THE CHEMISTRY OF *Vicia sativa* L. SELECTION

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Doctor of Philosophy

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A NEEDLE IN A HAYSTACK

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ABSTRACT

This thesis describes the development of two novel and complementary analytical approaches for assaying cyanoalanine non-protein amino acids. These assays were used to determine the distribution of these compounds both within and between plants. Subsequently, the assays were applied to identify accessions of common vetch which contain low levels of the cyanoalanine non-protein amino acids in germplasm collections. In collaboration with colleagues these analytical tools have been used to correlate toxicity observed in animal feeding experiments with the cyanoalanine content.

The limited knowledge base for Diffuse Reflectance Using Infrared Dispersive Spectrometry (DRUIDS) and the Micellar Electrokinetic Chromatography (MEKC) analytical methodologies have been examined and expanded. This thesis covers the first report of the use of diffuse reflectance using dispersive infrared spectrometry for the *in situ* quantification of specific organic components from plant tissue. It also reports the first use of micellar electrokinetic chromatography for the quantitative analysis of 9-fluorenylmethyl chloroformate (FMOC) derivatised and non-derivatised components of extracts from plant material.

Major aspects include:

- Isolation and characterisation by NMR, IR and X-ray crystallography of *N*-γ-L-glutamyl-β-cyano-L-alanine as its ammonium salt from the seeds of common vetch (*Vicia sativa* L.).
- Development a rapid and simple *in situ* method for the quantitative analysis of γ-glutamyl-β-cyanoalanine by diffuse reflectance using dispersive infrared spectrometry from homogenised vetch powder.
- Development of a quantitative separation by micellar electrokinetic chromatography of 9-fluorenylmethyl chloroformate (FMOC) derivatised and non-derivatised components of extracts from plant material.
- Determination of the distribution of γ-glutamyl-β-cyanoalanine, throughout and between plants of the same cultivar grown at the same site, by diffuse reflectance using dispersive infrared spectrometry.
- Screening of the germplasm collection of *Vicia sativa* L. from the International Centre for Agricultural Research in Dry Areas (ICARDA Aleppo, Syria) for γ-glutamyl-β-cyanoalanine by diffuse reflectance using dispersive infrared spectrometry and micellar electrokinetic chromatography to assess the occurrence of "zero cyanoalanine non-protein amino acid" accessions in the native population.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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PUBLICATIONS ARISING FROM THIS THESIS:

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Tate, M. E., Enneking, D., Delaere, I. M., Malaterre, C., Glatz, P. C. (1995). "Towards the detoxification of *Vicia sativa* L." *Lathyrus sativus* and Human Lathyrism: Progress and Prospects., University of Dhaka, Bangladesh.: 93-100.

Delaere, I. M., Rathjen, J. M., Asenstorfer, R. E., Paull, J. G., Tate, M. E. (1996). "Needle in a Haystack. The chemistry of *in situ* quantitation." RACI Medicinal & Agricultural Division 13th National Conference "Up and coming research in Australia", Monash University, Australia, Royal Australian Chemical Institute. (in press).

RELATED PUBLICATIONS

Tate, M. E., Delaere, I. M., Jones, G. P., Tiekink, E. R. T. (1995). "Crystal and molecular structure of cycasin, (*Z*)-β-D-Glucopyranosyloxy-*NNO*-azoxymethane." Australian Journal of Chemistry. **48**: 1059-1063.

Chapter One

Introduction and aims of the study

1.0 Introduction

The seeds of grain legumes, or pulses, are a primary source of protein for 1.2 billion people on the Indian subcontinent (van Rees *et al.* 1995). Due to the undeniable economic importance and increasing affluence of this market, grain legume crops suitable for servicing the protein requirements of this region are being actively researched in Australia. In 1993 the Australian Grains Research Development Corporation (GRDC) initiated a project to determine the potential for development of common vetch (*Vicia sativa* L.) (Petterson and Mackintosh 1994) as a grain legume crop suitable for human consumption.

The visual appearance of the split oiled pulse of common vetch can easily be mistaken for that of the red lentil (*Lens culinaris* Mill.) (Figure 1.0). However, common vetch contains three distinct families of low molecular weight, relatively thermostable, anti-nutritional factors which readily distinguish it from red lentil. Most notable are the cyanoalanine non-protein amino acids present as free β-cyanoalanine or more commonly as its bound form, γ-glutamyl-β-cyanoalanine. The cyanoalanine non-protein amino acids can be present at a concentration in excess of 1% (dry weight) in the cotyledon of common vetch and are considered unsuitable for human consumption (Tate and Enneking 1992).

It has long been established that the cyanoalanine non-protein amino acids are neurotoxic in chickens (Ressler *et al.* 1967) and the rat (Ressler *et al.* 1964). On a molar basis, γ-glutamyl-β-cyanoalanine and β-cyanoalanine have been shown to be equally toxic (Ressler *et al.* 1969a). Common vetch has also been described as toxic to ducks, monkeys and humans (Anderson *et al.* 1925; Anderson 1939; Shah 1939). The description of the neurological disorder attributed to the consumption of common vetch in humans is distinct from the irreversible form of lathyrism which is associated with the consumption of the grasspea (*Lathyrus sativus* L.).

1.1 Aims of this study

This thesis describes the development of two novel and complementary analytical approaches for assaying cyanoalanine non-protein amino acids. These assays were used to determine the distribution of these compounds both within and between plants. Subsequently, the assays were applied

Figure 1.0 A visual comparison of red lentil (*L. culinaris*) (Bottom) with commercially split common vetch cv. "Blanchefleur" (*V. sativa*) (Top right) and commercially split and oiled common vetch cv. "Blanchefleur" (Top left).

to identify accessions of common vetch which contain low levels of the cyanoalanine non-protein amino acids present in germplasm collections. In collaboration with Mr. Ali Darwish (University of Giessen), Ms. Jane Rathjen (University of Adelaide) and Dr Steven Valentine (South Australian Research and Development Institute, Flaxley Research Station, Flaxley S.A) these analytical tools have been used to correlate toxicity observed in animal feeding experiments with the cyanoalanine content.

In order to satisfy the above aims, it was first necessary to expand on the limited knowledge base for Diffuse Reflectance Using Infrared Dispersive Spectrometry (DRUIDS) and the Micellar Electrokinetic Chromatography (MEKC) analytical methodologies. This thesis covers the first report of the use of diffuse reflectance using dispersive infrared spectrometry for the *in situ* quantification of specific organic components from plant tissue. It also reports the first use of micellar electrokinetic chromatography for the quantitative analysis of 9-fluorenylmethyl chloroformate (FMOC) derivatised and non-derivatised components of extracts from plant material.

Specific aims of these investigations were:

- To isolate and fully characterise *N*-γ-L-glutamyl-β-cyano-L-alanine as its ammonium salt from the seeds of common vetch (*V. sativa*) (Chapter Three).
- To develop a rapid and simple *in situ* method for the quantitative analysis of γ-glutamyl-β-cyanoalanine by diffuse reflectance using dispersive infrared spectrometry from homogenised vetch powder (Chapter Four).
- To develop a quantitative separation by micellar electrokinetic chromatography of 9-fluorenylmethyl chloroformate (FMOC) derivatised and non-derivatised components of extracts from plant material (Chapter Five).
- To quantitatively determine the distribution of γ-glutamyl-β-cyanoalanine, throughout and between plants of the same cultivar grown at the same site, by diffuse reflectance using dispersive infrared spectrometry (Chapter Six).
- To quantitatively analyse the International Centre for Agricultural Research in Dry Areas (ICARDA Aleppo, Syria) germplasm collection of *V. sativa* for γ-glutamyl-β-cyanoalanine by diffuse reflectance using dispersive infrared spectrometry and micellar electrokinetic chromatography to assess the occurrence of "zero cyanoalanine non-protein amino acid" accessions in the native population (Chapter Seven).

Chapter Two

Literature review

2.0 Introduction

There is currently a need for alternative crops in the drier areas of the Southern Australian cereal belt, where cereal/fallow or cereal/pasture rotations are widely practised. Vetches are multi-purpose crops allowing for either fodder conservation or immediate cash returns through hay or grain production, while at the same time fixing nitrogen and providing green manure and grazing options. In addition they are beneficial with respect to weed control as crops prior to pastures (Enneking 1995).

The major development of vetches as Australian crops occurred after the establishment of the CSIRO plant introduction station at Muresk W.A. in 1943, as a part of a deliberate search for alternative grain legumes to replace field peas (*Pisum sativum* L.). The first introductions of common vetch (*V. sativa*) were made by Bailey in 1945 (Bailey 1952). A direct result of Bailey's work has been the release in Australia of the *V. sativa* cultivars Languedoc, Blanchefleur, Golden Tares and Nyabing (Oram 1990).

In Australia vetches are grown in mixtures with cereals as hay, forage and green manure crops. Common vetch was identified as the best adapted *Vicia* species for Australian grain legume production under the low rainfall conditions of the drier margins of the cereal belt (Enneking 1995). *V. sativa* has also been shown to perform well as a disease break crop and has a positive effect on grain yield and protein levels in subsequent wheat crops (Bull and Mayfield 1988; Cummins 1994).

2.1 Human consumption of common vetch

The literature contains few references which support a role for *V. sativa* in the human diet, and has been extensively reviewed (Enneking 1995). Most reports describe human consumption of common vetch as undesirable. For example, the consumption of common vetch has been highlighted as a causal agent of a debilitating neurological disorder in India with symptoms similar to, but distinguishable from those of lathyrism (Anderson *et al.* 1925; Anderson 1939; Shah 1939). More recently the consumption of common vetch (*V. sativa* ssp. *obovata* (Ser) Gaudin) has been

reported in the rural peasants of Chile. References to culinary preparative techniques and neurological disturbances were not made (Araya *et al.* 1990). However, the report noted adverse weight gains in rats fed a diet containing common vetch and warned that further investigations were required to determine the interaction of the low molecular weight anti-nutritional factors including the cyanoalanine non-protein amino acids present in vetch with the animal study model (Araya *et al.* 1990).

Australia continues to sell common vetch or "Australian orange vetch" (*V. sativa* cv. "Blanchefleur") in the Indian subcontinent as a promising protein rich and price attractive alternative pulse to the common lentil (*L. culinaris*) (van Rees *et al.* 1995). The literature identifies three distinct chemical families of thermostable, low molecular weight anti-nutritional factors present in the pulse of common vetch. Most notable are the cyanoalanine non-protein amino acids present as free β-cyanoalanine or more commonly as its bound form, γ-glutamyl-β-cyanoalanine. The cyanoalanine non-protein amino acids can be present at concentration well in excess of 1% (dry weight) in the cotyledon of common vetch and are considered unsuitable for human consumption (Tate and Enneking 1992). As a result, in 1993 *V. sativa* was banned from importation and sale in India and Egypt (Bhat and Raghuram 1993).

2.2 Taxonomy

Vetches are members of the Leguminosae and are included in the legume tribe *Vicieae*. Leguminosae are characterised by their fruit, the legume. A legume is basically a dehiscent fruit that develops from a single carpel and splits into valves that surround the fruit (Heywood 1971). Members of the *Vicieae* illustrate a wide range of phenomena, namely the ability to fix nitrogen by symbiosis with *Rhizobium*, the exemplification of Vavilov's homologous series (Vavilov 1922), and considerable structural diversity in the synthesis of low molecular weight secondary plant metabolites. The generic classification of the *Vicieae* are now generally accepted as a grouping of five genera (Kupicha 1981): *Vicia* L., *Lathyrus* L., *Lens* Mill., *Pisum* L., and *Vavilovia* A. Fedorov.

The taxonomic history of subgenus *Vicia* is extensive, 20 major classifications of the group having been produced since the work of Linnaeus (Maxted 1991). A recent revision of *Vicia* L. subgenus *Vicia* (Leguminosae, Vicieae) (Maxted 1995) has placed common vetch (*V. sativa*) in Section *Vicia* which is composed of five species, one of which is the pan-temperate and semi-tropical weed *V. sativa*. *V. sativa* is the most ubiquitous and polymorphic species of

Vicia. The other four species, *Vicia pyrenaica, Vicia qatmensis*, *Vicia grandiflora* and *Vicia barbazitae* are geographically restricted, suggesting that they may have evolved separately from a *V. sativa* like form (Maxted 1995). Successful crossing and hybridisation of *V. sativa* with the other four species within Section *Vicia* has not been reported. Interspecific hybridisation could have important implications for the introgression of traits from the other species to *V. sativa*, e.g., a gene conferring a zero or low cyanoalanine non-protein amino acid profile.

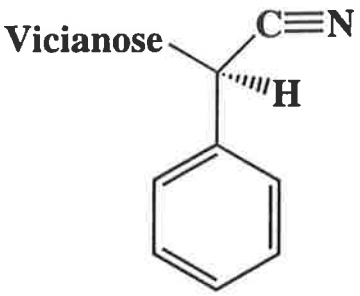
An elaborate morphological key to *Vicia* subgenus *Vicia*, section, series and species has been presented for plant identification in Maxted (1995). Identification of plants solely on morphological characteristics is not always straightforward leading to a difficult and sometimes ambiguous classification of some *Vicia* species in botanical collections. A number of chemotaxonomic characters have been identified which are useful in aiding in the classification of *Vicia* (Bell and Tirimanna 1965; Tschiersch and Hanelt 1967). This has involved the subdivision of *Vicia* into groups of species characterised by associations of non-protein amino acids and unusual glycosides occurring in high concentrations in their seeds.

2.3 Anti-nutritional factors found in common vetch

The plant kingdom has evolved an arsenal of enzyme inhibitors, lectins, phytates, saponins, phytoalexins, tannins, oligosaccharides and other potentially toxic anti-nutritional factors to deter predation. These compounds can have the effect of lowering the nutritional value of a food. For example, compounds such as tannins may lower digestibility of proteins. In many foods, processes have been developed to remove anti-nutritional factors. This can be achieved by continuous soaking and discarding of leachate for water soluble anti-nutritional factors or by cooking to decompose thermally labile anti-nutritional factors.

The literature identifies three distinct chemical families of thermostable, low molecular weight anti-nutritional factors present in the pulse of common vetch which have the potential to cause injury when ingested. These are the cyanogenic glycoside vicianine, the β-D-glucopyranosyl glycosides vicine and convicine, and the cyanoalanine non-protein amino acids, β-cyanoalanine and γ-glutamyl-β-cyanoalanine (Figure 2.0).

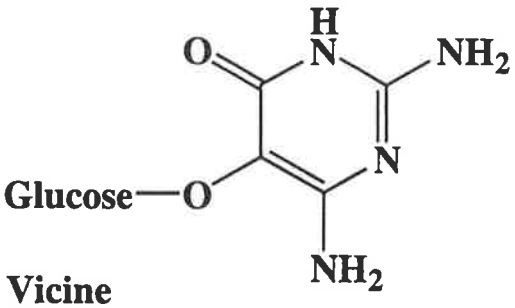
Cyanogenic glycosides



Vicianine

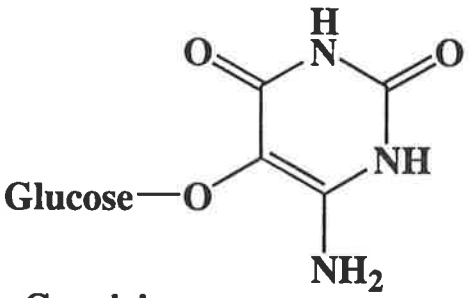
Mandelonitrile 6-O-α-L-Arabino-pyranosyl-D-glucose

β-D glucopyranosyl glycosides



Vicine

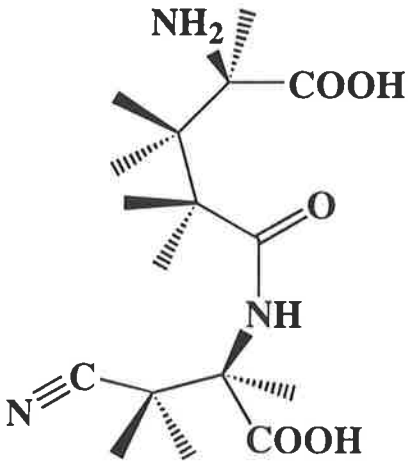
2,6-diamino-5-(β-D glucopyranosyl-oxy)-4-(1H)-pyrimidinone



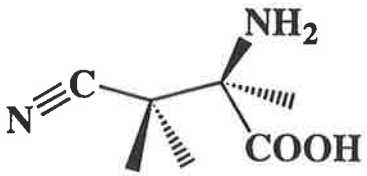
Convicine

6-amino-5-(β-D glucopyranosyloxy) -2,4(1H,3H)-pyrimidinedione

β-cyanoalanine non-protein amino acids



γ-L-glutamyl-β-cyano-L-alanine



β-cyano-L-alanine

Figure 2.0 The chemical structures of the low molecular weight thermostable anti-nutritional factors found in common vetch (*V. sativa* L.).

2.4 The biochemistry of anti-nutritional factors found in common vetch

2.4.1 Cyanogenic glycosides

The ability of organisms to produce hydrogen cyanide is termed cyanogenesis (Conn 1979). Cyanogenic glycosides are a group of secondary plant products which, upon hydrolysis, release hydrogen cyanide and are found in more than 2000 plant species, including sorghum, cassava and common vetch (Sibbesen *et al.* 1995). Vicianine, the cyanogenic glycoside found in common vetch, was first isolated by Bertrand (1906) from *V. sativa* var. *angustifolia* and contains an unusual disaccharide vicianose composed of glucose and arabinose (Dyson 1950). The β-D-gentiobioside analogue of vicianine, amygdalin is found in the seeds of the *Rosaceae*, particularly in bitter almonds, and is commercially available.

2.4.1.1 Biosynthesis

There are a number of excellent reviews describing the biosynthesis of the cyanogenic glycosides in plants (Liener 1966; Conn 1974, 1981; Sibbesen *et al.* 1995). The formation of cyanogenic glycosides was initially studied by injecting radioactively labelled amino acid precursors into intact seedlings (Akazawa *et al.* 1960). Only the precursor amino acid and the cyanogenic glycoside accumulated in the seedlings. The primary precursors of cyanogenic glycosides are restricted to the five hydrophobic protein amino acids valine, leucine, isoleucine, phenylalanine and tyrosine and a single non-protein amino acid, cyclopentenylglycine (Sibbesen *et al.* 1995).

Subsequent work using microsomal preparations has shown that the production of cyanogenic glycosides in plants involves a dehydration of the amino acid precursor via an aldehyde oxime intermediate leading to the formation of the nitrile. The cytochrome P-450 catalyses the conversion from amino acid to aldehyde oxime (Figure 2.1). All intermediates except 2-nitro-3-(*p*-hydroxyphenyl)propionic acid, which is a very labile compound, have been shown to be produced and metabolised by the microsomal preparation (Sibbesen *et al.* 1995). This work established that the synthesis of the nitrile group in cyanogenic glycosides is independent of 1-amino-cyclopropane-1-carboxylic acid (ACC), a precursor in ethylene synthesis responsible for the nitrile unit in the β-cyanoalanine non-protein amino acids found in *V. sativa* (Peiser *et al.* 1984).

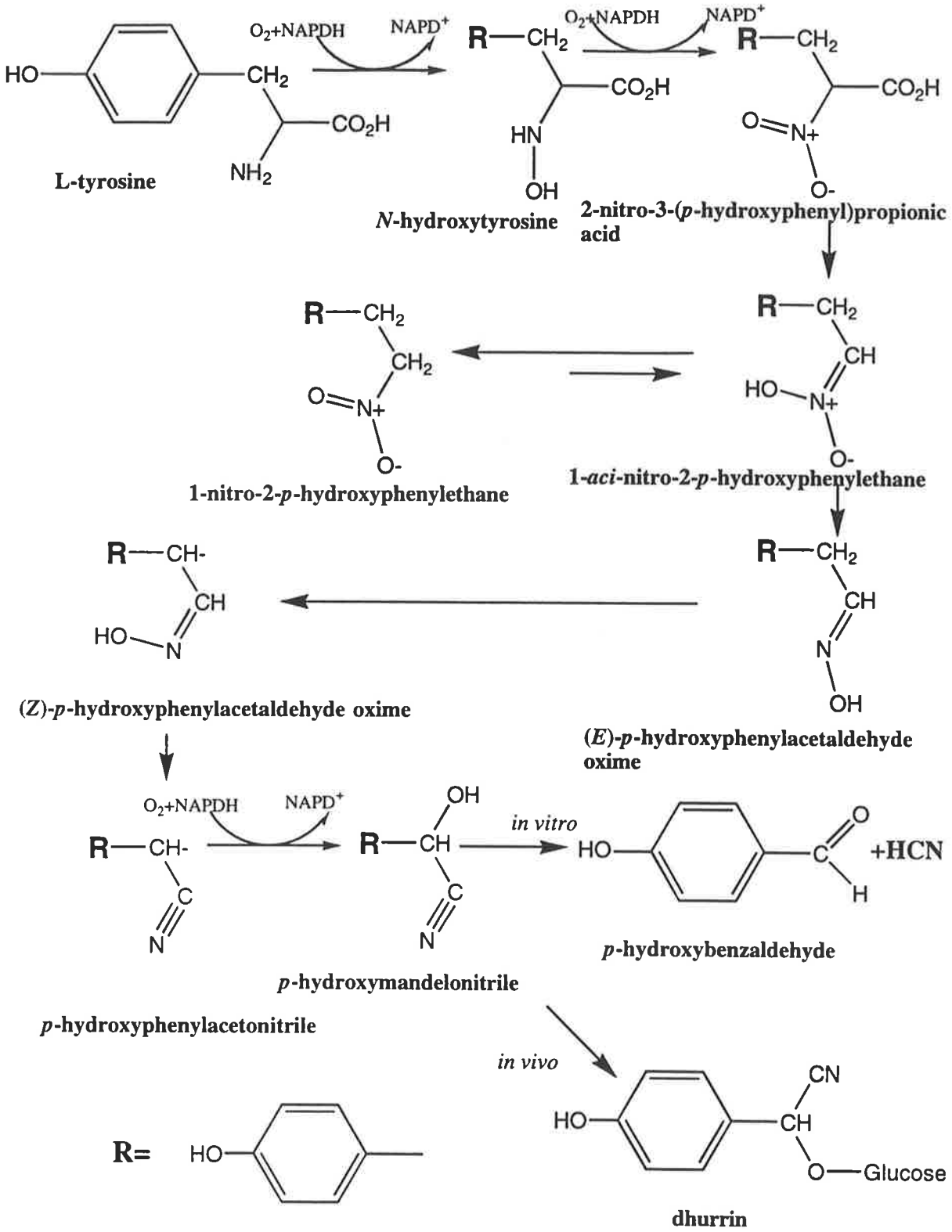


Figure 2.1 The biosynthetic pathway for the cyanogenic glycoside dhurrin for sorghum (Sibbesen *et al.* 1995).

2.4.1.2 Genetic variation

Vicianine is the only low molecular weight anti-nutritional factor in common vetch which has been studied for its potential to be eliminated from elite cultivars through plant breeding (Milczak 1968, 1971a, 1971b, 1971c, 1972). Cyanogenesis was shown to vary among different cultivars of common vetch. In the initial study a sensitive quantitative colorimetric determination for the presence of hydrogen cyanide in solution was used as an indicator of vicianine in the sample. A majority of the 271 *V. sativa* accessions tested had 10-150μg hydrogen cyanide g^{-1} (dry weight) while 51 cultivars were free of hydrogen cyanide. *V. sativa* cv. "Blanchefleur" (*V. sativa* convar. consentini) was found to be free of hydrogen cyanide (Tschiersch and Hanelt 1967).

2.4.1.3 Animal Toxicology

Cyanide is known as a rapid acting poison. However, it is not usually recognised that low level chronic intoxication of cyanide does exist and can be quite incapacitating (Way 1981). Ingestion of cyanide containing staple foods such as cassava (Osuntokun 1970) accounts for most of the instances of chronic cyanide exposure in man. There are various correlations which have implicated the effects of low level intoxication by cyanide with specific diseases such as Nigerian nutritional neuropathy, Leber's optical atrophy, retrobulbar neuritis, pernicious anaemia, tobacco amblyopia, ataxic tropical neuropathy (Way 1981) and the tropical myelopathy Konzo. Konzo is an upper motor neuron disease entity resulting in a non-progressive spastic parapesis (Howlett *et al.* 1990) which has some clinical similarities to lathyrism.

2.4.2 β-D-glucopyranosyl glycosides

There are two major pyrimidine β-D-glucopyranosides found in the seeds of common vetch (*V. sativa*), vicine (Ritthausen and Kreusler 1870) and convicine (Ritthausen 1881). A phytochemical examination (Griffiths and Ramsay 1992) has shown that vicine and convicine are only associated with *Vicia* species among the legumes and are not present in beet roots and other common legumes including peas and *Lupinus albus* as had been previously reported (Pitz *et al.* 1980). Vicine has been isolated and fully characterised from seeds of *Momordica charantia*, a member of the *Cucurbitaceae*, indicating that its synthesis is not unique to the *Vicieae* tribe (Dutta *et al.* 1981).

2.4.2.1 Biosynthesis

The formation of vicine and convicine was studied by injecting radioactively labelled precursors into immature pods attached to developing plants (Brown and Roberts 1972). This study determined that synthesis of the glycosides was through the orotic acid pathway and was localised to the seed and pod. The glycosides were found to be synthesised early in the development of the seed and the rate of glycoside synthesis, defined as the proportion of vicine to convicine, was constant throughout the development of the seed. This suggests that the two glycosides are synthesised simultaneously at fairly constant rates throughout ontogeny of the seed (Pitz *et al.* 1981).

2.4.2.2 Genetic variation

Reference to the genetic variation of vicine and convicine in common vetch has not been reported in the literature. The content of vicine and convicine in different faba bean cultivars using the mature seed has been reported by a number of workers (Jamalian 1978; Gardiner *et al.* 1982). Extensive studies with 242 (Pitz *et al.* 1981) and 364 (Bjerg *et al.* 1985) cultivars of widely diverse faba bean strains found that a majority of the investigated *Vicia faba* cultivars contained a total glycoside content of more than 1% of the seeds. Great variation was observed in the individual components of the total glycoside measurement. It is important to note that these extensive studies were unable to identify faba bean cultivars with negligible concentrations of either vicine or convicine in the native population.

2.4.2.3 Animal Toxicity

The consumption of faba beans (broad beans, *Vicia faba*) under certain circumstances results in the metabolic disease favism (Escobar *et al.* 1964). Favism has been recognised since antiquity, but the first authentic description of the disease in the medical literature dates back to the mid 1850's. It is most prevalent in the islands and coastal regions of the Mediterranean Sea, in the Middle East and is frequently encountered in China (Marquardt 1989).

Favism occurs in genetically predisposed individuals who express an isozyme of glucose-6-phosphate dehydrogenase (G-6-PD) which limits the rate of erythrocytic glutathione replenishment. Favism has the potential to manifest as acute erythrocytic haemolytic anaemia and haemolytic crisis when the erythrocyte is exposed to persistent free radical generators. Acute favism may be fatal and usually occurs in children, especially young males. It accounts for as many as 1 to 2% of paediatric

admissions in Cairo and Alexandria, Egypt (Belsey 1973) and is readily treated by a blood transfusion.

The β-D-glucopyranosyl glycosides, vicine and convicine and their aglycones, have been the focus of intense international research efforts strongly implicating them as the causal agents in favism and has been well reviewed (Marquardt 1989). The aglycones, divicine and isouramil, have been shown to affect the metabolism of several compounds, particularly glutathione and NADPH. The oxidised form of the β-D-glucopyranosyl glycosides are able to react with reducing reagents such as glutathione, NADPH and ascorbate in the red blood cell. This results in the regeneration of the reduced aglycones (Figure 2.2). While this research has centred exclusively on the faba bean, as common vetch also contains vicine and convicine it might be expected that consumption of common vetch would lead to favism in predisposed individuals.

The redox cycle of the aglycones produces large quantities of free radicals and peroxides which require neutralisation. Four molecules of glutathione are consumed each redox cycle. In glucose-6-phosphate dehydrogenase (G-6-PD) deficient red blood cells, the redox cycle continues to operate until the aglycones either spontaneously decompose, form irreversible adducts, or until all of the reducing equivalents are exhausted. At this stage irreversible damage to the erythrocyte occurs (Marquardt 1989).

2.4.3 Cyanoalanine non-protein amino acids

β-Cyanoalanine was first isolated from common vetch (*V. sativa*) by Ressler (1962). The identification of β-cyano-L-alanine was based on a comparison of physical, chemical and biological properties of synthesised material (Ressler and Ratzkin 1961). Subsequently the bound form γ-glutamyl-β-cyanoalanine was isolated from seeds of common vetch (*V. sativa*) (Ressler *et al.* 1963). A complete synthesis has been described for γ-glutamyl-β-cyanoalanine (Ressler *et al.* 1969a).

A phytochemical examination has shown that γ-glutamyl-β-cyanoalanine is only associated with a subsection of *Vicia* species and one species of *Lathyrus* among the legumes and is not present in beet roots and other common legumes (Bell and Tirimanna 1965). Investigations into the toxic effects of the fungus *Clitocybe acromelalga*, a member of the Tricholomataceae resulted in the isolation and complete characterisation of β-cyanoalanine and γ-glutamyl-β-cyanoalanine indicating synthesis of γ-glutamyl-β-cyanoalanine is not unique to the *Vicieae* tribe (Fushiya *et al.* 1993).

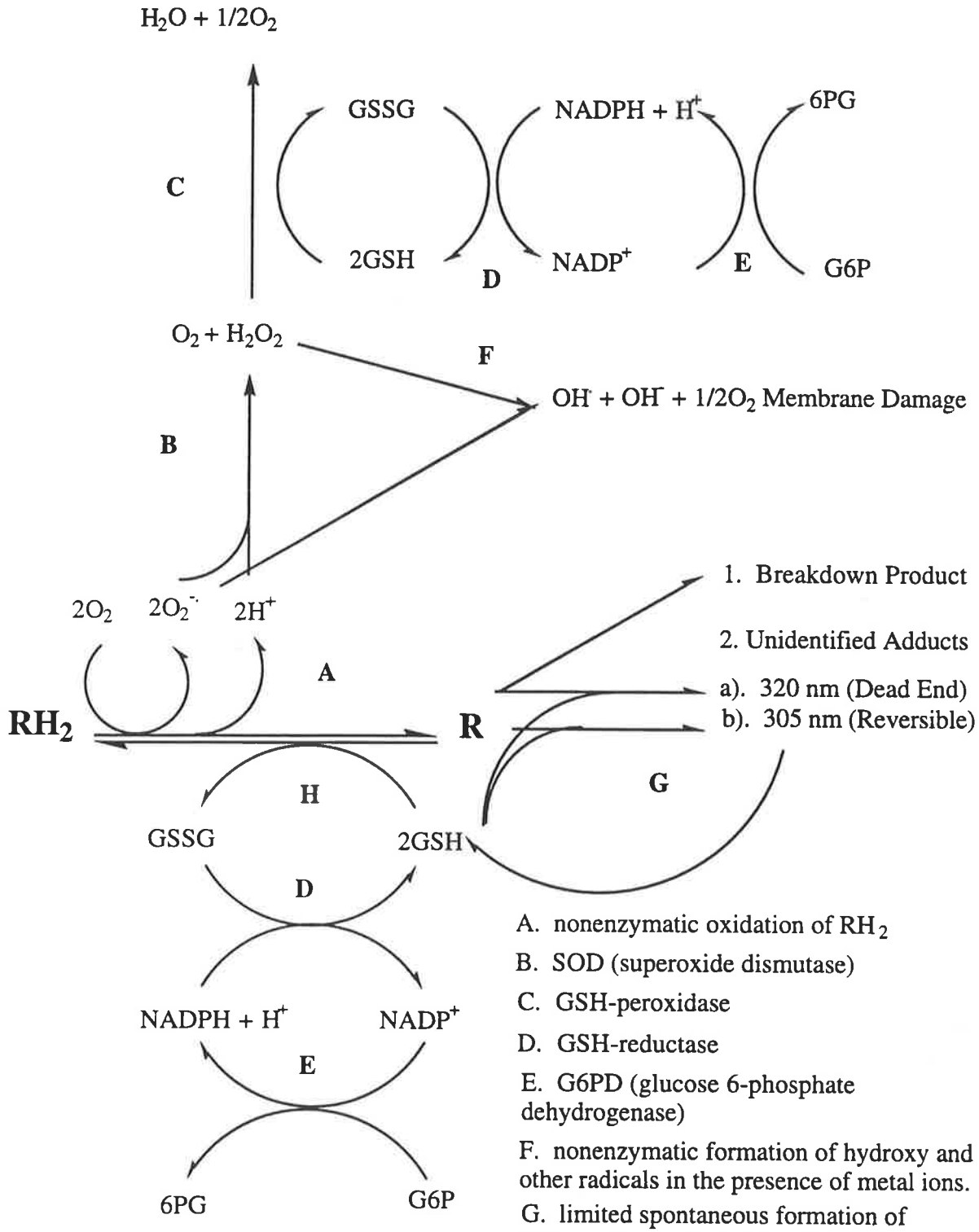


Figure 2.2 Nature of the interaction of Divicine and Isouramil with glutathione (GSH) in the biological system: Establishment of a futile oxidative-reduction cycle (Marquardt 1989).

2.4.3.1 Biosynthesis

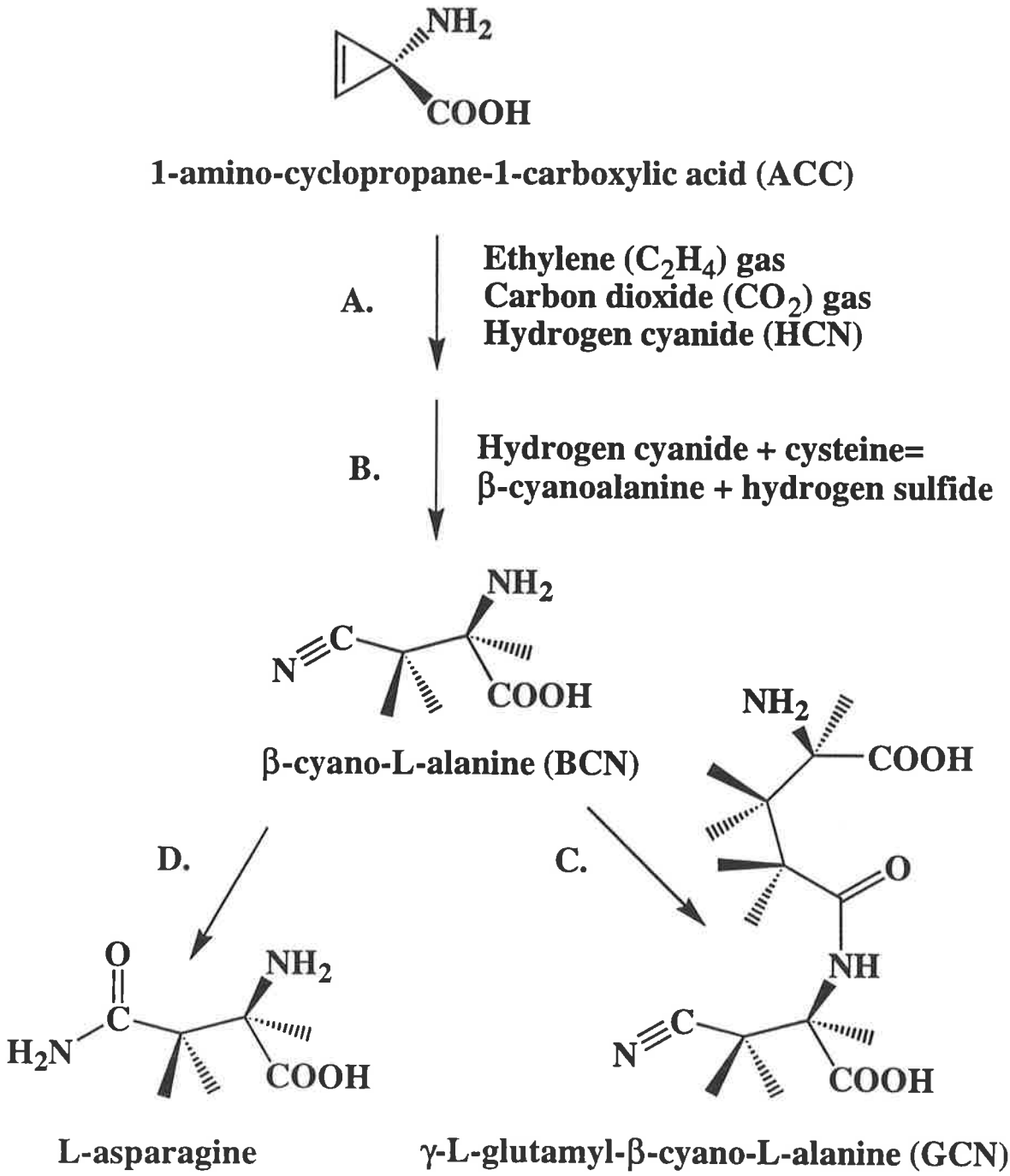
There are numerous reports about the fate of cyanide in the metabolism of higher plants. The first evidence that β-cyanoalanine was the principle product of cyanide assimilation was determined in kinetic experiments with *Chlorella pyrenoidosa* which were treated with $H^{14}CN$ (Fowden and Bell 1965). It was then shown that *V. sativa* contains an active enzyme which can catalyse the formation of γ-glutamyl-β-cyanoalanine in this and several other vetches (Fowden and Bell 1965). Incorporation of cyanide into the nitrile group of γ-glutamyl-β-cyanoalanine in *V. sativa* was initially studied using radioactively labeled $K^{14}CN$ and $K^{14}C^{15}N$ with L-serine-3-^{14}C acting as a substrate (Nigam and Ressler 1964; Nigam and McConnell 1968; Ressler *et al.* 1969b).

However, in a study on the seedlings of blue lupin, sorghum and common vetch, radioactively labelled $H^{14}CN$ and L-cysteine-3-^{14}C were extensively converted into asparagine in lupins and sorghum and into γ-glutamyl-β-cyanoalanine in common vetch at a rate far in excess of that obtained using L-serine-3-^{14}C as a substrate. This indicated that in higher plants cysteine was a far better substrate than serine for the formation of β-cyanoalanine (Blumenthal *et al.* 1968). The enzyme was isolated, characterised and named β-cyanoalanine synthetase (ECO 4.4.1.9). It was another 15 years before it became apparent that there was a stoichiometric relationship between ethylene formation and the production of cyanide metabolites from (1-^{14}C) 1-amino-cyclopropane-1-carboxylic acid (Peiser *et al.* 1984) (Figure 2.3). An alternative biosynthetic pathway for β-cyanoalanine utilizing cysteine synthase (CSase) with O-acetyl-serine and cyanide as substrates has been reported (Ikegami and Murakoshi 1994).

The literature has reported the presence of β-cyanoalanine and γ-glutamyl-β-cyanoalanine in plant tissue during the lifecycle of common vetch (Ressler *et al.* 1969b). This report only describes seed germination and the juvenile plant; information at flowering and seed pod set was not provided.

2.4.3.2 Genetic variation

Variation in the content of γ-glutamyl-β-cyanoalanine between cultivars of *V. sativa* has been reported (Ressler *et al.* 1969b). The study involved 14 cultivars/lines of *V. sativa* and found that all cultivars contained significant amounts of free β-cyanoalanine. However, two cultivars had significantly less γ-glutamyl-β-cyanoalanine than found in the commercial varieties.



A. Ethylene forming enzyme (EFE) (Peiser *et al.* 1984)
B. β-cyanoalanine synthetase, addition of cysteine to hydrogen cyanide (HCN) (Blumenthal *et al.* 1968).
C. γ-glutamyltransferase, addition of glutamic acid to β-cyanoalanine (Fowden and Bell 1965).
D. β-cyanoalanine hydratase, addition of water to β-cyanoalanine.

Figure 2.3 The biosynthetic fate of cyanide in higher plants produced from the enzymatic cleavage of 1-amino-cyclopropane-1-carboxylic acid (ACC) during the synthesis of ethylene.

2.4.3.3 Animal Toxicity

Congenital cystathioninuria is a familial disease first reported by Harris *et al.* (1958). It is caused by a specific defect in the activity of the enzyme cystathioninase responsible for the cleavage of cystathionine (Finkelstein *et al.* 1966). Cystathionine is known to be involved in one biochemical reaction, the transfer of sulfur from methionine to cysteine. The enzyme has been characterised (E.C. 4.2.1.15) and pyridoxal phosphate is the coenzyme (Matsuo and Greenberg 1958).

Cystathioninuria appears to be associated with developmental and mental abnormalities (Frimpter *et al.* 1963; Efron 1965) and has also been observed in patients with neural tumours (Gjessing 1963). It is an uncommon defect in adult humans and is characterised by a grossly increased urinary excretion of the amino acid, cystathionine. Administration of high doses of pyridoxine hydrochloride (Vitamin B6) resulted in a marked reduction in the concentration of cystathionine in urine and blood (Frimpter 1965) in patients with cystathioninuria.

The connection between cystathioninuria and common vetch intoxication is that pyridoxal hydrochloride had been found to delay the onset and alleviate the severity of the symptoms of β-cyanoalanine toxicity (hyperkinesis, convulsions, and depression) and to decrease greatly the mortality of rats injected with a single lethal dose of β-cyanoalanine. It was also observed that the rats fed β-cyanoalanine excreted large amounts of cystathionine, a substance not generally found in the urine of the rat (Ressler *et al.* 1964).

Subsequently it was demonstrated that β-cyanoalanine was a specific agent for inducing cystathioninuria in the rat. Continuous supplementation of the diet with high levels of pyridoxal hydrochloride along with β-cyanoalanine afforded no protection to rats against hyper-irritability, retardation of growth, and high mortality. β-cyanoalanine acted as a structural inhibitor of cystathionase *in vivo*, and it was concluded that β-cyanoalanine was not acting as an antagonist to Vitamin B6 (Ressler *et al.* 1967).

In rats fed a diet containing β-cyanoalanine; β-cyanoalanine, γ-glutamyl-β-cyanoalanine and the unique tripeptide, γ-glutamyl-β-cyanoalanylglycine, a structural analogue of glutathione, were found distributed in the liver, blood, brain and muscles (Sasaoka *et al.* 1968). Significant amounts of β-cyanoalanine were found in the kidney and the urine while only minute amounts of γ-glutamyl-β-cyanoalanine were observed in the urine. γ-glutamyl-β-cyanoalanylglycine was not observed in the kidney or the urine (Ressler *et al.* 1967). This suggests that γ-glutamyl-β-cyanoalanine is a successful mimic of the γ-glutamylcysteine, a substrate in cellular glutathione biosynthesis, and that the resultant product is readily sequestered at a cellular level and not rapidly excreted.

Studies have demonstrated that feeding common vetch (*V. sativa*) is harmful to the rat, chicken and monkey (Anderson *et al.* 1925; Ressler *et al.* 1964; Ressler *et al.* 1967; Ressler *et al.* 1969a; Araya *et al.* 1990; Enneking 1995). The mode of cyanoalanine toxicity has not been elucidated. β-cyanoalanine and γ-glutamyl-β-cyanoalanine have been shown to be quantitatively responsible on a molar basis for the observed toxicity when added to a basal diet in the rat (Ressler *et al.* 1969a). Fortification of the diet with Vitamin B6 does not afford long term protection in the rat against toxicity of a diet containing β-cyanoalanine. However, supplementation of the diet with methionine has been shown to alleviate some of the symptoms observed with common vetch intoxication (Araya *et al.* 1990). Hence the mode of toxicity associated with common vetch intoxication may be similar to those observed in disorders of sulphur metabolism.

2.4.3.3.1 The glutathione model for the toxicity of common vetch

A direct consequence of a deficiency in cystathionase activity is a reduction of the transulfuration of methionine. This leads to decreased cysteine formation, which in turn limits the synthesis of glutathione. Patients with these conditions typically have symptoms and signs of central nervous system dysfunction (Meister and Larsson 1989). A mouse model has been developed to offer an experimental system for the modeling of diseases of the adult human in which glutathione synthesis is deficient (Jain *et al.* 1992).

The mouse model has been applied to the study of pre-term babies with respiratory distress. In the developing human, cystathionase activity does not appear until after birth (Sturman *et al.* 1970). It has been observed that pre-term infants have significantly lower levels of peripheral venous blood plasma total glutathione compared to full term infants. The glutathione deficient mouse has successfully modelled this phenomenon (Jain *et al.* 1992).

The administration of L-buthionine-(S,R)-sulfoximine, a transition state inhibitor of γ-glutamylcysteine synthetase, to the adult rat leads to marked oxidative damage in several tissues, myofiber degeneration in skeletal muscle (Martensson and Meister 1989), damage to the type 2 cell lamellar bodies and capillary endothelial cells in the lungs (Jain *et al.* 1992), and epithelial-cell damage to the jejunum and colon (Martensson *et al.* 1990): no effects were seen in the liver or the kidney. In contrast, newborn rats had lens epithelial cell degradation and cataract formation, focal necrosis (Calvin *et al.* 1986; Martensson *et al.* 1989), decreased extramedullary haematopoiesis in the liver, renal proximal-tubular degeneration, mitochondrial damage in the cerebral cortex (Martensson *et al.* 1991) and hind leg paralysis (Calvin *et al.* 1986) as well as the effects previously

observed in the adult mouse. Similar results were found in the newborn rat and in guinea pig (Griffith *et al.* 1991).

L-Buthionine-(S,R)-sulfoximine-treated newborn rat exhibits definite signs of neurological disturbance, including lethargy, intermittent tremors (accentuated by perceptual and physical stimuli), and minor fits (Jain *et al.* 1991). Mortality in newborn rats treated with L-buthionine-(S,R)-sulfoximine-induced glutathione deficiency was 91% (Martensson and Meister 1991). However, it is of special interest that ascorbate, in high doses, protects newborn rats (which apparently do not synthesise ascorbate) as well as guinea pigs against the lethal effects of glutathione deficiency. It was also shown that ascorbate protects against cellular damage in the lung and skeletal tissue of glutathione deficient adult mice (Jain *et al.* 1992). The protection afforded by ascorbate was quantified in increased mitochondrial glutathione levels. This suggests that glutathione and ascorbate function together as a part of a physiologically significant antioxidant system (Jain *et al.* 1992).

Mitochondria normally produce a substantial amount of hydrogen peroxide. The tissue damage produced by glutathione deficiency was invariably accompanied by markedly decreased mitochondrial glutathione levels. Low mitochondrial glutathione levels are associated with mitochondrial swelling with vacuolation and rupture of cristae and mitochondrial membranes as seen by electron microscopy (Jain *et al.* 1992). On a cellular level, inhibition of glutathione synthesis leads to the accumulation of the substantial amounts of hydrogen peroxide in the mitochondria and oxidative damage to the mitochondria, resulting in significant deleterious effects in the subject.

2.4.3.3.2 Biological significance of cyanoalanine induced glutathione deficiency

The mode of cyanoalanine toxicity may not be lathyrogenic as defined by neurolathyrism but a direct consequence of the cascade effect of a disrupted sulfur metabolism. This argument could easily be resolved in the manner described in the observations made in the glutathione deficient rat. If consistent then this highlights an intriguing hypothesis concerning the co-evolutionary biology of plant/animal interactions and the development of plant anti-nutritional factor defences.

Common vetch has developed two structurally unique families of antinutritional factors, the β-D-glucopyranosyl glycosides and the cyanoalanine non-protein amino acids which both appear to target the glutathione component of the physiologically significant antioxidant system. The cyanoalanine non-protein amino acids may inhibit global glutathione metabolism through enzymatic

inhibition of substrate synthesis and at the same time the β-D-glucopyranosyl glycosides, vicine and convicine, stress the glutathione metabolism in erythrocytes and may cause acute illness in genetically susceptible individuals. The damage observed in both systems is ultimately due to the production of hydrogen peroxide which then oxidises critical components of the cell or organelle where the redox system has been damaged.

2.5 Conclusion

In 1993 the Australian Grains Research Development Corporation (GRDC) initiated a project to determine the potential for development of common vetch (*V. sativa*) (Petterson and Mackintosh 1994) as a grain legume crop suitable for human consumption. The universal nature of the disorder associated with the intoxication of cyanoalanine non-protein amino acid determined that the first step in achieving this goal would be the identification of "zero cyanoalanine non-protein amino acid" accessions of *V. sativa*.

Chapter Three

A Solid Picture

3.0 Introduction

The *Leguminosae* are known to have evolved a wide range of non-protein amino acids and many are present as the γ-glutamyl dipeptide derivatives (Bell and Tirimanna 1965; Fowden and Bell 1965; Kasai and Larsen 1980; Lambein *et al.* 1992). This chapter describes the isolation, purification and the chemical characterisation of two peptides found in *Vicia* species, γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine. Both compounds have been shown to act as potent feed intake inhibitors in monogastric animals (Ressler *et al.* 1969a; Enneking 1995).

