0.514 | 0.061 |

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