γ-Glutamyl-β-cyanoalanine was isolated from the seeds of common vetch (*V. sativa* L., cv. "Blanchefleur" Australian Temperate Field Crops Collection (ATFCC) 60333). The isolation procedure of Ressler *et al.* (1969a) was modified, resulting in significant improvements in yield and ease of crystallisation. A full chemical characterization is described and a crystal structure was published (Delaere *et al.* 1995). Assignment of NMR data for γ-glutamyl-β-cyanoalanine was in agreement with the literature (Fushiya *et al.* 1993) and supported by NMR simulation (Hagele *et al.* 1993).

Crude γ-glutamyl-S-ethenyl-cysteine supplied by Dr. Dirk Enneking was isolated from the seeds of the Narbon bean (*Vicia narbonensis* L., ATFCC 60105) (Enneking 1995). A purification procedure has been developed and a full chemical characterisation for γ-glutamyl-S-ethenyl-cysteine is described. Assignments of NMR data for γ-glutamyl-S-ethenyl-cysteine were in full agreement the NMR simulation (Hagele *et al.* 1993) and were supported by assignments made to a related compound S-ethenyl-cysteine (SEC) (Thumfort *et al.* 1993).

3.1 Purification

3.1.1 Isolation and purification of γ-glutamyl-β-cyanoalanine

The anion-exchange procedure of Ressler *et al.* (1969a) was modified by elution with ammonium acetate instead of pyridine. 1 kg of split vetch seed was extracted in 1:10 w/v 30% v/v ethanol (Analar) at room temperature for twelve hours. The eluent was passed through an Amberlite IR

400 Acetate anion-exchange column with a total column volume of 625meq at a rate of 60mL/minute. Fractions were collected off the bottom of the column and were monitored by paper electrophoresis (0.75M formic acid/1M acetic acid, pH 1.76) and stained with 0.25% ninhydrin in acetone heated for 3-5 minutes at 90°C to determine column saturation, described in section 3.2.8. The column was eluted with 0.1M ammonium acetate pH 6.60, 500mL fractions collected and monitored by paper electrophoresis. Fractions 1-7 were pooled and rotary evaporated to dryness (22.2g d.w.) in a 50°C waterbath. Small additions of n-butanol (Analar) was used as an antifoaming agent. The dried material was extracted with boiling methanol (Analar) and filtered through a sintered glass funnel to remove ammonium acetate and glutamate. The precipitate (7.8g) was checked for purity by paper electrophoresis, redissolved in a minimum amount of water, adjusted to pH 9.0 with ammonia and crystallized from propanol-water (1:1) solution yielding 5.6g of crystals. Recrystallization yielded crystals suitable for structural elucidation by X-ray crystallography (Delaere *et al.* 1995).

3.1.2 Purification of γ-glutamyl-S-ethenyl-cysteine

A crude sample of γ-glutamyl-S-ethenyl-cysteine was obtained from Dr. D. Enneking. 0.960g of crude γ-glutamyl-S-ethenyl-cysteine was dissolved in 1:10 w/vol water with 68mg activated charcoal (BDH) and was left at room temperature for 15 minutes. The solution was gravity filtered using Whatman No.541 filter paper. The resulting yellow coloured solution was chilled to 4°C, left overnight and decanted leaving a yellow oily residue. Aliquots of 30, 20, and 20 mL of isopropanol (Analar propan-2-ol) were added, chilled overnight and decanted leaving a yellow oil. Crystal formation was stimulated by agitation and refrigeration resulting in a crop (150mg) of long linear and twinned crystalline material. Subsequent refrigeration and addition of 50mL of isopropanol (Analar) resulted in the isolation of white amorphous material (350mg) containing no appreciable ninhydrin-positive components other than γ-glutamyl-S-ethenyl-cysteine as determined by paper electrophoresis, described in section 3.2.8. This resulted in a total yeild of 500mg (52%) from 960mg of crude product.

3.2 Characterisation

3.2.1 Elemental analysis

Elemental analysis was carried out by the Australian Microanalytical Service (National Analytical Laboratories Pty. Ltd. 585 Blackburn Road, Notting Hill, Victoria, Australia 3168). See section 3.3.

3.2.2 Optical rotation

Measurements were made at pH 8.37 for γ-glutamyl-β-cyanoalanine and pH 7.51 for γ-glutamyl-S-ethenyl-cysteine in water. The pH was measured with a Radiometer pHM 64 Research pH meter equipped with a glass electrode and calibrated according to standard procedures. The optical rotation data were collected at 25°C at concentrations of 1.99% ($3.66x10^{-4}$ M) for γ-glutamyl-β-cyanoalanine and 1.99% ($3.57x10^{-4}$ M) for γ-glutamyl-S-ethenyl-cysteine. Measurements were taken on a Perkin Elmer 141 Polarimeter illuminated using a sodium lamp at 589nm in a Kovetten Hochster Präzision 100mm, 1mL quartz microcell.

3.2.3 Infrared spectroscopy

3.2.3.1 Apparatus

All measurements were taken on a Perkin Elmer 983G double beam dispersive infrared spectrometer fitted with a Harrick Praying Mantis Diffuse Reflectance attachment (Model 3SP, Harrick Scientific Corporation, Ossining, NY) operating in double beam mode. The PE 983G was linked to an Acer 466DX2 PC clone (Acer Tech. Corp., Taiwan R.O.C.) and controlled through a modified PE 983 emulator/controller (MDS Engineering Associates, Inc., Brookfield, CT). The sample compartment was continually purged throughout the duration of the sample collection using a Perkin Elmer CD 3 circulatory air drier.

3.2.3.2 Sample preparation and data collection and manipulation

50mM of crystalline γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine were rigorously ground and mixed in KCl using a mortar and pestle. The samples were poured into the large sample cup and smoothed using a spatula. Considerable attention was placed on achieving a smooth surface and sample alignment was adjusted to maximise reflectance. Sample collection parameters were Scan Mode 1, Filter Noise 4, in the region 4000-650cm^{-1}. Reflectance data were converted to absorbance units, baseline-corrected and offset using the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) data manipulation software.

3.2.4 Fast atom bombardment-mass spectroscopy

Mass spectral analyses were performed with a Finnigan Mat TSQ 70 triple stage quadrupole mass spectrometer of the Australian Wine Research Institute, tuned for operation under fast atom bombardment mode using Xe as the bombardment gas. The established voltage was -10keV, and the ion current, 0.5 mA. Sample concentrations were approximately 1 mg/mL in glycerol. Glycerol was

used as the liquid matrix for excitation in both the positive and negative ionisation state modes. The assistance of Dr. Yogi Hayasaka (Australian Wine Research Institute), with the analyses is gratefully acknowledged.

3.2.5 X-ray crystallography

The conditions for data collection are summarized in Table 1 (Appendix A). Cell parameters were determined by least-square fit to the diffractometer settings for 18 general reflections. Hydrogen atoms were not refined and were assigned the B_{eq} value of the atom to which they were bonded. Data were collected on *MSC/AFC Diffractometer Control Software* (Molecular Structure Corporation 1988). Cell refinement: *MSC/AFC Diffractometer Control Software.* Data reduction: *TEXAN PROCESS* (Molecular Structure Corporation 1992). Program used to solve the structure: *SHELXS86* (Sheldrick 1985). Program used to refine the structure: *TEXAN LS.* All postional parameters and anisotropic temperature factors for heavy atoms were refined by least squares methods, giving R=0.060 and wR=0.054, with goodness of fit S=2.08. The weighting scheme was based on measured e.s.d.'s $(\delta/\sigma)_{max}$=0.001, $\delta\rho_{max}$=0.39 eÅ^{-3}, $\delta\rho_{min}$= -0.44 eÅ^{-3}. Atomic scattering factors were from International Tables for X-ray Crystallography (1974, Vol. IV). Software used to prepare material for publication: *TEXAN FINISH.* The assistance of Dr. E.R.T. Tiekink (Department of Chemistry, University of Adelaide) in the analysis of the sample is gratefully acknowledged. Dr. G.P. Jones (Department of Horticulture, Viticulture and Oenology, Waite Agricultural Research Institute, University of Adelaide) is acknowledged for the growing of the x-ray crystallographic quality crystal.

3.2.6 Nuclear magnetic resonance spectroscopy

The assistance of Mr. Phil Clements (Department of Chemistry, University of Adelaide) is acknowledged for acquisition of solution nuclear magnetic resonance (NMR) spectra during the course of this study.

3.2.6.1 Preparation of solutions

Measurements were made at pD 6.8 and pD 4.9 for γ-glutamyl-β-cyanoalanine and pD 6.0 and pD 4.8 for γ-glutamyl-S-ethenyl-cysteine. The pD was measured with a pH meter equipped with a glass electrode and calibrated according to standard procedures. The pD was adjusted by adding 1 volume 0.1M sodium bisulfite buffer in D_2O. Spectra were collected at concentrations of 0.24M and 0.12M for γ-glutamyl-β-cyanoalanine and 0.28M and 0.14M for γ-glutamyl-S-ethenyl-cysteine.

3.2.6.2 NMR measurements

All measurements were collected on a Bruker ACP-300, 300MHz NMR spectrometer at a probe temperature of 297°K except for the high temperature decoupling experiment involving γ-glutamyl-S-ethenyl-cysteine which was conduced at 333°K. Data were interpreted and manipulated on an Brukert ASPECT 3000 computer system running DISNMR91. Field strength of the magnet was 7.05 Tesla.

3.2.6.3 NMR experiments

3.2.6.3.1 ^{1}H and ^{13}C experiments

Proton NMR experiments were collected at 300.13 Mhz. Chemical shift assignments were internally referenced to HOD set to 4.8 ppm. Carbon NMR experiments were collected at 75.47 Mhz. Chemical shift assignments were externally referenced to t-butanol, methyl group set to 31.6 ppm. Carbon experiments were collected at pD 6.8 and pD 4.9 for γ-glutamyl-β-cyanoalanine and pD 6.0 and pD 4.8 for γ-glutamyl-S-ethenyl-cysteine.

3.2.6.3.2 2D-NMR experiments; HETCOR and COSY

Proton/carbon connectivity correlations were collected using the 2D HETCOR pulse sequence. Proton/proton connectivity correlations were collected using the 2D COSY pulse sequence.

3.2.6.3.3 ^{1}H decoupling experiments

Proton data were collected on γ-glutamyl-S-ethenyl-cysteine with protons irradiated at 6.42, 4.41, 3.75, 2.48 and 2.16 ppm at 297°K and 6.86 ppm at 333°K. Proton NMR data were collected on γ-glutamyl-β-cyanoalanine with protons irradiated at 4.52, 3.81, 2.55 and 2.19 ppm at 297°K. Chemical shift assignments were supported by use of NMR simulation software (Hagele *et al.* 1993).

3.2.7 Micellar electrokinetic chromatography

3.2.7.1 Apparatus

Capillary electrophoresis was performed using a P/ACE 2100 system (Beckman Instruments Incorporated, Fullerton, CA) fitted with a thermostatically controlled liquid cooled capillary cartridge using the Beckman P/ACE System 2000 capillary liquid coolant. The capillary cartridge contained a 50μm internal diameter untreated fused silica capillary tubing of 57cm total length, 50cm effective length connected to a tunable ultraviolet diode array detector (UV-DAD). The P/ACE 2100 was linked

to an IBM PS/2 clone (Dell 466DX2, 66MHz, 8Mb RAM, Dell Computer Company, USA) and controlled through the Beckman System GOLD 7.0 software sytem (Beckman Instruments Incorporated, Fullerton, CA).

3.2.7.2 Buffers and derivatisation of samples

The separation buffer was 20mM sodium tetraborate, 19.5mM sodium dodecylsulphate (SDS) buffer, adjusted to pH 9.52 with 1M NaOH. The derivatising buffer was identical to the separation buffer except for the addition of 2-deoxycytidine ($4.5x10^{-4}$ M) (Sigma Chemical Company) and the non-protein amino acid norvaline ($1.5x10^{-4}$ M) (Fluka Chemika Biochemika).

A solution containing $1.25x10^{-3}$ M, vicine (VIC), convicine (CON), authentic samples kindly provided by Prof. Ron Marquart (University of Manitoba, Winnipeg, Manitoba, Canada), β-cyanoalanine (BCN), canavanine (CAN) (Sigma Chemical Company), γ-glutamyl-β-cyanoalanine (GCN), and γ-glutamyl-S-ethenyl-cysteine (GEC) was made in 18mW Milli-Q water (Millipore, Bedford, MA) containing sodium azide (0.1%). Samples were derivatised by adding 5mL of the sample solution to 50mL of the derivatising buffer then adding 5mL 1% 9-flourenylmethyl chloroformate (FMOC) in acetonitrile (Fluka Chemika Biochemika), gently shaken and left for one minute at room temperature.

3.2.7.3 Data collection

Detection was accomplished by an on-column ultraviolet diode array detector (UV-DAD) set to monitor absorbance at 272±10nm. Separations by micellar electrokinetic chromatography were performed with the following conditions: T= 30°C, V=23.7 kV, polarity was positive (Albin *et al.* 1991). The column was pretreated with 0.1M NaOH for 10 minutes and then equilibrated for 3 minutes with the separation buffer. Samples were loaded onto the column using a 3 second pressure injection. The run time was 20 minutes.

3.2.7.4 Data manipulation

The Beckman GOLD format data files were converted to the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) format using Beckman Gold Chromatogram (BKMNGCHR) and Beckman Gold Diode Array Spectra (BKMNGOLD) file converters associated with the GRAMS/386 II package. The files were baseline-corrected and the relative retention times were realigned by the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) data manipulation package using three internal standards, 2-deoxycytidine (3.80minutes), FMOC reagent peak R1 (12.00minutes), FMOC

reagent peak R2 (15.70minutes) using the Baseline and X-Shift functions. The identity and elution order of each compound was determined through sample spiking and component elimination.

3.2.8 High-voltage paper electrophoresis

High-voltage paper electrophoresis (HVPE) followed the procedure of Tate (1968, 1981). The aqueous paper electrophoretic buffer was 1M formic acid/0.75M glacial acetic acid, pH 1.76. Samples were loaded in the middle of the paper using glass capillaries. High-voltage paper electrophoresis was run for 15 minutes at 3500 volts, the paper was dried in a 90°C oven to remove perclean (perchloroethylene), stained with 0.25% ninhydrin in acetone and dried for 3-5 minutes at 90°C.

3.3 Results

Elemental analysis: γ-L-Glutamyl-β-cyano-L-alanine as its ammonium salt: $C_9H_{12}N_3O_5$ requires (found); C, 41.6 (40.8); H, 6.1 (6.1); N, 21.5 (20.6); O, 30.8 (32.9) T, 100 (100.4). FAB-MS (positive ion mode): $[M+H]^+$=245, (negative ion mode) $[M-H]^-$=243*m/z*. Optical rotation (-33.6°).

Elemental analysis: γ-Glutamyl-S-ethenyl-cysteine: $C_{10}H_{16}N_2O_5S$ requires (found); C, 43.5 (43.4); H, 5.8 (6.0); N, 10.1 (10.3); S, 11.6 (11.7). FAB-MS (positive ion mode): $[M+H]^+$=277, (negative ion mode) $[M-H]^-$=275*m/z* (Enneking 1995). Optical rotation (-5.0°).

3.3.1 X-ray crystallographic data

N-γ-L-Glutamyl-β-cyano-L-alanine as its ammonium salt $NH_4^+.C_9H_{12}N_3O_5^-$; M=260.3, Monoclinic, *a*=9.347 (8) Å, *b*=4.772 (2) Å, *c*=12.967 (5) Å, β=90.49 (5)°, V=578.3 (8) Å^3, Z=2, space group $P2_1$, D_x=1.500Mg m^{-3}. Figure 3.0 shows the molecular structure of the anion of *N*-γ-L-glutamyl-β-cyano-L-alanine and clearly shows an extended conformation of the main chain C(1-5)-N(2)-C(6)-C(7). The end of the molecule possessing the C(1) carboxylate group is zwitterionic with the N(1) atom protonated. The charge balance for the C(7) carboxylate group is provided by the ammonium cation. There are extensive hydrogen-bonding contacts in the lattice; detailed analysis of these was precluded as the lattice ammonium N(4) H atoms are not located in the X-ray study (Appendix A, Table 4). The occurrence of three hydrogen-bonding contacts involving the O(2) atom is responsible for the relatively long C(1)-O(2) distance of 1.267 (9) Å (Delaere *et al.* 1995).

3.3.2 Infrared data

Figure 3.1 shows the diffuse reflectance infrared spectrum of 50mM crystalline γ-glutamyl-β-cyanoalanine as its ammonium salt mixed in KCl while Figure 3.2 shows the diffuse reflectance

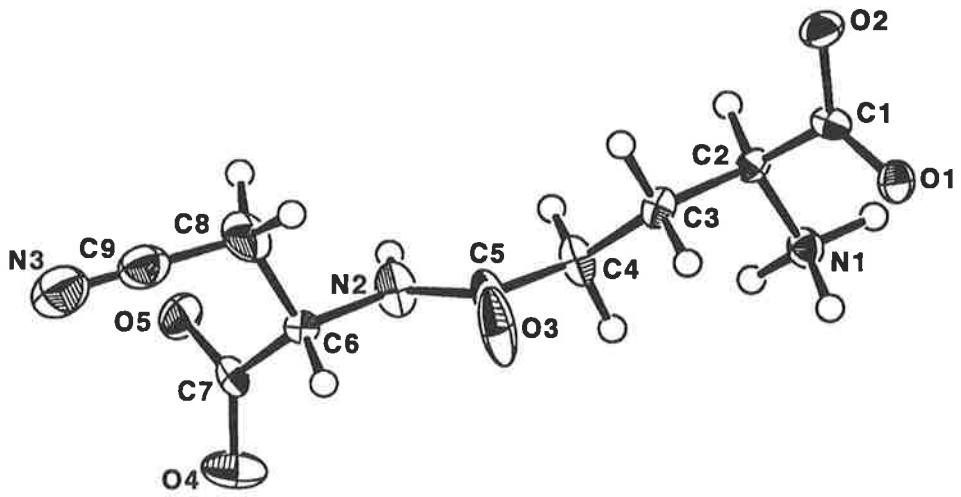


Figure 3.0 *ORTEP*II (Johnson, 1976) plot of the molecular structure the anion of N-γ-L-glutamyl-β-cyano-L-alanine showing 40% probability ellipsoids (Delaere, 1995).

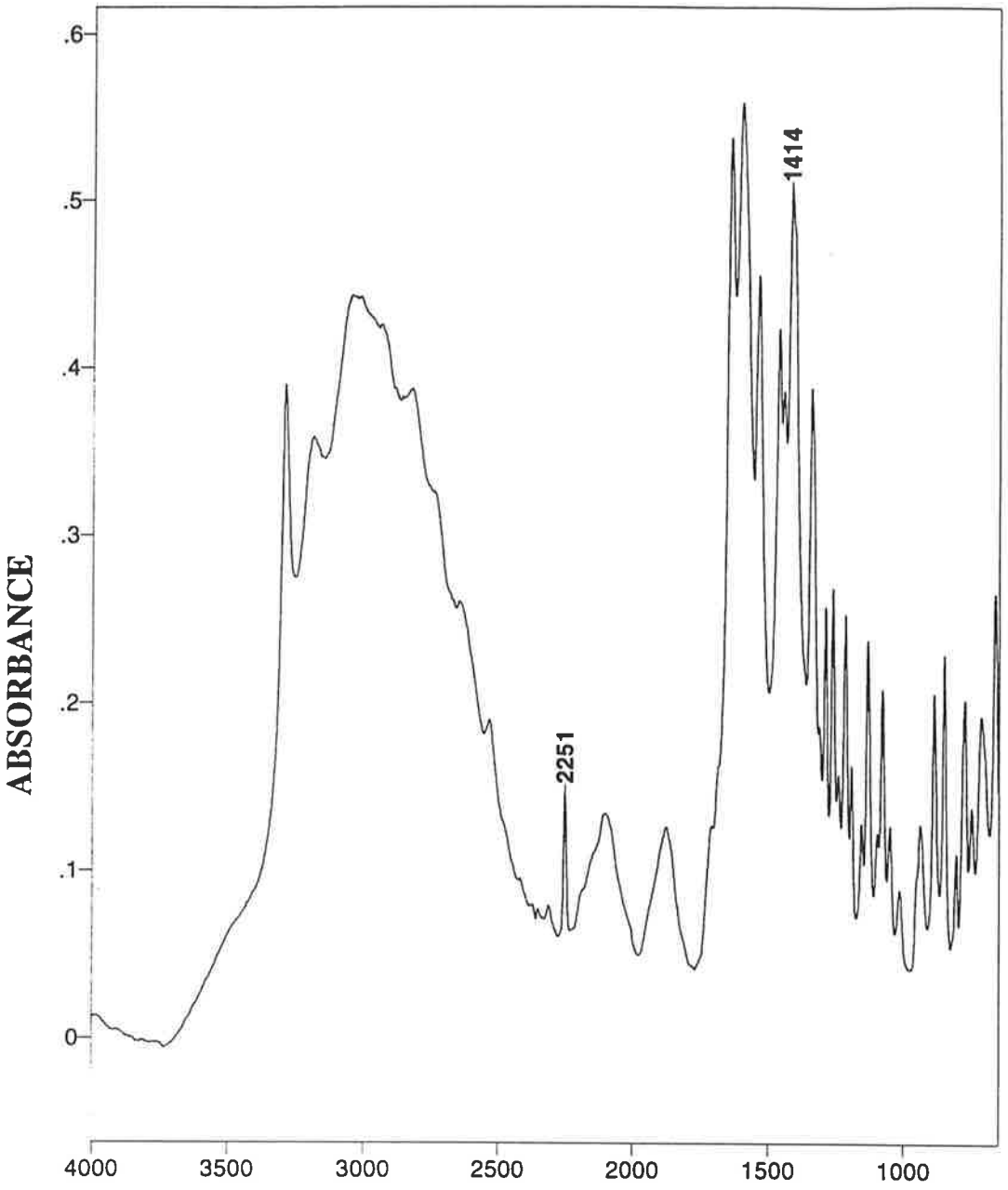


Figure 3.1 Diffuse reflectance infrared spectra of 50mM γ-glutamyl-β-cyanoalanine as its ammonium salt mixed in KCl; Scan Mode 1, Filter Noise 4.

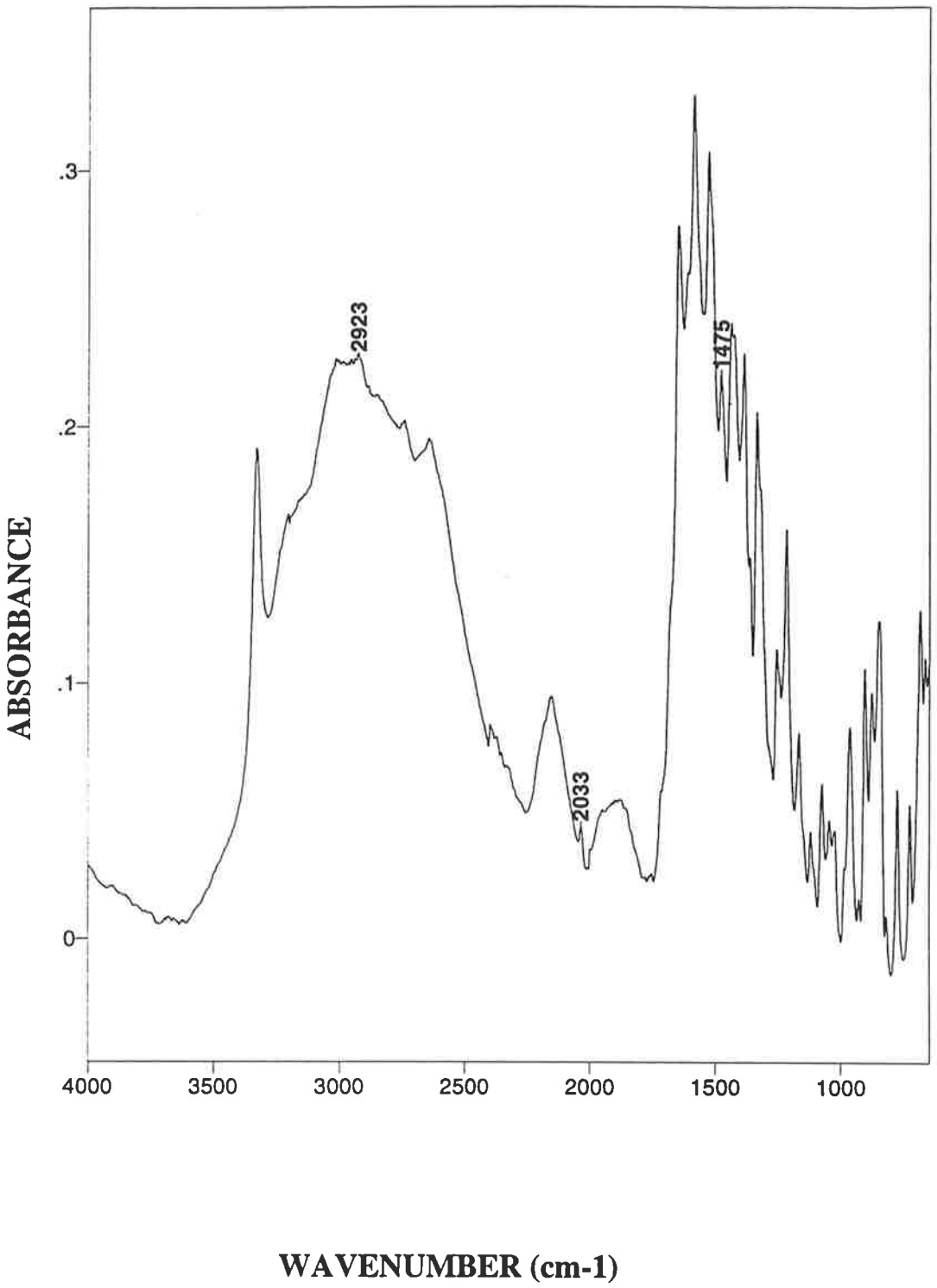


Figure 3.2 Diffuse reflectance infrared spectra of 50mM γ-glutamyl-S-ethenyl-cysteine mixed in KCl; Scan Mode 1, Filter Noise 4.

Table 3.1 Assignment of infrared stretching frequencies for the diffuse reflectance infrared spectra of 50mM γ-glutamyl-β-cyanoalanine as its ammonium salt mixed in KCl.

Frequency (cm^{-1})	Assignment	Vibration	Intensity
774	NH_2	dipole	strong
849	C-C	stretch	strong
1078	CH	rocking	strong
1132	NH_2	rocking	strong
1215	CH_2	t	strong
1260	CO	assymetric stretch	strong
1288	CH	bend	strong
1340	OH	bend	strong
1414	**NH_4^+**	**bend**	**very strong**
1442	CH_2	d-a	strong
1459	CH_2	bend	strong
1536	Amide II		strong
1596	carboxylate ion		very strong
1637	Amide I		strong
2251	**CN**	**stretch**	**strong**
3289	NH_2	stretch	very strong

Table 3.2 Assignment of infrared stretching frequencies for the diffuse reflectance infrared spectra of 50mM γ-glutamyl-S-ethenyl-cysteine mixed in KCl.

Frequency (cm^{-1})	Assignment	Vibration	Intensity
775	NH_2	dipole	strong
846	C-C	stretch	strong
1074	CH	rocking	strong
1121	NH_2	rocking	strong
1217	CH_2	t	strong
1250	CO	assymetric stretch	strong
1332	OH	bend	strong
1434	CH_2	d-a	strong
1475	**S-CH=CH_2**	**bend**	**strong**
1523	Amide II		strong
1581	carboxylate ion		very strong
1645	Amide I		strong
2033	**S-CH=CH_2**	**stretch**	**weak**
2923	**-CH=CH_2**	**stretch**	**weak**
3327	NH_2	stretch	very strong

infrared spectrum of 50mM crystalline γ-glutamyl-S-ethenyl-cysteine mixed in KCl. Both spectra have been background subtracted to remove infrared frequencies for CO_2 and water vapour. Assignment of the characteristic stretching frequencies associated with crystalline γ-glutamyl-β-cyanoalanine as its ammonium salt (Table 3.1) and crystalline γ-glutamyl-S-ethenyl-cysteine (Table 3.2) were made with the aid of tables (Randall *et al.* 1959; Alpert *et al.* 1970; Williams and Fleming 1989).

There are many similarities in the fingerprint region. The major differences being the S-ethenyl absorbance at 1474cm^{-1} in γ-glutamyl-S-ethenyl-cysteine and the very strong ammonium salt stretching frequency at 1414 cm^{-1} in γ-glutamyl-β-cyanoalanine as its ammonium salt. Other unique assignments are the nitrile stretching frequency at 2251cm^{-1} in γ-glutamyl-β-cyanoalanine, the S-ethenyl stretching at 2033cm^{-1}, and the weak ethylenic stretching frequency at 2923cm^{-1} in γ-glutamyl-S-ethenyl-cysteine.

3.3.3 Nuclear magnetic resonance data

Proton and carbon NMR data provided basic resonance information (Appendix B). Assignment of the carboxylate anion from carbon NMR data was facilitated by lowering the pD. 2D HETCOR analysis was used to determine proton/carbon connectivity and the 2D COSY analysis derived proton/proton connectivity. Evaluation of the coupling constants was through direct interpretation of the proton data or in the more complex determinations through use of proton pulse experiments (Appendix B).

Assignment of protons and coupling constants at 297°K are summarised in Table 3.3 for γ-glutamyl-β-cyanoalanine, and Table 3.4a for γ-glutamyl-S-ethenyl-cysteine. The proton assignments at 333°K for the high field ethylenic protons of γ-glutamyl-S-ethenyl-cysteine are in Table 3.4b. Assignment of proton coupling constants was supported by NMR simulation (Appendix B) (Hagele *et al.* 1993).

Carbon assignments for γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine are summarised in Table 3.5a. Assignment of the carboxylate anion of γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine by carbon NMR data was facilitated by lowering the pD and are in Table 3.5b.

3.3.4 Micellar electrokinetic chromatography data

Figure 3.3 shows the separation by micellar electrokinetic chromatography of a 9-fluorenylmethyl chloroformate (FMOC) derivatised standard solution containing *Vicia* anti-nutritional factors using a pH 9.52, 20mM borate/19.5mM SDS separation buffer. These data show that micellar electrokinetic chromatography is an effective means for the separation of *Vicia* anti-nutritional factors.

Table 3.3 Assignments for the D_2O solution ^1H NMR data of γ-glutamyl-β-cyanoalanine at 297°K.

δ ppm	Integration	Multiplicity (*J* Hz)	Assignment
2.19	2H	(m) 7.3	Glu 3Ha,Hb
2.55	2H	(t,d) 7.3, 9.0	Glu 4Ha,Hb
3.81	1H	(t) 6.1	Glu 2H
2.96	1H	(dd) 17.2, 7.2	Ala 3Ha
3.05	1H	(dd) 17.2, 5.5	Ala 3Hb
4.53	1H	(dd) 7.2, 5.5	Ala 2H

Table 3.4a Assignments for the D_2O solution ^1H NMR data of γ-glutamyl-S-ethenyl-cysteine at 297°K.

δ ppm	Integration	Multiplicity (*J* Hz)	Assignment
2.16	2H	(m) 7.4	Glu 3Ha, 3Hb
2.48	2H	(t,d) 7.4, 8.6	Glu 4Ha, 4Hb
3.75	1H	(dd) 6.7, 5.6	Glu 2H
3.00	1H	(dd) 14.0, 8.3	Cys 3Ha
3.24	1H	(dd) 14.0, 4.3	Cys 3Hb
4.41	1H	(dd) 8.3, 4.3	Cys 2H
5.28	1H	(br-d) 16.3	Eth 2Ha
5.29	1H	(br-d) 10.2	Eth 2Hb
6.41	1H	(dd) 16.8, 10.0	Eth 1H

Table 3.4b High field proton assignments for the D_2O solution ^1H NMR data of γ-glutamyl-S-ethenyl-cysteine at 333°K.

δ ppm	Integration	Multiplicity (*J* Hz)	Assignment
5.73	1H	(br-d) 16.8	Eth 2Ha
5.73	1H	(br-d) 10.0	Eth 2Hb
6.87	1H	(dd) 16.8, 10.0	Eth 1H

Table 3.5a Assignments for the D_2O solution ^{13}C NMR data for γ-glutamyl-β-cyanoalanine at pD 6.8 and γ-glutamyl-S-ethenyl-cysteine at pD 6.0.

γ-glutamyl-S-ethenyl-cysteine pD 6.0		γ-glutamyl-β-cyanoalanine pD 6.8	
δ ppm.	**Assignment**	**δ ppm.**	**Assignment**
28.36	Glu 3C	22.87	βAla 3C
33.72	Glu 4C	28.29	Glu 3C
35.58	Cys 3C	35.52	Glu 4C
56.27	Glu 2C	52.84	βAla 2C
56.49	Cys 2C	56.22	Glu 2C
114.94	Eth 2C	120.98	βAla 4C
133.37	Eth 1C	176.05	Glu 1C
176.04	Glu 1C	176.36	Glu 5C
176.20	Glu 5C	176.84	βAla 1C
178.65	Cys 1C		

Table 3.5b Assignments for the D_2O solution ^{13}C NMR by adjustment of pD for the elucidation of the carboxyl residues of γ-glutamyl-β-cyanoalanine at pD 4.9 and γ-glutamyl-S-ethenyl-cysteine at pD 4.8.

γ-glutamyl-S-ethenyl-cysteine pD 4.8		γ-glutamyl-β-cyanoalanine pD 4.9	
δ ppm.	**Assignment**	**δ ppm.**	**Assignment**
175.40	Cys 1C	175.72	βAla 1C
176.46	Glu 1C	176.18	Glu 1C
176.46	Glu 5C	176.40	Glu 5C

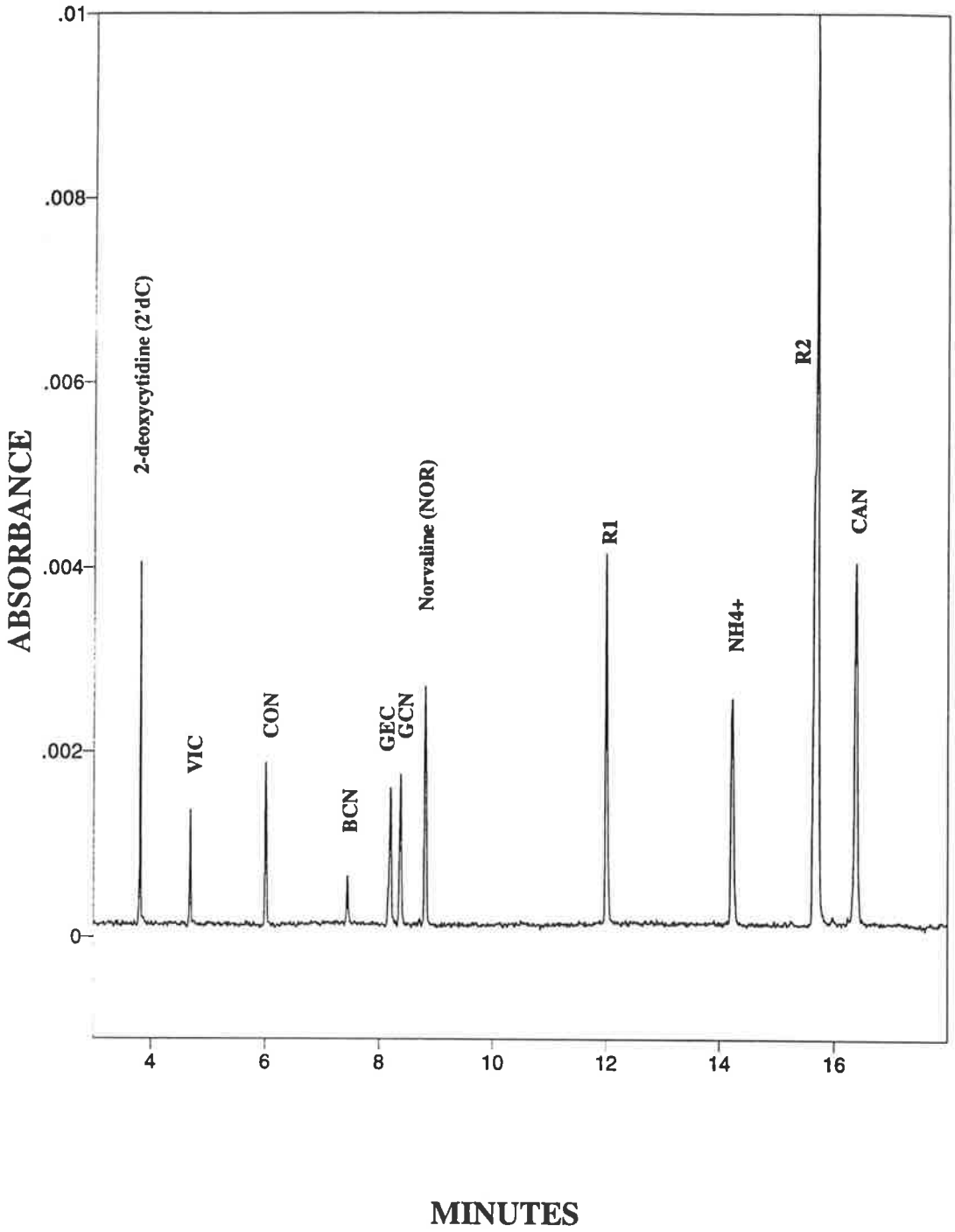


Figure 3.3 Separation by micellar electrokinetic chromatography of *Vicia* anti-nutritional factors using the 20mM borate/19.5mM SDS buffer, adjusted to pH 9.52.

3.4 Discussion

The method of Ressler *et al.* (1969a) for the isolation and purification of γ-glutamyl-β-cyanoalanine from the seeds of common vetch uses two organic solvents, pyridine for elution of acidic components from the anion-exchange column and dicyclohexylamine for crystallisation as its dicyclohexylammonium salt. Numerous laborious recrystallisations were involved resulting in a yield of 174mg of γ-glutamyl-β-cyanoalanine dicyclohexylammonium microcrystals from 200g common vetch. A simplified procedure for the isolation and purification of γ-glutamyl-β-cyanoalanine using ammonium acetate for the elution of acidic components from the anion-exchange column and ammonia for crystallisation as its ammonium salt has been described. Recrystallisation resulted in a yield of 5.6g (0.56%) of γ-glutamyl-β-cyanoalanine ammonium crystals from 1 kg of common vetch. A subsequent recrystallisation provided crystals suitable for structural elucidation by X-ray crystallography (Delaere *et al.* 1995).

Structural elucidation by X-ray crystallography studies the molecular orientation and structure of a compound at an atomic level in a crystal lattice. These data can be useful for correlating known biological activity to structure in a series of related compounds *in vivo*. For example, Figure 3.4 shows the molecular structure of the anion of the dipeptide γ-glutamyl-β-cyanoalanine (Delaere *et al.* 1995) overlayed on the molecular structure of the tripeptide γ-glutamyl-cysteinylglycine (Gorbitz 1987) commonly known as glutathione. The overlay shows a high degree of structural homology between the two crystal structures at an atomic level. Glutathione is known to be synthesised from γ-glutamyl-cysteine by the enzymatic addition of glycine to the terminal cysteine carboxyl residue. These data support the possible action of γ-glutamyl-β-cyanoalanine as a structural analogue for γ-glutamyl-cysteine and hence the presence of γ-glutamyl-β-cyanoalanylglycine in tissues of animals fed a diet containing vetch (Ressler *et al.* 1967; Sasaoka *et al.* 1968).

A number of unique infrared assignments were identified in infrared spectra of γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine with the potential to be useful in the characterisation of these molecules. The infrared spectrum of crystalline γ-glutamyl-β-cyanoalanine mixed in KCl exhibited a strong stretching frequency at 2251cm^{-1} for the nitrile functional group (Figure 3.1), while the S-ethenyl group of γ-glutamyl-S-ethenyl-cysteine showed absorbances at 1474cm^{-1} and 2033cm^{-1} for the S-ethenyl stretching and at 2923cm^{-1} for the weak ethylenic stretching frequency (Figure 3.2).

γ-Glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine were fully characterised by NMR spectroscopy. The validity of the chemical shift and proton coupling assignments were supported by NMR simulations (Hagele *et al.* 1993) and assignments made to related non-protein amino

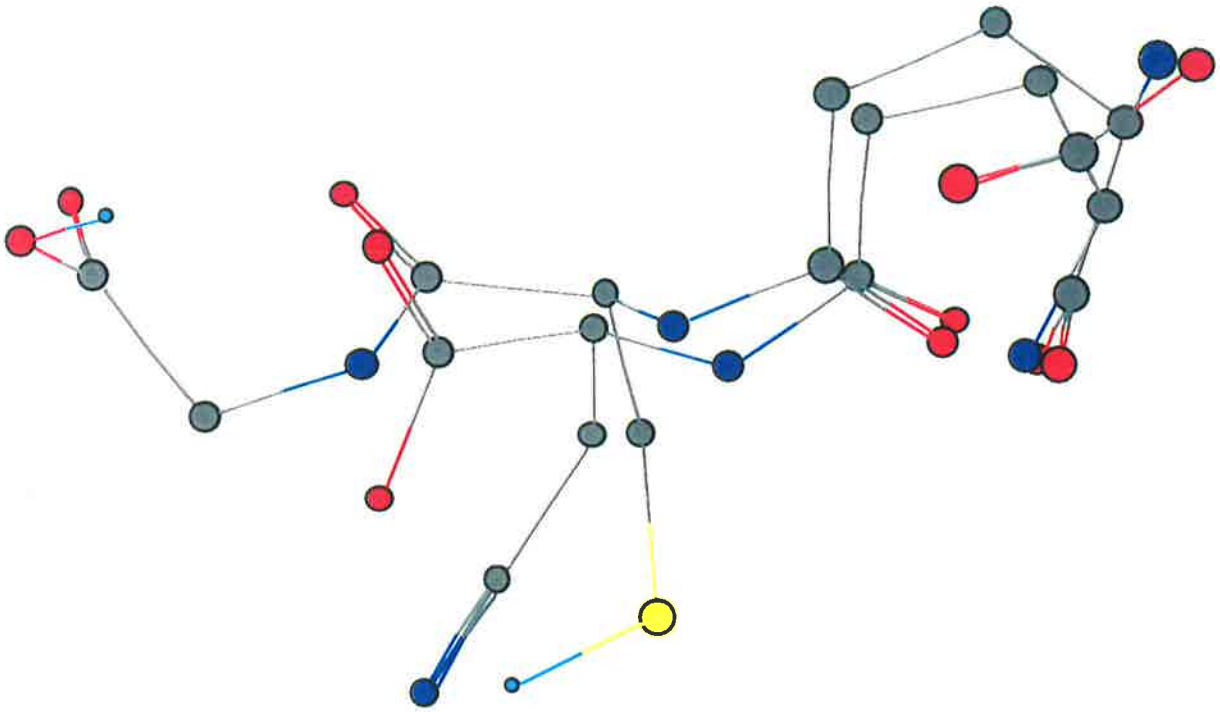
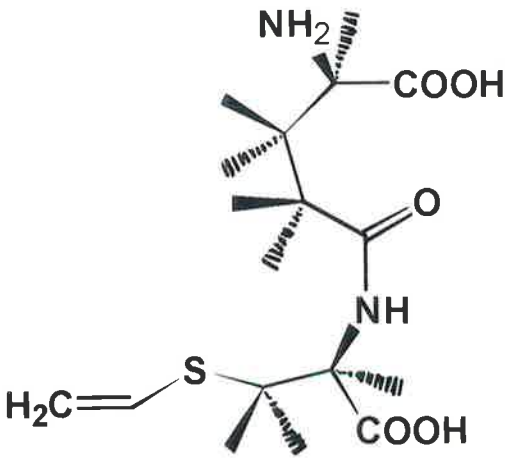


Figure 3.4 Crystal structure of γ-glutamyl-β-cyanoalanine overlayed on the crystal structure of the sulfur containing tripeptide γ-glutamyl-cysteinylglycine, glutathione.



γ-L-glutamyl-S-ethenyl-cysteine

S-ethenyl-cysteine non-protein amino acids

S-ethenyl-cysteine

Figure 3.5 The chemical structures of the low molecular weight S-ethenyl-cysteine non-protein amino acids found in *Vicia narbonensis* L.

acids β-cyanoalanine (Fushiya *et al.* 1993) (Figure 2.0) and S-ethenyl-cysteine (Thumfort *et al.* 1993) (Figure 3.5).

All the proton assignments were made relative to HOD, consequently absolute chemical shift values are not very stable and are influenced by changes in temperature and pD. The investigations into the assignments of protons for γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine indicated significant multiplicity associated with the glutamic acid residue . The glutamic acid multiplicity was resolved through the use of proton pulse experiments. Selective irradiation removes the influence of a proton from the system, reducing degeneracy and allowing for the determination of proton/proton coupling constants of interest. An example, is the irradiation of the γH protons of glutamic acid which results in a resolvable ABX system of 2βH protons interacting with an αH proton (Rahman 1986; Bovey 1988).

γ-Glutamyl-β-cyanoalanine exhibited an ABX system for the identification of the three protons associated with the β-cyanoalanine residue. Classically this is defined by the presence of 12 lines in the NMR spectra, 4 with the αH proton and 8 with the βH protons at two different chemical shifts. γ-Glutamyl-β-cyanoalanine was a classical example of an ABX system.

γ-Glutamyl-S-ethenyl-cysteine exhibited a similar ABX system for the three protons associated with the cysteine residue. However, down field at 297°C it also exhibited the presence of a degenerate not fully resolved ABX system associated the the three protons of the ethylenic residue (Figure 3.5). This was better resolved at 333°C which allowed for faster rotation of the αH around the double bond and provided clear resolution of the ABX system. The two βH protons were defined as doublets at the same chemical shift frequency, supporting the presence of a double bond.

The assignment of carbon chemical shift data were undertaken with the aid of 2D HETCOR analysis for carbons atoms in association with hydrogen atoms, and by alteration of pD for the determination of nonprotonated carbons. Changing pD resulted in changes to the relative chemical shift values of nonprotonated carbon residues due to partial protonation. The change in the chemical shift of peptide terminal carbons is largest, followed by the glutamic acid carbonyl, followed by the amide carbonyl. As a result, change of pD below the first ionisation of the terminal amino acid residue was used in the assignment of the amide carbonyl. However, change in pD had the effect of adding sulphite across the ethylenic double bond in γ-

glutamyl-S-ethenyl-cysteine, something which will be confirmed by proton assignments in a forthcoming publication.

3.5 Conclusion

This chapter describes the isolation, purification and full chemical characterisation of two γ-glutamyl peptides found in *Vicia* species, γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine. The structural elucidation by X-ray crystallography of γ-glutamyl-β-cyanoalanine has been published (Delaere *et al.* 1995). The infrared spectrum of crystalline γ-glutamyl-β-cyanoalanine mixed in KCl exhibited a strong stretching frequency at 2251cm^{-1} for the nitrile functional group. The full assignment of protons and coupling constants for γ-glutamyl-β-cyanoalanine and γ-glutamyl-S-ethenyl-cysteine was facilitated by proton decoupling and temperature variation experiments. The assignments were supported by the literature (Fushiya *et al.* 1993; Thumfort *et al.* 1993) and by NMR simulation (Hagele *et al.* 1993). Assignment of carbon NMR spectra was facilitated by HETCOR analysis and by pD variation experiments and was supported by the literature (Fushiya *et al.* 1993; Thumfort *et al.* 1993). Micellar electrokinetic chromatography was shown as a sensitive and effective method for the separation of *Vicia* anti-nutritional factors. The use of analytically pure and fully characterised reference standards provided chemical clues necessary for the development of tools required to assess the occurrence of low cyanoalanine non-protein amino acid accessions of *V. sativa* in the native population.

Chapter Four

Making Light Work

4.0 Introduction

Useful chemotaxonomic characters have been correlated for classification purposes in *Vicia* (Bell and Tirimanna 1965; Tschiersch and Hanelt 1967) and include the nitrile-containing anti-nutritional factors γ-glutamyl-β-cyanoalanine, β-cyanoalanine and vicianine in common vetch (*V. sativa*) (Bertrand 1906; Ressler and Ratzkin 1961; Ressler 1962; Ressler *et al.* 1963). The non-protein amino acids β-aminopropionitrile and β-(*N*-γ-L-glutamyl)-aminopropionitrile are present in the ornamental sweet pea (*Lathyrus odoratus* L.) (Dasler 1954; Schilling and Strong 1954; Schilling and Strong 1955). The seeds of common vetch (*V. sativa*) can contain in excess of 1% dry weight of γ-glutamyl-β-cyanoalanine (Ressler *et al.* 1969a).

To identify accessions of common vetch which contain low levels of cyanoalanine non-protein amino acids in germplasm collections, an efficient assay suitable for application to a plant breeding program had to be developed. Ideally this would be a simple, rapid and specific assay for the desired chemical trait requiring minimal sample preparation. Infrared spectrometry offered one of the most useful approaches for developing a rapid nondestructive analysis for the nitrile-containing compounds in the cotyledons of seeds.

Infrared radiation is usually defined as that electromagnetic radiation whose frequency is between 14,300 and 20cm^{-1} (0.7 and 500μm). Within this region of the electromagnetic spectrum, chemical compounds absorb infrared radiation providing that there is a dipole moment change during a normal molecular vibration or rotation. The frequencies and intensities of the infrared bands exhibited by a chemical compound uniquely characterise the material, and its infrared spectrum can be used to identify and quantify the particular substance in an unknown sample (Putzig *et al.* 1994).

Organic nitriles exhibit characteristic group stretching frequencies in the 2300-2100 cm^{-1} region of the mid-infrared (Alpert *et al.* 1970; Williams and Fleming 1989). This is a region of few vibrational and combinational overtone bands. An infrared band used to uniquely characterise γ-glutamyl-β-cyanoalanine, the major nitrile-containing anti-nutritional factor in common vetch, was the strongly absorbing nitrile stretching frequency at 2251cm^{-1}.

The development of efficient ellipsoidal collecting mirrors for diffuse reflectance infrared spectroscopy has facilitated the mid-infrared analysis of powders (Fuller and Griffiths 1978) (Figure 4.0). Most of the parameters which influence the diffuse reflectance process have been examined by earlier experimental studies. The general character of diffuse reflectance spectra has been shown to be a function of the particle size (Fuller and Griffiths 1978), the granulometric distribution (Yang and Mantsch 1987), the sample packing (Yeboah *et al.* 1984), and the scattering coefficient of the sample (Fraser and Griffith 1990). Other factors discussed in the literature include optical geometries (Yang *et al.* 1986), optical parameters (Brimmer *et al.* 1986), polarization effects (Brimmer and Griffith 1987) and Kubelka-Munk function dependence (Brimmer and Griffith 1988).

A general theory for diffuse reflectance at scattering layers within powdered samples was developed by Kubelka and Munk and widely supported with experimental measurements (Kubelka and Munk 1931; Kubelka 1948). The Kubelka-Munk equation is the diffuse reflectance analogue to Beer's law for transmission measurements and can be used to obtain quantitative information (Fuller and Griffiths 1978). It contains two parameters - K, the absorption coefficient, and S, the diffusion coefficient - neither of which is directly related to the physical or optical parameters of the powder (Moradi *et al.* 1994).

The Kubelka-Munk equation is valid only for systems where no further reflectance takes place at the boundary between the semi-infinite medium (e.g. KBr powder) and air (Reinecke *et al.* 1988). However, in many applications the characteristics of the sample or the matrix do not follow the assumptions made in the derivation of the Kubelka-Munk equation. Diffuse reflectance infrared spectra of organic analytes in strongly absorbing matrices demonstrate nonlinearity for Kubelka-Munk values with respect to concentration. In these cases Beer's law better approximates the linearity over the region of interest (Olinger and Griffiths 1988).

Spectral interferences, frequently encountered with *in situ* diffuse reflectance infrared measurements on strongly absorbing matrices, have slowed the application of quantitative diffuse reflectance. As a result the literature contains few reports of the use of diffuse reflectance infrared spectroscopy for the quantitative determination in the mid-infrared of organic compounds from strongly absorbing matrices (Beauchemin and Brown 1989), and there are no reports on quantitative diffuse reflectance mid-infrared analysis derived from organic matrices.

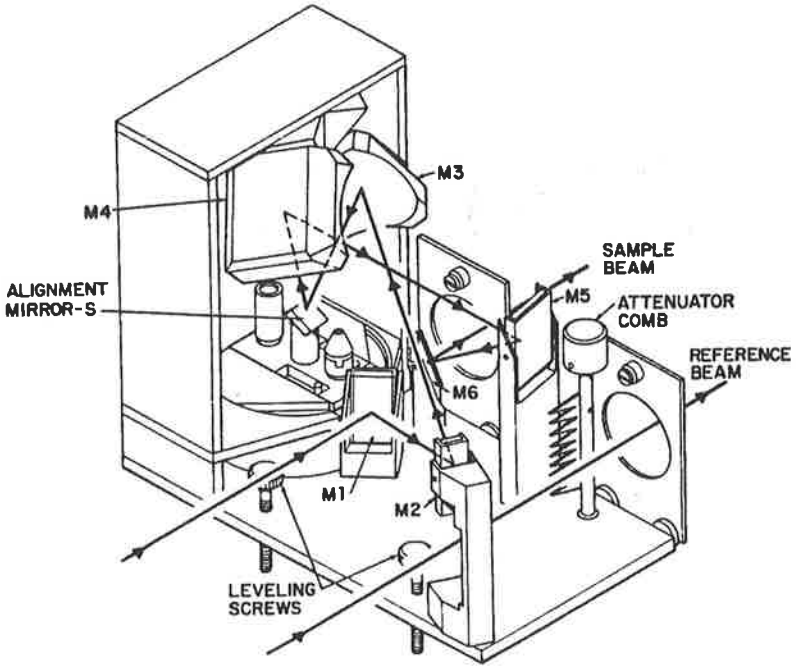


Figure 4.0 The optical arrangement of the ellipsoidal mirror array of the Harrick Model 3SP "Praying Mantis" diffuse reflectance attachment used in the diffuse reflectance infrared spectroscopy from the Perkin Elmer 983G dispersive infrared spectrometer. The large and small cups are either side of the alignment mirror on the sample carriage.

The work in this chapter sets out to study the various attributes of quantitative analysis of individual organic compounds present in a strongly absorbing organic matrix by mid-infrared diffuse reflectance using infrared dispersive spectrometry (DRUIDS). This was achieved by measuring the spectral characteristics of nitrile-containing compounds by diffuse reflectance infrared spectrometry in both absorbing and non-absorbing matrices. These data established the importance of preparing appropriate model matrices for the quantitative mid-infrared diffuse reflectance infrared analysis of nitrile-containing organic molecules in biological tissue. Two quantitative assays for γ-glutamyl-β-cyanoalanine in homogenised cotyledons of common vetch (*V. sativa*) have been developed.

4.1 Instrumentation

4.1.1 Diffuse Reflectance Using Infrared Dispersive Spectrometry (DRUIDS)

4.1.1.1 DRUIDS apparatus

A double beam dispersive infrared spectrometer (Model PE 983G (50 Hz instrument), Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) fitted with a Harrick Praying Mantis Diffuse Reflectance attachment (Model 3SP, Harrick Scientific Corporation, Ossining, NY) operating in double beam mode was used to obtain diffuse reflectance infrared spectra. The Model PE983G was linked to an IBM PS/2 clone (Acer 466DX2, Intel 486, 66 MHz, 8Mb RAM, Acer Technologies Corporation, Taiwan R.O.C.) and controlled through a modified PE983G emulator/controller (MDS Engineering Associates, Brookfield, CT). The sample compartment was continually purged throughout the duration of sample collection using a circulatory air drier (Model CD3, Bodenseewerk Perkin-Elmer & Co GmbH, Uberlingen, Bundeesrepublik Deutschland). The small cup assay samples were loaded into a modified microsample holder (Model DRA-SX3, Harrick Scientific Corporation, Ossining, NY), diameter 3mm, depth adjusted to 1mm using a teflon plug (Figure 4.1). The large cup assay samples were loaded into a large sample holder (Model DRA-SX3) diameter 11mm, depth 3mm (Figure 4.1).

4.1.1.2 DRUIDS data collection

The small sample cup procedure used <5mg and the large sample cup procedure used <70mg of homogenised powdered sample. Samples were poured into the sample cups and

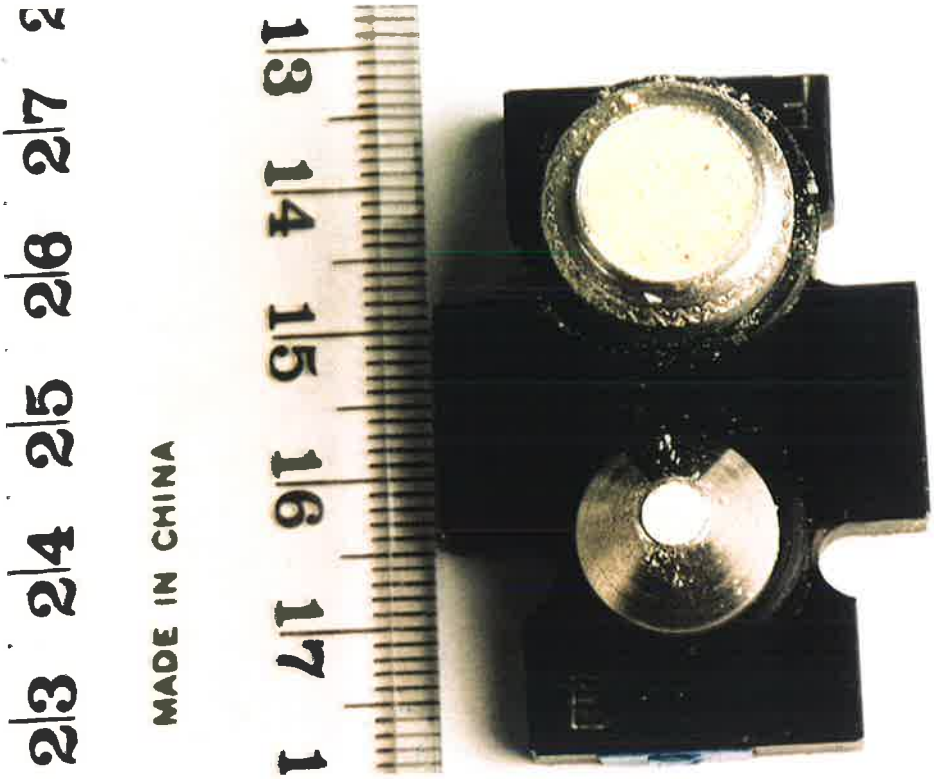


Figure 4.1 The small and large sample cup arrangement of the Harrick Model DRA-SX3 sample carriage associated with the Harrick "Praying Mantis" diffuse reflectance attachment for the Perkin Elmer 983G dispersive infrared spectrometer. Smallest scale divisions are 1mm.

smoothed using a polished spatula. Considerable attention was placed on achieving a smooth surface. Sample alignment was adjusted to maximized reflectance (%"Transmission" > 20.0). Collection parameters for the small sample cup procedure were Scan Mode 1 (Resolution 10 cm^{-1}, Relative Noise %T, 0.03), Filter Noise 64 (Noise %T, 0.03), and Scan Mode 1, Filter Noise 16 (Noise %T, 0.05%) for the large sample cup procedure (Perkin-Elmer 1983). Samples were scanned from 2300-2200cm^{-1}.

4.1.1.3 DRUIDS data manipulation

Reflectance data were converted to absorbance units, baseline-corrected and spectra offset using the GRAMS/386 II data manipulation program (Galactic Industries Corporation, Salem, NH); Absorbance, Auto-baseline and Offset functions. Peak areas and heights were determined at 2251cm^{-1} using a valley to valley integration mode. Quantitative absorbance and Kubelka-Munk data were obtained. The results were exported to an Excel 5.0 spreadsheet (Microsoft Corporation, Redmond, WA) for tabulation. During large scale screenings, samples were barcoded (Code 39) and entered using a REB Barcode Systems keyboard wedge barcode reader.

4.1.2 Diffuse Reflectance Infrared Fourier Transform Spectrometry (DRIFTS)

4.1.2.1 DRIFTS apparatus

Diffuse reflectance infrared Fourier transform spectrometry (DRIFTS) spectra were collected on a Bio-Rad Digilab FTS-80 rapid-scan Fourier transform spectrometer using a germanium coated KBr beam splitter, a high intensity globar source, a liquid nitrogen cooled wide-band mercury cadmium telluride (MCT) detector operating at 2 scans per second and fitted with a Harrick off-axis diffuse reflectance accessory (DRA-3SO). The Bio-Rad Digilab FTS-80 was linked to an Digilab 3200 computer with the IDRIS operating system. The sample compartment was continually purged for carbon dioxide and water throughout the duration of sample collection using a circulatory air drier (Puregas Corporation, U.S.A). The samples were loaded into a large sample holder.

4.1.2.2 DRIFTS sample preparation

Samples were poured into a 10mm diameter stainless steel sample holder and smoothed using a spatula. Considerable attention was placed on achieving a smooth surface. Spectra were

recorded from 4000 to 500cm^{-1} at 1.92cm^{-1} intervals (maximum range 7000-430cm^{-1}) and a resolution of 4cm^{-1}.

4.1.1.3 DRIFTS data manipulation

Data files were moved from the Digilab 3200 to a PC, using an RS-232 cable and communication software before file conversion. Digilab uses a KERMIT transfer protocol for the Digilab 3200 data and an application program, AP9, which writes data directly onto MS-DOS formatted disks. The data files containing the spectral data points were translated into GRAMS/386 II format using the Bio-Rad Digilab FTS Series FT-IR, 3200 Data Station (DIGILAB) file converter associated with the GRAMS/386 II package (Galactic Industries Corporation, Salem, NH). Reflectance data were converted to absorbance units and truncated and saved for the region of 2300-2200cm^{-1}. The spectra were baseline-corrected and offset using the GRAMS/386 II data manipulation program (Galactic Industries Corporation, Salem, NH); Auto-baseline and Offset functions. Peak areas and heights were determined at 2251cm^{-1} using a valley to valley integration mode. Quantitative absorbance and Kubelka-Munk data were obtained. The results were exported to an Excel 5.0 spreadsheet (Microsoft Corporation, Redmond, WA) for tabulation.

4.2 Experimental

4.2.1 Reagents

All additions of γ-glutamyl-β-cyanoalanine to various matrices were of its crystalline ammonium salt, isolated and characterized from common vetch (*V. sativa*) (Delaere *et al.* 1995). β-cyanoalanine, β-aminopropionitrile monofumarate and amygdalin were obtained from the Sigma Chemical Company. The reagents used in the diffuse reflectance infrared studies were spectroscopic quality KCl, 18MΩcm^{-1} Milli-Q water (Millipore, Bedford, MA), phyto-certified common vetch cv. "Blanchefleur" (*V. sativa*), red lentil (*L. culinaris*) and sweet pea, Yates cv. "Colorcade" (*L. odoratus*). All samples were milled through a 0.5mm aluminium sieve. The milled samples were dried (1 hr@110°C) to remove residual water and enzymatic activity.

4.2.2 Quantitative diffuse reflectance infrared spectrometry

4.2.2.1 Quantitation function

Instrumental error in diffuse reflectance infrared spectrometry was determined by acquiring common vetch cv. "Blanchefleur" (*V. sativa*) nine times. The accuracy of the dispersive infrared

spectrophotometer (DRUIDS) data acquired in a small cup was compared with the corresponding results obtained using a Fourier transform spectrophotometer (DRIFTS). The quantitation functions were compared from percentage relative standard deviation data derived from Kubelka-Munk and absorbance unit data for peak height and integrated peak area.

4.2.2.2 Quantitative Analysis

Known amounts of γ-glutamyl-β-cyanoalanine at 9 concentrations were added to the milled samples (1 g) of red lentil (*L. culinaris*) and vigorously ground and mixed using a mortar and pestle. 1mL Milli-Q water was added to 200mg of each sample mixture, shaken overnight and dried *in vacuo* to produce the absorbed γ-glutamyl-β-cyanoalanine/red lentil matrix. All samples were again homogenized using a mortar and pestle, dried overnight at 40°C and stored in a desiccator over silica gel prior to use. The analysis was repeated 7 times in accordance with a randomised block design.

4.3 Results

All infrared spectra displayed in the results section were collected between 2300-2200cm^{-1} using dispersive infrared parameters Scan Mode 1, Filter Noise 64, unless otherwise stated.

4.3.1 Diffuse reflectance infrared properties of organic nitriles found in *Vicia* and *Lathyrus* species observed in a non-absorbing KCl matrix

The diffuse reflectance infrared spectra for the nitrile stretching frequency associated with 50mM γ-glutamyl-β-cyanoalanine, β-cyanoalanine (>98%), amygdalin, and β-aminopropionitrile monofumarate, mixed and absorbed in a non-absorbing KCl matrix were collected (Figures 4.2, 4.3, 4.4, and 4.5). γ-Glutamyl-β-cyanoalanine exhibited a peak maximum at 2251cm^{-1} when mixed as its crystalline ammonium salt or absorbed in the non-absorbing KCl matrix (Figure 4.2). β-Cyanoalanine (>98%) showed polymorphic nitrile stretching frequencies when mixed with KCl exhibiting peak maxima at 2264cm^{-1} and 2255cm^{-1} and a uniform nitrile stretching frequency at 2254cm^{-1} when present in an amorphous state absorbed in KCl (Figure 4.3). Whereas β-aminopropionitrile exhibited a crystalline and amorphous nitrile stretching frequency in a non-absorbing matrix at 2254cm^{-1} (Figure 4.5).

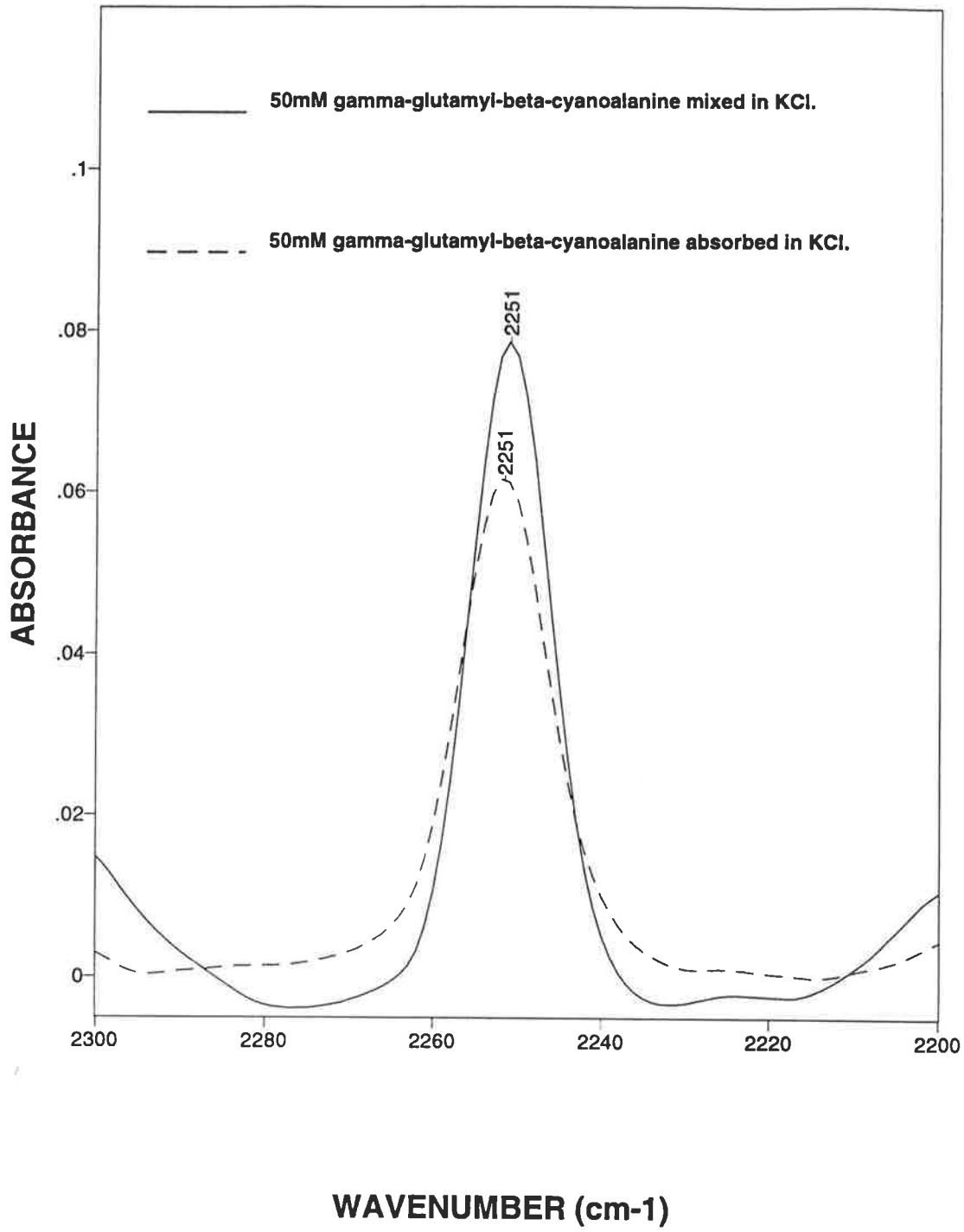


Figure 4.2 The diffuse reflectance infrared spectra of 50mM γ-glutamyl-β-cyanoalanine mixed in KCl, overlayed with 50mM γ-glutamyl-β-cyanoalanine absorbed in KCl.

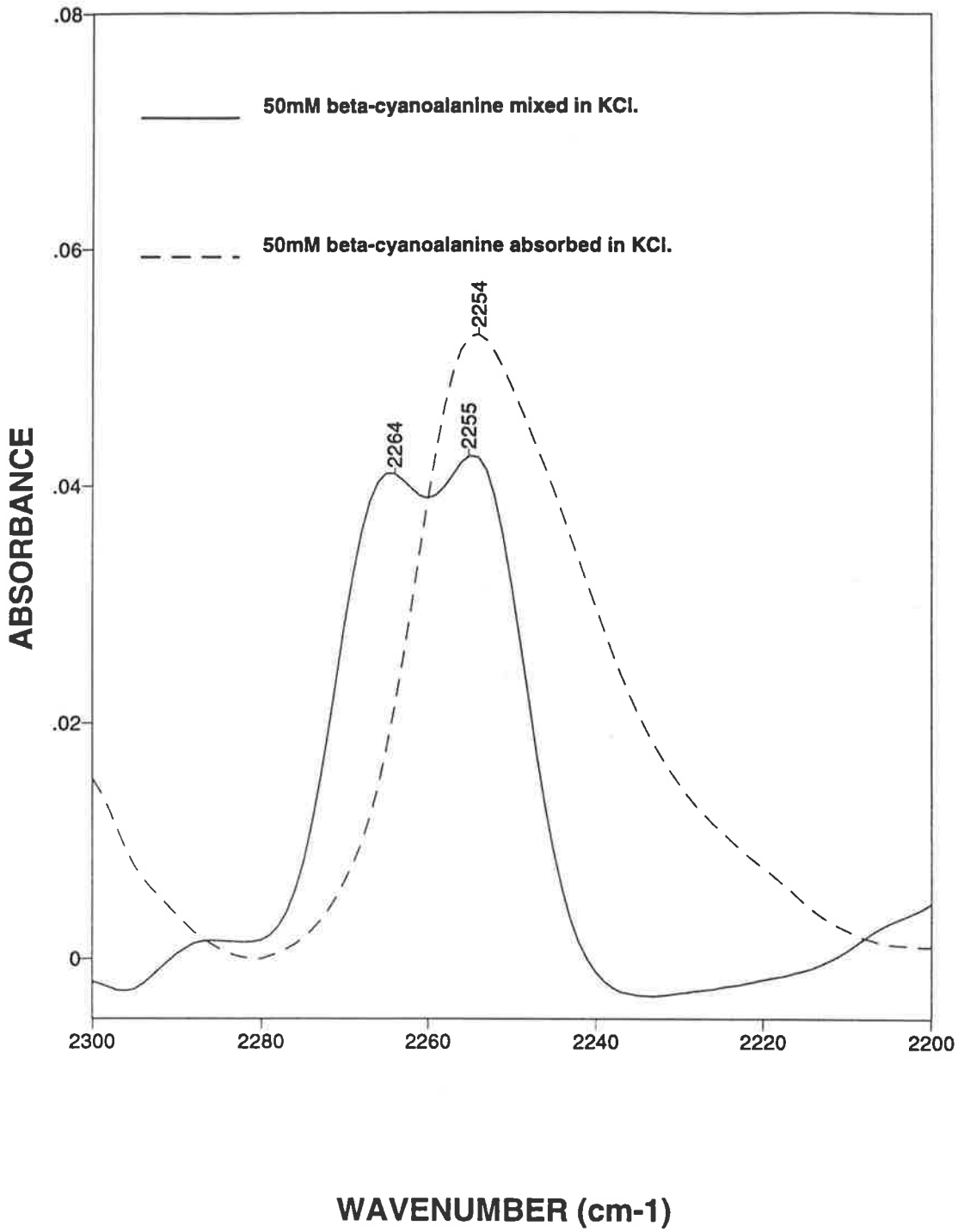


Figure 4.3 The diffuse reflectance infrared spectra of 50mM >98% pure β-cyanoalanine mixed in KCl overlayed with 50mM >98% pure β-cyanoalanine absorbed in KCl.

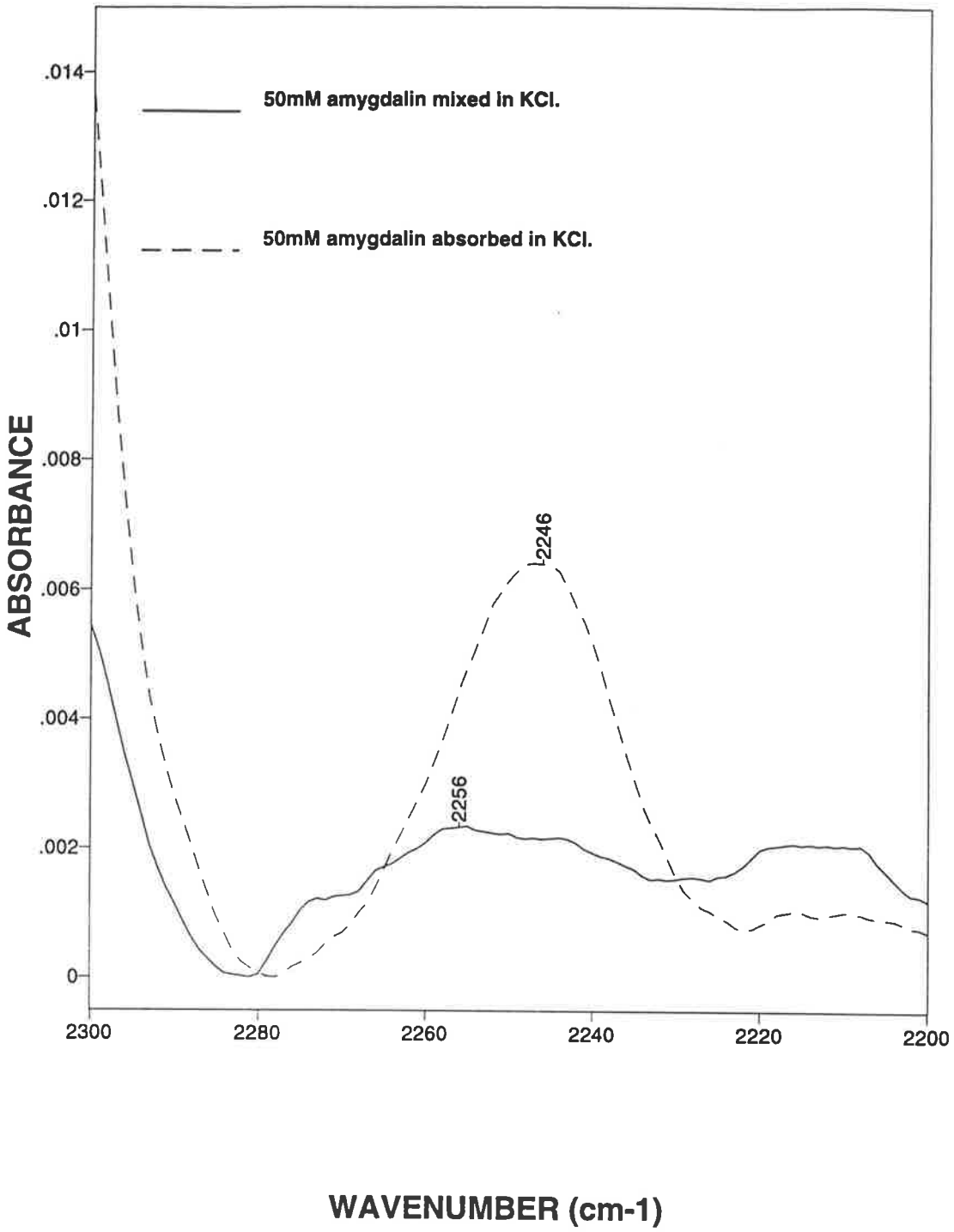


Figure 4.4 The diffuse reflectance infrared spectra of 50mM amygdalin, a cyanogenic glycoside, mixed in KCl, overlayed with 50mM amygdalin absorbed in KCl.

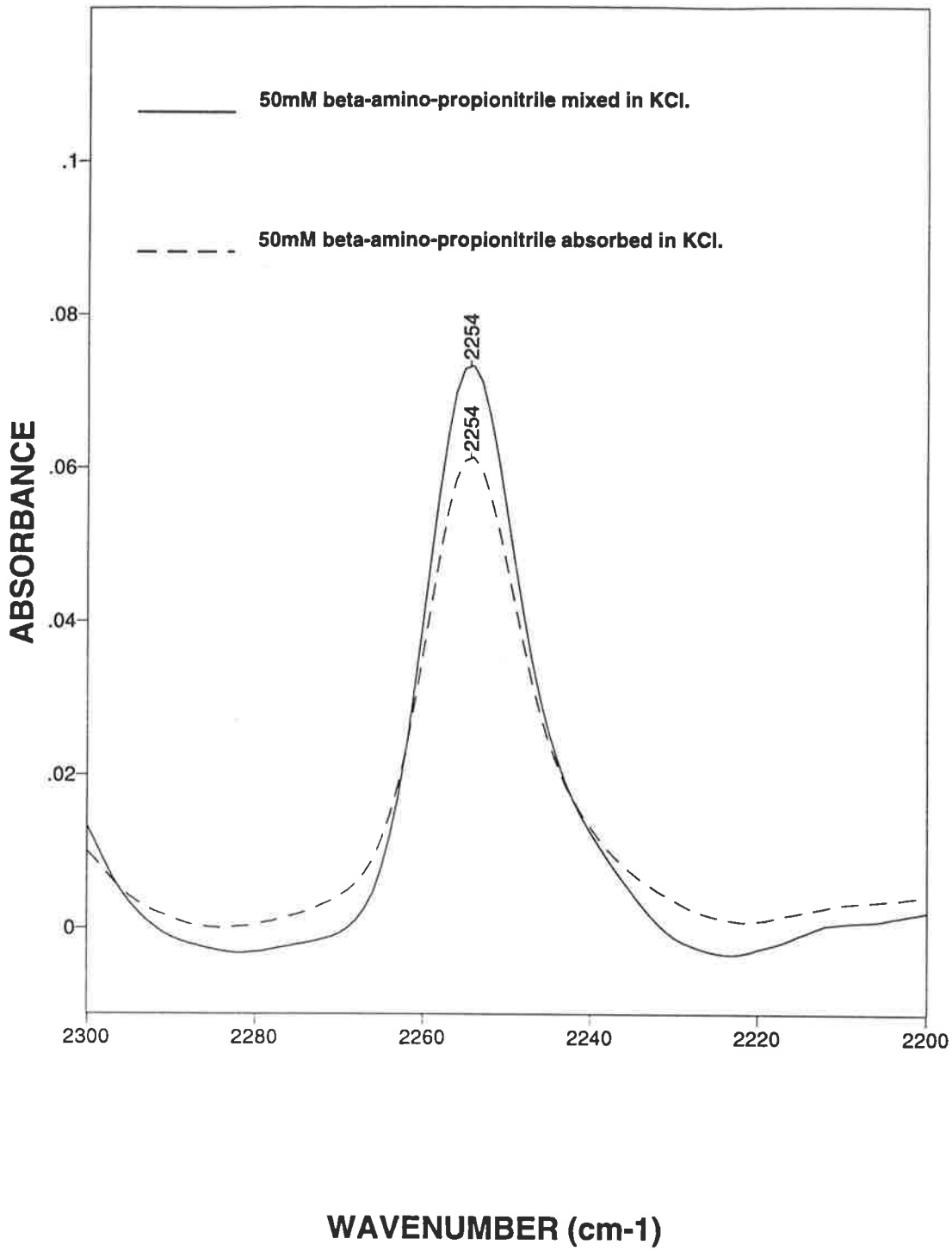


Figure 4.5 The diffuse reflectance infrared spectra of 50mM β-amino-propionitrile mixed in KCl, overlayed with 50mM β-amino-propionitrile absorbed in KCl.

These data show that the nature of the physical state of the nitrile-containing non-protein amino acids found in *Vicia* and *Lathyrus* species may influence the resultant diffuse reflectance infrared spectra.

In the absence of a vicianine standard, the results obtained from amygdalin, a close structural analogue of vicianine, were used to simulate the expected nitrile absorbance in vicianine. The effect of physical state was shown to be particularly important in the identification of the nitrile stretching frequency in the cyanogenic glycoside amygdalin (Figure 4.4). Crystalline amygdalin exhibited a poorly defined nitrile stretching frequency at 2256cm^{-1} when mixed in a non-aborbing matrix. However, when present in an amorphous state, absorbed in the KCl matrix, amygdalin exhibited a weak but measurable nitrile stretching frequency at 2246cm^{-1} (Figure 4.4, Table 4.1a and Table 4.1b).

Table 4.1a The molal absorption coefficients of nitrile standards, either mixed or absorbed in KCl derived from Absorbance units.

Peak Height Peak Maxima	GCN 2251cm^{-1}	BCN 2255cm^{-1}	BCN 2264cm^{-1}	Amygdalin 2256cm^{-1}	Amygdalin 2246cm^{-1}	BAPN 2254cm^{-1}
Mixed	1.64	0.91	0.88	0.04		1.53
Adsorbed	1.23	1.05			0.12	1.22

Table 4.1b The molal absorption coefficients of nitrile standards, either mixed or absorbed in KCl derived from Kubelka-Munk units.

Peak Height Peak Maxima	GCN 2251cm^{-1}	BCN 2255cm^{-1}	BCN 2264cm^{-1}	Amygdalin 2256cm^{-1}	Amygdalin 2246cm^{-1}	BAPN
Mixed	0.33	0.10	0.09	2.26×10^{-4}		0.29
Adsorbed	0.20	0.15			2.15×10^{-3}	0.20

This study showed that physical properties and the effect of the matrix environment can affect the diffuse reflectance infrared spectra of nitrile-containing compounds in a non-absorbing matrix. Both the absorbance maximum and the molal absorbance coefficients of the nitrile-containing standards (Table 4.1a and Table 4.1b), when expressed in either absorbance or Kubelka-Munk units, are influenced by the physical state of the compound.

4.3.2 Qualitative analysis of γ-glutamyl-β-cyanoalanine in strongly absorbing organic matrices by DRUIDS

Figure 4.6 shows the diffuse reflectance infrared spectrum of powdered common vetch cv. "Blanchefleur" (*V. sativa*) overlayed with the spectrum of 1% γ-glutamyl-β-cyanoalanine absorbed in KCl. Both spectra have been background substracted to remove the infrared frequencies for CO_2 and water vapour. Prominent in both spectra is the stretching frequency at 2251cm^{-1}, associated with the nitrile functional group in γ-glutamyl-β-cyanoalanine. It is this 2251cm^{-1} absorbance which has provided the key to monitoring the concentration of γ-glutamyl-β-cyanoalanine in homogenised powdered common vetch.

Figure 4.7 shows the diffuse reflectance infrared spectrum of 1% γ-glutamyl-β-cyanoalanine mixed in red lentil (*L. culinaris*) overlayed with the spectrum of 1% γ-glutamyl-β-cyanoalanine absorbed in red lentil as well as the spectrum of powdered cotyledon of red lentil acting as a nitrile negative control. These data show that there are significant qualitative differences in shape and height of the nitrile stretching frequency between γ-glutamyl-β-cyanoalanine samples absorbed and mixed in red lentil. This indicates that the effect of the matrix on the nitrile stretching frequency, as observed in the non-absorbing KCl environment, is present in strongly absorbing organic matrices. It also shows, as expected, that there is no nitrile stretching frequency associated with red lentil (*L. culinaris*).

Figure 4.8 shows the diffuse reflectance infrared spectrum of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*) overlayed with the spectrum of 1% γ-glutamyl-β-cyanoalanine absorbed in red lentil (*L. culinaris*) as well as the spectrum of powdered and exhaustively extracted (30% ethanol/water) cotyledon of common vetch cv. "Blanchefleur". These data show that there are qualitative similarities in the shape of the nitrile stretching frequency associated with 1% γ-glutamyl-β-cyanoalanine absorbed in red lentil (*L. culinaris*) to that found for γ-glutamyl-β-cyanoalanine in the matrix of native common vetch cv. "Blanchefleur" (*V. sativa*). It also shows that the nitrile in common vetch is extractable and the diffuse reflectance infrared spectrum of the extracted vetch residue is similar to that observed in the negative control, red lentil (*L. culinaris)* (Figure 4.7).

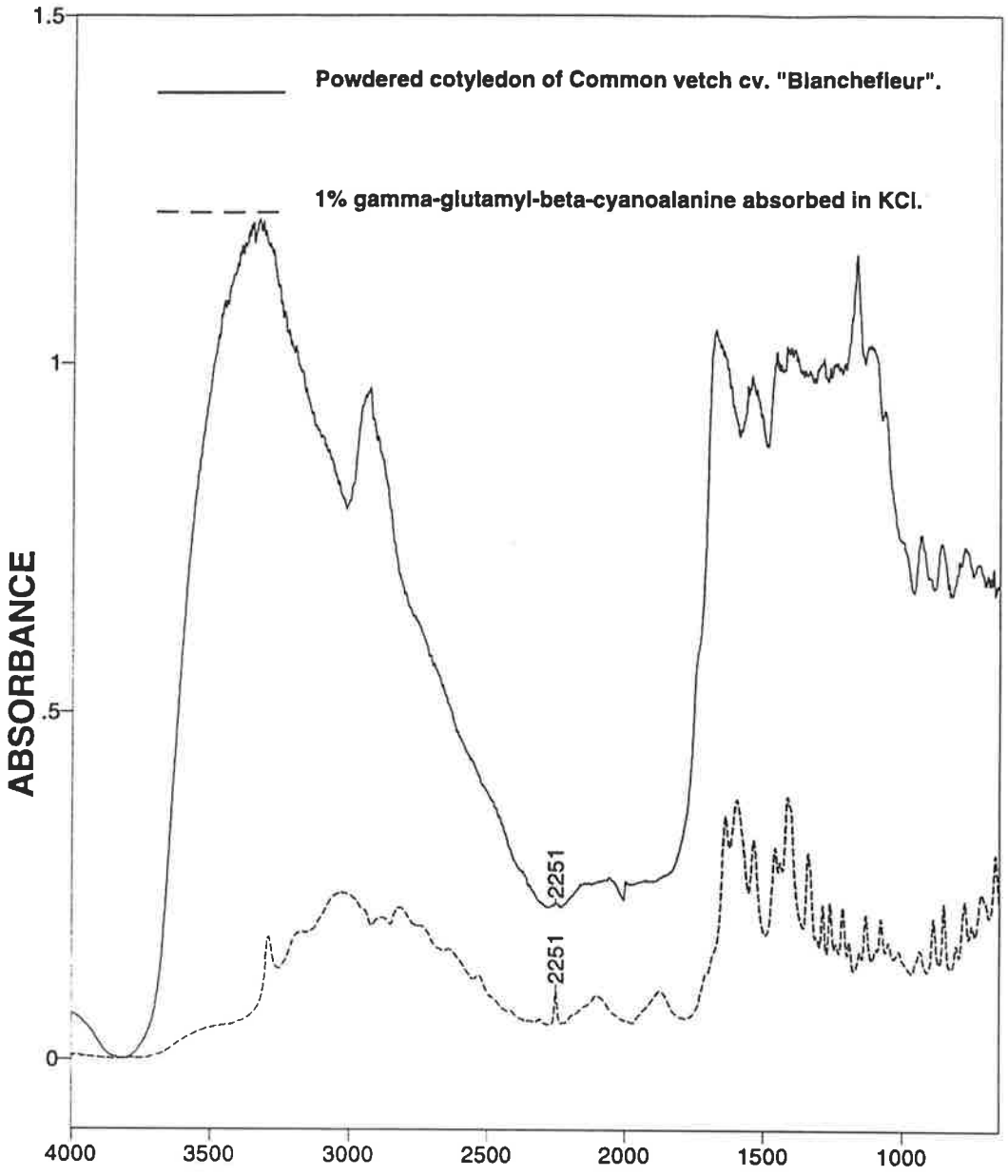


Figure 4.6 The diffuse reflectance infrared spectra of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*) overlayed with 1% γ-glutamyl-β-cyanoalanine absorbed in KCl. The dispersive infrared spectrometer collection parameters were; Scan Mode 1, Filter Noise 4.

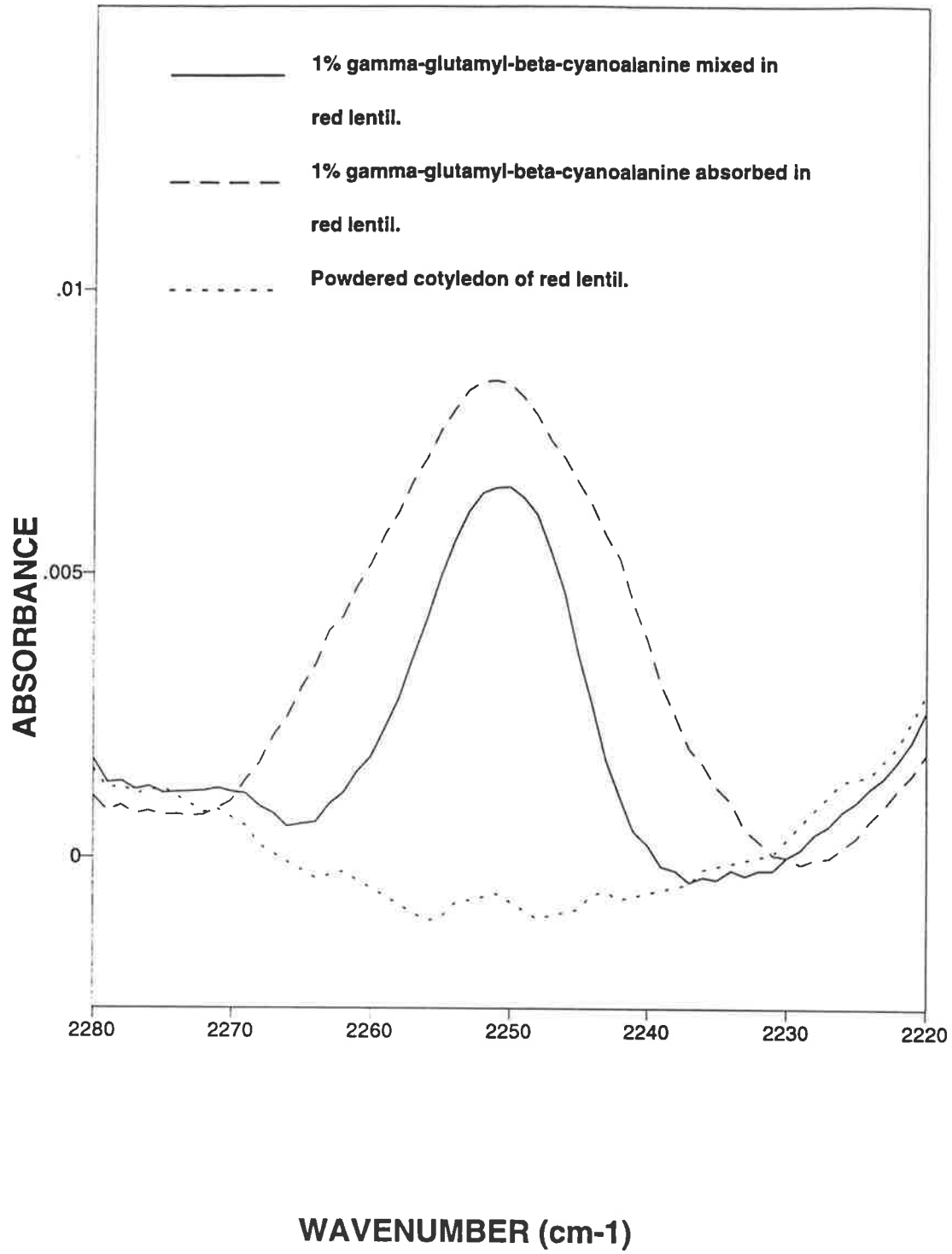


Figure 4.7 The diffuse reflectance infrared spectra of 1% γ-glutamyl-β-cyanoalanine mixed in red lentil (*L. culinaris*) overlayed with 1% γ-glutamyl-β-cyanoalanine absorbed in red lentil, and powdered cotyledon of red lentil acting as a nitrile negative control.

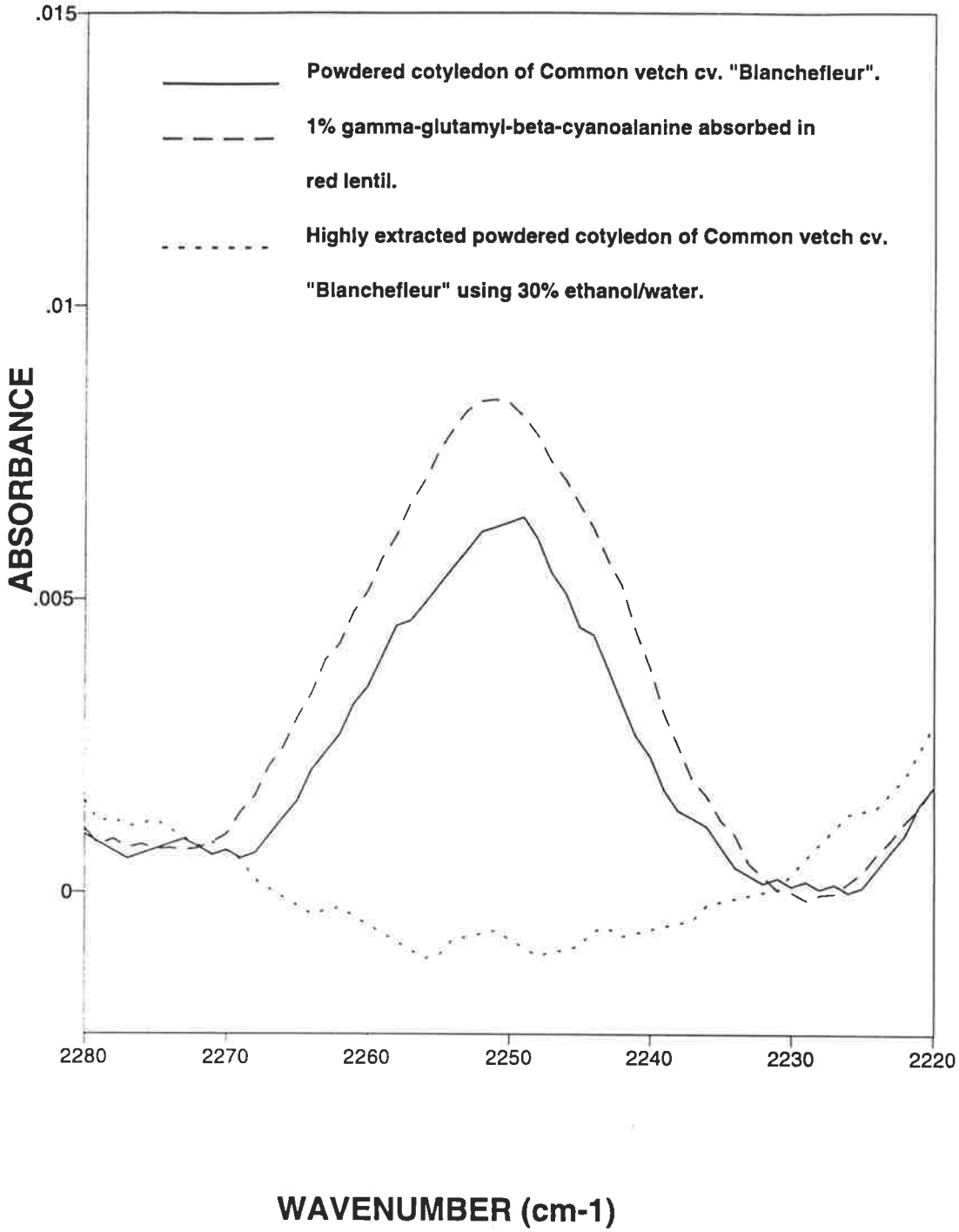


Figure 4.8 The diffuse reflectance infrared spectra of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*), overlayed with 1% γ-glutamyl-β-cyanoalanine absorbed in red lentil (*L. culinaris*) and highly extracted powdered cotyledon of common vetch cv. "Blanchefleur" using 30% ethanol/water acting as a nitrile negative control.

4.3.3 Quantitative diffuse reflectance infrared spectrometry in a strongly absorbing organic matrix

4.3.3.1 Mathematical function for quantitation by diffuse reflectance infrared spectrometry in a strongly absorbing organic matrix

Measurements were calculated from absorbance and Kubelka-Munk units collected by dispersive infrared (DRUIDS) and Fourier transform infrared (DRIFTS) spectrometers. The measurements were analysed as the percentage relative standard deviation (%RSD) for the peak height and integrated peak area of the nitrile stretching frequency at 2251cm^{-1} of γ-glutamyl-β-cyanoalanine obtained from the diffuse reflectance infrared spectra of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*) (Table 4.2a and Table 4.2b). Reflectance data calculated by absorbance units were substantially more precise than derived by Kubelka-Munk units. The precision of peak height measurements was better than the area measurements while the precision in absorbance peak height measurements was better when obtained by the Fourier transform infrared (DRIFTS) instrument rather than the dispersive infrared (DRUIDS) spectrometer.

Table 4.2a The percentage relative standard deviation (%RSD) for the peak height and integrated peak area of the nitrile stretching frequency at 2251cm^{-1} of γ-glutamyl-β-cyanoalanine obtained from the diffuse reflectance infrared spectra of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*). The measurements are Absorbance unit data collected by DRUIDS and DRIFT spectrometers.

Measurement	DRUIDS (%RSD).	DRIFT (%RSD).
Peak Height	4.43	1.91
Peak Area	11.04	2.14

Table 4.2b The percentage relative standard deviation (%RSD) for the peak height and integrated peak area of the nitrile stretching frequency at 2251cm^{-1} of γ-glutamyl-β-cyanoalanine obtained from the diffuse reflectance infrared spectra of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*). The measurements are Kubelka-Munk unit data collected by DRUIDS and DRIFT spectrometers.

Measurement	DRUIDS (%RSD).	DRIFT (%RSD).
Peak Height	10.09	5.26
Peak Area	14.18	7.99

4.3.3.2 Matrix effects on quantitation using diffuse reflectance infrared spectrometry

Figure 4.9 shows the linear regression lines of the mean absorbance height of the nitrile stretching frequency at 2251cm^{-1} for γ-glutamyl-β-cyanoalanine both mixed with and absorbed in a red lentil matrix, and employing either the large or small cup sampling procedures. The slope of the regression lines for the absorbance height (molar absorption coefficient) for mixed and absorbed samples are clearly different due to the effect of the environment of the nitrile constituent in the strongly absorbing organic matrix. There was no evidence to suggest that the molar absorption coefficient of the nitrile varied with respect to sample holder or collection conditions.

4.3.3.3 Quantitative analysis by DRUIDS of γ-glutamyl-β-cyanoalanine in a strongly absorbing organic matrix

Figure 4.10 shows the standard curve for quantitation of γ-glutamyl-β-cyanoalanine, with error bars, for the single seed analysis using the small cup procedure (sample size <5mg). Figure 4.11 shows the standard curve for the quantitation of γ-glutamyl-β-cyanoalanine, with error bars, suitable for the larger homogenised germplasm screening assay using the large cup procedure (sample size <70mg).

4.4 Discussion

To develop a simple and rapid chemical assay for a desired chemical trait suitable for application in a plant breeding program, issues such as ease of use, specificity, sample size and rate of throughput need to be addressed. Diffuse reflectance infrared spectrometry (Fuller and Griffiths 1978) has been used in this thesis to produce a rapid, efficient, quantitative, and non-extractive procedure for the analysis of γ-glutamyl-β-cyanoalanine in common vetch cotyledons. The quantitation of γ-glutamyl-β-cyanoalanine in powders derived from *V. sativa* utilizes the unique infrared characteristics of the nitrile functional group associated with γ-glutamyl-β-cyanoalanine resulting in a method with intrinsic molecular selectivity, high sensitivity, and rapid throughput while requiring minimal sample preparation. Recent advances in Raman spectroscopic instrumentation could facilitate a similar approach for the *in situ* quatitation of a diverse range of natural products.

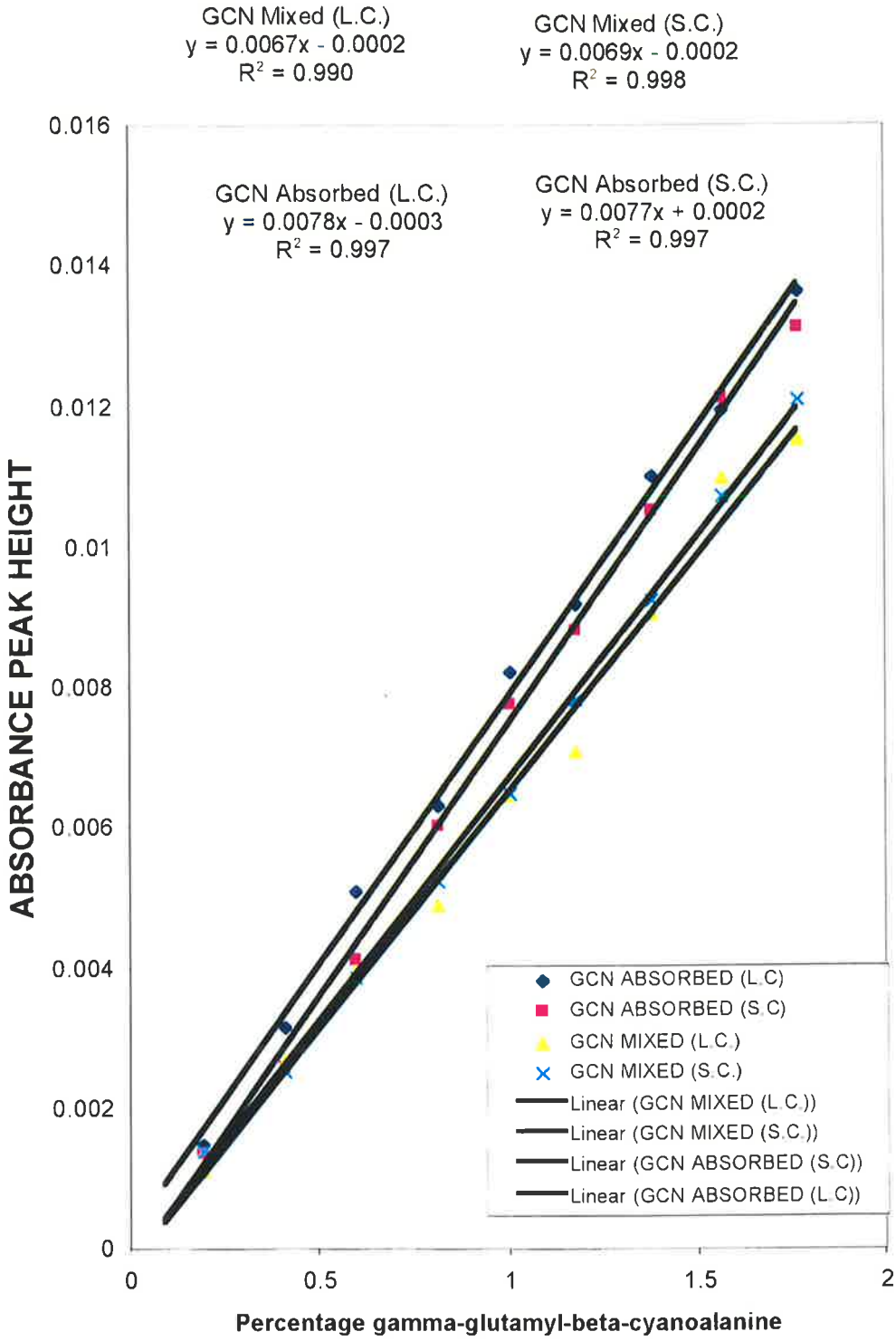


Figure 4.9 Graph of the linear regression lines of the mean absorbance height for the nitrile stretching frequency at 2251 cm^{-1} of γ-glutamyl-β-cyanoalanine (GCN) mixed and absorbed in red lentil (*L. culinaris*) for the large (L.C) and small cup (S.C) assays.

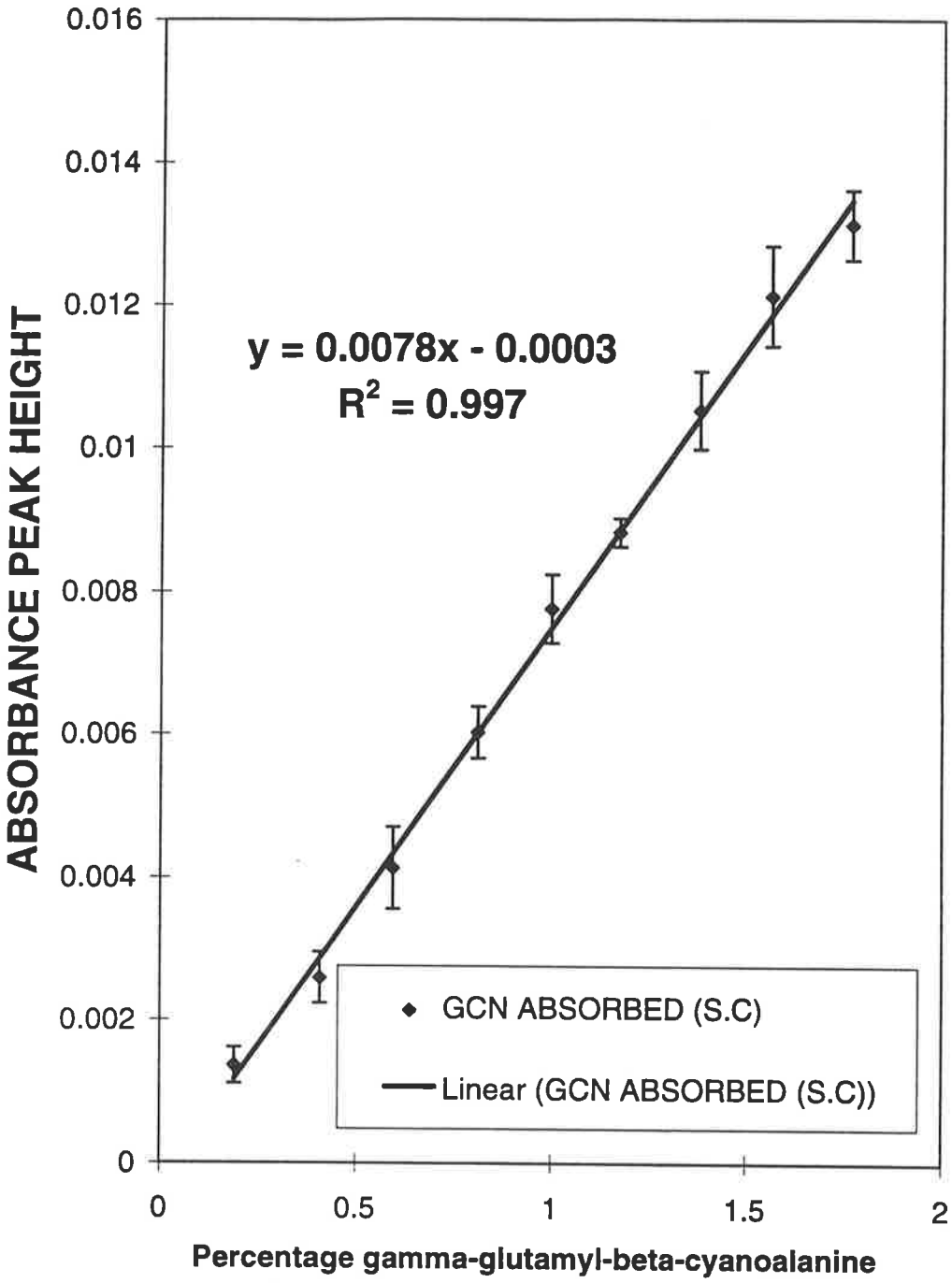


Figure 4.10 Graph of the linear regression of the mean absorbance height, with error bars, for the nitrile stretching frequency at 2251cm^{-1} of γ-glutamyl-β-cyanoalanine (GCN) absorbed in red lentil (*L. culinaris*) for the small cup (S.C) assay.

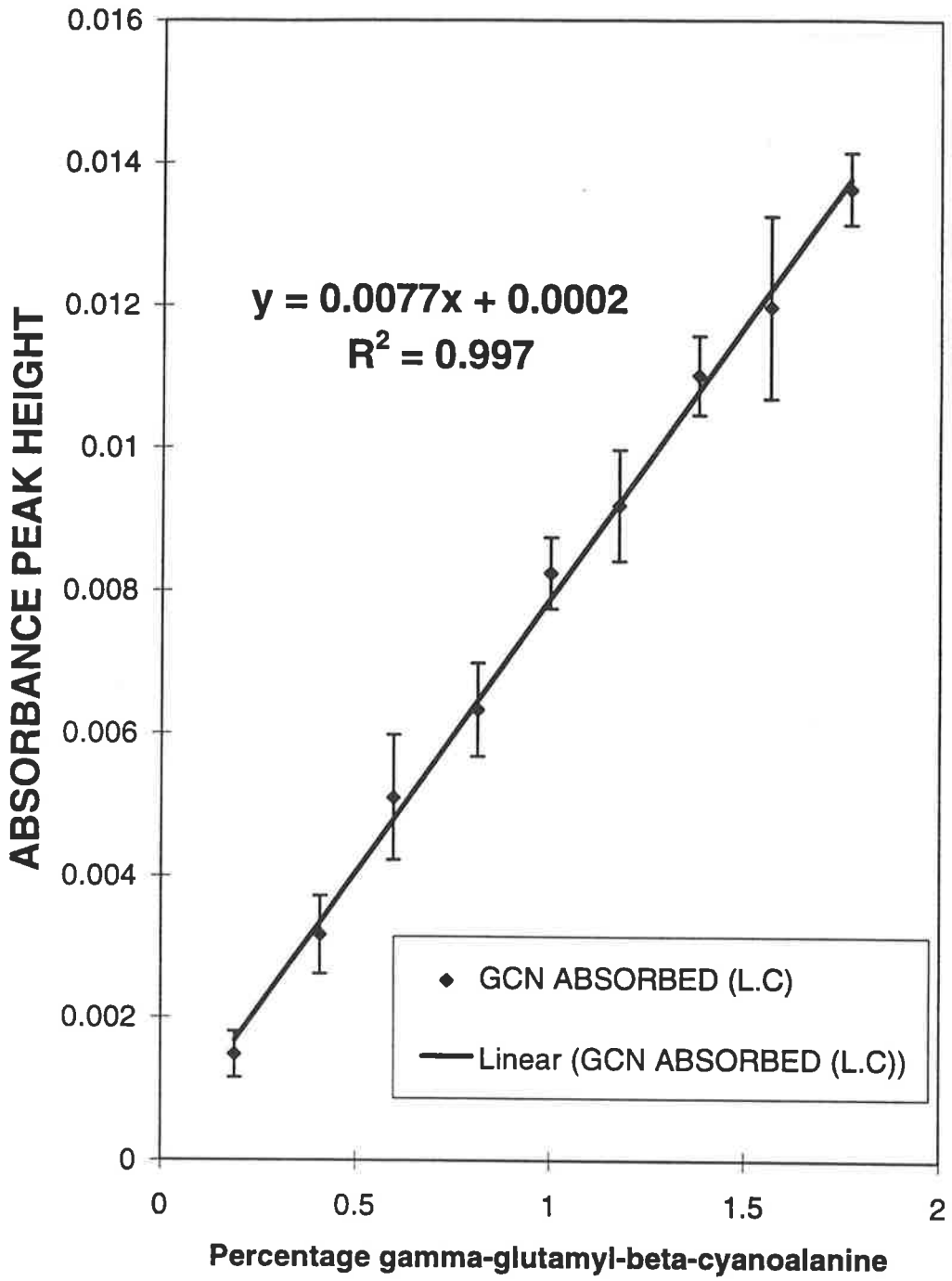


Figure 4.11 Graph of the linear regression of the mean absorbance height, with error bars, for the nitrile stretching frequency at 2251cm^{-1} of γ-glutamyl-β-cyanoalanine (GCN) absorbed in red lentil (*L. culinaris*) for the large cup (L.C) assay.

The diffuse reflectance infrared properties of nitrile-containing anti-nutritional factors associated with *Vicia* and *Lathyrus* species were initially measured in a non-absorbing KCl matrix environment. These data showed that the individual nitrile-containing compounds could be identified by the presence of the nitrile stretching frequencies with reference to the physical state of the compound (Figures 4.2, 4.3, 4.4, and 4.5). Subsequently, four compounds were identified with the potential to interfere with the quantitation by diffuse reflectance mid-infrared nitrile frequency analysis of γ-glutamyl-β-cyanoalanine in common vetch. These were the precursor of γ-glutamyl-β-cyanoalanine, β-cyanoalanine, the cyanogenic glycoside vicianine present in *V. sativa*, and the non-protein amino acids β-aminopropionitrile and its peptide β-(γ-glutamyl)-aminopropionitrile present in *L. odoratus*.

The nitrile of free β-cyanoalanine has a relatively high molal absorptivity in its amorphous state (Table 4.1a and Table 4.1b). Therefore the potential for free β-cyanoalanine to interfere with the quantitation of γ-glutamyl-β-cyanoalanine in common vetch is concentration dependent. Free β-cyanoalanine has been reported in low concentrations (0.15%) in the cotyledon of *V. sativa* (Ressler *et al.* 1969b) and monitored by an extractive chromatographic-ninhydrin procedure. The present study has been unable to provide confirmatory evidence for the presence of free cyanoalanine in unextracted vetch cotyledons from the band shape of the diffuse reflectance infrared spectra of homogenised vetch cotyledon (Figure 4.8). However, the low concentrations observed by Ressler *et al.* (1969b) were supported by results obtained from rapidly extracted (<2 hr) samples derivatised with 9-fluorenylmethyl chloroformate (FMOC), an amino acid derivatising reagent, and examined by micellar electrokinetic chromatography, described in chapter five, suggesting that free β-cyanoalanine may be an artifact of the isolation. With negligible amounts of free β-cyanoalanine present in the cotyledon of common vetch it was clear that free β-cyanoalanine was not a significant interfering factor in the quantitation of γ-glutamyl-β-cyanoalanine in common vetch or in the overall observed toxicity of the pulse due to the quantitative molar resposes observed by the cyanoalanine non-protein amino acids when added to a basel diet in the rat (Ressler *et al.* 1969a).

In the absence of vicianine, amygdalin, a close structural analogue of vicianine, was studied to identify infrared spectroscopic characteristics with the potential to interfere with the quantitation of γ-glutamyl-β-cyanoalanine in common vetch. The cyanogenic glycoside vicianine has been reported at concentration varying between 10-1000μM (Bell and Tirimanna 1965), with it being absent in many selections including *V. sativa* cv. "Blanchefleur". It became apparent that at these reported concentrations, the low molal absorptivity observed in cyanogenic glycosides (Table 4.1a and Table 4.1b) ensured that vicianine would be unlikely to be resolvable by diffuse reflectance

infrared and as such would not have potential to interfere in the quantitation of γ-glutamyl-β-cyanoalanine.

The structurally related β-aminopropionitrile non-protein amino acids, and in particular β-(γ-glutamyl)-aminopropionitrile (Dasler 1954; Schilling and Strong 1954; Schilling and Strong 1955), have the potential to interfere with the quantitative mid-infrared analysis of γ-glutamyl-β-cyanoalanine (Figure 4.12). However, both the β-aminopropionitrile and its γ-glutamyl peptide are readily separable from the β-cyanoalanine and its γ-glutamyl peptide by paper electrophoresis at pH 1.76, described in chapter three, and neither has been reported as present in *V. sativa.*

The interaction between the physical state of a compound and the matrix results in pronounced effects on the measurement of the peak maxima and the corresponding molecular absorption coefficient of the peak when measured by diffuse reflectance infrared spectrometry (Table 4.1a and Table 4.1b). This finding has significant implications for quantitative correlations drawn from *in situ* monitoring and quantitation in native matrices.

The *in situ* analysis of individual compounds in strongly absorbing matrices can be facilitated by the identification of a unique characteristic stretching frequency associated with the compound of interest and has been successfully applied to the quantitation of diazonaphthoquinones on thin-layer chromatographic (TLC) plates by DRIFTS (Beauchemin and Brown 1989). This approach has now been applied to the plant breeding objective of the *in situ* quantification of γ-glutamyl-β-cyanoalanine in the homogenised cotyledons of common vetch. The diffuse reflectance infrared spectra of homogenised cotyledon of common vetch cv. "Blanchefleur' and 1% γ-glutamyl-β-cyanoalanine absorbed in KCl show that the 2251cm^{-1} peak is common to both infrared spectra and can act as a unique identifying frequency for the presence of γ-glutamyl-β-cyanoalanine in a common vetch tissue sample (Figure 4.6). The 2251 cm^{-1} nitrile stretching frequency of γ-glutamyl-β-cyanoalanine in homogenised vetch seed tissue is particularly useful because it is measured in a region with the greatest stationary phase reflectivity in the mid-infrared and consequently provides a high signal-to-noise ratio (Figure 4.6).

The diffuse reflectance infrared spectrum of powdered, exhaustively extracted (30% ethanol/water) cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*) shows that γ-glutamyl-β-cyanoalanine is an extractable infrared spectral constituent of common vetch (Figure 4.8). Red lentil (*L. culinaris*) shows an infrared spectrum similar in stationary phase reflectivity and infrared spectral characteristics (Figure 4.7) to that found in exhaustively extracted common vetch (Figure 4.8) and consequently provides a readily available matrix for producing a standard curve.

The diffuse reflectance infrared spectrum of 1% γ-glutamyl-β-cyanoalanine mixed and absorbed in red lentil shows that there are qualitative differences in the shape and height of the

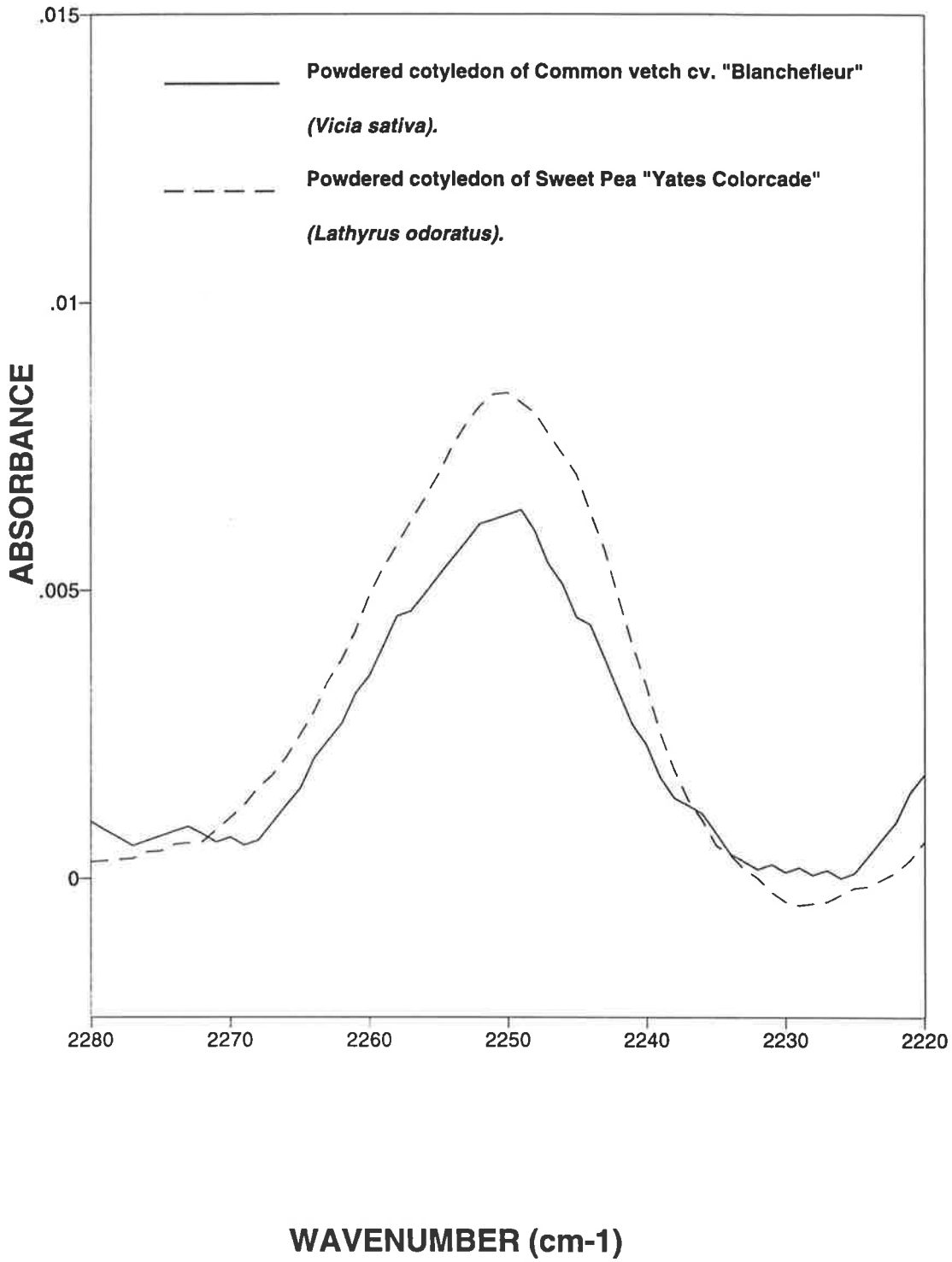


Figure 4.12 The diffuse reflectance infrared spectra of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*), overlayed with the powdered cotyledon of sweet pea "Yates Colorcade" (*L. odoratus*).

nitrile stretching frequency between the treatments (Figure 4.7). The diffuse reflectance infrared spectrum of 1% γ-glutamyl-β-cyanoalanine absorbed in red lentil overlayed on the spectrum of homogenised common vetch cotyledons demonstrates that there are close similarities in the overall shape of both nitrile stretching frequencies (Figure 4.8).

Alternative mathematical treatments for the determination of γ-glutamyl-β-cyanoalanine in a strongly absorbing environment from reflectance data had to be assessed. Measurements were calculated using both absorbance and Kubelka-Munk units and were collected by both dispersive infrared (DRUIDS) and Fourier transform infrared (DRIFTS) spectrometers. These measurements were analysed as the percentage relative standard deviation (%RSD) for the peak height and integrated peak area of the nitrile stretching frequency at 2251cm^{-1} obtained from the diffuse reflectance infrared spectra of powdered cotyledon of common vetch cv. "Blanchefleur" (*V. sativa*) (Table 4.2a and Table 4.2b). These data showed that infrared reflectance data calculated as absorbance units were substantially more precise than with the Kubelka-Munk units, a finding also noted by Beauchemin and Brown (1989).

The precision of peak height measurements was better than the area measurements (Table 4.2a and Table 4.2b). Peak area measurements were influenced by contributions from the tail of the CO_2 peak in the region of 2270cm^{-1} which increases between regenerations of the circulatory airdryer. In addition, the precision in absorbance peak height measurements was better when obtained by the Fourier transform infrared (DRIFTS) instrument rather than the older dispersive infrared (DRUIDS) spectrometer (Table 4.2a and Table 4.2b). The major difference in the two diffuse reflectance infrared methodologies was attributed to the intensity of the nitrile absorption frequency, resulting in a higher signal-to-noise ratio and hence greater precision for the Fourier transform instrument.

Having defined absorbance peak height as the most appropriate mathematical treatment for the determination of γ-glutamyl-β-cyanoalanine in a strongly absorbing matrix enviroment, the effect of the physical environment of γ-glutamyl-β-cyanoalanine was studied to determine the effect on the molar absorptivity of the nitrile stretching frequency over a range of concentrations. γ-Glutamyl-β-cyanoalanine was either mixed or absorbed in a red lentil matrix at nine concentrations between 0.2 and 1.8% and analysed using both the large and small sample cup procedures, to determine the dependence of sample holder and collection conditions on the molar absorptivity of the nitrile stretching frequency. Graphs of the linear regression lines of the mean absorbance peak height of the nitrile stretching frequency at 2251cm^{-1} showed that the slope (molar absorption coefficient) clearly varied due to the effect of the environment of the constituent in the strongly

absorbing matrix (Figure 4.9). However, there was no evidence to suggest that the molal absorption coefficient of the nitrile varied with respect to sample holder or sample collection conditions.

These data provide the first statistically validated evidence quantifying matrix effects on diffuse reflectance infrared quantitation of a compound in a strongly absorbing environment. Hence, the importance of selecting appropriate model matrices for quantitative mid-infrared diffuse reflectance infrared analysis of specific organic molecules in biological tissue matrices was clearly established.

Examination of the Australian *V. sativa* germplasm collection, described in chapter seven, established that many accessions consisted of mixtures species. A nondestructive single seed assay was developed to overcome the problems associated with seed mixtures by permitting the analysis of individual seeds. Quantification of γ-glutamyl-β-cyanoalanine in a single seed uses the small cup procedure and requires <5mg of homogenised common vetch cotyledon. The homogenised common vetch is obtained by carefully drilling a small hole in the cotyledon of the vetch seed, which does not impair the viability of the seed, described in chapter six.

With the small surface area of the small cup (Figure 4.1), precision is dependent on signal-to-noise maximisation through improved particle homogeneity (sieve <0.5mm) and control of the noise-filter function (Filter Noise 64). This method has become a highly precise diffuse reflectance infrared quantitation for γ-glutamyl-β-cyanoalanine between 0.2 and 1.8% (Figure 4.10) in common vetch. Below 0.2% γ-glutamyl-β-cyanoalanine signal-to-noise considerations make quantitation impractical. The slope of the equation yields a negative y-axis intercept due to the concave nature of the diffuse reflectance infrared spectra of both red lentil and exhaustively extracted common vetch when a straight line is drawn between 2272-2232cm^{-1} (Figure 4.7 and Figure 4.8). A disadvantage of this approach is its relatively long (5 minute) data acquisition time.

The application of diffuse reflectance infrared spectrometry to a plant breeding program was aimed at identifying plant material, which expresses a low or zero γ-glutamyl-β-cyanoalanine concentration in the cotyledon of common vetch. This program required a high sample throughput, and access to well maintained and documented collections of *V. sativa*. Australia's strict quarantine laws make it expensive to import all available accessions from the large and well maintained *V. sativa* collections held in various international germplasm collections. However, the quarantine regulations permit the importation of non-viable milled seed without cost, for screening purposes, if handled by a registered quarantine facility. As a result, close ties have been forged with the

international germplasm collections which have supplied gram quantities of milled non-viable *V. sativa* samples to be screened for γ-glutamyl-β-cyanoalanine content, described in chapter seven.

The size of the surface area and hence total reflectivity is not a controlling factor in the precision of the large cup procedure (Figure 4.1). The purpose of the large cup procedure was the rapid screening of large collections of *V. sativa* for γ-glutamyl-β-cyanoalanine in the powder of homogenised common vetch cotyledon. In this situation assay time was the main limiting factor. The milled common vetch samples were sieved (<100μm) to increase both the total reflectivity and the signal-to-noise ratio, while acquisition time was reduced to <2minutes per sample using a quicker and consequently less precise averaging of the background spectroscopic noise (Noise-filter 16). The slope of the equation was the same as for the small cup assay, however it yielded a positive y-axis intercept and greater standard deviation about the mean due to the less precise nature of the signal noise suppression system (Figure 4.11). Each accession was analysed in duplicate and for accessions where the standard deviation was in excess of 0.1%, the samples were reanalysed, described in chapter seven.

4.5 Conclusion

This chapter records the successful development of the rapid *in situ* diffuse reflectance infrared assay for γ-glutamyl-β-cyanoalanine in powdered single seed and bulk samples of common vetch (*V. sativa*). The diffuse reflectance infrared properties of the nitrile-containing anti-nutritional factors associated with *Vicia* and *Lathyrus* species have been studied and discussed. Important findings on the effect of the physical state of a compound in association with a matrix and the pronounced effects of these interactions on the molecular absorption coefficient and peak shape were highlighted. This led to the first statistically validated evidence demonstrating the effect of the matrix on the quantitation of a compound in a strongly absorbing environment. The success of the screening procedure relied heavily on preparing a suitable model matrix for the vetch cotyledon. The screening procedure has now established a sound foundation for the breeding of the world's first low, or perhaps zero, cyanoalanine containing *V. sativa* plant.

Chapter Five

Separation by S(u)DS

5.0 Introduction

Quantification of the cyanoalanine anti-nutritional factors at low levels (<0.2%) in single seeds or in large germplasm samples by diffuse reflectance using infrared dispersive spectrometry (DRUIDS) is ineffective due to signal to noise considerations. These chemical species can be qualitatively monitored in aqueous extracts of plant tissues by paper electrophoresis (Bell and Tirimanna 1965; Enneking 1995). However, the applicability of paper electrophoresis for routine use in a plant breeding program is limited due to the non-quantitative nature of the analysis.

The use of narrow bore capillaries has facilitated the development of capillary electrophoresis (Mikkers *et al.* 1979; Jorgenson and Lukacs 1981) into a highly efficient separation method for qualitative purposes. However, the application of capillary electrophoresis to routine analysis of samples other than reference compounds has been slow due to sensitivity and repeatibility problems (Lee and Yeung 1991; Leube and Roeckel 1994; Schneede and Ueland 1995).

Capillary electrophoresis has been used to efficiently resolve charged as well as neutral species during a separation. This has been achieved by micellar electrokinetic chromatography (MEKC) which employs high electric field strength and fused silica capillaries filled with surfactant rich electrolyte solutions for the separation (Terabe *et al.* 1984; Terabe *et al.* 1985). By incorporating charged micelles in the capillary system, neutral species in addition to charged species can be separated on their differential distribution between electrokinetically migrating mobile and micellar phases (Holland and Sepaniak 1994). The fact that micellar electrokinetic chromatography is regarded as a chromatographic technique is primarily a result of the partitioning mechanism. However, the exact physico-chemical nature of the solute-micellar interaction is unknown (Yang and Khaledi 1995).

Micellar electrokinetic chromatography is characterised by an elution range or retention window that extends from the migration time (t_0) of an unretained solute, to the migration time (t_{mc}) of another solute which is completely solubilised by the micelle (Tsai *et al.* 1993). The magnitude of the retention window largely influences the peak capacity, resolution, and analysis time. With

surfactants that are charged in their natural state the magnitude of the retention window is usually predetermined by the characteristics of the surfactant and cannot be varied systematically (Smith *et al.* 1994). The retention time window of SDS has been shown to be largely unaffected by pH (Nichi *et al.* 1989).

It is common practice in micellar electrokinetic chromatography to work under conditions where the electro-osmotic flow is greater than the electrophoretic velocity of the micelles in the opposite direction (Terabe *et al.* 1985). Therefore, the micelles are not infinitely retained within the capillary but elute in a finite period of time (the retention window). It is obvious that the possibility of two solutes being resolved from one another is controlled not only by the column efficiency, separation selectivity and the degree of retention by the micellar phase but also by the relative amount of time these solutes experience in the separation mechanism (Ahuja *et al.* 1995).

Pre-column derivatisation of amino acids with sensitive fluorescent tags can improve the detection limits and quantitative precision of the analyte in capillary electrophoresis. Derivatisation methods for amino acids have been well developed for high pressure liquid chromatography (HPLC) with many commercial reagents available. These derivatisation reagents are hydrophobic fluorescent compounds which react with the primary and secondary amine functional groups in the amino acids to form fluorescent conjugates. This substantially changes the charge, solubility and ultra-violet absorption profiles of the original moiety and are ideally suited for quantitative micellar electrokinetic chromatographic separation.

The work in this chapter sets out to study the various attributes of quantitative analysis by micellar electrokinetic chromatography while addressing sensitivity and repeatibility issues. Long-term stability and reproducibility required for routine quantitative analysis of plant samples as distinct from reference standards was obtained. The methods developed emphasise the use of appropriate internal standards and data manipulation to overcome inherent run to run variation in retention times. With the aid of this approach a quantitative micellar electrokinetic chromatographic assay has been developed for the 9-fluorenylmethyl chloroformate (FMOC) derivatisable amino acids and anti-nutritional factors present in *Vicia* and *Lathyrus* species.

5.1 Micellar Electrokinetic Chromatography (MEKC)

5.1.1 Apparatus

Capillary electrophoresis was performed using a P/ACE 2100 system (Beckman Instruments Incorporated, Fullerton, CA) fitted with a thermostatically controlled liquid cooled

capillary cartridge using the Beckman P/ACE system 2000 capillary liquid coolant. The capillary cartridge contained a 50μm internal diameter untreated fused silica capillary tubing of 57cm total length, 50cm effective length, connected to a tunable ultraviolet diode array detector (UV-DAD). The P/ACE 2100 was linked to an IBM PS/2 clone (Dell 466DX2, 66MHz, 8Mb RAM, Dell Computer Company, USA) and controlled through the Beckman System GOLD 7.0 software system (Beckman Instruments Incorporated, Fullerton, CA).

5.1.2 Buffers and derivatisation of samples

The micellar surfactant separation buffer contained 20mM sodium tetraborate, adjusted to pH 9.52 with 1M NaOH. The derivatising buffer was identical to the separation buffer except for the addition of 2-deoxycytidine ($4.5x10^{-4}$ M) (Sigma Chemical Company) and the non-protein amino acid norvaline ($1.5x10^{-4}$ M) (Fluka Chemika Biochemika). The optimised separation buffer was 20mM sodium tetraborate with 19.5mM sodium dodecylsulphate (SDS). Samples were derivatised by adding 5μL of the sample solution to 50μL of the derivatising buffer then adding 5μL 1% 9-fluorenylmethyl chloroformate (FMOC) in acetonitrile (Fluka Chemika Biochemika), gently shaken, and left for one minute at room temperature.

5.1.3 Data collection

Detection was accomplished by an on-column ultraviolet diode array detector (UV-DAD) set to monitor absorbance at 272±10nm. Separations by micellar electrokinetic chromatography were performed with the following conditions: T= 30°C, V=23.7 kV, polarity was positive (Albin *et al.* 1991). The column was pretreated with 0.1M NaOH for 10 minutes and then equilibrated for 3 minutes with the separation buffer. Samples were loaded onto the column using a 3 second pressure injection. The run time was 20 minutes.

5.1.4 Data manipulation

The Beckman GOLD format data files were converted to the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) format using Beckman Gold Chromatogram (BKMNGCHR) and Beckman Gold Diode Array Spectra (BKMNGOLD) file converters associated with the GRAMS/386 II package. The files were baseline-corrected and the relative retention times were realigned by the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) data manipulation package using three internal standards, 2-deoxycytidine (3.80minutes), FMOC reagent peak R1 (12.00minutes) and FMOC reagent peak R2 (15.70minutes) using the Baseline and X-Shift

functions. Chromatographic events were selected and stored using the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) Peak Pick and Save Peak Table functions. The chromatographic peak tables were opened in Excel 5.0 (Microsoft Corporation, Redmond, WA).

5.2 Experimental

5.2.1 Qualitative analysis of the effect of surfactant concentration on the elution order of standard solutions

All micellar electrokinetic chromatograms were realigned by the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) data manipulation package using three internal standards, 2-deoxycytidine (3.80minutes), FMOC reagent peak R1 (12.00minutes) and FMOC reagent peak R2 (15.70minutes). Chromatographic peak table data were exported to Excel 5.0 (Microsoft Corporation, Redmond, WA). The identity and elution order of each compound was determined through sample spiking and component elimination at five surfactant concentrations (30, 25, 20, 18, and 15mM SDS). Relative retention time data was determined from the mean sample retention time in minutes of the constituents in the standard solution at the five surfactant concentrations.

The standard solutions were made in $18M\Omega cm^{-1}$ Milli-Q water (Millipore, Bedford, MA) containing sodium azide (0.1%). The elution order of amino acids in a solution was determined from solutions containing at $2.50x10^{-4}$ M, L-Ala, L-Asp, L-Asn, L-Arg, L-Cys, L-Cyd, L-Gln, L-Glu, L-Gly, L-His, L-Hyp, L-Iso, L-Leu, L-Lys, L-Met, L-Phe, L-Pro, L-Ser, L-Thr, L-Try, L-Tyr, L-Val, L-Homoarginine.HCl (Sigma Chemical Company) and at $1.25x10^{-4}$ M, β-cyanoalanine (BCN) (Sigma Chemical Company), γ-glutamyl-β-cyanoalanine NH_3 (GCN), γ-glutamyl-S-ethenyl-cysteine (GEC), an authentic sample of canavanine 2HCl, kindly provided by Dr. Dirk Enneking, β-N-oxalylamino-L-alanine (BOAA), β-aminopropionitrile monofumarate (BAPN) and 2-4-diamino-n-butyric acid 2HCl (DABA) (Sigma Chemical Company).

5.2.2 Quantification by micellar electrokinetic chromatography

5.2.2.1 Calibration standards

Quantification of amino acids used a commercial standard amino acid solution (Hewlett Packard) containing 17 amino acids. Anti-nutritional factors of *Vicia* were quantified from a standard solution containing $2.50x10^{-3}$ M, authentic samples of vicine and convicine, kindly

provided by Prof. Ron Marquardt (University of Manitoba, Winnipeg, Manitoba, Canada), β-cyanoalanine (BCN) (Sigma Chemical Company), γ-glutamyl-β-cyanoalanine.NH_3 (GCN), γ-glutamyl-S-ethenyl-cysteine (GEC) and an authentic sample of canavanine 2HCl, kindly provided by Dr. Dirk Enneking. Anti-nutritional factors of *Lathyrus* were quantified from a standard solution containing $2.50x10^{-3}$ M, commercial samples of β-N-oxalylamino-L-alanine (BOAA), β-aminopropionitrile monofumarate (BAPN) and 2-4-diamino-n-butyric acid 2HCl (DABA) (Sigma Chemical Company). All standard solutions were made in 18mΩ Milli-Q water (Millipore, Bedford, MA) containing (0.1%) sodium azide.

5.2.2.2 Quantitative retention time repeatability of micellar electrokinetic chromatographic separations using standard solutions

All micellar electrokinetic chromatograms were realigned by the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) data manipulation package using three internal standards, 2-deoxycytidine (3.80minutes), FMOC reagent peak R1 (12.00minutes) and FMOC reagent peak R2 (15.70minutes). Chromatographic peak table data were exported to Excel 5.0 (Microsoft Corporation, Redmond, WA). Mean sample relative retention time, percentage relative standard deviation and variance in retention time were derived from analysis of variance (ANOVA) calculated in Excel 5.0 using relative retention time for each constituent.

5.2.2.3 Quantitation of constituent concentration by micellar electrokinetic chromatographic separations using standard solutions

All micellar electrokinetic chromatograms were realigned by the GRAMS/386 II (Galactic Industries Corporation, Salem, NH) data manipulation package using three internal standards, 2-deoxycytidine (3.80minutes), FMOC reagent peak R1 (12.00minutes) and FMOC reagent peak R2 (15.70minutes). Chromatographic data used in the quantification of amino acids were collected on seven consecutive days from calibrated standards at six concentrations (1250, 625, 312.5, 156.25, 78.125, and 39 pmol/μL). Chromatographic data used in the quantification of the *Vicia* and *Lathyrus* anti-nutritional factors were collected on seven consecutive days at seven concentrations (2500, 1250, 625, 312.5, 156.25, 78.125, and 39 pmol/μL).

Chromatographic peak table data were exported to Excel 5.0 (Microsoft Corporation, Redmond, WA). Linear regression lines for the standard curves used in the quantification of FMOC derivatised calibration standards were derived from the mean absorbance peak height ratio of a constituent relative to the absorbance peak height of FMOC derivatised norvaline at the measured concentrations. The linear regression lines for the standard curves used in the quantification of vicine and convicine constituents in the calibration standards were derived from the mean absorbance peak area ratio relative to the peak area of 2-deoxycytidine. The regression coefficient r^2, which described the error associated with each standard curve, was derived from the plots of the linear regression lines of the standard curves in Microsoft Excel 5.0.

5.2.4 Identification of anti-nutritional factors in aqueous extracts of *V. sativa* and *V. narbonensis* by MEKC relative retention time data

5mg of homogenised common vetch (*V. sativa*) and narbon bean (*V. narbonensis*) cotyledon was extracted using 50µL 30% ethanol/water at room temperature, vigorously shaken for 2 hours. Samples were spun in an Eppendorf centrifuge at 7000rpm for 10 minutes and 5µL of supernatant was added to 50µL derivatising buffer and derivatised with 1% FMOC in acetonitrile. Micellar electrokinetic chromatographic data were collected and manipulated as described above.

5.3 Results

5.3.1 Internal standards

Figure 5.1 shows the micellar electrokinetic chromatogram of the derivatising buffer after the addition of 5µL 1% FMOC (9-fluorenylmethyl chloroformate) in acetonitrile. The derivatizing buffer was identical to the running buffer except for addition of the non-protein amino acid norvaline ($1.5x10^{-4}$ M) and 2-deoxycytidine ($4.5x10^{-4}$ M). These data show the realignment peaks 2-deoxycytidine, FMOC reagent peak R1, FMOC reagent peak R2 and the FMOC derivatising internal standard norvaline.

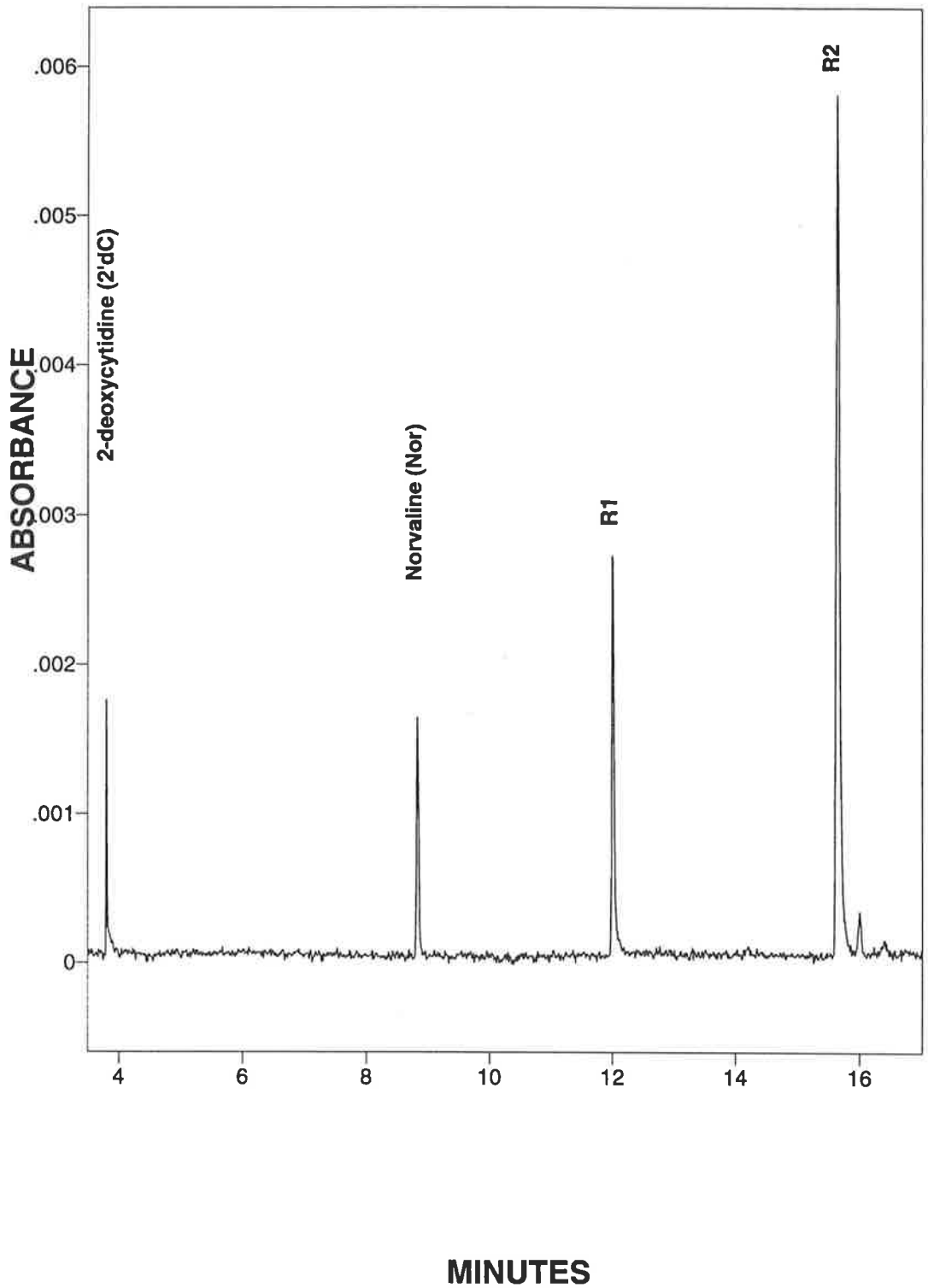


Figure 5.1 The separation by micellar electrokinetic chromatography of a reference sample containing the internal standards, 2-deoxycytidine (2'dC), norvaline (Nor) and the 9-fluorenylmethyl chloroformate (FMOC) reagent peaks R1 and R2, used in the realignment of run-to run variation between separations.

5.3.2 Effect of the micellar surfactant concentration on elution order of constituents in a standard solution

Figure 5.2 shows the separation by micellar electrokinetic chromatography of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids between 5.00 minutes and 11.00 minutes using a 30mM SDS separation buffer. Figure 5.3 shows the separation by micellar electrokinetic chromatography of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids between 5.00 minutes and 11.00 minutes using an 18mM SDS separation buffer. These data show that relative migration times, elution order and co-elution of FMOC derivatised constituents in a standard solution are a function of surfactant concentration.

Figure 5.4 shows a graph of the variation in the mean relative retention times of dicarboxylate constituents of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids with respect to concentration of the micellar surfactant SDS in the separation by micellar electrokinetic chromatography. These data show that surfactant concentration had a pronounced effect on relative retention times of the dicarboxylate species and that the more anionic components were retained for shorter times in the micelle with increasing concentration of SDS. This is consistent with an anion exclusion mechanism.

Figure 5.5 shows a graph of the variation in the mean relative retention times of some monocarboxylate constituents of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids with respect to concentration of the micellar surfactant SDS in the separations by micellar electrokinetic chromatography. These data show that the effect of surfactant concentration on the relative retention times of the FMOC derivatised monocarboxylate constituents, which have a smaller net anionic charge, was less pronounced. However, there was a trend toward increased retention of the monocarboxylate derivatives in the micelle in the range 25-30mM SDS.

Figure 5.6 shows a graph of the variation in the mean relative retention times for some monocarboxylate and dicarboxylate constituents of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids with respect to concentration of SDS in the separations by micellar electrokinetic chromatography. These data show the micellar surfactant concentrations where co-elution of FMOC derivatives occurs. The optimised separation buffer was 20mM sodium tetraborate, 19.5mM sodium dodecylsulphate (SDS) buffer, adjusted to pH 9.52 with 1M NaOH.

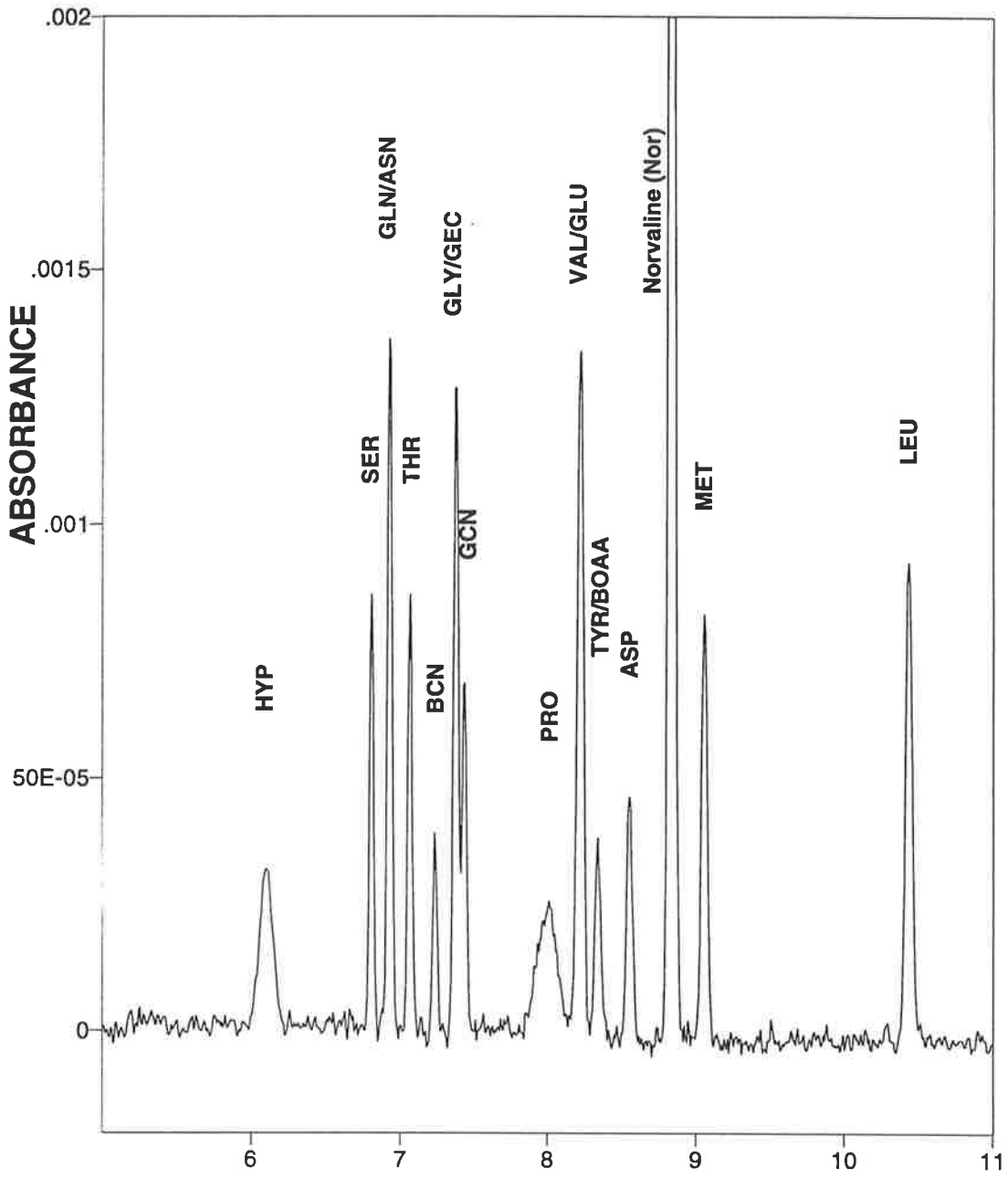


Figure 5.2 The separation by micellar electrokinetic chromatography of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids, between 5.00 minutes and 11.00 minutes using a 30mM SDS running buffer.

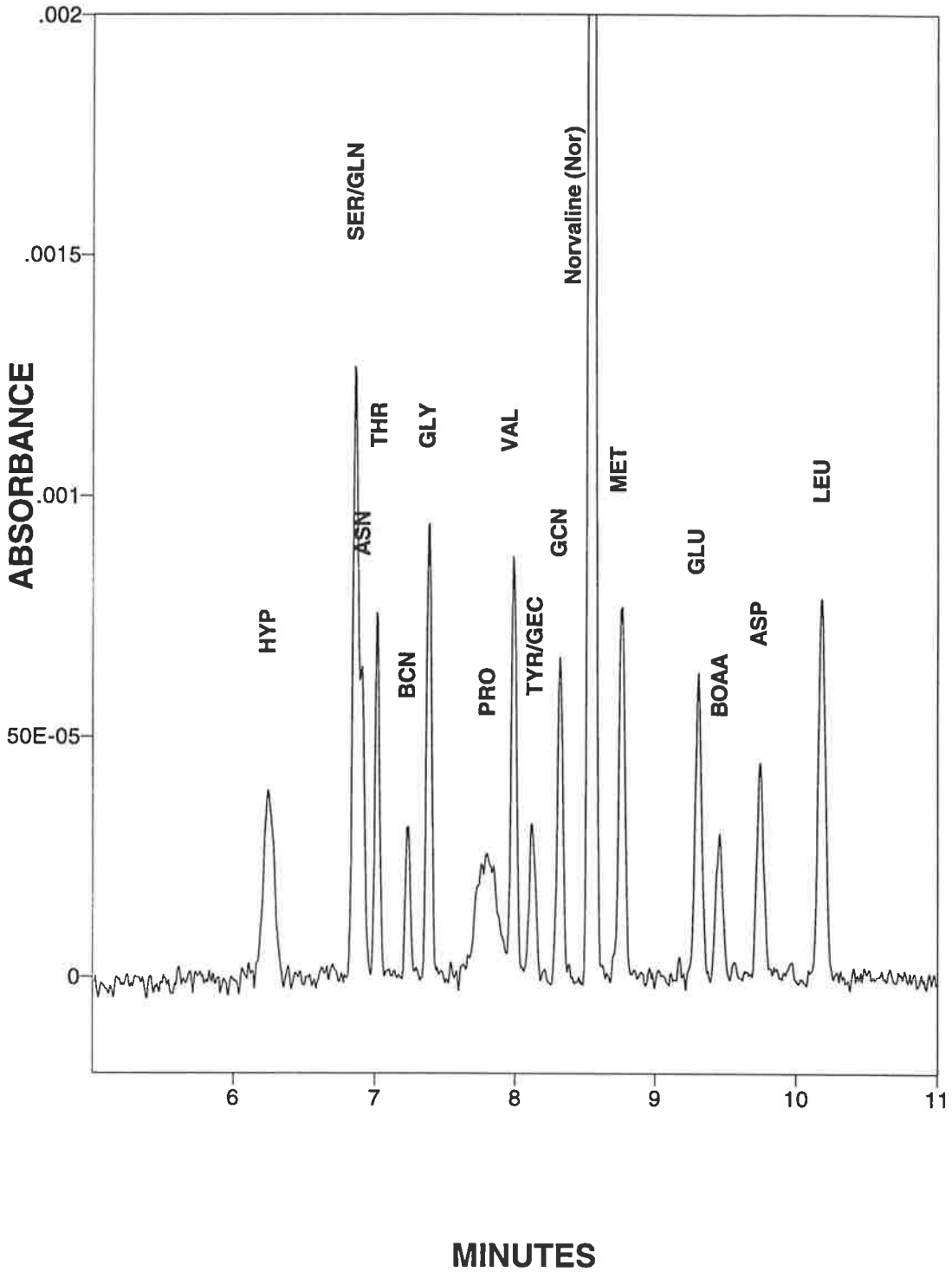


Figure 5.3 The separation by micellar electrokinetic chromatography of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids, between 5.00 minutes and 11.00 minutes using a 18mM SDS running buffer.

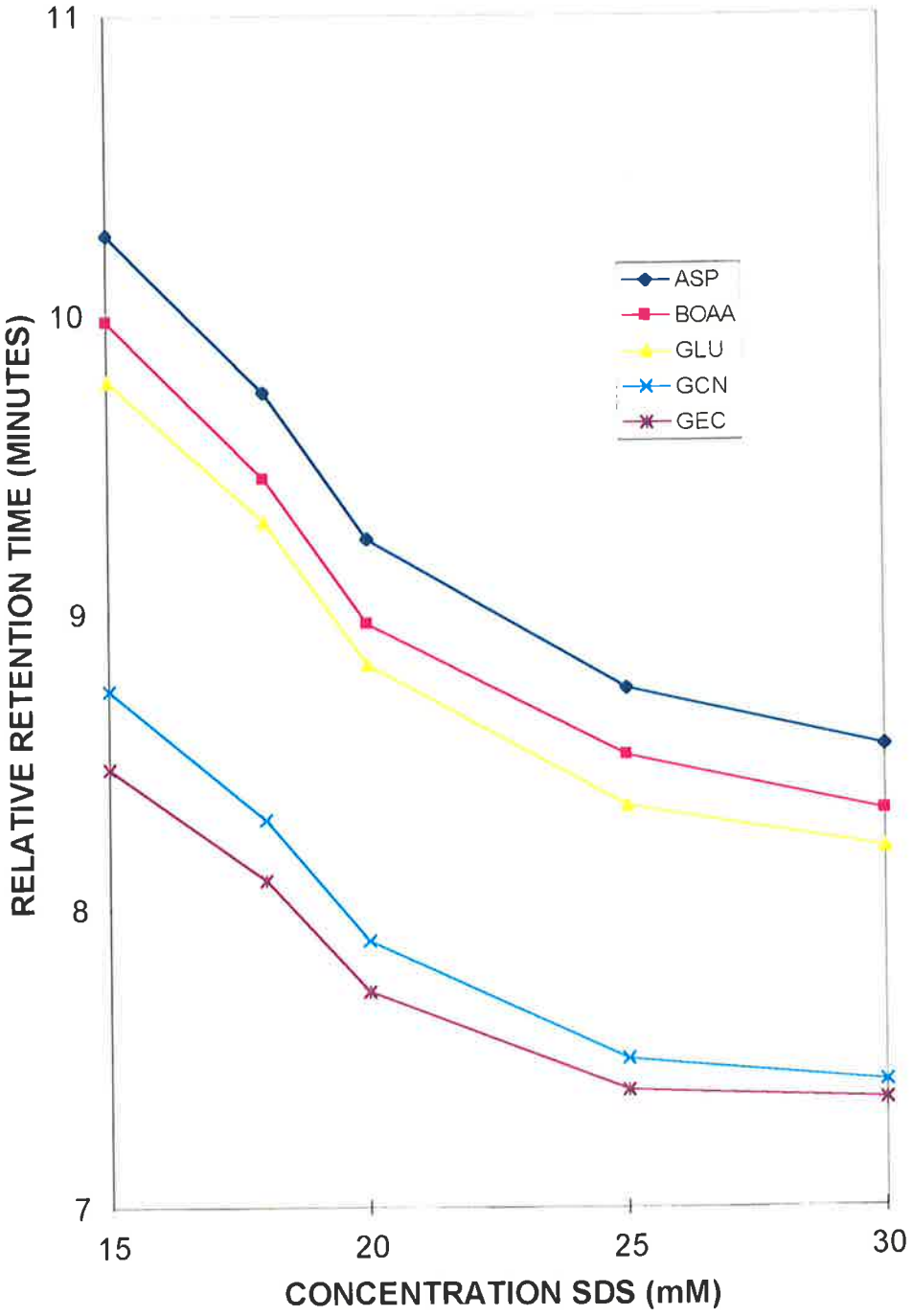


Figure 5.4 Graph of the variation in the mean relative retention times for dicarboxylate components of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids with respect to variation in the concentration of the micellar surfactant SDS in separations by micellar electrokinetic chromatography.

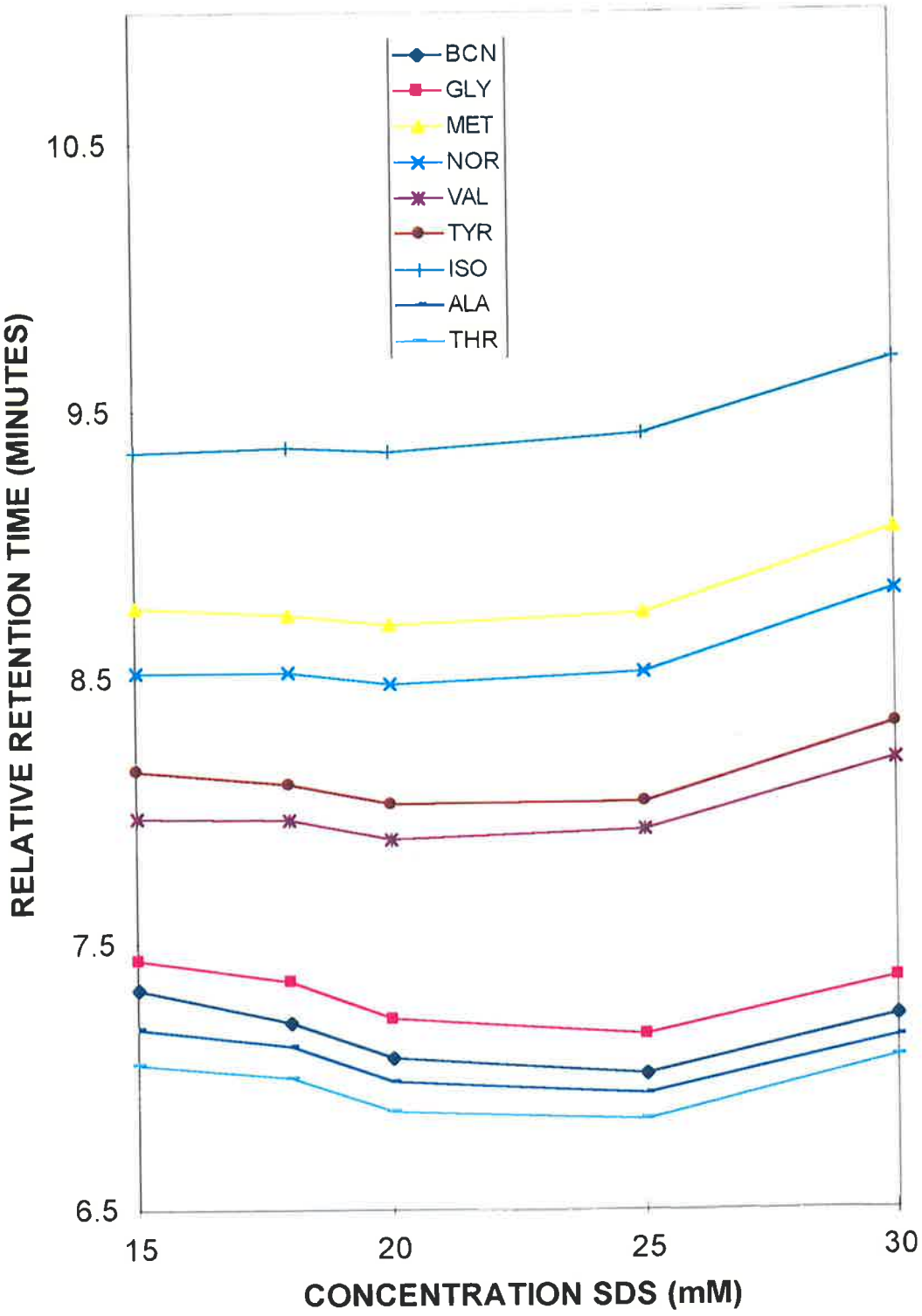


Figure 5.5 Graph of the variation in the relative mean retention times for monocarboxylate components of a standard solution of amino acids and *Vicia* and *Lathyrus* non-protein amino acids with respect to variation in the concentration of the micellar surfactant SDS in separations by micellar electrokinetic chromatography.

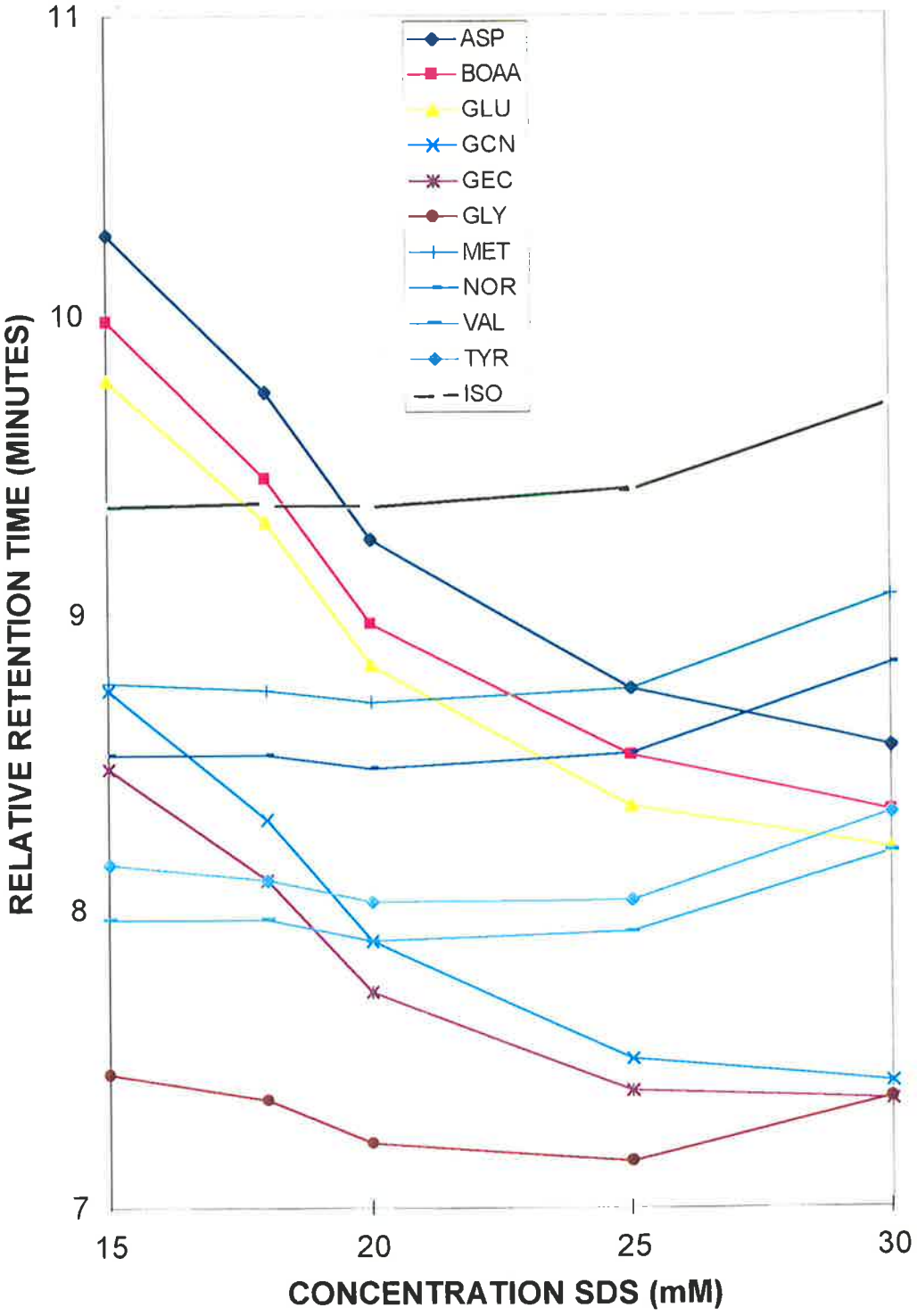


Figure 5.6 Graph of the variation in the mean relative retention times for the monocarboxylate and dicarboxylate components of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids with respect to variation in the concentration of the micellar surfactant SDS in separations by micellar electrokinetic chromatography.

5.3.3 Quantification of calibration standards

5.3.3.1 Quantitative retention time repeatability of micellar electrokinetic chromatographic separations using standard solutions

Table 5.1 shows data for the repeatability of separations by micellar electrokinetic chromatography of relative retention times in terms of relative standard deviation about the mean retention times for constituents of a standard solution containing amino acids as well as *Vicia* and *Lathyrus* non-protein amino acids, using a 19.5mM SDS separation buffer. The low percentage relative standard deviation (<1%) is a measure of the high precision obtained in the retention times of the constituents of the standard solution using internal standards to fix retention time indices.

5.3.3.2 Quantitation of constituent concentration by micellar electrokinetic chromatographic separations using standard solutions

Table 5.2 summarises data for the linear regression lines for the standard curves used in the quantitation of individual constituents of a standard solution containing amino acids and *Vicia* and *Lathyrus* non-protein amino acids, using a 19.5mM SDS separation buffer. The limits of detection for each constituent is shown (Table 5.2). The regression coefficient r^2 described the error associated with each standard curve. These data show that absorbance peak height ratio of a FMOC derivatised constituent relative to the absorbance peak height of the non-protein amino acid norvaline provided satisfactory quantitation of the FMOC derivatised amino acids and non-protein amino acids examined in this study. These data also show that the absorbance peak area ratio of vicine and convicine relative to the absorbance peak area of 2-deoxycytidine provided satisfactory quantitation of the non-derivatised fluorogenic constituents.

5.3.4 Identification of anti-nutritional factors in aqueous extracts of *V. sativa* and *V. narbonensis* by MEKC relative retention time data

Figure 5.7 shows the separation by micellar electrokinetic chromatography of an aqueous extract from the seed of common vetch (*V. sativa*), using a 19.5mM SDS separation

Table 5.1 The repeatability of separations by micellar electrokinetic chromatography in terms of percentage relative standard deviation about the mean retention times of the samples of amino acids and low molecular weight *Vicia* and *Lathyrus* anti-nutritional factors, using the 19.5 mM SDS running buffer.

Sample name	Mean sample retention time (Minutes)	Percent Relative Standard Deviation (%RSD)	ANOVA Variance	Number of Replicates
VIC	4.62	1.45	4.46E-03	29
CON	5.95	0.79	2.18E-03	26
HYP	6.41	0.19	1.55E-04	6
SER	7.06	0.19	1.77E-04	30
ASN	7.10	0.18	1.58E-04	6
GLN	7.13	0.12	7.88E-05	6
THR	7.25	0.19	1.84E-04	30
ALA	7.37	0.17	1.60E-04	30
BCN	7.42	0.41	9.11E-04	29
GLY	7.61	0.18	1.95E-04	30
PRO	8.07	0.15	1.49E-04	19
GEC	8.13	0.74	3.64E-03	29
VAL	8.28	0.19	2.46E-04	30
GCN	8.34	0.57	2.24E-03	19
TYR	8.40	0.19	2.49E-04	19
NOR	8.84	0.16	1.99E-04	30
MET	9.05	0.18	2.78E-04	30
GLU	9.28	0.23	4.37E-04	30
BOAA	9.32	0.92	7.33E-03	22
ISO/ASP	9.67	0.15	2.14E-04	30
LEU	10.41	0.14	2.09E-04	30
PHE	11.81	0.10	1.38E-04	30
TRY	13.07	0.06	5.47E-05	6
NH3	14.25	0.10	2.17E-04	30
BAPN	14.98	0.24	1.35E-03	37
CYS	15.59	0.13	3.87E-04	30
DABA	16.32	0.31	2.52E-03	38
ARG/LYS	16.37	0.17	7.76E-04	30
CAN	16.40	0.22	1.30E-03	25

List of non-IUPAC abbreviations:
Vicine (VIC), convicine (CON), β-cyanoalanine (BCN), γ-glutamyl-S-ethenyl-cysteine (GEC), γ-glutamyl-β-cyanoalanine NH_3 (GCN), β-N-oxalylamino-L-alanine (BOAA), isoleucine (ISO), β-aminopropionitrile monofumarate (BAPN), 2-4-diamino-n-butyric acid 2HCl (DABA), and canavanine 2HCl (CAN).

Table 5.2 The linear regression lines for the standard curve of each of the amino acids and *Vicia* and *Lathyrus* anti-nutritional factors measured and the regression coefficient r^2, the error associated with the standard curve. The limit of detection for each constituent is shown. These data were obtained from separations by micellar electrokinetic chromatography using the 19.5mM SDS running buffer.

Sample name	Mean sample retention time (Minutes)	Linear regression equation for the standard curve for each sample	Limit of Detection ($<x10^{-6}$ M)	Regression coefficient r^2	Number of Replicates
VIC	4.62	0.0003x-0.0219 (a)	5	0.9966	27
CON	5.95	0.0004x+0.0993 (a)	10	0.9901	27
SER	7.06	0.0003x-0.0045	5	0.9974	30
THR	7.25	0.0003x-0.0042	5	0.9984	30
ALA	7.37	0.0002x+0.0025	5	0.9958	30
BCN	7.42	0.0002+0.0217	5	0.999	19
GLY	7.61	0.0005x-0.021	5	0.9915	24
GEC	8.13	0.0004+0.0313	5	0.9999	19
VAL	8.28	0.0005x - 0.0223	5	0.9961	28
GCN	8.34	0.0005+0.0323	5	0.9994	19
TYR	8.40	0.0005x - 0.0861	20	0.9884	17
MET	9.05	0.0004x - 0.0261	5	0.9922	24
GLU	9.28	0.0002x + 0.0036	5	0.9981	30
BOAA	9.32	0.0005x - 0.0011	10	0.9885	32
LEU	10.41	0.0005x - 0.0183	5	0.998	28
PHE	11.81	0.0008x - 0.0898	5	0.9833	28
BAPN	14.98	0.0036x + 0.0204	2.5	0.9948	22
CYS	15.59	0.0005x - 0.0136	10	1	11
DABA	16.32	0.0028x + 0.0436	2.5	0.9879	17
CAN	16.40	0.001x+0.1217	5	0.992	19

(a) quantified relative to the peak area of 2 deoxycytidine.

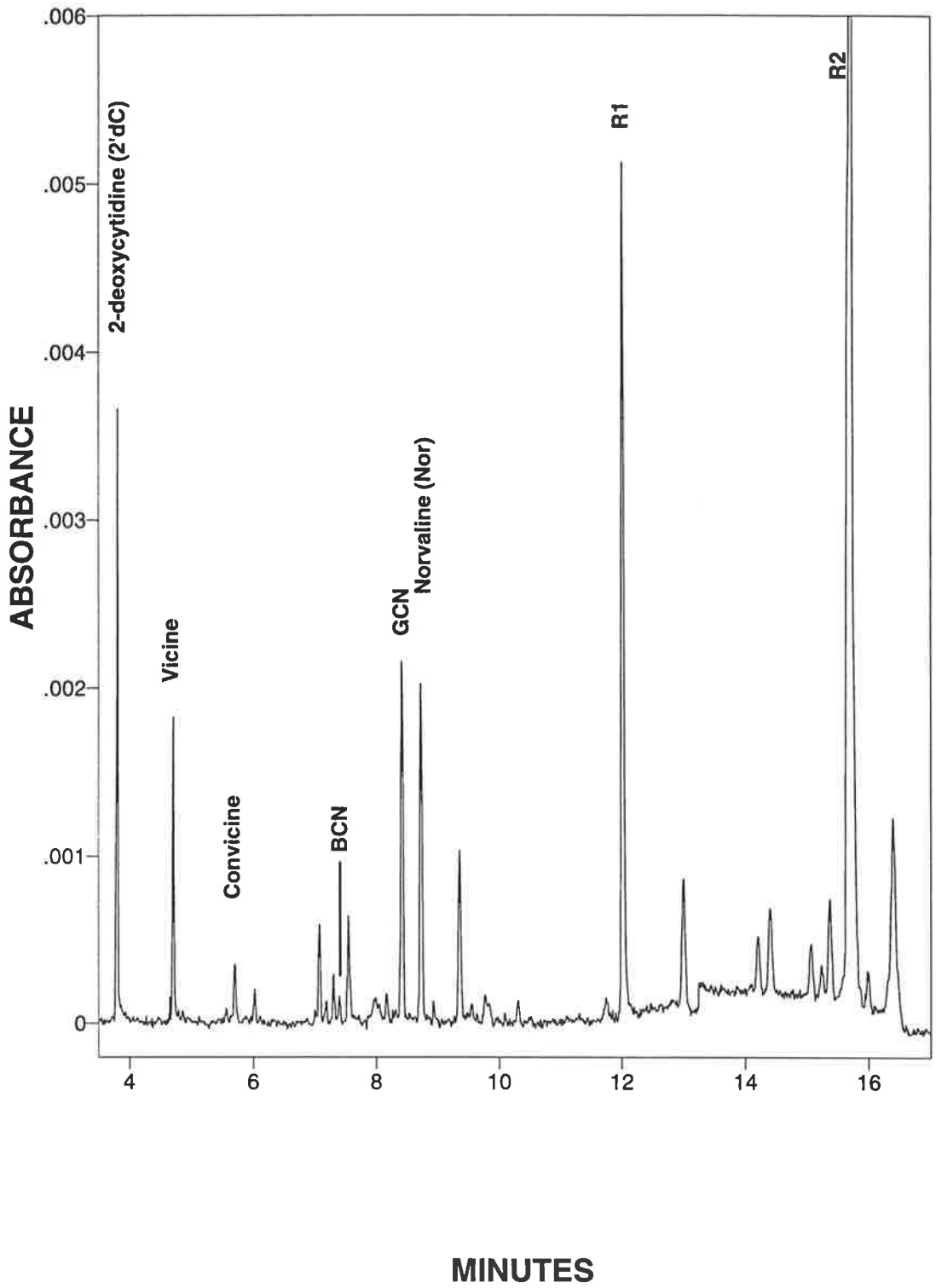


Figure 5.7 The separations by micellar electrokinetic chromatography of a 9-fluorenylmethyl chloroformate (FMOC) derviatised ethanolic extract from common vetch cv. "Blanchefleur" (*V. sativa*), using a 19.5mM SDS running buffer.

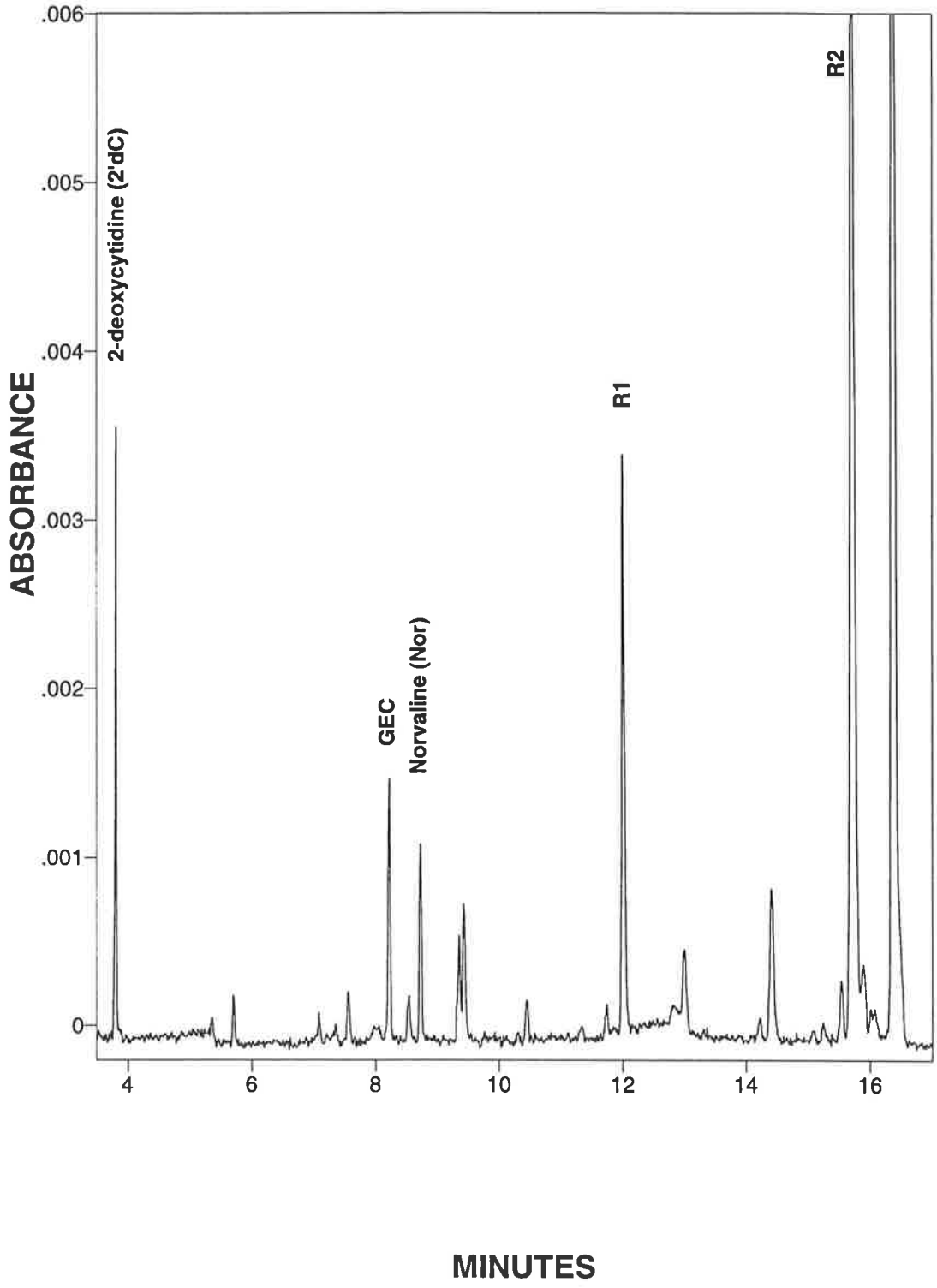


Figure 5.8 The separations by micellar electrokinetic chromatography of 9-fluorenylmethyl chloroformate (FMOC) derivatised ethanolic extract from narbon bean AFTCC 60105 (*V. narbonensis*), using a 19.5mM SDS running buffer.

buffer. Figure 5.8 shows the separation by micellar electrokinetic chromatography of an aqueous extract from the seed of the narbon bean (*V. narbonensis*), using a 19.5mM SDS separation buffer. These data show the identification of the low molecular weight anti-nutritional factors associated with common vetch and the narbon bean from relative retention time data.

5.4. Discussion

The focus of this study has been to identify low cyanoalanine containing *V. sativa* germplasm suitable for use in a plant breeding program. The complementary technique of diffuse reflectance using infrared dispersive spectrometry is an extremely efficient preliminary screening process for identifying low cyanoalanine accessions in the germplasm. However, within the range for low cyanoalanine common vetch plant material (<0.2%), the plant breeder is also interested in a wider range of chemical traits such as the amino acid and β-glucopyranosyl glycoside profiles of this plant material.

Capillary electrophoresis has the potential to provide quantitative information on the amino acids, non-protein amino acids and unusual glycosides found in *Vicia* and *Lathyrus* species by the analysis of aqueous extracts of tissue samples. The literature includes many reports describing promising separations of derivatised amino acids by micellar eletrokinetic chromatography. Most of these reports deal with the separation of a few amino acids and with purchased amino acid derivatives of various types (Michaelsen *et al.* 1994). However, the application of capillary electrophoresis to routine analysis of samples other than reference compounds has been slow due to sensitivity and repeatibility problems (Lee and Yeung 1991; Leube and Roeckel 1994; Schneede and Ueland 1995).

Pre-column derivatisation of amino acids with sensitive fluorescent tags such as 9-fluorenylmethyl chloroformate (FMOC) overcomes the sensitivity problem while at the same time improving the selectivity of the separation. The highly sensitive identification and quantitative separation of a standard solution of FMOC derivatived amino acids by micellar electrokinetic chromatography using laser induced fluorescence has been reported (Albin *et al.* 1991; Chan *et al.* 1993). In particular, Chan *et al.* (1993) suggested the utility for the highly sensitive detection of biological amino compounds in urine but no quantitative data has since been published.

Separation by micellar electrokinetic chromatography is based on the partitioning of analytes between a mobile liquid electrolyte phase, whose motion arises from field-induced electro-osmotic flow, and a micellar phase of electrically charged micelles, whose lesser motion arises from the

combined field-induced actions of the electro-osmotic flow and electrophoresis. The differential migration of the two phases spatially separates the components of a mixture when the components partition coefficients differ (Davis 1989). Hence the stability and quantitative reproducibility of separations are influenced by the electro-osmotic flow and the resolution by the partitioning coefficient defined as the capacity factor (*k'*) of the analyte/micellar separation mechanism.

Electro-osmotic flow is difficult to control (Schneede and Ueland 1995). The electro-osmotic flow in micellar electrokinetic chromatography can be monitored and corrected for by the use of internal standards, data manipulation and realignment of the internal standards to fixed time indices (Figure 5.1). Importantly the use of realignment peaks 2-deoxycytidine, FMOC reagent peak R1 and FMOC reagent peak R2 as an internal relative retention time calibration system yields analyte retention times which are insensitive to variations in the electro-osmotic flow between separations, giving relative standard deviations (<1.0%) for the mean retention times of analytes (Table 5.1). This effective control over the electo-osmotic flow has facilitated an indepth study of the interactions involved in the analyte/micelle separation mechanism and subsequently led to the identification of optimised separation conditions (Figure 5.6).

The most significant difference between micellar electokinetic chromatography and conventional chromatography lies in the fact that the relative retention times of compounds are limited to the region between the migration time of an unretained solute t_0 and the migration time of a solute completely retained in the micelle t_{mc} (Terabe *et al.* 1985). Hence the type of micellar surfactant in the separation buffer strongly influences the relative retention time, migration order and selectivity of constituents separated in the analyte/micelle separation mechanisms (Figure 5.2 and Figure 5.3). These effects are probably due to a combination of changes in the shape and size of the micelle, as well as micellar surface charge associated with the presence of counter ions, and an increased ratio of the volume of the micellar phase to that of the aqueous phase (Michaelsen *et al.* 1994).

The effect of surfactant concentration between 15-30mM SDS on the relative retention time of dicarboxylate and monocarboxylate FMOC derivatised constituents was studied (Figure 5.4 and Figure 5.5). Increased anionic surfactant concentration between 15-25mM SDS led to reduced retention of the dicarboxylate FMOC derivatived constituents in the anionic micellar phase and consequently to quicker detector response times (Figure 5.4). This

could be due to increased micellar electrical repulsion of the dianionic constituents resulting in these components spending less time residing in the anionic micelles and more time in the running buffer. Surfactant concentration did not strongly influence the relative retention times of the monocaboxylate derivatised constituents in the range15-25mM SDS (Figure 5.5).

However, between 25-30mM SDS a decrease in the rate of change in the relative retention time of the dicarboxylate FMOC derivatives was observed (Figure 5.4). Correspondingly, in the monocarboxylate FMOC derivatives, a trend toward increased micellar retention was observed (Figure 5.5). These data were consistant with an increase in the hydrophobicity of the micelle around 25-30mM SDS.

The relative retention times of strongly hydrophobic constituents of the standard solution (relative retention time >12.00 minutes) were not influenced by minor changes in surfactant concentration as these constituents are strongly retained in the micellar separation system (Table 5.1). In particular the bis-FMOC derivatives elute near the migration time of a solute completely retained in the micelle t_{mc} and were not well resolved. Hence, this system is not appropriate for the quantitative study of *Vicia* and *Lathyrus* species containing canavanine, diaminobutyric and diaminopropionic acid or for the study of arginine and lysine.

Quantitative precision by micellar electrokinetic chromatography can be influenced by the repeatibility of the sample injection and by the sensitivity of the detection system. Both these factors were compensated for by the use of internal standards in the derivatising buffer (Figure 5.1). 2-Deoxycytidine peak area was used to compensated for the variation in the absolute sample volume injected onto the column by the hydrodynamic injection system. Hence, calibration with 2-deoxycytidine peak area provided the basis for the precise and sensitive quantitation of the non-derivatised UV absorbing constituents of the standard solutions (Table 5.2). Quantification relative to the peak height of 9-fluorenylmethyl chloroformate (FMOC) derivatised norvaline was shown to provide a precise and sensitive quantitation for derivatisable amino acid constituents of the standard solutions (Table 5.2). The use of FMOC norvaline peak height as the response variate for the quantitation of FMOC derivatised constituents was found to be unbiased for absolute sample volume loaded onto the column as the ratio of peak heights with respect to norvaline peak height is due to competition for the derivatising reagent and not the precise amount of sample injected onto the column.

Quantification of FMOC derivatised amino acids has been reported by micellar electrokinetic chromatography using laser induced fluorescence (Albin *et al.* 1991; Chan *et al.*

1993). Chan *et al.* (1993) showed similar separation resolution with respect to relative retention time and migration order of the derivatised amino acids as that observed in this study. The limit of detection for laser induced fluorescence (LIF) of FMOC glycine was shown to be $5x10^{-10}$ M (Albin *et al.* 1991) compared with $5x10^{-6}$ M with the absorbance detection used in this study. However, neither CE-LIF study has been applied to the problem of identification or quantitation of constituents from biological material.

This study has focused on the development of suitable compensation mechanisms for the variation of electro-osmotic flow. This has been achieved through the use of internal standards, and data manipulation to establish long-term stability (Figure 5.1 and Table 5.1) and quantitative reproducibility in micellar electrokinetic chromatography (Figure 5.1, Table 5.1 and Table 5.2). The stability of relative retention times associated with derivatised and underivatised compounds has facilitated the identification of chemotaxonomic characters associated with *V. sativa* (γ-glutamyl-β-cyanoalanine, β-cyanoalanine, vicine and convicine) and *V. narbonesis* (γ-glutamyl-S-ethenyl-cysteine) present in aqueous extracts from plant tissue samples (Table 5.1, Figure 5.7 and Figure 5.8). The limit of detection achieved for the cyanoalanine anti-nutritional factors by micellar electrokinetic chromatography nicely complements detection by diffuse reflectance using infrared dispersive spectrometry (DRUIDS) and has been extensively employed for the study of low cyanoalanine accessions identified by DRUIDS, described in chapter seven and Appendix C1-C10. As a result, micellar electrokinetic chromatography has been adopted as an important secondary mechanism for studying the chemical traits of *V. sativa* samples used in the plant screening program.

5.5 Conclusion

This chapter records the successful development of a quantitative separation by micellar electrokinetic chromatography of FMOC derivatised plant extracts. Parameters necessary for long-term stability and reproducibility required for routine quantitative analysis of biological samples are described. The importance of appropriate internal standards and data manipulation to compensate for run to run variability is emphasised. Quantitation of non-derivatised UV absorbing components of an aqueous extract was facilitated through compensating for variation in the absolute volume of sample injected onto the column by reference to the peak area of 2-deoxycytidine. In the case of FMOC derivatised components

quantitation was optimised by measurement of peak height ratios relative to the reference peak height of FMOC norvaline as the internal standard. The micellar electrokinetic chromatography procedure in conjunction with the diffuse reflectance infrared method has now established a sound foundation for the breeding of the world's first low or zero cyanoalanine content, *V. sativa* plant. Furthermore, it presents solutions for the hitherto perceived shortcomings of capillary electrophoresis that have inhibited the ready adoption of this powerful methodology to the quantitative analysis of hydrophobic analytes from an array of natural product samples.

Chapter Six

Peas in a Pod

6.0 Introduction

Examination of the local *V. sativa* germplasm, described in chapter seven, established that many accessions consisted of mixtures of species. Analysis of single seeds from these accessions by diffuse reflectance using dispersive infrared spectrometry and paper electrophoresis showed substantial variation within and between accessions in the Australian germplasm collection. This clearly showed the need to investigate the source of the variation in the concentration of cyanoalanine non-protein amino acids found in single seeds.

The distribution of the cyanoalanine non-protein amino acids in the cotyledons and between seeds from fourteen stems of matured plants of *V. sativa* cv. "Blanchefleur" was examined. Position of the seed within the pod was shown to have a significant influence on cyanoalanine non-protein amino acid content in the seed, while variation between the means of the pods was not significant.

6.1 Non-destructive single seed analysis

6.1.1 Plant material and sample collection

Seeds of *V. sativa* cv. "Blanchefleur" were held firmly in a pair of tweezers and drilled, using an Arlec jeweller's drill fitted with an engraving drill bit, through the cotyledons taking care not to damage the embryo (Figure 6.0). Drill cuttings were sieved (<0.5mm) to remove seed coat. Quantification of the cyanoalanine non-protein amino acids in the seed samples was by diffuse reflectance using dispersive infrared spectrometry with the small sample cup procedure, described in chapter four.

6.1.2 Germination conditions

The drilled seeds were surface sterilised by immersion (1 minute) in either 70% ethanol, 2.0g/L Benlate or 1.25g/L Captan to compare effects on germination. No surface sterilisation was used as a control. Whatman Number 1 filter paper, Difco agar (0.6%), and rinsed autoclaved vermiculite were

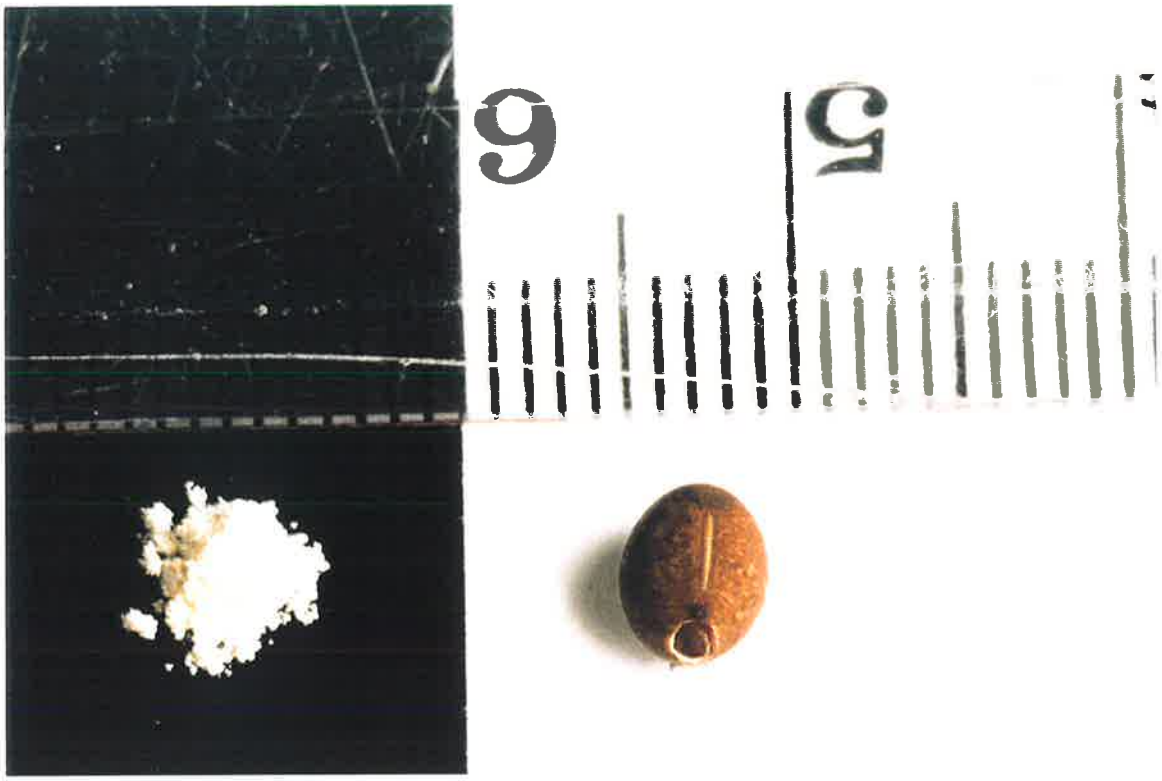


Figure 6.0 A single drilled seed of *V. sativa* cv. "Blanchefleur" and collected drillings. The embryo is situated on the top of the hylem and the drilling transect passes through the cotyledon in a way not to damage the embryo. Smallest scale divisions are 1 mm.

compared as growth media. The growth medium was placed in a 9cm petri dish, 10mL of water was added to the Whatman Number 1 filter paper and the rinsed autoclaved vermiculite. Germination was conducted in a dark incubator set at 27°C over 5 days. Seedlings were then transferred to larger vessels (4 inch U.C pots) for continued growth in the containment glasshouse.

6.2 Distribution of the cyanoalanine non-protein amino acids in the seeds of mature *V. sativa*

6.2.1 Distribution of the cyanoalanine non-protein amino acids in the cotyledon of *V. sativa* cv. "Blanchefleur"

6.2.1.1 Plant material and sample collection

Homogenised plant material was collected by drilling three perpendicular transects through the cotyledons of ten seeds of *V. sativa* cv. "Blanchefleur". The samples were collected in duplicate, on two consecutive days, by diffuse reflectance using dispersive infrared spectrometry with the small sample cup procedure, described in chapter four.

6.2.1.2 Data analysis

Quantitative cyanoalanine non-protein amino acid data were exported to Excel 5.0 (Microsoft Corporation, Redmond, WA) and analysed using Residual Estimation of Maximum Likelihood (REML) to estimate the three components of variance (Genstat 5.0).

6.2.2 Distribution of the cyanoalanine non-protein amino acids within the seeds of plants of *V. sativa* cv. "Blanchefleur"

6.2.2.1 Plant material and sample collection

Fourteen stems from three mature plants of *V. sativa* cv. "Blanchefleur", grown in the birdcage at the Northfield Research Station (SARDI, Northfield, S.A.) were collected, dried and stored at room temperature in a large plastic bag. The 14 stems consisting of 92 pods containing 462 seeds. Seed pod mass and individual seed masses were recorded from a Mettler H20T five figure balance. Seed order within the pod was noted. Homogenised seed material was collected and analysed using the procedure described in section 6.1.1.

6.2.2.2 Data Analysis

In collaboration with Ms. Lynne Giles (Waite Institute, Biometrical Statistical Consultant) analysis of variance was determined using the GENSTAT 5 statistical analysis program. Each stem was treated independently. Several different approaches were taken with the data.

6.2.2.2.1 Component of variance

A stem with 4 pods, each containing 6-7 seeds, was used to identify components of variation for the content of the cyanoalanine non-protein amino acids within the plant. Variation between pods, variation between seeds within the pod and the variation between measurements within seeds were examined. Residual Estimation of Maximum Likelihood (REML) was used to estimate the three components of variance.

6.2.2.2.2 Spearman's Correlation Coefficient

A comprehensive correlation involving 462 seeds from 14 stems using the Spearman Correlation Coefficient was conducted. Correlations between cyanoalanine content and mass, cyanoalanine content and position, and mass and position were examined.

6.2.2.2.3 Analysis of Variance

An analysis of variance (ANOVA) involving 462 seeds from 14 stems was conducted. The position of the seed in the pod was examined further by partioning the sources of variance to include the effect of the proximal and distal "terminal" seeds from the "other" seeds in the pod. The analysis of variance for these components were derived for the percent cyanoalanine non-protein amino acid content, seed mass and the mass of cyanoalanine non-protein amino acids in each seed.

6.3 Results

6.3.1 Non-destructive single seed analysis

The drilled seeds were surface sterilised by immursion (1 minute) in either 70% ethanol, 2.0g/L Benlate or 1.25g/L Captan to compared for effects on germination. Immersion in 70% ethanol failed to control fungal infection. Immersion in either 2.0g/L Benlate or 1.25g/L Captan were equally effective in the control of subsequent fungal infestations in drilled seeds.

Initially seeds were successfully germinated on filterpaper (Figure 6.1). Subsequently Whatman Number 1 filter paper, nonsterilised agar (0.6%) and rinsed, autoclaved vermiculite were

Figure 6.1 A drilled seed of *V. sativa* cv. "Blanchefleur" and a germinated seed of *V. sativa* cv. "Blanchefleur" using Whatman Number 1 filter paper as the germination medium. Smallest scale divisions are 1 mm.

compared as growth media. Seeds grown on agar showed greater initial vigour compared to those grown on vermiculite and filter paper. However by Day 4, root growth and shoot vigour was stronger in the vermiculite than observed in agar and filter paper.

Seeds grown on vermiculite were easily transferred to larger pots (4 inch U.C pots) for continued growth in the containment glasshouse. The experiment concluded on Day 30 with all plant growing well. The optimum germination conditions for drilled *V. sativa* cv. "Blanchefleur" seeds in this experiment were those surface sterilized by exposure to 2g/L Benlate in water for 2 minutes and placed in a 9cm petri dish containing autoclaved vermiculite and 10mL of water and incubation at 27°C in the dark.

6.3.2 Distribution of the cyanoalanine non-protein amino acids in the cotyledon of *V. sativa* cv. "Blanchefleur"

Table 6.1 shows three components of variance in the content of the cyanoalanine non-protein amino acids in the seeds of a stem quantitated by diffuse reflectance using infrared dispersive spectrometry and calculated by Residual Estimation of Maximum Likelihood (REML). The largest component of variance was attributed to variance of the seed. Variation between measurements and measurements within the seed were small compared with the variation between seeds.

Table 6.1 Three components of variance in the concentration of γ-glutamyl-β-cyanoalanine as fitted by Residual Estimation of Maximum Likelihood.

Component of variance	estimate ($x10^{-5}$)	standard error ($x10^{-6}$)	% total variance
between measurement within seed	-0.0132	0.0443	7
measurement between seeds	0.114	0.445	72
measurement within seed	0.0577	0.129	21

6.3.3 Distribution of the cyanoalanine non-protein amino acids within the seeds of plants of *V. sativa* cv. "Blanchefleur"

Table 6.2 shows three components of variance in the content of the cyanoalanine non-protein amino acids in the seeds of a stem quantitated by diffuse reflectance using infrared dispersive spectrometry and calculated by Residual Estimation of Maximum Likelihood (REML). The largest component of variance was attributed to variance of the seed within the pod. Variation between

measurements and between pods on the stem is small compared with the variation between seeds within the pod.

Table 6.2 Three components of variance in the concentration of γ-glutamyl-β-cyanoalanine as fitted by Residual Estimation of Maximum Likelihood.

Component of variance	estimate	standard error	% total var
pod	0.001475	0.000313	9
seed within pod	0.01343	0.004345	82
measurement within seed within pod	0.001548	0.0003419	9

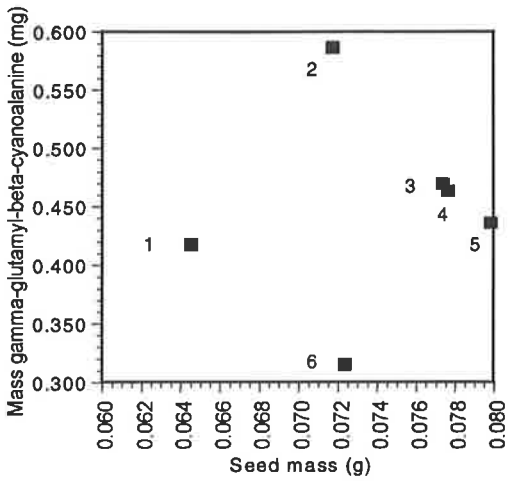
Figure 6.2 shows the graphs of a number of pods for the content of cyanoalanine non-protein amino acids in a seed against seed position which are significant for the Spearman's correlation coefficient. These data establish a relationship between the content of cyanoalanine non-protein amino acids in a seed and seed position in a pod.

Table 6.3 shows analysis of variance with mass of the cyanoalanine non-protein amino acids in the seed as the response variate and with seed position included in the model. The analysis of variance indicated that there is a significant effect of position. Partitioning of variance between the proximal and distal "terminal" seeds from the "other" seeds in the pod showed that there is a highly significant difference between the "terminal" and "other" seeds. A further examination indicated that there is significant differences between the first and last seed in a pod.

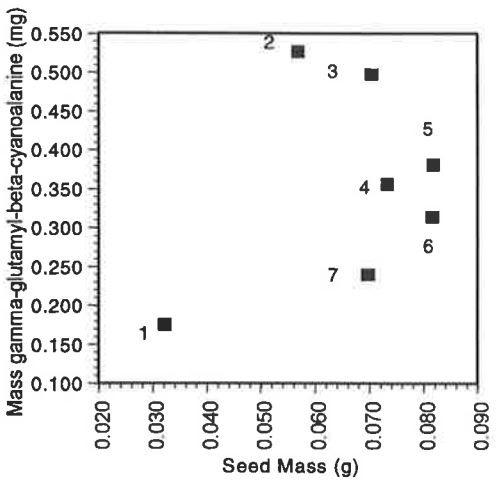
Table 6.3 The analysis of variance (ANOVA) table with the mass of γ-glutamyl-β-cyanoalanine in the seed as a response variate.

Source	Degrees of freedom	Mean Square	Variance ratio	F	Significance level
Pod	10	0.035	1.24	P<0.243	NS
Position	2	0.726	26.17	P<0.001	***
α vs. ω	1	0.114	4.11	P<0.05	*
Other vs. α/ω	1	1.339	48.25	P<0.001	***
Residual	298	0.028			

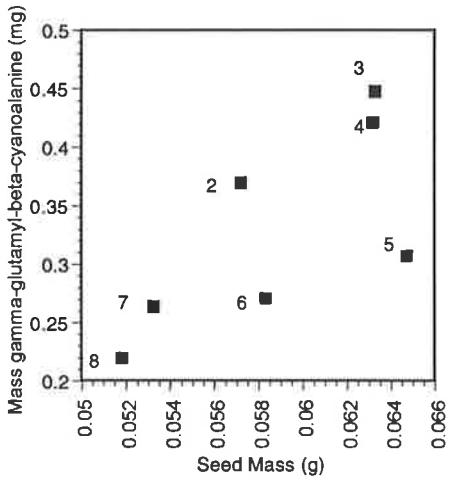
*** P<0.001
** P<0.01
* P<0.05
NS Not Significant



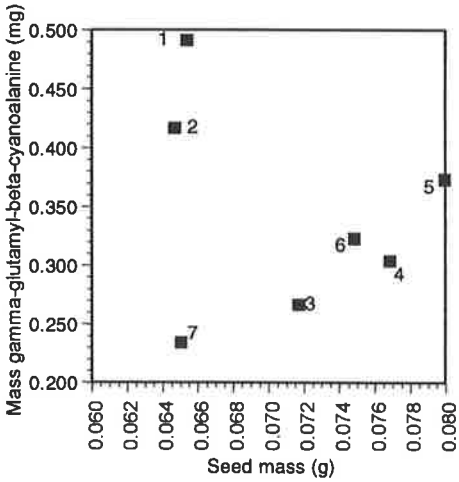
Spearman Rank Correlation
γ-Glutamyl-β-cyanoalanine vs. position
Correlation: -0.9429



Spearman Rank Correlation
γ-Glutamyl-β-cyanoalanine vs. position
Correlation: -0.8929



Spearman Rank Correlation
γ-Glutamyl-β-cyanoalanine vs. position
Correlation: -0.7857
Note: Seed 1 was missing.



Spearman Rank Correlation
γ-Glutamyl-β-cyanoalanine vs. position
Correlation: -0.8929

Figure 6.2 The graphs of pods which are significant for the Spearman correlation coefficient for the content of cyanoalanine non-protein amino acids in a seed against seed position in the pod. The position in the pod is indicated where 1 is the proximal seed.

6.4. Discussion

A single seed quantification for the cyanoalanine non-protein amino acids by diffuse reflectance using dispersive infrared spectrometry which did not impair the viability of the seed was developed. Sample collection from various transects of the seed identified that physical injury to the embryo terminated seed viability. Also, sample collection compromised the integrity of the seed coat resulting in increased levels of opportunistic fungal infection. Optimisiation of germination and collection conditions has facilitated the single seed selection of plant material from mixtures and has provided a basic tool for the study of the inheritance of the cyanoalanine non-protein amino acids in common vetch.

Variation in the cyanoalanine content between accessions of common vetch has been reported (Ressler *et al.* 1969b). An examination on a single seed basis of variation within a commercial cultivar of common vetch (*V. sativa* cv. "Blanchefleur") established that substantial variation was observed in the cyanoalanine concentration between individual seeds (Table 6.1). Variation attributed to drilling position, though greater than the observed error between measurements within individual seeds (Table 6.1), was shown to be a minor component of the observed variation within the population.

The distribution of cyanoalanine non-protein amino acids within in a population was further investigated by studying the distribution of cyanoalanine content within individual seeds between pods on a single stem. It was recognised that there are several sources of variation contributing to the overall variance observed in the cyanoalanine content within the stem. Variation between pods, variation between seeds within a pod, and the variation between measurements within the seed were examined. The components of variation were analysed as random events from a random sample within a population. These data were not balanced, hence the use of Residual Estimation of Maximum Likelihood (REML) to estimate the three components of variance. The analysis of variance attributed the largest component of variance to the seed in the pod (Table 6.2).

Subsequently a correlation involving 462 seeds from 14 stems was conducted using the Spearman's correlation coefficient. This method is based on ranks and does not depend on the assumptions about normal distributions. Correlations between cyanoalanine content and seed mass, cyanoalanine content and seed position, and seed mass and seed position were examined. The Spearman's correlation coefficient established a correlation between cyanoalanine content and position of the seed within the pod (Figure 6.2).

Having established that seed position in the pod was an important factor in the content of cyanoalanine non-protein amino acids in the seed, a comprehensive analysis of variance was conducted with this factor included in the model. Cyanoalanine content and seed mass, cyanoalanine content and seed position, and seed mass and seed position were examined. Furthermore, the analysis of variance was partitioned between the proximal and distal "terminal" seeds from the "other" seeds in the pod. The analysis of variance indicated that there was a significant effect of position and that there was a highly significant difference between the "terminal" and "other" seeds. Further examination indicated that there was a significant difference between the first and last seed in a pod (Table 6.3). Variation between pods was not significant.

6.5 Conclusion

This chapter describes the physical treatment of the single seed necessary for the non-destructive quantification for the cyanoalanine non-protein amino acids by diffuse reflectance using dispersive infrared spectrometry. Variation on a single seed basis of the cyanoalanine content between individual seeds in accessions was showed that substantial variation in the cyanoalanine non-protein amino acid concentration was observed within an accession. Variation within the seed was a minor component of the variation observed within a population. The sources of variation for the cyanoalanine content in a seed were studied. ANOVA showed that variation between pods was not significant but variation between seeds in a pod was significant. Importantly, partitioning of the variance within the pod showed that there was a significant difference between the "terminal" and "other" seeds within a pod. This work established that caution must be observed in the use of single seed quantifications as a suitable representative of the mean cyanoalanine content within a pod or throughout a plant. However, it has been shown that the mean cyanoalanine content within a pod is an appropriate model for the determination of the mean cyanoalanine content within a plant.

Chapter Seven

Needle in a Haystack

7.0 Introduction

The literature identifies three distinct chemical families of relatively thermostable, low molecular weight anti-nutritional factors present in the pulse of common vetch (*V. sativa*) which have the potential to cause injury when ingested. These are the cyanogenic glycoside vicianine, the β-D-glucopyranosyl glycosides vicine and convicine, and the cyanoalanine non-protein amino acids, β-cyanoalanine and γ-glutamyl-β-cyanoalanine. The application of post-harvest detoxification for the non-protein amino acids in common vetch has been reported (Arscott and Harper 1964; Tate *et al.* 1994; Enneking 1995) but has been considered uneconomic.

In order to safely use common vetch as a food for monogastric animals, the concentration of the cyanoalanine non-protein amino acids in the pulse must be reduced. A plant breeding approach for the removal of an undesirable trait involves the screening of vast numbers of plants to identify parental material with low or zero expression of the undesired trait. This approach has lead to the successful release of varieties with low levels of the bitter alkaloids in *Lupinus luteus* and *Lupinus angustifolia* (Gladstone 1994), the lathyrogen β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP) in *Lathyrus sativus* (Campbell *et al.* 1994) and the cyanogenic glycoside vicianine in common vetch (*V. sativa*) (Milczak 1972). These examples are characterised by the use of a simple and rapid chemical assay for the undesirable chemical trait and access to large collections of documented and well maintained germplasm.

This chapter details collaboration with Dr. Larry Robertson, (Curator, Grain and Pasture Legumes), International Centre for Agricultural Research in Dry Areas (ICARDA Aleppo, Syria), and the evaluation of all available accessions of *V. sativa* for low or zero cyanoalanine non-protein amino acid plant material. Initial screenings were conducted by the large cup diffuse reflectance using infrared dispersive spectrometry method described in chapter four. Material identified as either low (<0.2%) or high (>2.0%) in cyanoalanine non-protein amino acids were subsequently examined by the micellar electrokinetic chromatography method described in chapter five. These accessions were imported under quarantine regulations as whole seed. Individual seeds were classified using morphological traits (Ms. Jane Rathjen and Dr. Jennifer Gardener, University of Adelaide) and examined for chemical traits on a single seed basis using the small cup diffuse reflectance using infrared dispersive spectrometry and

micellar electrokinetic chromatography methods. Seed material sourced by Mr Rade Matic, South Australian Research and Development Institute (SARDI) from the Australian Temperate Field Crops Collection (ATFCC Horsham, Victoria) and the Vavilov Institute (St. Petersburg, Russia) were examined by diffuse reflectance using infrared dispersive spectrometry.

7.1 Plant Material

7.1.1 Australian Temperate Field Crops Collection

Common vetch from the ATFCC was provided by Mr. Rade Matic SARDI, with available passport data. The plant material frequently consisted of three to five seeds of unknown age and in some cases morphological heterogeneity was noted. Reference to the passport data showed that many of the accessions had not been grown out since introduced to the collection. Several accessions had been in storage for >35 years.

7.1.2 International Centre for Agricultural Research in Dry Areas

Common vetch from ICARDA was provided by Dr. Larry Robertson via Dr. Clive Francis at the Co-operative Research Centre for Legumes in Mediterranean Agriculture (CLIMA Perth, WA). All accessions analysed were grown as single plots at ICARDA and were harvested in June/July 1995. The plant material was received as dried, threshed and milled (<0.5mm) homogenised seed in vacuum sealed plastic envelopes with the plot number associated with the sample clearly written on the exterior of the package. All available passport data was provided from the ICARDA genetic resource unit via Dr. Dirk Enneking, CLIMA.

7.1.3 Vavilov Institute

Common vetch from the Vavilov Institute was provided by Mr. Rade Matic SARDI, without passport data. The plant material of unknown age had been milled (<0.5mm).

7.1.4 International Centre for Agricultural Research in Dry Areas, enviromental replication

Three accessions of *V. sativa* ssp. *sativa*, IFVI 1416, IFVI 2541, and IFVI 2627 from the ICARDA collection were each grown at ICARDA in multiple plots and were individually harvested to determine the influence of environment on the cyanoalanine non-protein amino acid content within populations of accessions.

7.2 Analysis

7.2.1 Australian Temperate Field Crops Collection

Quantification of cyanoalanine non-protein amino acids was conducted by the small cup diffuse reflectance using dispersive spectrometry method on a single seed basis as described in chapters four and six. Where possible, samples were collected on three morphologically similar seeds. Prior to the development of the micellar electrokinetic chromatography procedure, accessions with low concentrations (<0.2%) of cyanoalanine non-protein amino acids were examined using high-voltage paper electrophoresis, as described in chapter three, to check for the presence of canavanine.

7.2.2 International Centre for Agricultural Research in Dry Areas

The milled samples were sieved and sievings (<100μm) were placed in bar coded zip lock polyurethane bags. Quantification of cyanoalanine non-protein amino acids was conducted by the large cup diffuse reflectance using infrared dispersive spectrometry method as described in chapter four. Samples were analysed in duplicate using a modified carriage containing two large cups. Data management was facilitated through the use of bar code entered sample names. The bar coded sample names corresponded to the ICARDA plot number for each of the *V. sativa* accessions. Each plot was supplied with the corresponding IFVI accession number to allow correlation with the ICARDA passport data. The percentage cyanoalanine non-protein amino acid concentrations were calculated by both area and height for the nitrile stretching frequency at 2251cm^{-1} for duplicate samples from each accession. In accessions where the standard deviation for the four measurements was in excess of 0.1%, the samples were reanalysed. The bar code system facilitated the rapid and error free transfer of quantitative cyanoalanine non-protein amino acid data from the sample bags to the ICARDA passport database in Microsoft Access 2.0 (Microsoft Corporation, Redmond, WA).

Accessions with either low (<0.2%) or high (>2.0%) cyanoalanine non-protein amino acid concentrations were then examined by micellar electrokinetic chromatography, as described in chapter five. These accessions were imported under quarantine regulations as whole seed. Individual seeds were classified using morphological traits (Ms. Jane Rathjen and Dr. Jennifer Gardener, University of Adelaide) and examined for chemical traits on a single seed basis using the small cup diffuse reflectance using infrared dispersive spectrometry and micellar electrokinetic chromatography methods. Where possible, errors detected in the ICARDA classification were supported by Dr. Robertson and the appropriate taxonomic revisions were made to the ICARDA passport database.

7.2.3 Vavilov Institute

The samples were sieved (<100μm) and placed in bar coded zip lock polyurethane bags. Quantification of cyanoalanine non-protein amino acids was by the diffuse reflectance using infrared dispersive spectrometry method, as described in section 7.2.2. The bar coded sample names were supplied by Mr Rade Matic, SARDI.

7.3 Results

7.3.1 Australian Temperate Field Crops Collection

Figure 7.1 shows the frequency distribution of γ-glutamyl-β-cyanoalanine in the ATFCC *V. sativa* collection indicating a number of low or zero cyanoalanine accessions in the population. Paper electrophoresis invariably showed that accessions with zero cyanoalanine non-protein amino acid content had the non-protein amino acid canavanine, suggesting taxonomic misclassification. Accessions that exhibited low cyanoalanine non-protein amino acid concentrations (<0.2%) invariably had γ-glutamyl-β-cyanoalanine and vicine in the aqueous ethanolic extracts. Attempts at growing many of these old accessions were unsuccessful, except in the case of CPI 60218 (Ms. Jane Rathjen). The progeny of this plant exhibited a concentration of 0.7% γ-glutamyl-β-cyanoalanine in the cotyledon.

7.3.2 International Centre for Agricultural Research in Dry Areas

A slightly skewed normal curve was observed for the frequency distribution of γ-glutamyl-β-cyanoalanine in the ICARDA *V. sativa* collection (Figure 7.2). Micellar electrokinetic chromatography showed that accessions which exhibied low cyanoalanine non-protein amino acid concentrations (<0.2%) had γ-glutamyl-β-cyanoalanine and vicine in trace amounts, while containing other non-protein amino acids (Appendix C1-10). These accessions were imported under quarantine regulations as whole seed and were shown to consist of mixtures of species. *V. sativa* was a minor component of these mixtures. The authentic *V. sativa* single seeds in these mixtures contained a concentration in the order of 0.7% γ-glutamyl-β-cyanoalanine in the cotyledon. Seeds from four accessions expressioning low cyanoalanine non-protein amino acid concentrations (0.45-0.55%) have been imported under quarantine regulations and are being used in a plant breeding program (Ms. Jane Rathjen).

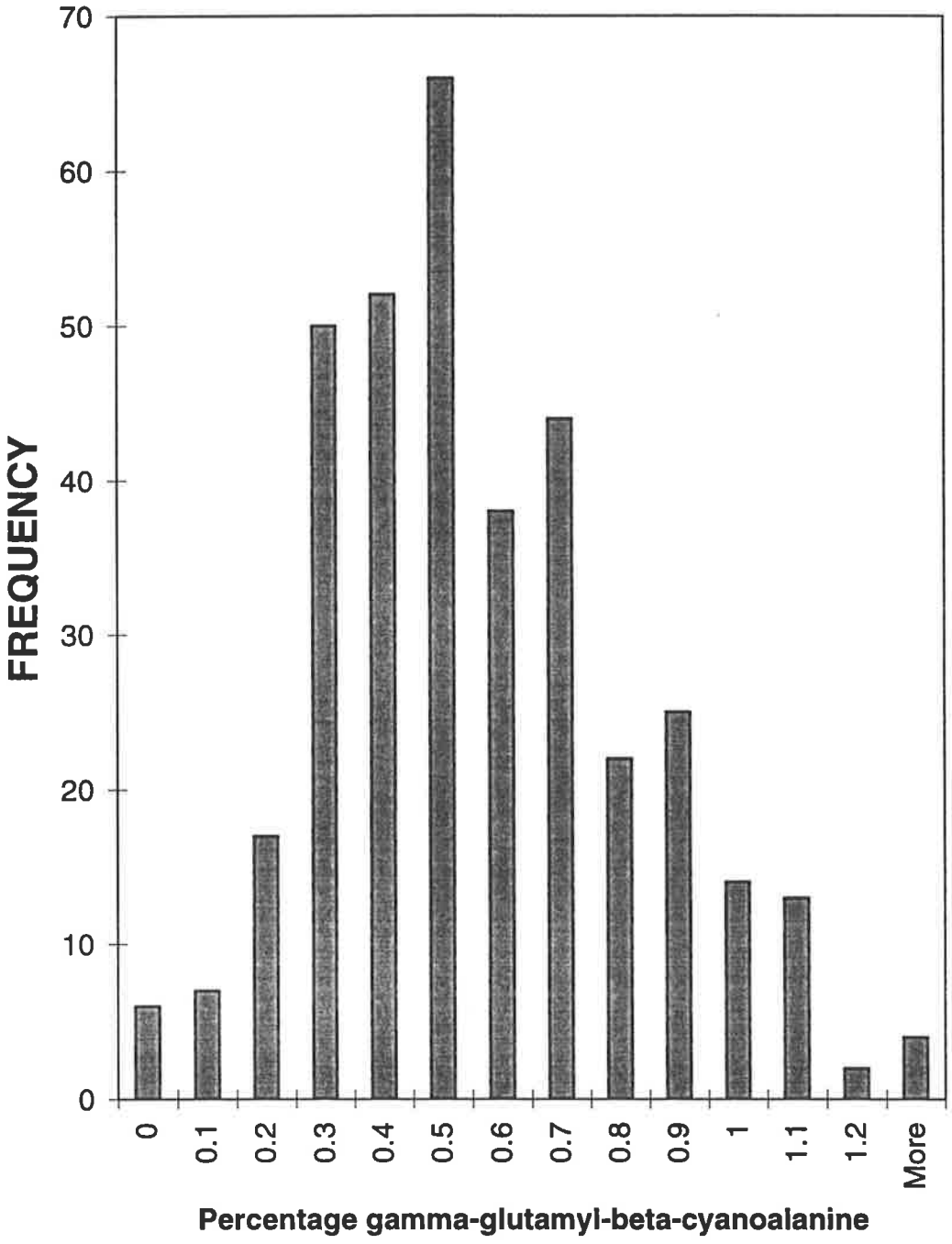


Figure 7.1 The frequency distribution of γ-glutamyl-β-cyanoalanine in 360 accessions of *V. sativa* sourced from the Australian Temperate Field Crops Collection (ATFCC Horsham, Victoria. Australia).

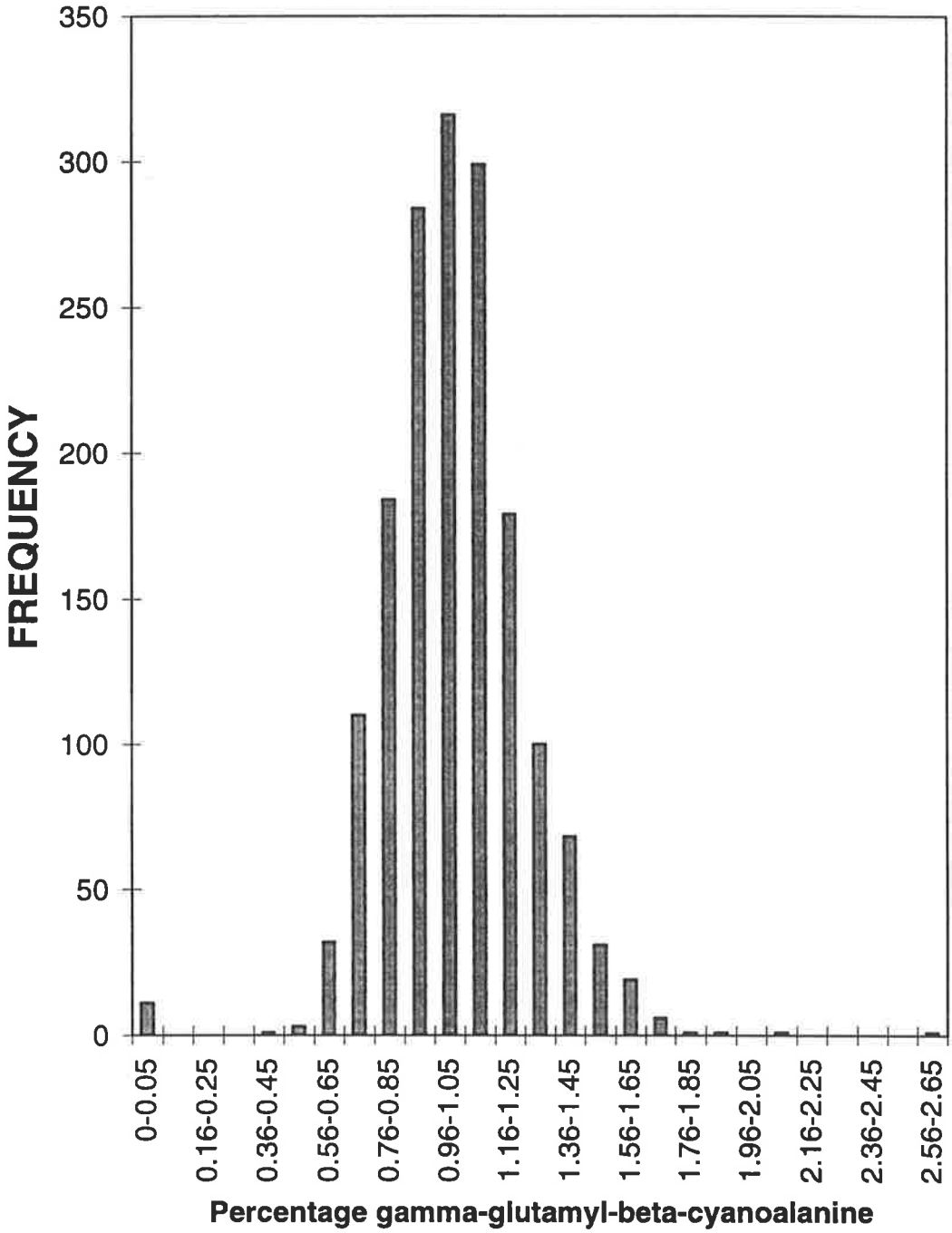


Figure 7.2 The frequency distribution of γ-glutamyl-β-cyanoalanine in 1647 accessions of *V. sativa* ssp. *sativa* sourced from the International Centre for Agricultural Research in Dry Areas (ICARDA Aleppo, Syria) Genetic Resource Unit collection.

7.3.3 Vavilov Institute

Figure 7.3 shows the frequency distribution of γ-glutamyl-β-cyanoalanine in the Vavilov Institute *V. sativa* collection indicating a number of low or zero cyanoalanine accessions in the population.

7.3.4 International Centre for Agricultural Research in Dry Areas, enviromental replication

The frequency distributions of γ-glutamyl-β-cyanoalanine in three accessions of *V. sativa* grown in multiple replicates were used to deduce the effect of enviroment on γ-glutamyl-β-cyanoalanine deposition within populations of *V. sativa* at the ICARDA field site. Figures 7.4, 7.5 and 7.6 show a narrow range in the concentration of cyanoalanine non-protein amino acid toxins across plots of each accession.

7.4 Discussion

Prior to the establishment of a germplasm resources plan for *Vicia* at the Australian Temperate Field Crops Collection by Dr. Alan McIntyre, many of the disparate *V. sativa* collections in Australia had not been satisfactorily documented or maintained. Examination of the local *V. sativa* germplasm identified that many accessions contained mixtures of morphologically similar, but clearly different species. Single seed selections from these accessions showed substantial variation within the population. Numerous low cyanoalanine non-protein amino acid containing seeds were identified. Aqueous ethanolic extracts analysed by paper electrophoresis showed that seeds in accessions with zero cyanoalanine non-protein amino acid content invariably contained the non-protein amino acid canavanine consistent with other *Vicia* species. However, it was found that seeds in accessions with low cyanoalanine non-protein amino acid concentrations (<0.2%) clearly showed γ-glutamyl-β-cyanoalanine and vicine in their aqueous ethanolic extracts and were consistent with the *V. sativa* classification.

Subsequent work on the regeneration of these old low cyanoalanine non-protein amino acid containing accessions found that most of the seeds were non-viable. Inspection of the passport and genetic resource data indicated that many of the accessions had not been grown out since introduction into Australia. However, Ms. Jane Rathjen (University of Adelaide, Waite Institute)

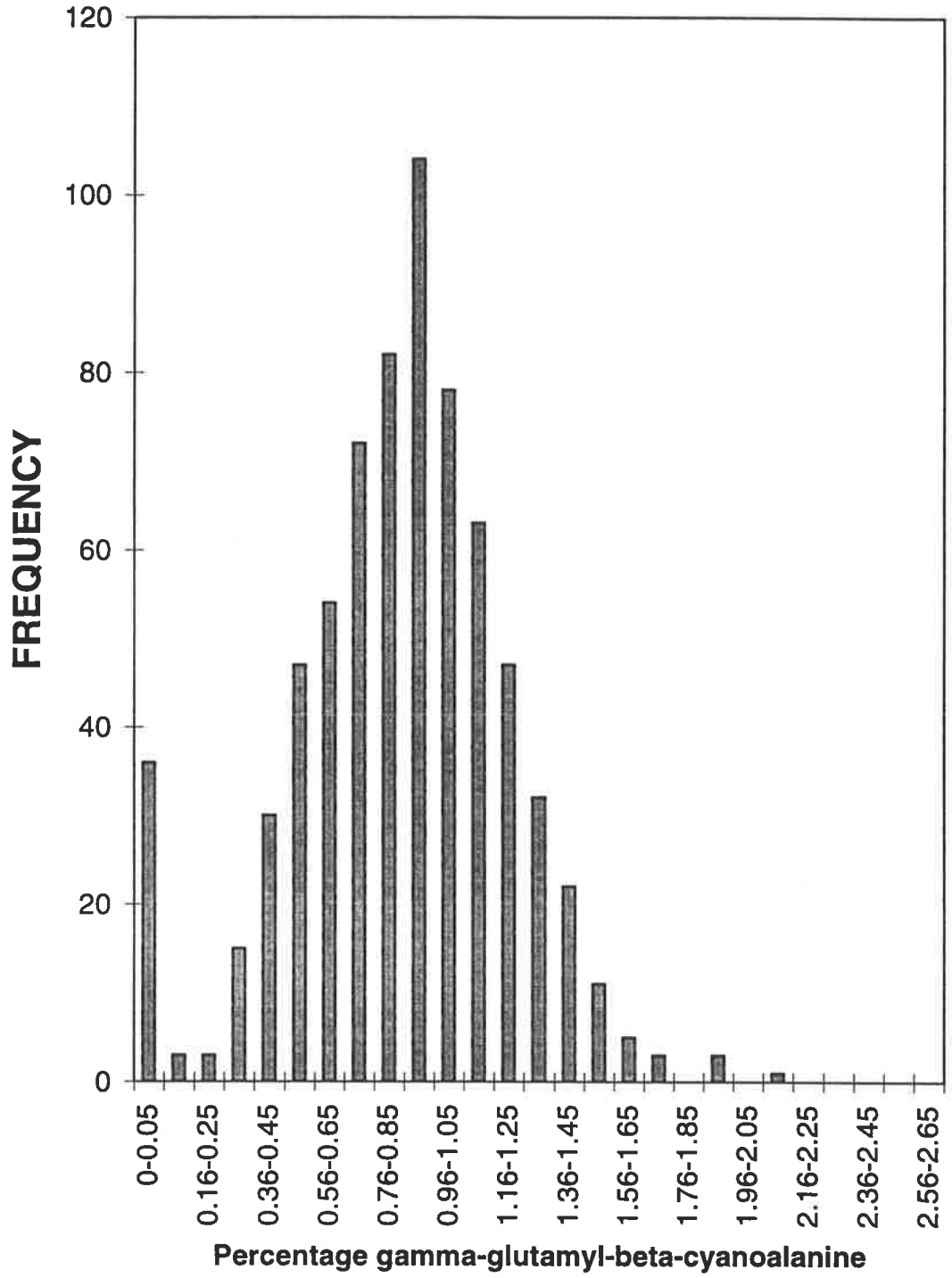


Figure 7.3 The frequency distribution of γ-glutamyl-β-cyanoalanine in 711 accessions of *V. sativa* sourced from the Vavilov Institute (St. Petersburg, Russia).

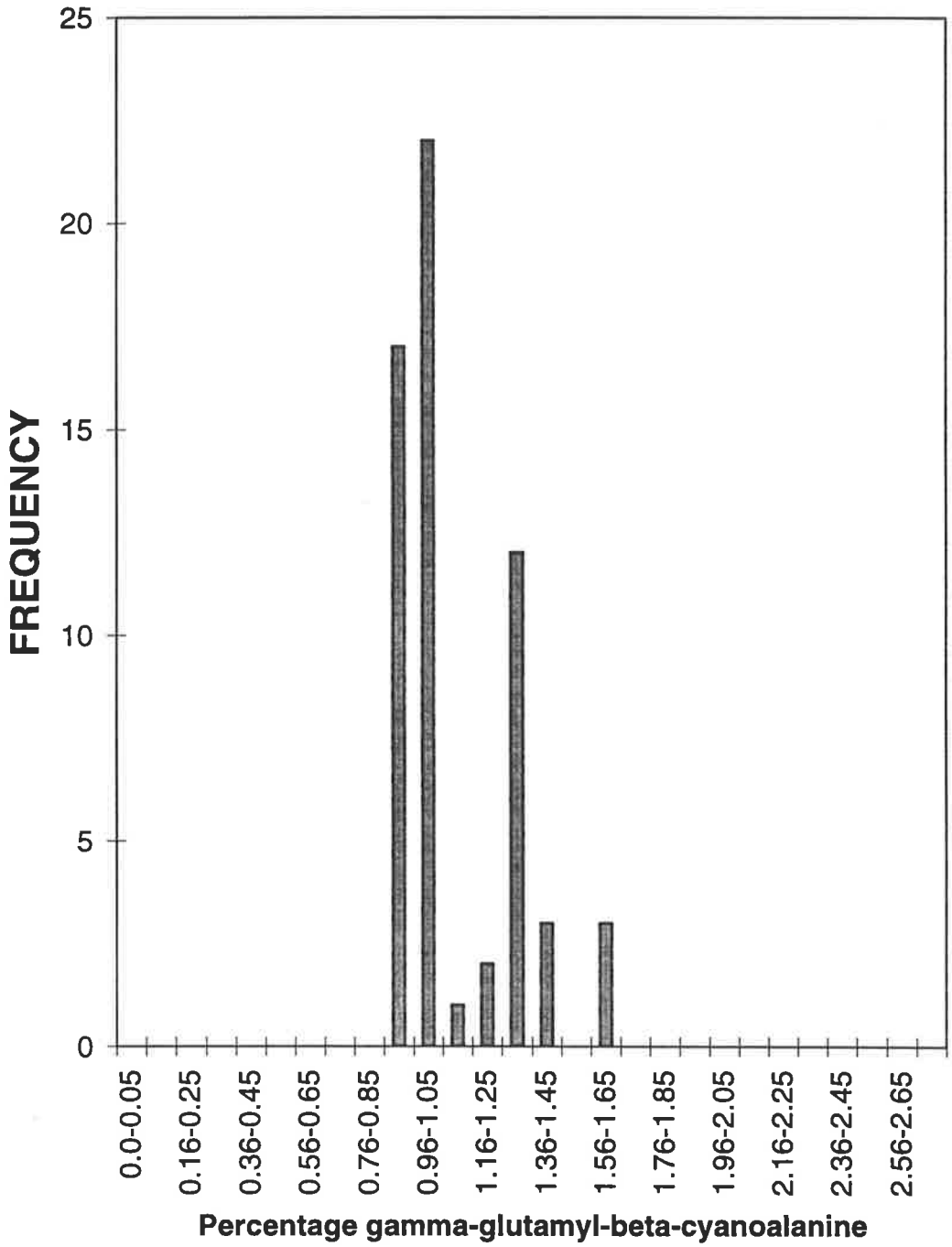


Figure 7.4 The frequency distribution of γ-glutamyl-β-cyanoalanine within multiple replicates of accession IFVI 1416 (*V. sativa* ssp. *sativa*) sourced from the International Centre for Agricultural Research in Dry Areas (ICARDA Aleppo, Syria). Total number of replicates, 60.

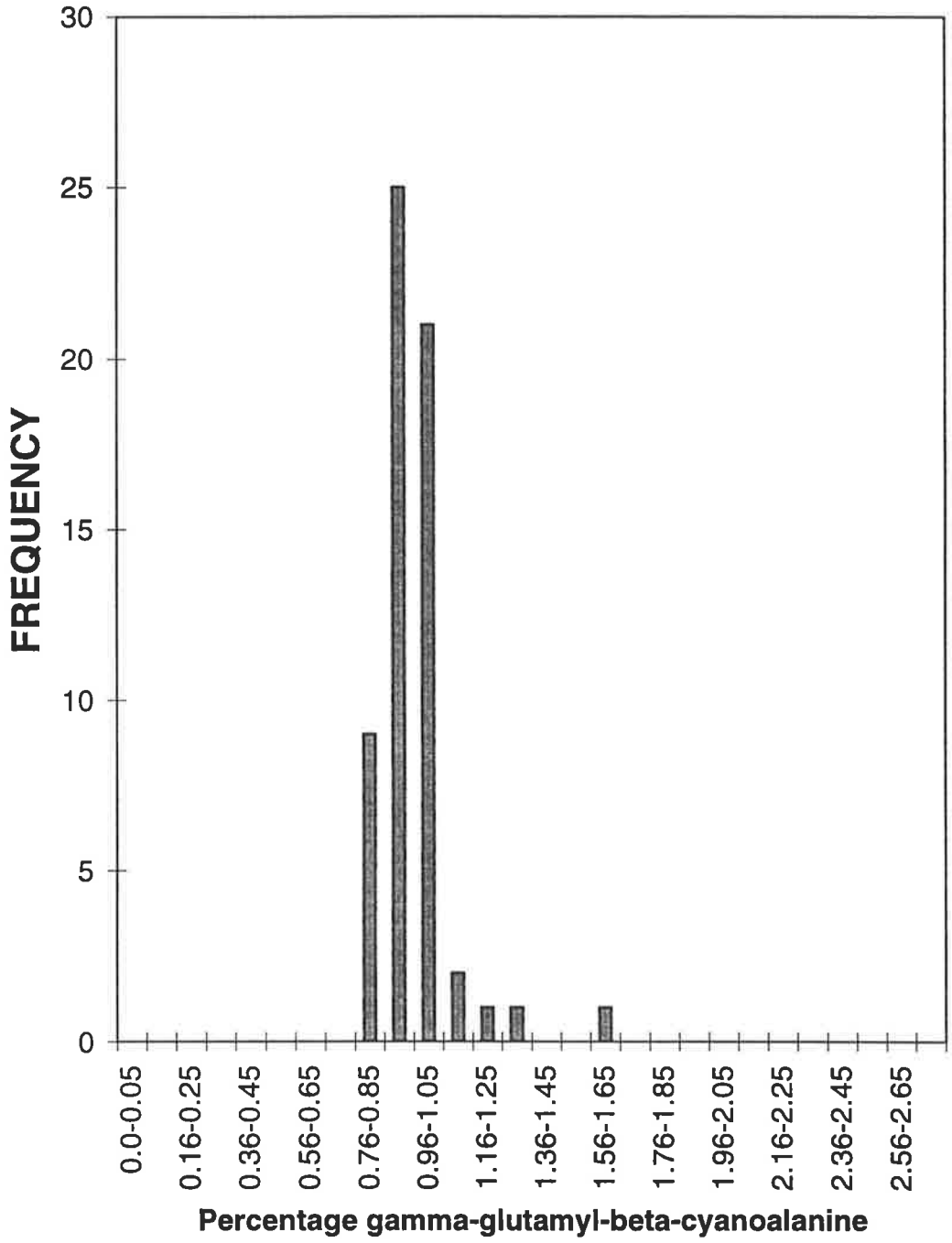


Figure 7.5 The frequency distribution of γ-glutamyl-β-cyanoalanine within multiple replicates of accession IFVI 2541 (*V. sativa* ssp. *sativa*) sourced from the International Centre for Agricultural Research in Dry Areas (ICARDA Aleppo, Syria). Total number of replicates, 60.

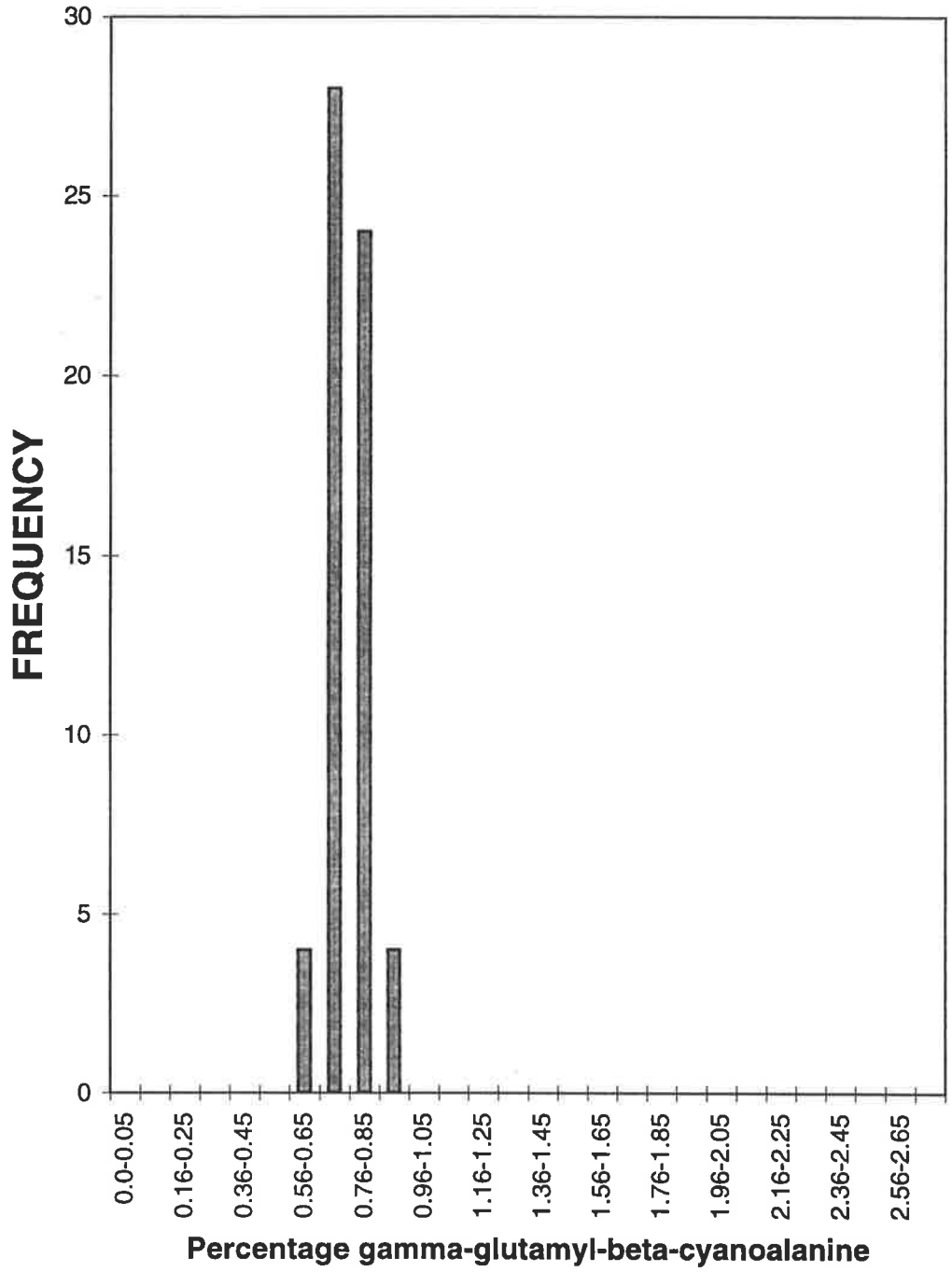


Figure 7.6 The frequency distribution of γ-glutamyl-β-cyanoalanine within multiple replicates of accession IFVI 2627 (*V. sativa* ssp. *sativa*) sourced from the International Centre for Agricultural Research in Dry Areas (ICARDA Aleppo, Syria). Total number of replicates, 60.

was able to germinate several seeds from CPI 60218 which had shown a low cyanoalanine non-protein amino acid content. The progeny of these old seeds once more expressed a higher concentration in the order of 0.7% γ-glutamyl-β-cyanoalanine in the cotyledons. This indicates that the cyanoalanine non-protein amino acids degrade during long term storage and that the age of the seed tested could have a large influence on the results and the interpretation of analysis of germplasm collections for these compounds.

In collaboration with Dr. Larry Robertson, single plots of 1647 accessions of *V. sativa* were grown at ICARDA, Aleppo, Syria in 1995. Seed of the accessions was dried, milled and sent to be examined for cyanoalanine concentration. This material showed a near normal distribution in the concentration of cyanoalanine non-protein amino acids throughout the germplasm collection. The initial examination by diffuse reflectance using dispersive infrared spectrometry identified ten potential low cyanoalanine non-protein amino acid (<0.2%) containing accessions. These accessions were examined by micellar electrokinetic chromatography to investigate the amino acid and non-protein amino acid profiles of the homogenised plant material and showed that the ten accessions contained γ-glutamyl-β-cyanoalanine and vicine while also containing other non-protein amino acids (Appendix C1-10).

The ten accessions were imported under quarantine regulations as whole seed. Individual seeds were classified using morphological traits (Ms. Jane Rathjen and Dr. Jennifer Gardener, University of Adelaide) and examined for chemical traits on a single seed basis using the small cup diffuse reflectance using infrared dispersive spectrometry and micellar electrokinetic chromatography methods. The accessions were shown to consist of mixtures of species and that *V. sativa* was a minor component of these mixtures. The authentic *V. sativa* single seeds in these mixtures contained in the order of 0.7% γ-glutamyl-β-cyanoalanine in the cotyledons. Hence, the screening of 1647 plots of *V. sativa* ssp. *sativa* from ICARDA for low cyanoalanine accessions was unable to identify an accession expressing a very low cyanoalanine non-protein amino acid content.

However, quantification of cyanoalanine non-protein amino acids by diffuse reflectance using infrared spectrometry identified one accession with 0.36-0.45% and three accessions with 0.36-0.55% cyanoalanine content. This plant material is now in the *V. sativa* breeding program (Ms. Jane Rathjen). Subsequently, examinations of the *V. sativa* ssp. *macrocarpa* and *V. sativa* ssp. *nigra* collections from ICARDA have been initiated to identify low or zero cyanoalanine non-protein amino acid containing accessions within these taxonomic subspecies.

The effect of environment on the deposition of γ-glutamyl-β-cyanoalanine in mature plants in a population of *V. sativa* has not previously been reported. The enviromental checks show that a narrow range of cyanoalanine non-protein amino acid concentrations were expressed within accessions due to the effect of different plots at a single site (Figures 7.4, 7.5 and 7.6). Therefore, although the samples

of ICARDA germplasm were obtained from single plots, the low level of enviromental variation within a site indicates that variation observed across accessions was due to genetic, rather that environmental effects. This result contrasts the variation of γ-glutamyl-β-cyanoalanine deposition between seeds within a pod, described in chapter six.

The 711 accession of *V. sativa* sourced from the Vavilov Institute through Mr Rade Matic, SARDI were of unknown age and were without passport data. Quantification of cyanoalanine non-protein amino acids by diffuse reflectance using infrared spectrometry identified thirty six accessions with 0.0-0.05%, three accessions with 0.06-0.16%, and three accessions with 0.16-0.25% cyanoalanine content. Limitations with respect to documentation and age of this material means that its usefulness for breeding must await its regeneration, taxonomic verification and a re-evaluation of the cyanoalanine non-protein amino acid concentration in the progeny of the identified plant material.

7.5 Conclusion

This chapter demonstrates the successful application of the diffuse reflectance using infrared dispersive spectrometry and micellar electrokinetic chromatography methods in the identification of low cyanoalanine non-protein amino acid containing accessions from *V. sativa* germplasm collections. The complementary analyses correctly identified ten potential low cyanoalanine non-protein amino acid containing accessions from the ICARDA collection and provided crucial evidence for the reselection and reclassification of that particular plant material. Although the "Holy Grail" of a low cyanoalanine non-protein amino acid (<0.2%) accession has not been identified among the ICARDA accessions, this chapter shows that the tools are now in place to rapidly identify low cyanoalanine containing plant material wherever it may be, in any of the world's *V. sativa* germplasm collections. This work also establishes the importance of proper passport documentation and maintenance of germplasm collections to preserve genetic diversity before it is lost through ageing. The international collaboration typified by the interaction of ICARDA, CLIMA and the University of Adelaide in the analysis of the *V. sativa* collection, has established a sound basis for the ultimate breeding of a new legume crop, the low or zero cyanoalanine non-protein amino acid containing common vetch.

Appendix A

Table 1 Experimental conditions for X-ray crystallographic data collection.

Instrument	AFC-6R diffractometer
Measured reflections	1638
Independent reflections	1548
Observed reflections [I>3.0σ(I)]	698
Scanning mode	ω/2θ
Radiation	Monochromated MoKα, λ=0.7107 A.
Temperature	290°K
Crystal dimensions (mm)	0.57 x 0.08 x 0.02
Crystal discription	Monoclinic, needle, colourless
θ range/°	7.6-22.4

Appendix A

Table 2. Fractional atomic coordinates and equivalent isotropic displacement parameters (A2)

$$Ueq = (1/3)\Sigma_i\Sigma_j U_{ij} a_i^* a_j^* \mathbf{a}_i.\mathbf{a}_j.$$

	X	Y	Z	Ueq
O(1)	0.6086 (7)	0.3687	0.4588 (4)	0.030 (3)
O(2)	0.8095 (6)	0.134 (2)	0.4719 (4)	0.030 (3)
O(3)	0.7151 (10)	0.372 (2)	0.0487 (5)	0.060 (4)
O(4)	0.6194 (7)	-0.029 (2)	-0.2528 (5)	0.044 (4)
O(5)	0.7865 (7)	-0.344 (2)	-0.2230 (5)	0.036 (3)
N(1)	0.4857 (8)	-0.028 (2)	0.3447 (5)	0.025 (4)
N(2)	0.7372 (9)	-0.040 (2)	-0.0241 (5)	0.031 (4)
N(3)	1.0382 (9)	0.150 (3)	-0.3075 (6)	0.053 (5)
N(4)	0.1135 (7)	0.122 (2)	0.4261 (5)	0.025 (3)
C(1)	0.688 (1)	0.182 (3)	0.4321 (7)	0.021 (4)
C(2)	0.6420 (9)	-0.006 (3)	0.3428 (6)	0.021 (4)
C(3)	0.6914 (9)	0.134 (3)	0.2436 (6)	0.024 (4)
C(4)	0.642 (1)	-0.017 (3)	0.1466 (6)	0.032 (4)
C(5)	0.700 (1)	0.118 (3)	0.0545 (6)	0.028 (5)
C(6)	0.784 (1)	0.060 (3)	-0.1218 (6)	0.022 (4)
C(7)	0.724 (1)	-0.116 (3)	-0.2074 (6)	0.021 (4)
C(8)	0.948 (1)	0.061 (3)	-0.1228 (7)	0.046 (6)
C(9)	1.000 (1)	0.116 (3)	-0.2243 (8)	0.041 (5)

Appendix A

Table 3 Selected geometric parameters (A, °)

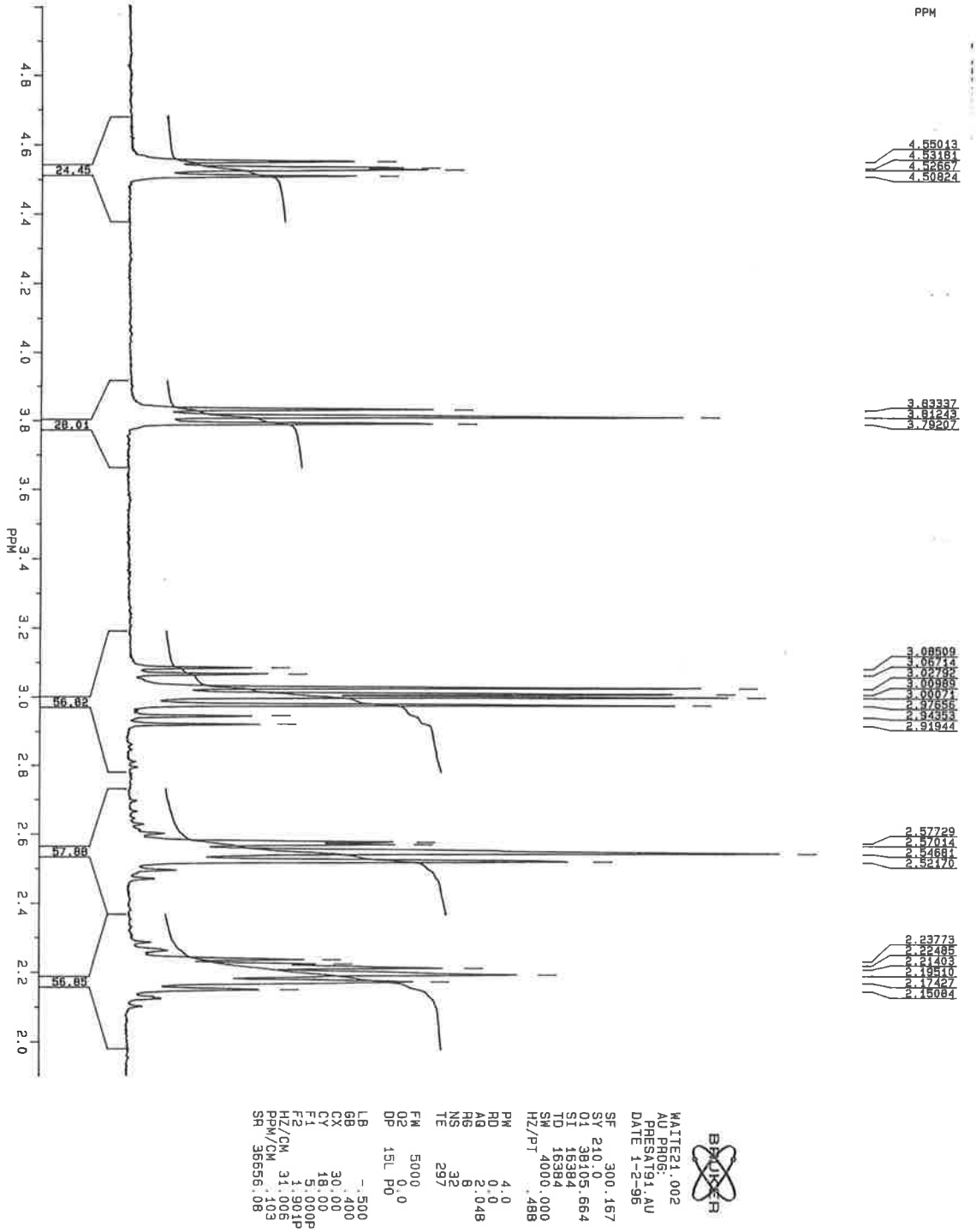
O(1)-C(1)	1.21 (1)	N(3)-C(9)	1.15 (1)
O(2)-C(1)	1.266 (9)	C(1)-C(2)	1.52 (1)
O(3)-C(5)	1.22 (1)	C(2)-C(3)	1.52 (1)
O(4)-C(7)	1.21 (1)	C(3)-C(4)	1.52 (1)
O(5)-C(7)	1.25 (1)	C(4)-C(5)	1.46 (1)
N(1)-C(2)	1.47 (1)	C(6)-C(7)	1.50 (1)
N(2)-C(5)	1.32 (1)	C(6)-C(8)	1.53 (1)
N(2)-C(6)	1.43 (1)	C(8)-C(9)	1.43 (1)
C(5)-N(2)-C(6)	125.4 (8)	O(3)-C(5)-C(4)	122.2 (8)
O(1)-C(1)-O(2)	124 (1)	N(2)-C(5)-C(4)	118.5 (8)
O(1)-C(1)-C(2)	118.9 (9)	N(2)-C(6)-C(7)	110.6 (8)
O(2)-C(1)-C(2)	116.7 (9)	N(2)-C(6)-C(8)	108.9 (8)
N(1)-C(2)-C(1)	107.6 (7)	C(7)-C(6)-C(8)	111.6 (8)
N(1)-C(2)-C(3)	110.7 (7)	O(4)-C(7)-O(5)	126.6 (10)
C(1)-C(2)-C(3)	107.4 (8)	O(4)-C(7)-C(6)	117.8 (10)
C(2)-C(3)-C(4)	113.5 (8)	O(5)-C(7)-C(6)	115.5 (9)
C(3)-C(4)-C(5)	110.8 (9)	C(6)-C(8)-C(9)	110.8 (8)
O(3)-C(5)-N(2)	119.3 (8)	N(3)-C(9)-C(8)	177 (1)

Appendix A

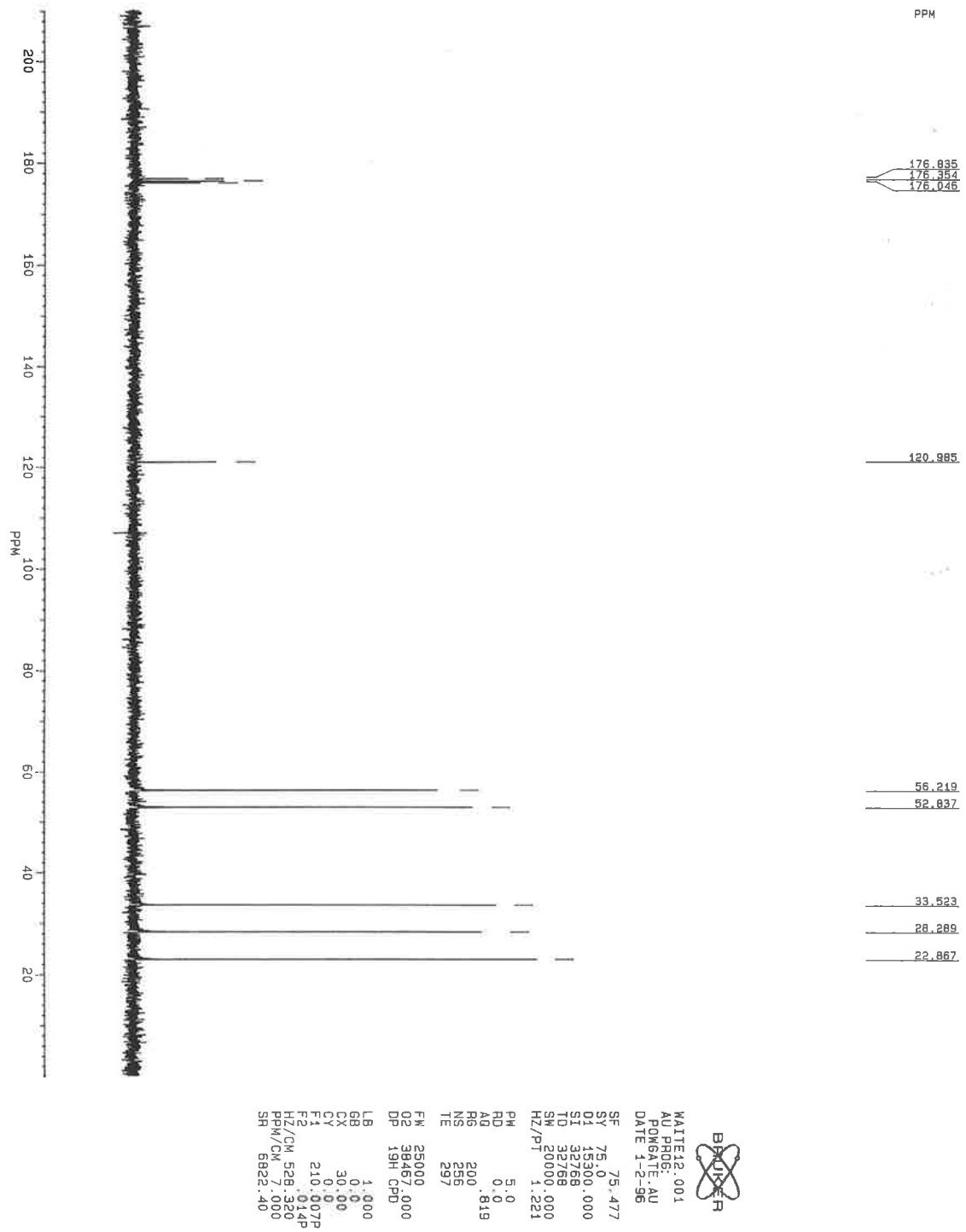
Table 4 Contact distances (A)

O(1)...N(1^{i})	2.748 (9)	O(4)...N(1^{v})	2.83 (1)
O(2)...N(4^{i})	2.77 (1)	O(4)...N(1^{vi})	2.84 (1)
O(2)...N(4^{ii})	2.86 (1)	O(5)...N(1^{v})	3.11 (1)
O(2)...N(4^{iii})	2.908 (9)	N(3)...N(4^{vi})	3.07 (1)
O(3)...N(2^{iv})	2.96 (1)	N(3)...N(4^{v})	3.27 (1)

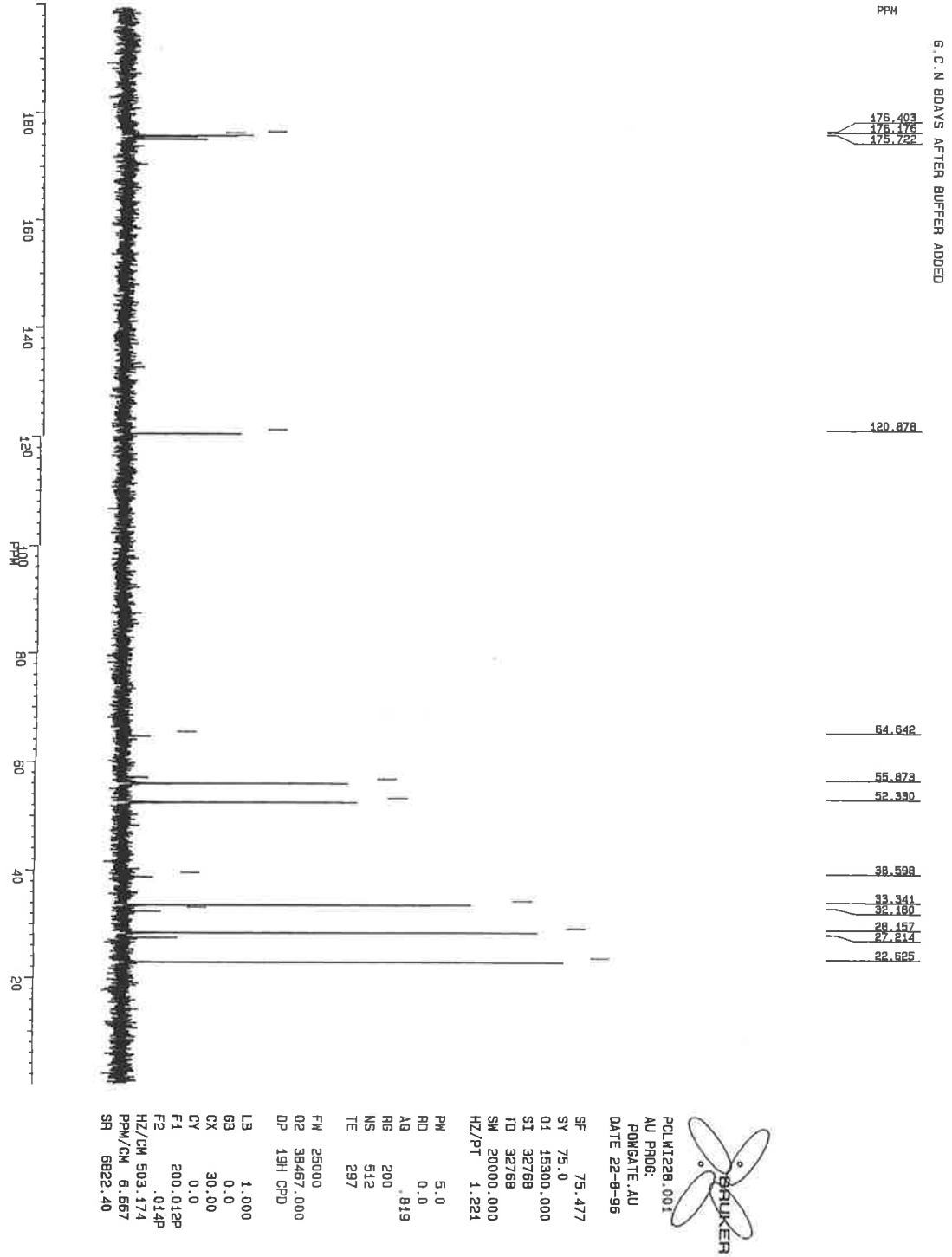
Symmetry codes: (i) 1-*x*, $^{1}/_{2}$+*y*, 1-*z*; (ii) 1-*x*, *y*-$^{1}/_{2}$, 1-*z*; (iii) 1+*x*, *y*, *z*; (iv) *x*, 1+*y*, *z*; (v) 1-x, *y*-$^{1}/_{2}$, -z; (vi) 1-*x*, $^{1}/_{2}$+*y*, -*z*.



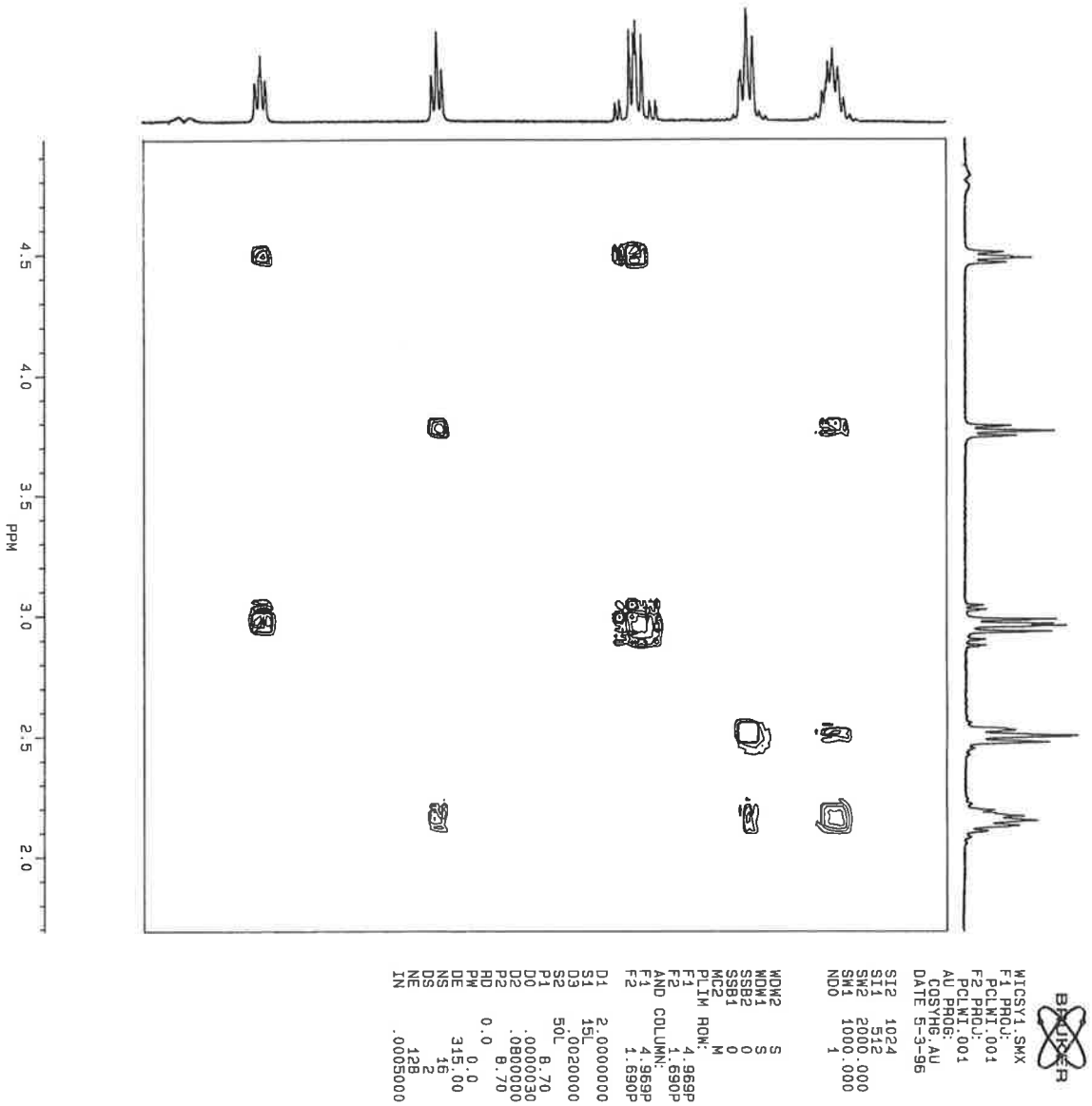
Appendix B1 Proton NMR of γ-glutamyl-β-cyanoalanine.



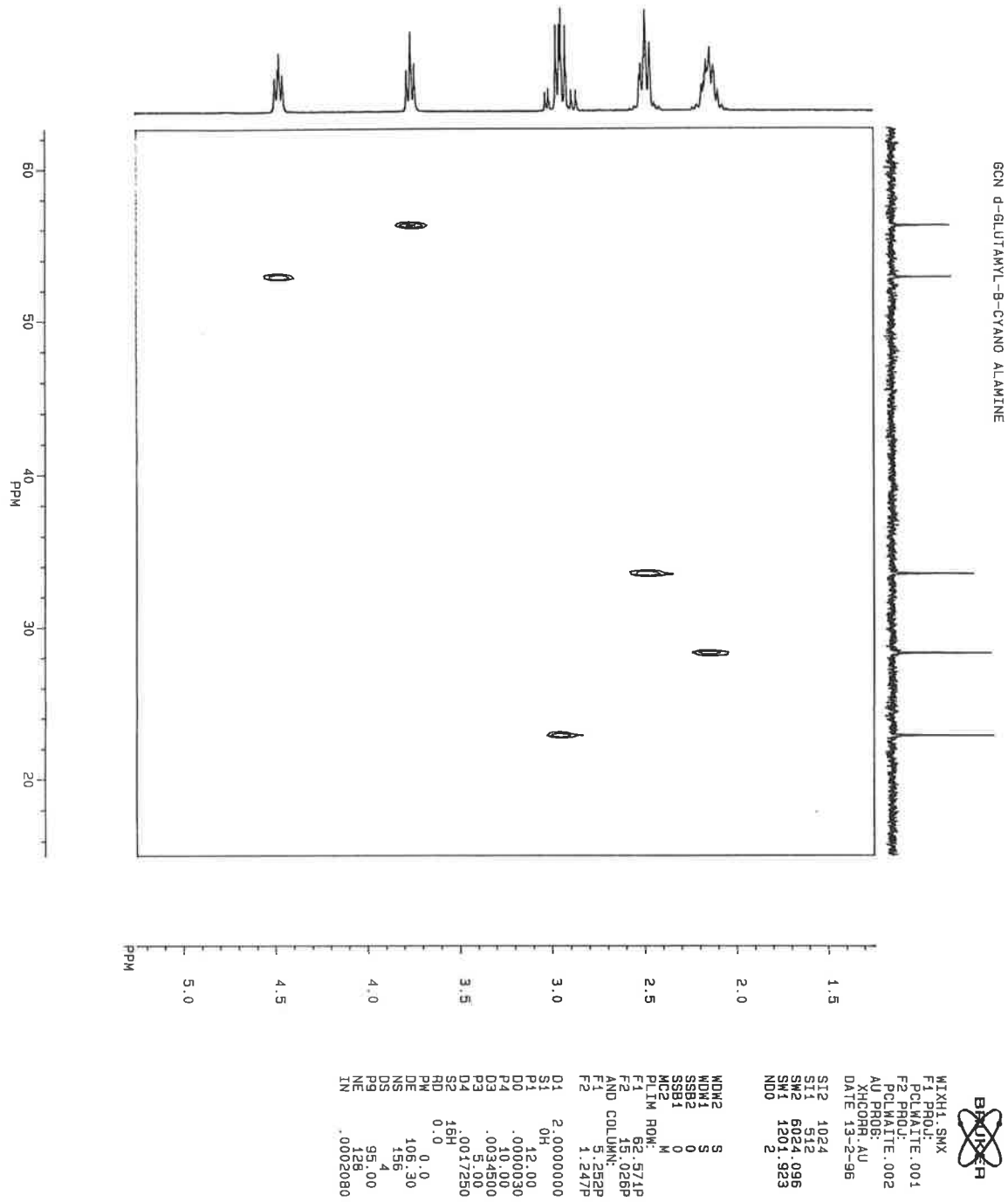
Appendix B2 Carbon NMR for γ-glutamyl-β-cyanoalanine at pD6.8.



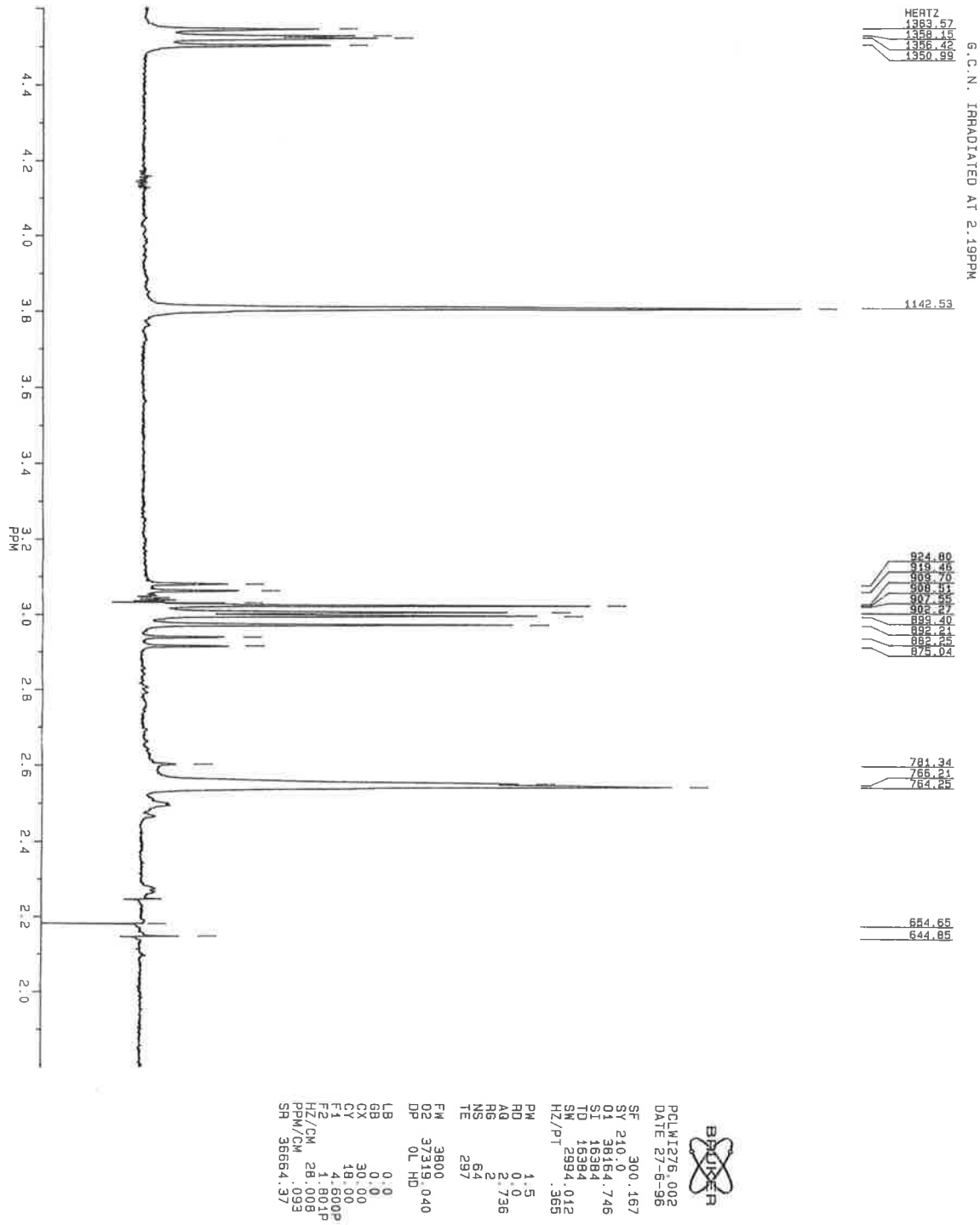
Appendix B3 Carbon NMR of γ-glutamyl-β-cyanoalanine at pD 4.9.



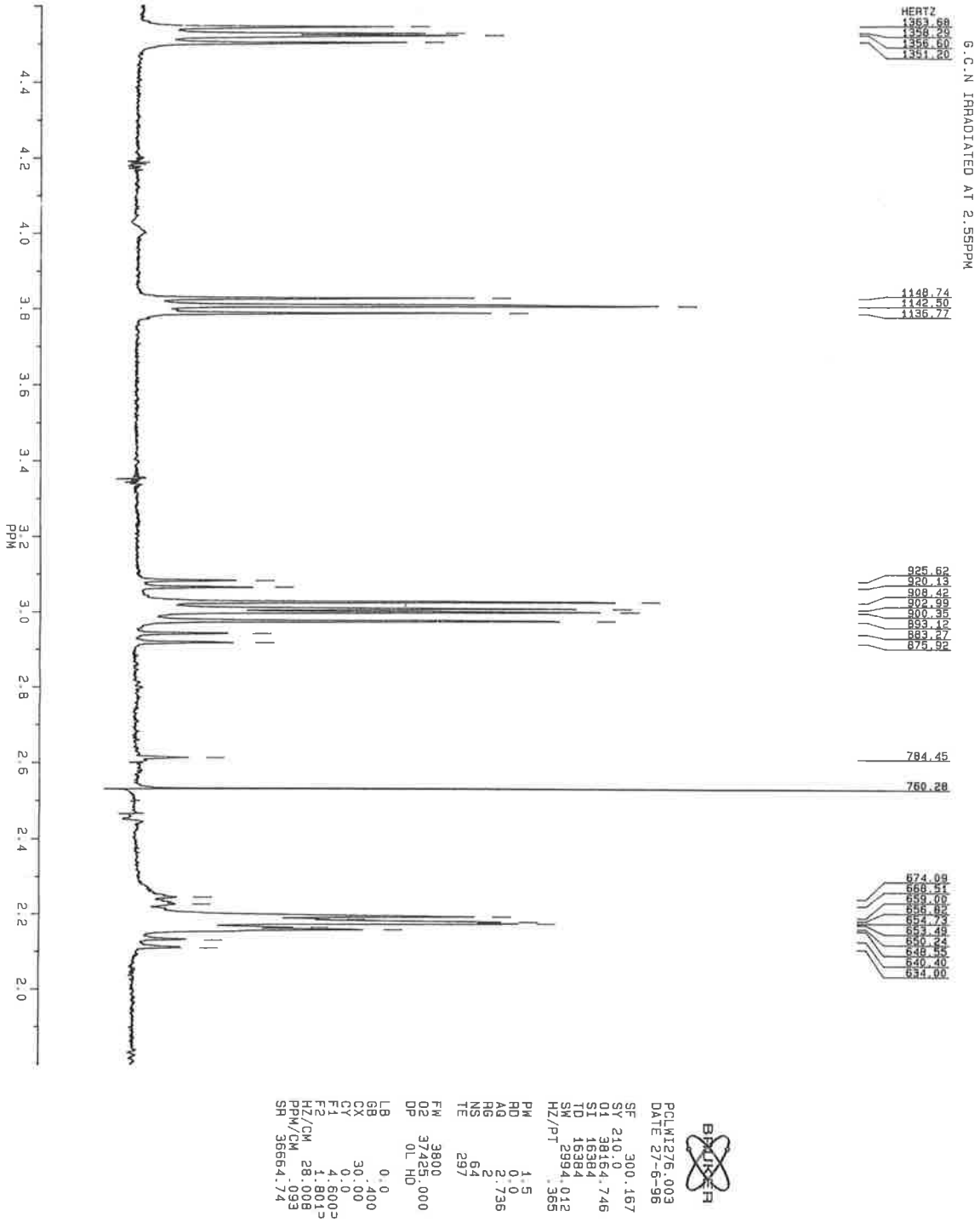
Appendix B4 Proton/proton connectivity correlations collected using the 2D COSY pulse sequence for γ-glutamyl-β-cyanoalanine.



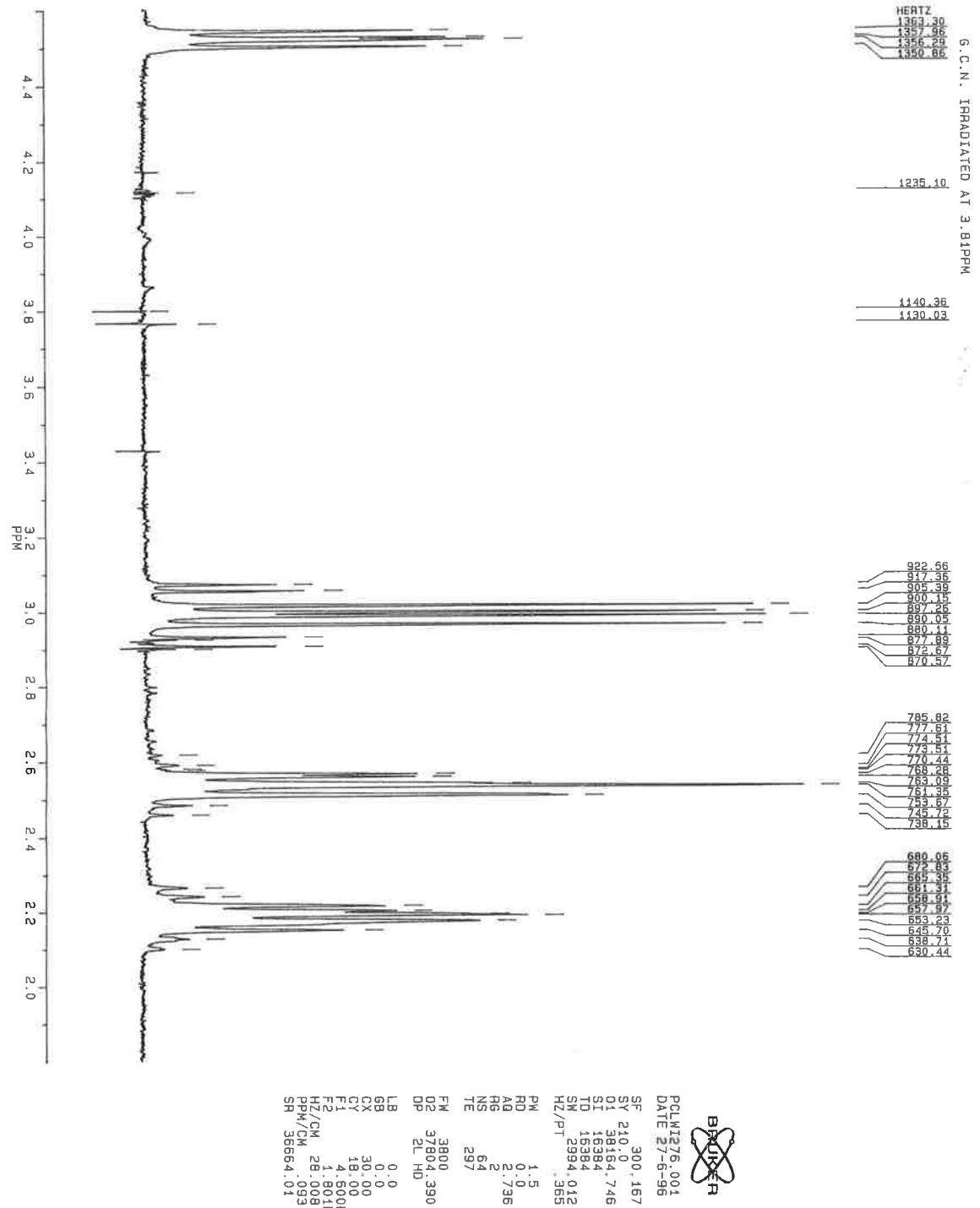
Appendix B5 Proton/carbon connectivity correlations collected using the 2D HETCOR pulse sequence for γ-glutamyl-β-cyanoalanine.



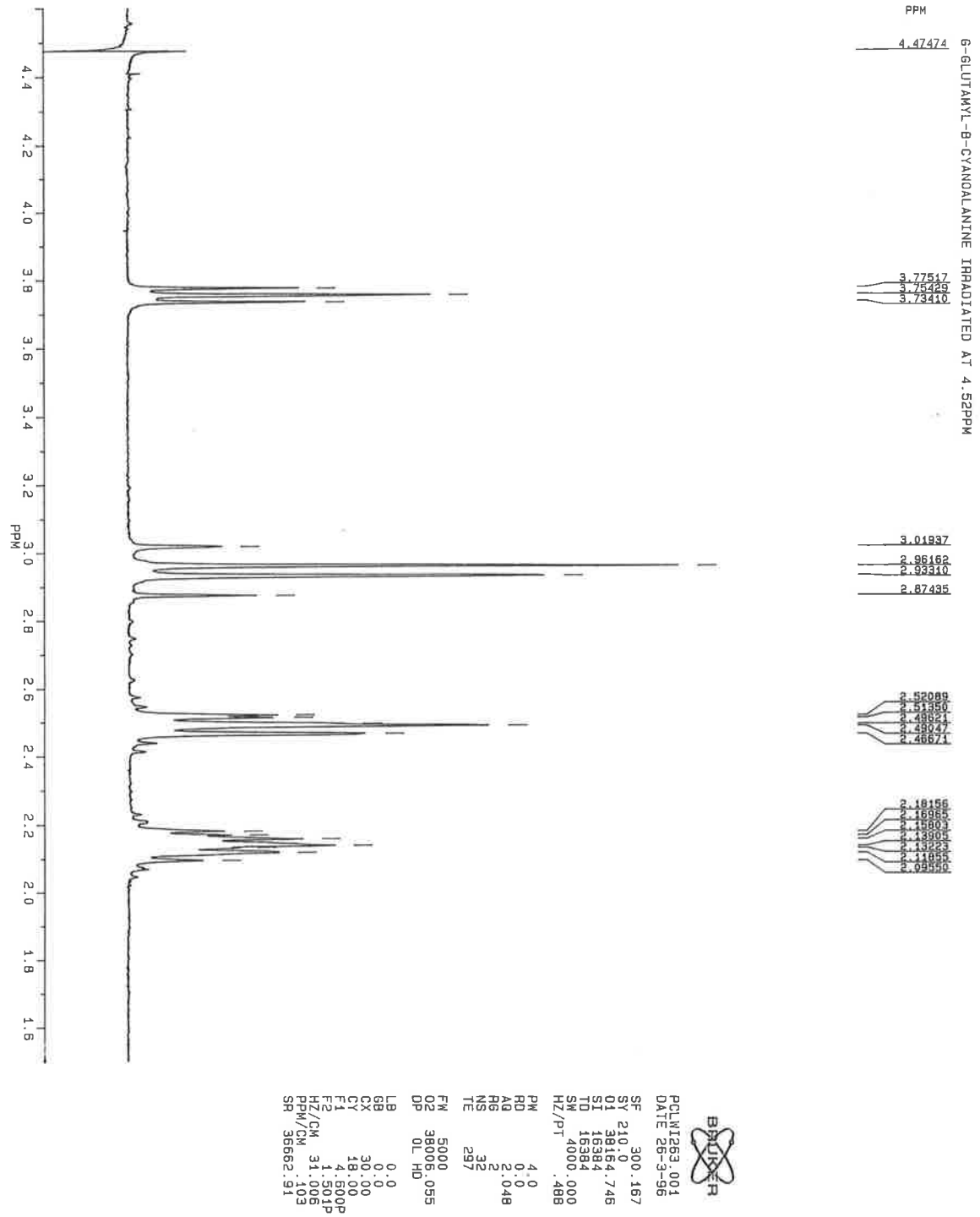
Appendix B6 Proton NMR for a proton decoupling experiment on γ-glutamyl-β-cyanoalanine irradiated at 2.19ppm.



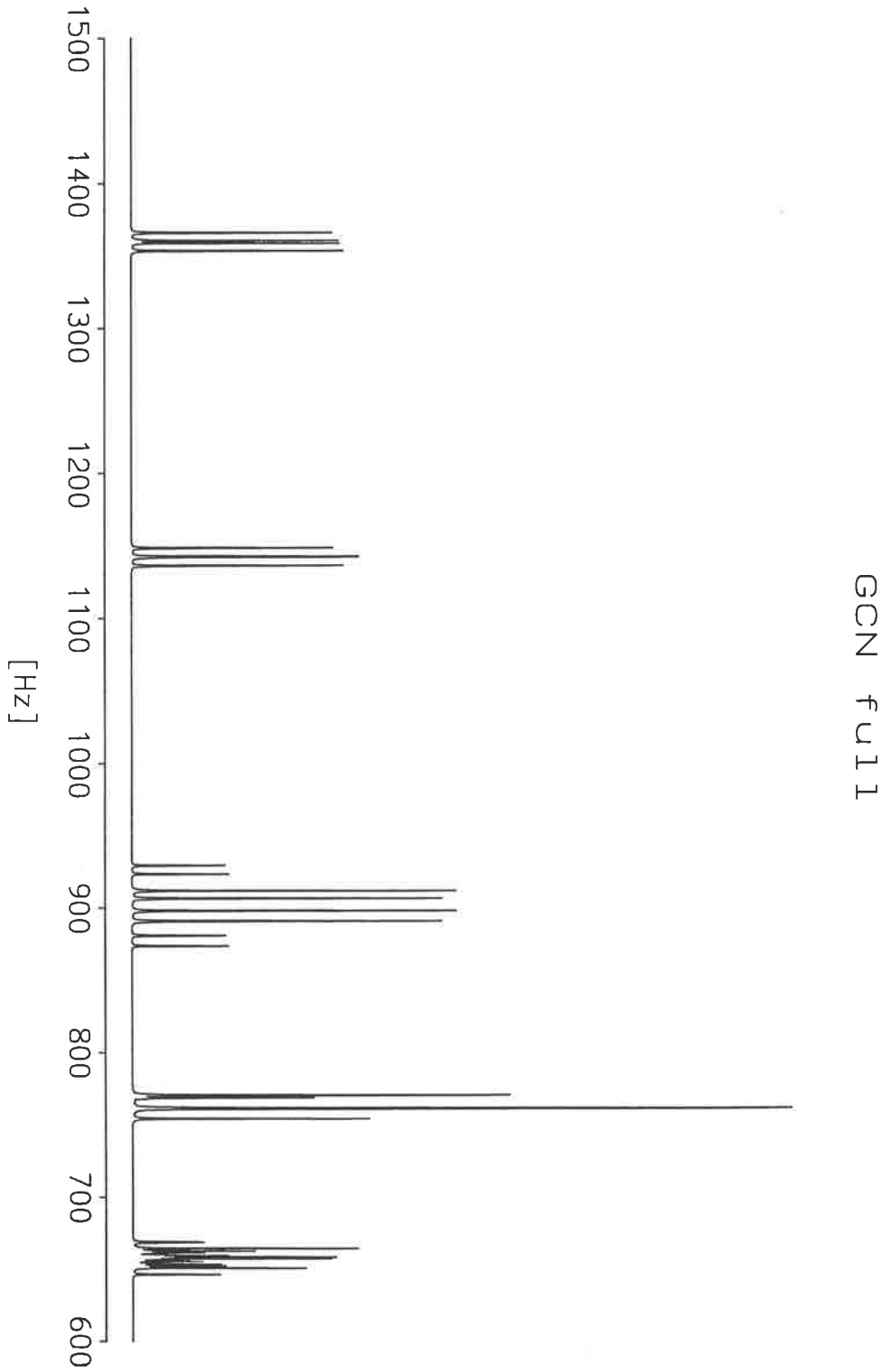
Appendix B7 Proton NMR for a proton decoupling experiment on γ-glutamyl-β-cyanoalanine irradiated at 2.55ppm.



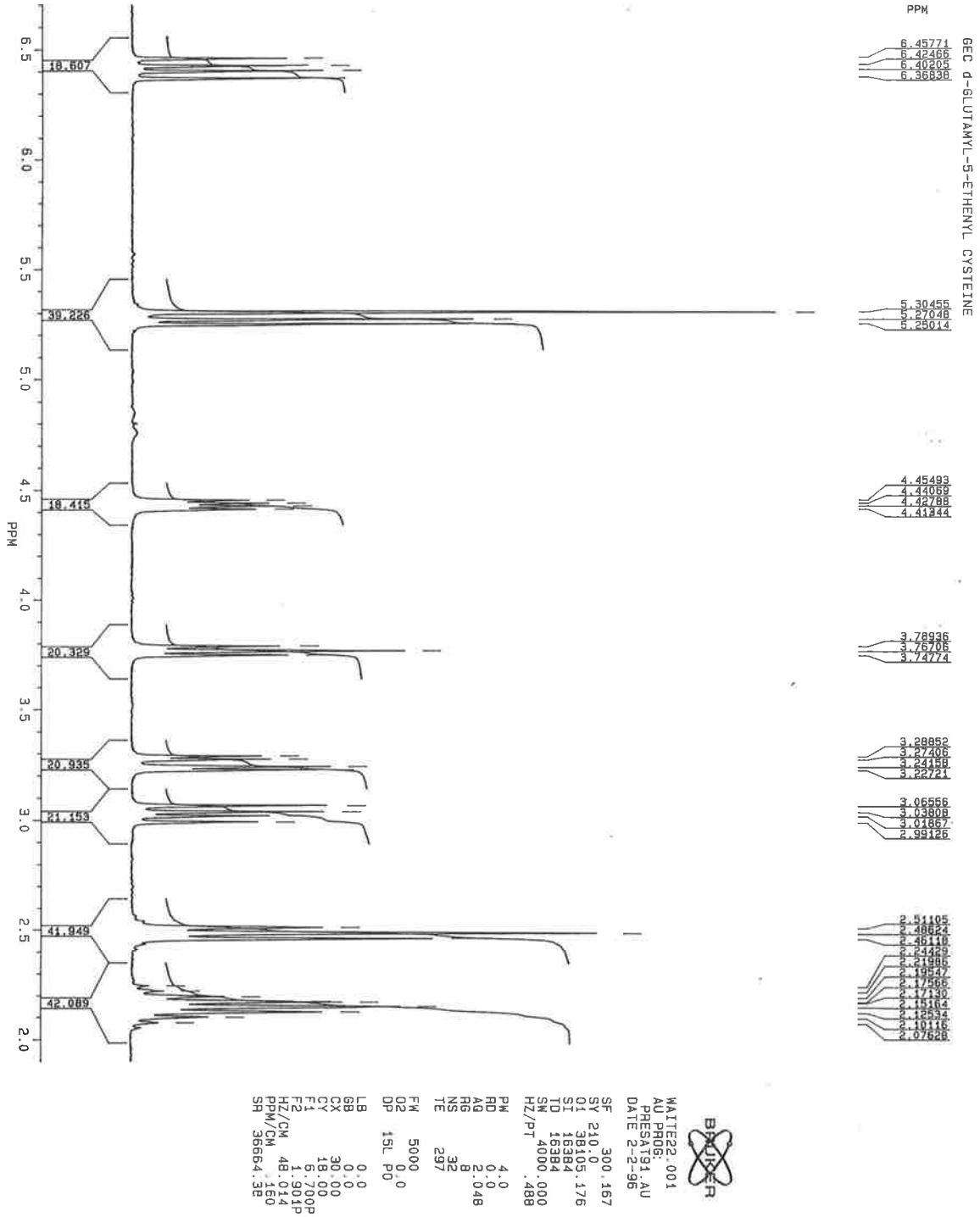
Appendix B8 Proton NMR for a proton decoupling experiment on γ-glutamyl-β-cyanoalanine irradiated at 3.81ppm.



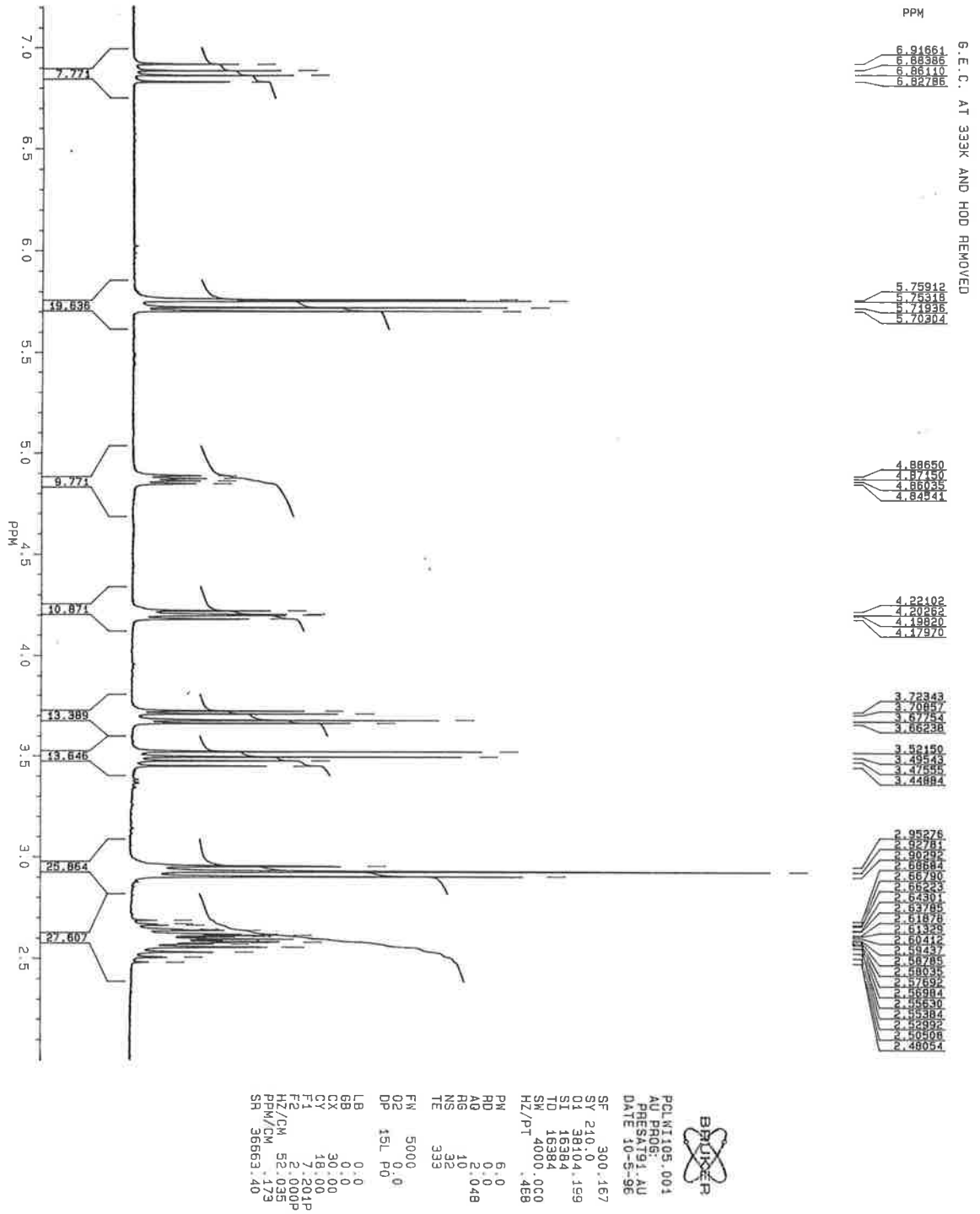
Appendix B9 Proton NMR for a proton decoupling experiment on γ-glutamyl-β-cyanoalanine irradiated at 4.52ppm.



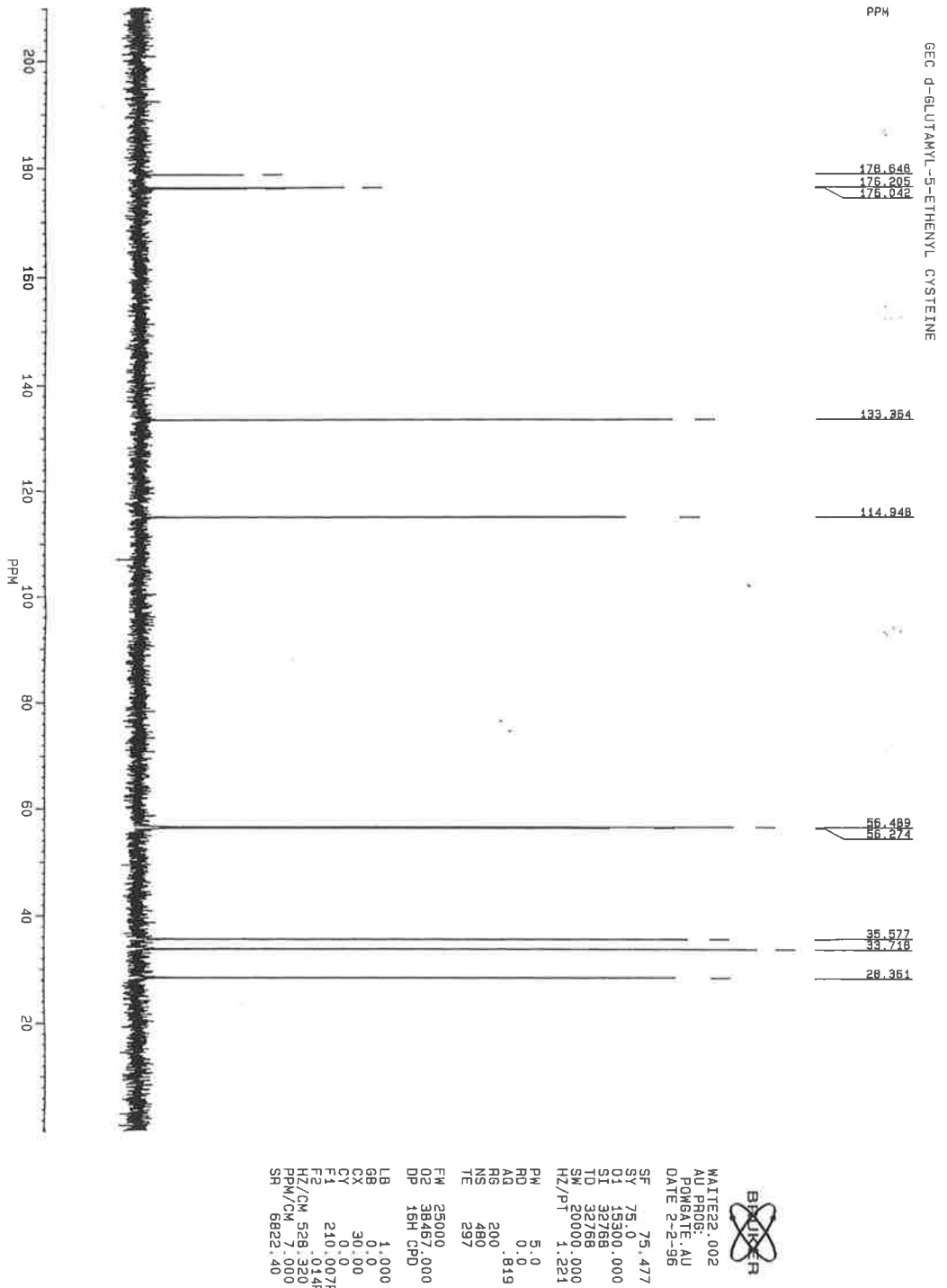
Appendix B10 Proton NMR simulation (Hagele *et al.* 1993) of γ-glutamyl-β-cyanoalanine using the chemical shift and coupling constant data in Table 3.3.



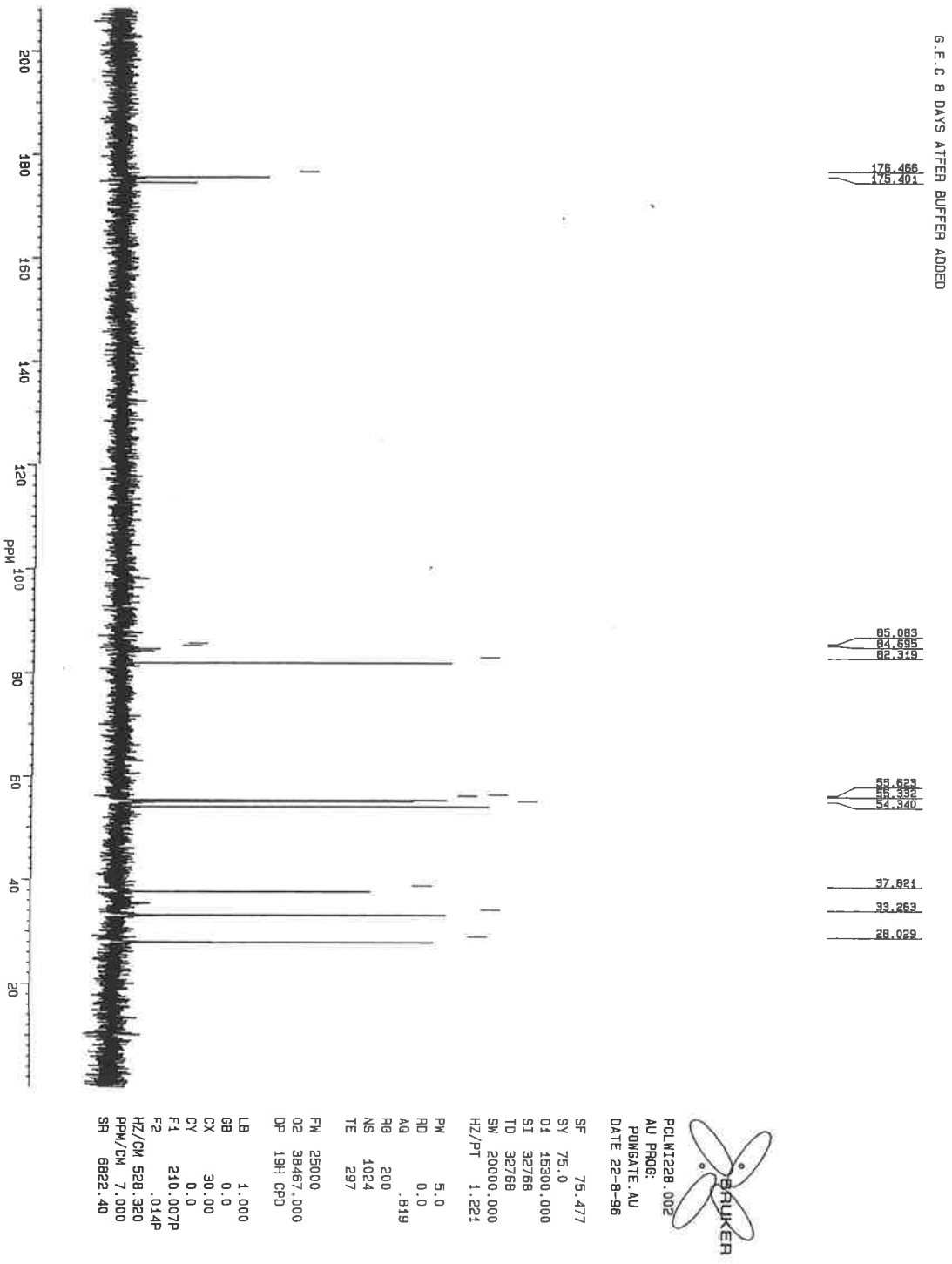
Appendix B11 Proton NMR of γ-glutamyl-S-ethenyl-cysteine at 297°K.



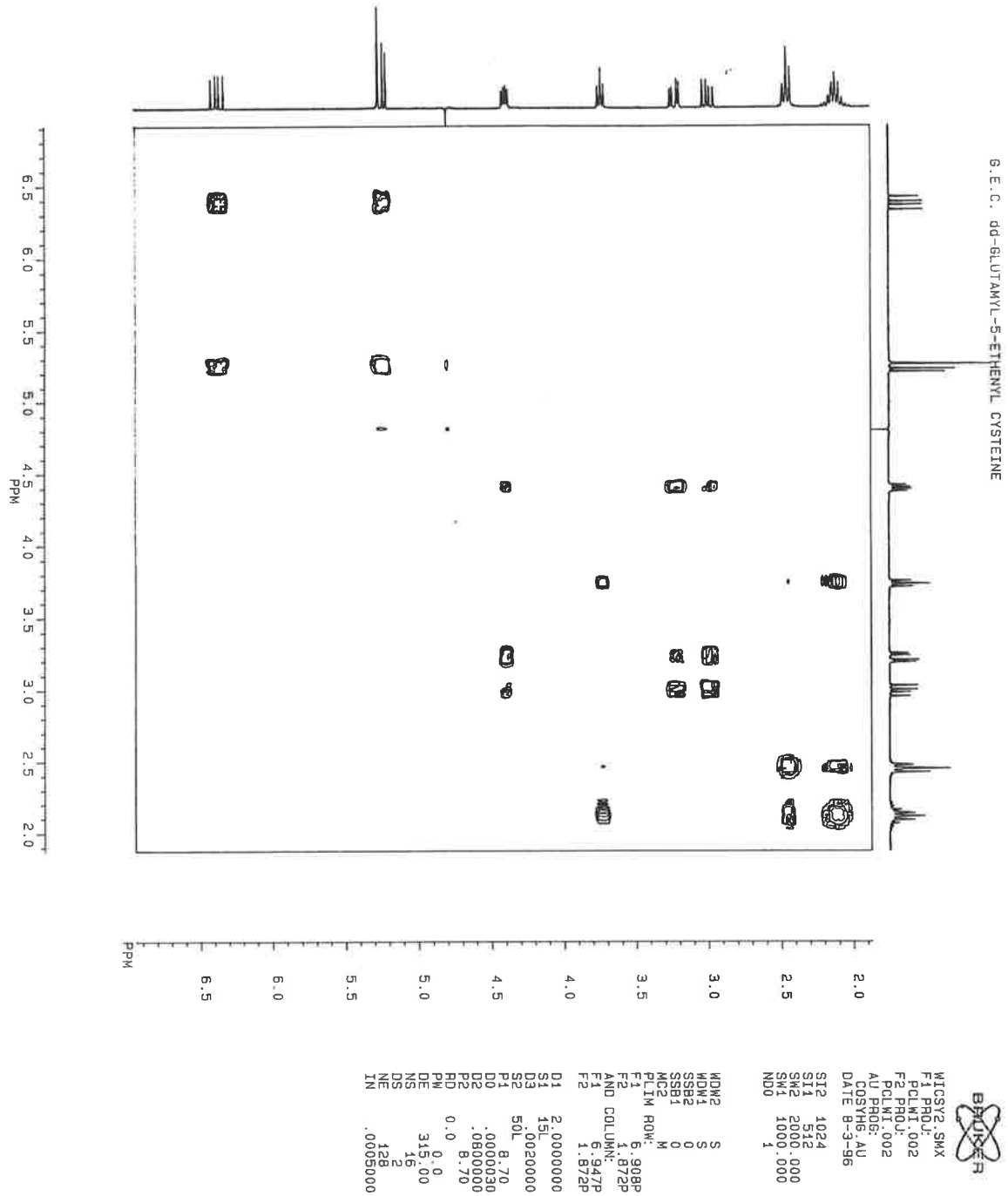
Appendix B12 Proton NMR of γ-glutamyl-S-ethenyl-cysteine at 333°K.



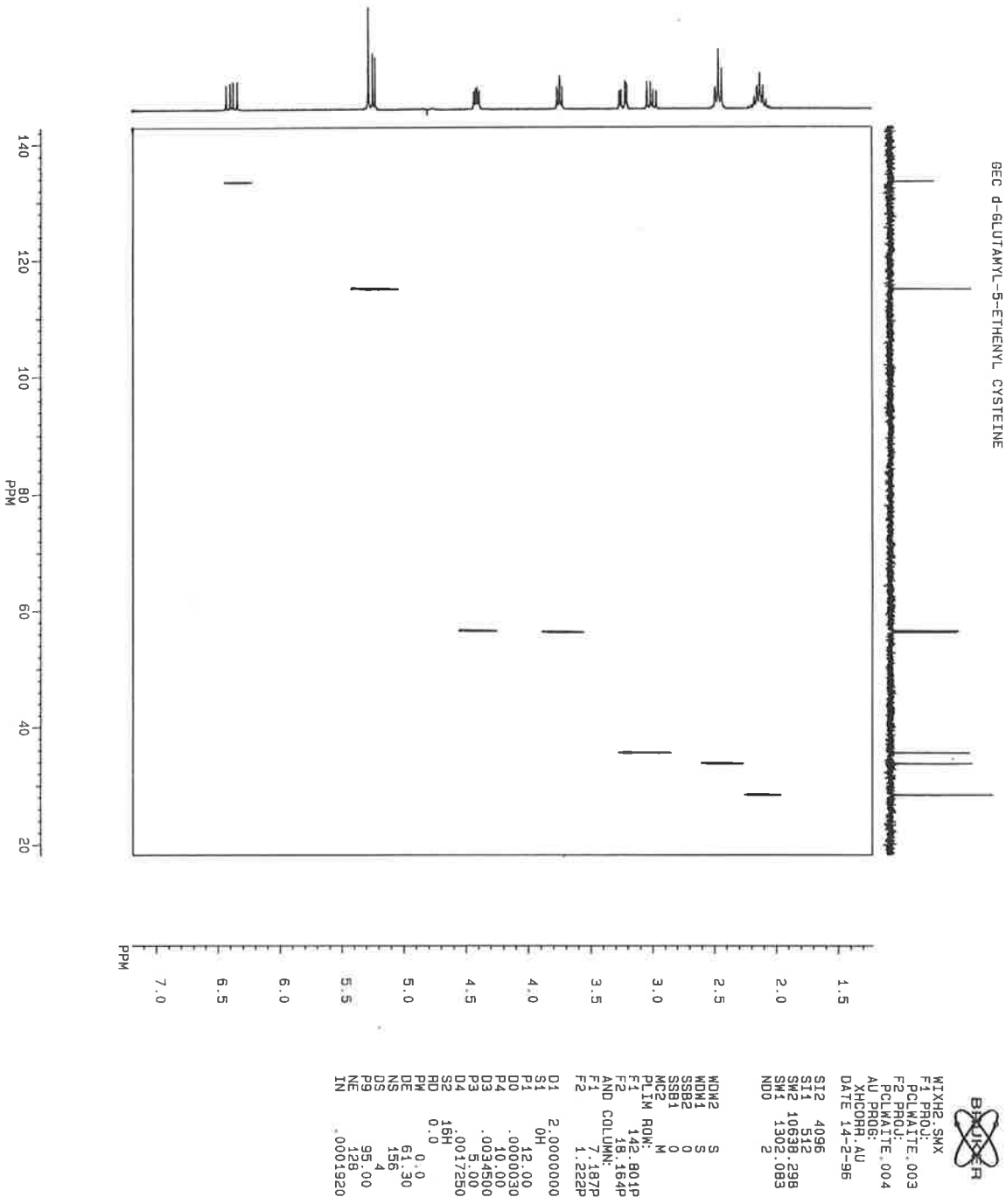
Appendix B13 Carbon NMR for γ-glutamyl-S-ethenyl-cysteine at pD 6.0.



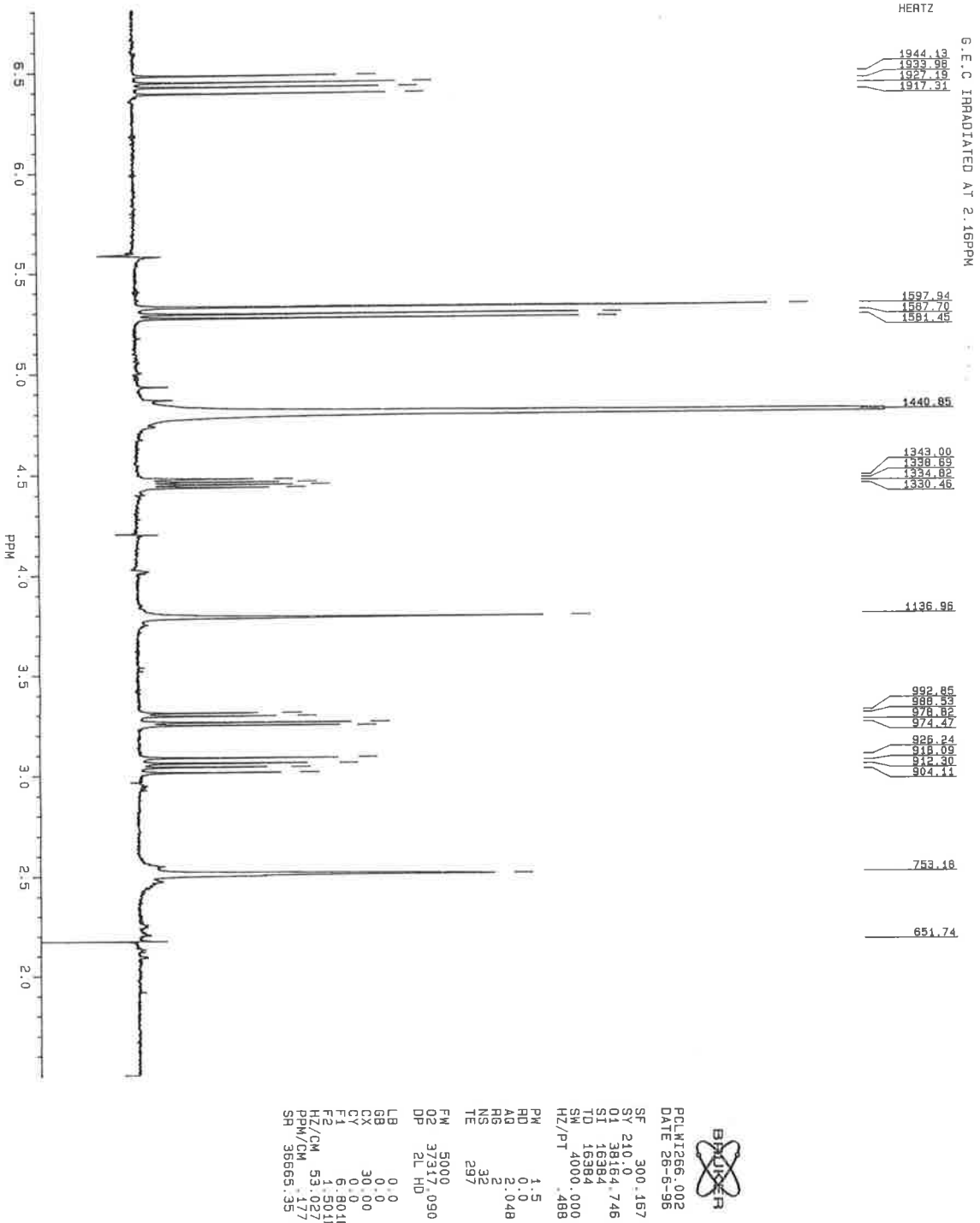
Appendix B14 Carbon NMR for γ-glutamyl-S-ethenyl-cysteine at pD 4.8.



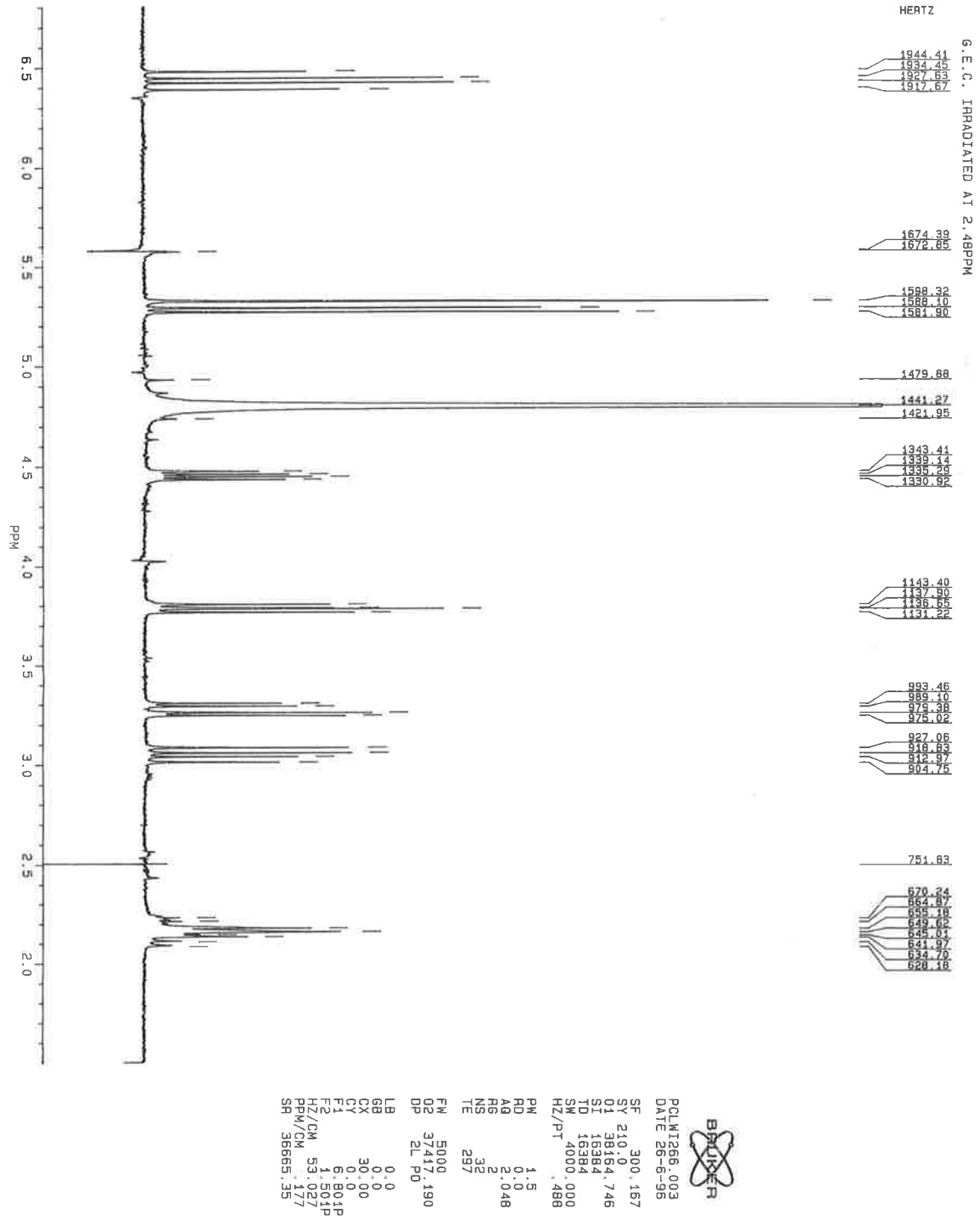
Appendix B15 Proton/proton connectivity correlations collected using the 2D COSY pulse sequence for γ-glutamyl-S-ethenyl-cysteine.



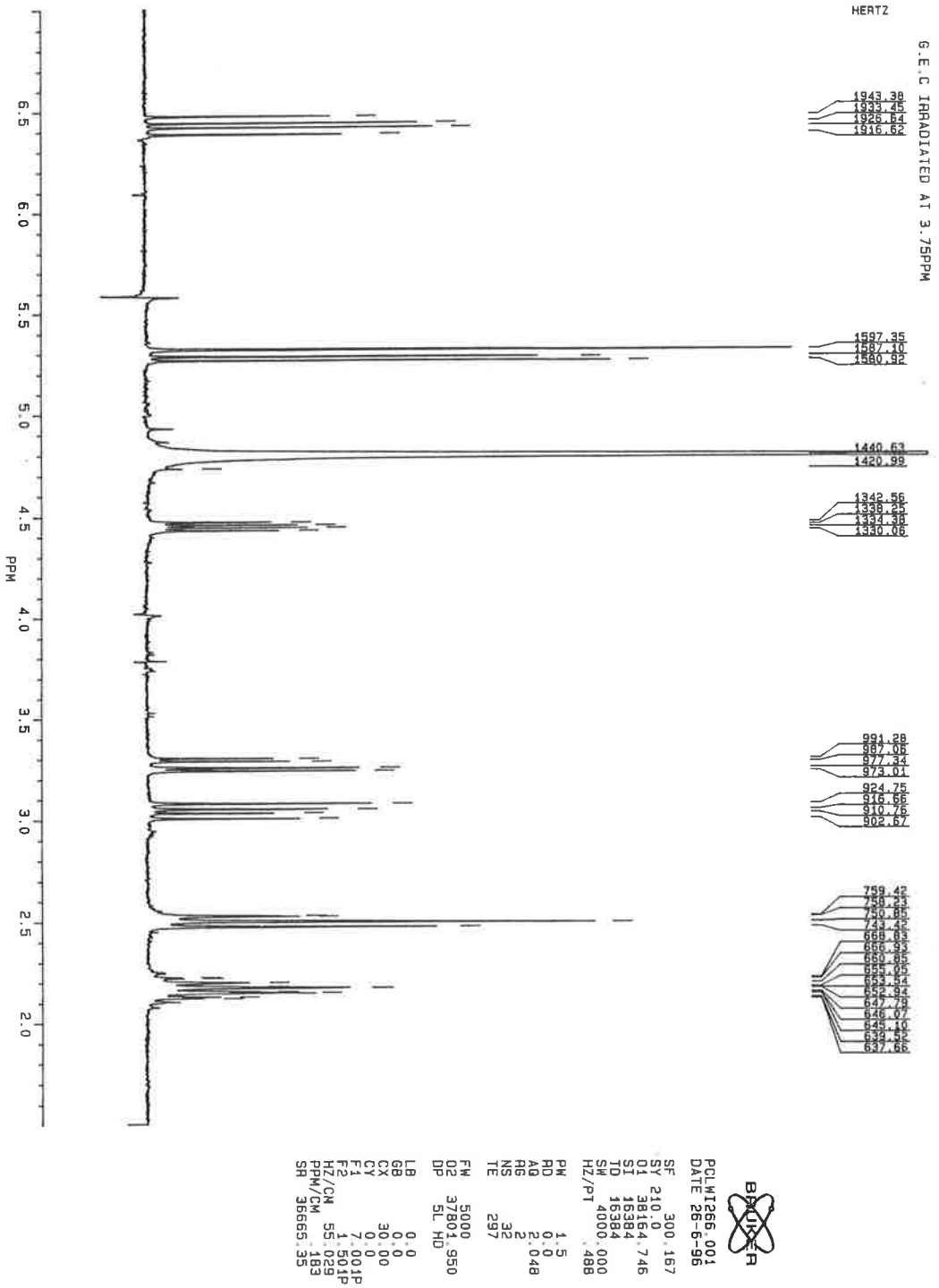
Appendix B16 Proton/carbon connectivity correlations collected using the 2D HETCOR pulse sequence for γ-glutamyl-S-ethenyl-cysteine.



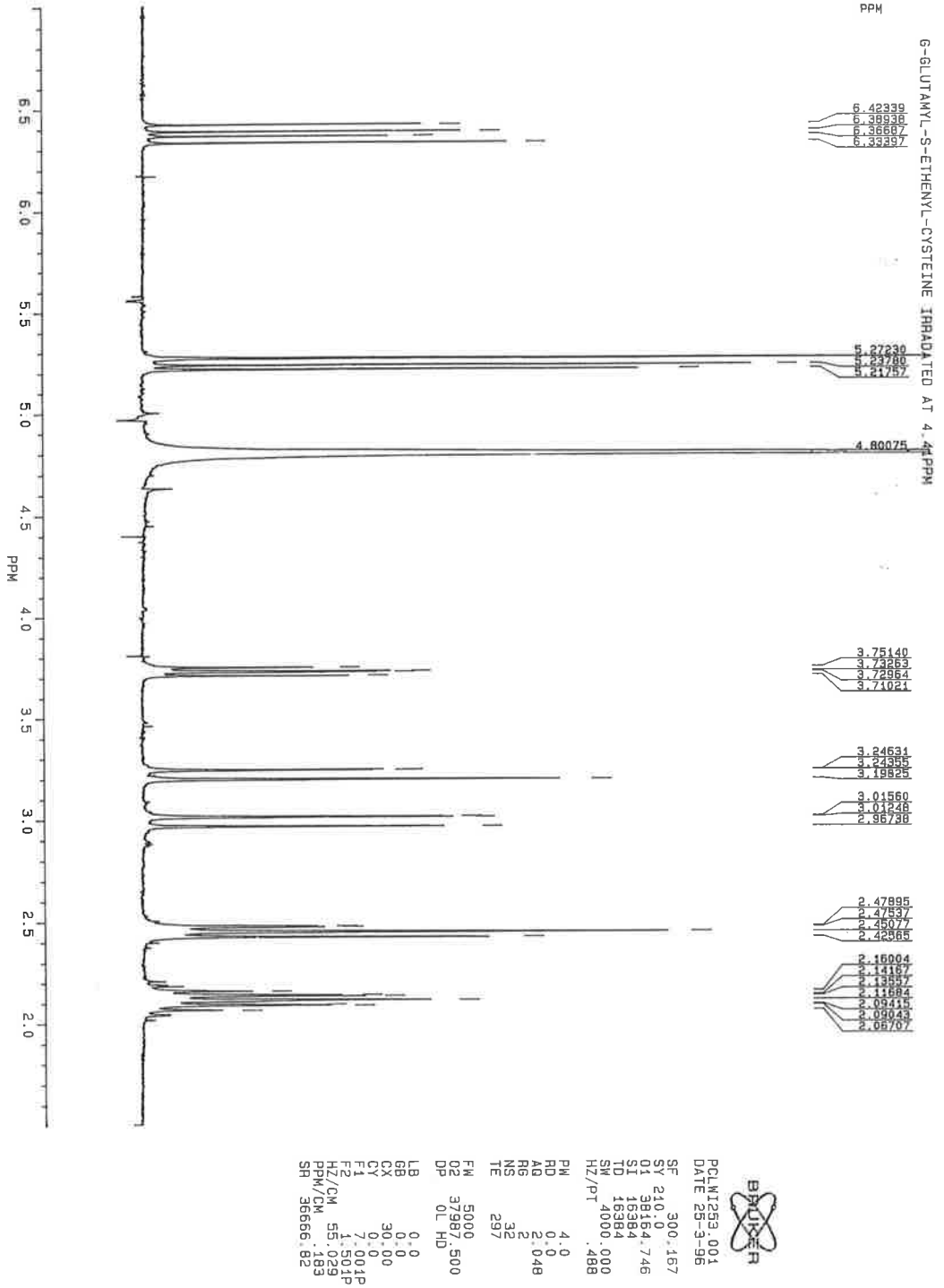
Appendix B17 Proton NMR for a proton decoupling experiment on γ-glutamyl-S-ethenyl-cysteine irradiated at 2.16ppm.



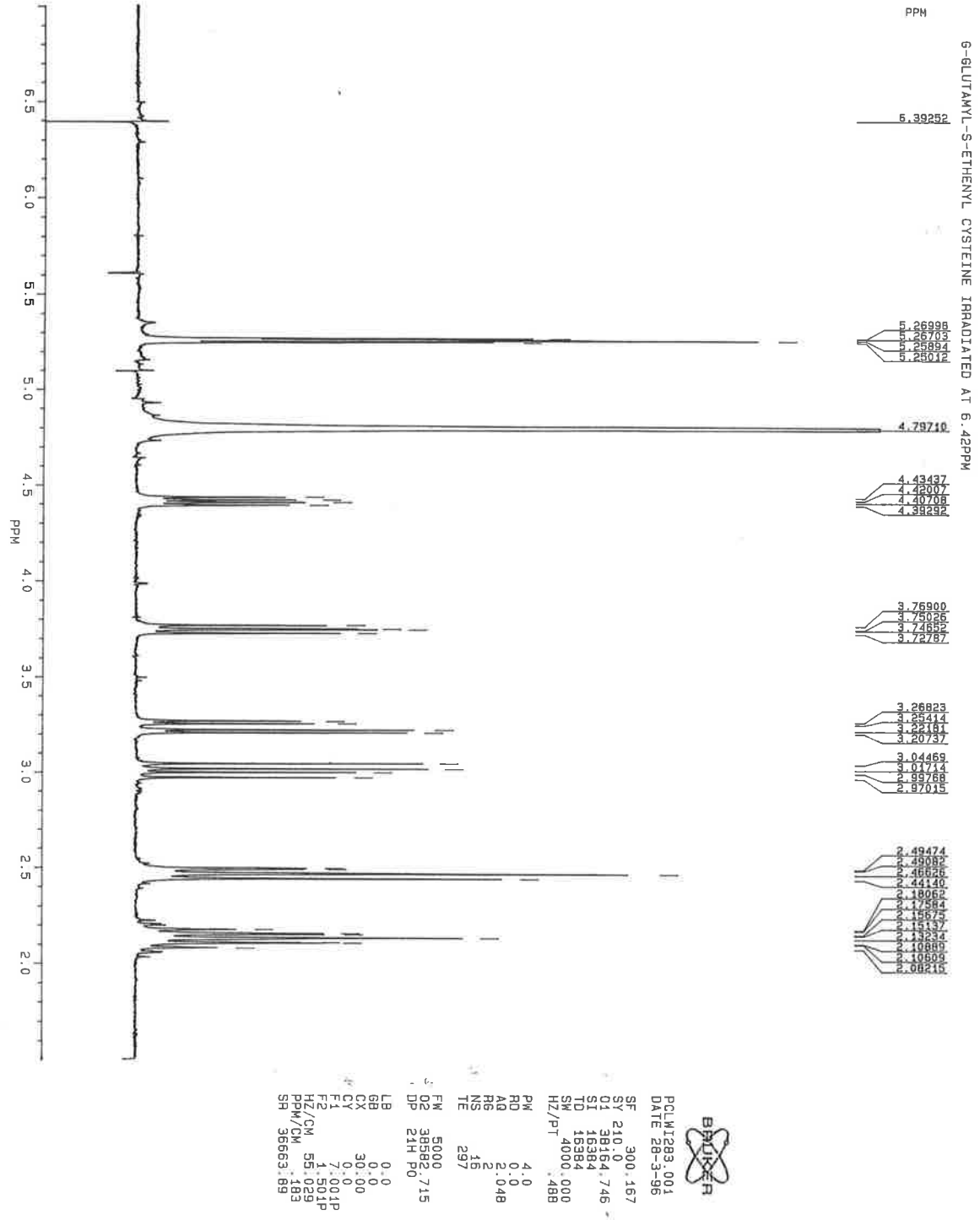
Appendix B18 Proton NMR for a proton decoupling experiment on γ-glutamyl-S-ethenyl-cysteine irradiated at 2.48ppm.



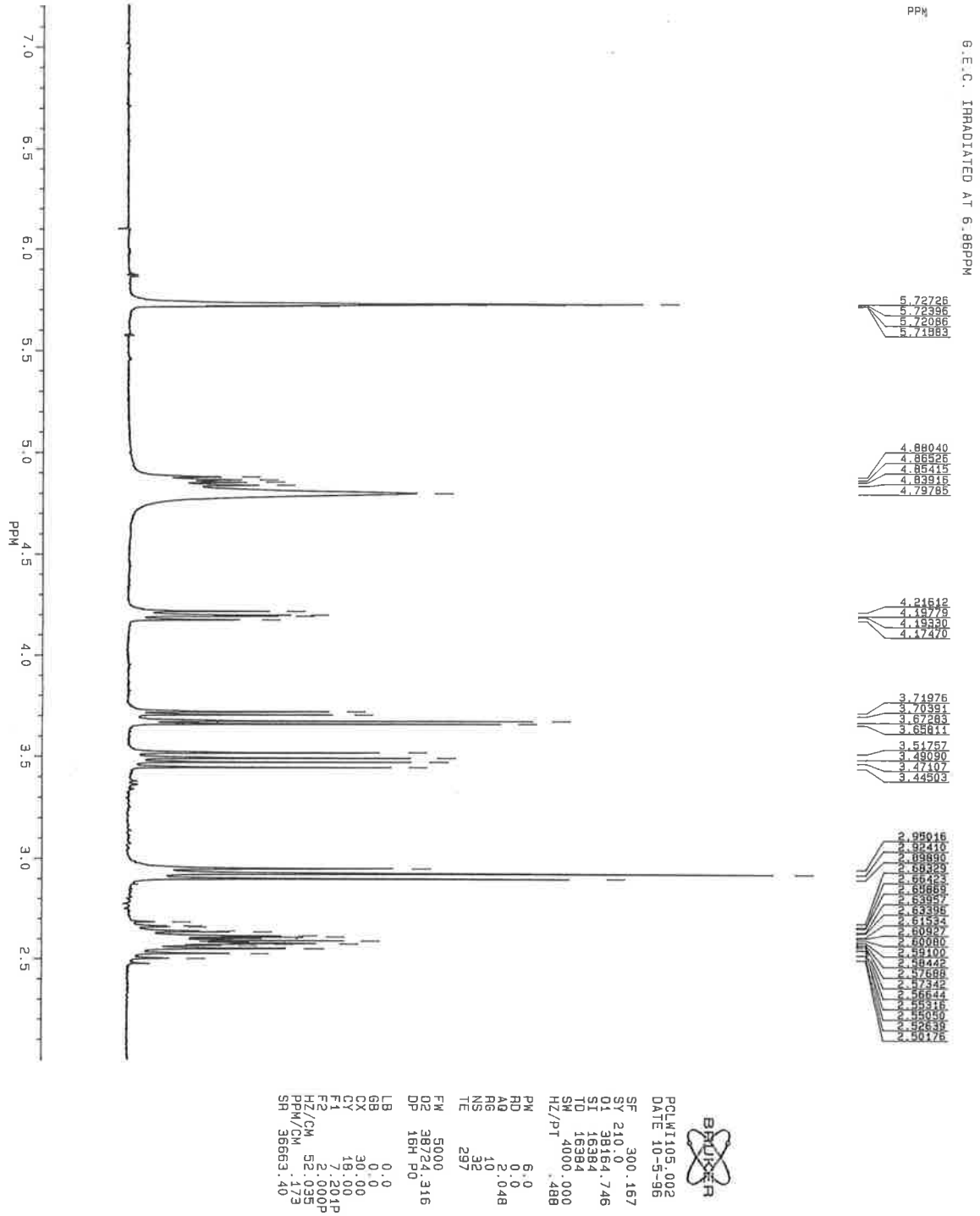
Appendix B19 Proton NMR for a proton decoupling experiment on γ-glutamyl-S-ethenyl-cysteine irradiated at 3.75ppm.



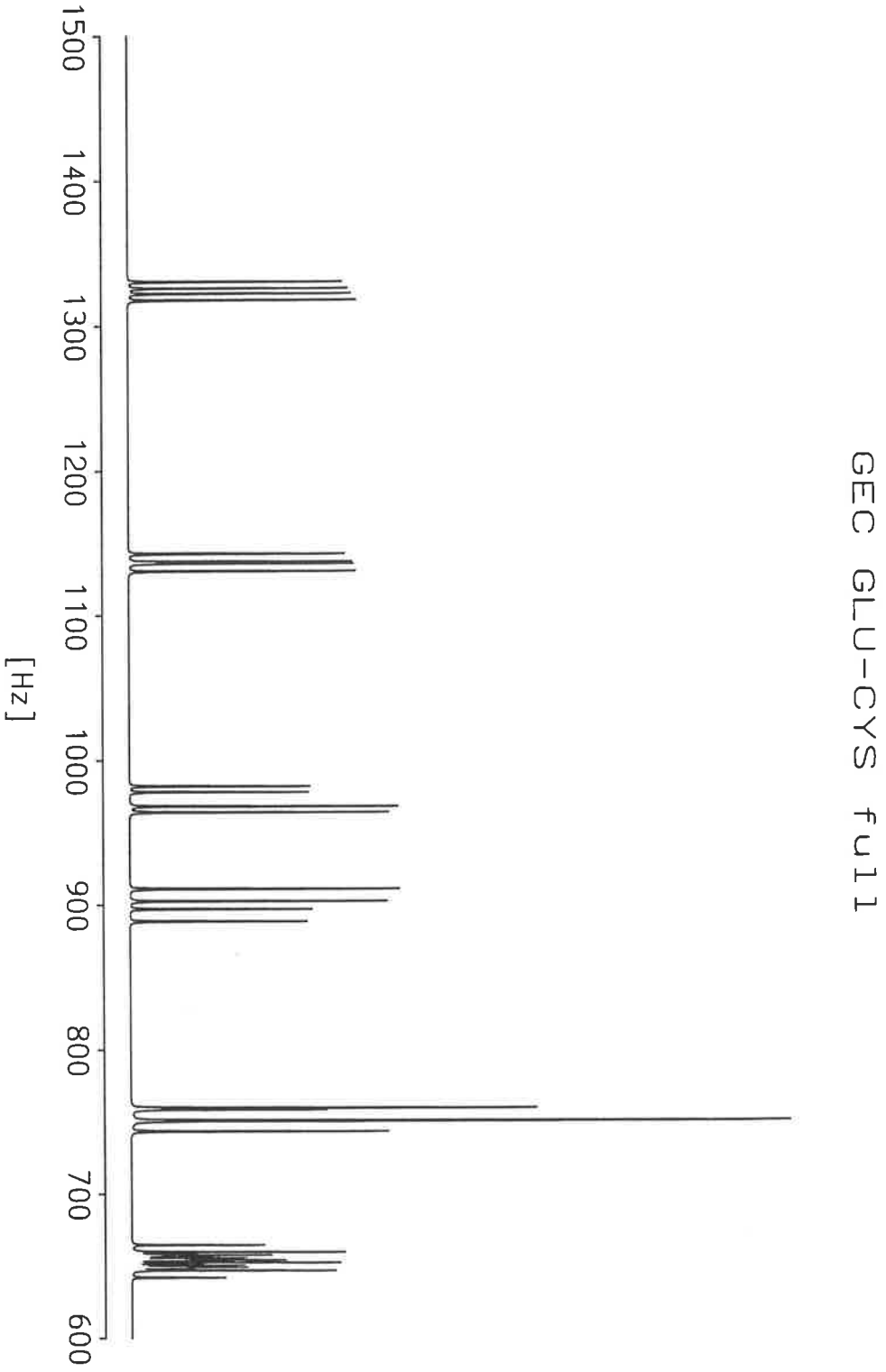
Appendix B20 Proton NMR for a proton decoupling experiment on γ-glutamyl-S-ethenyl-cysteine irradiated at 4.41ppm.



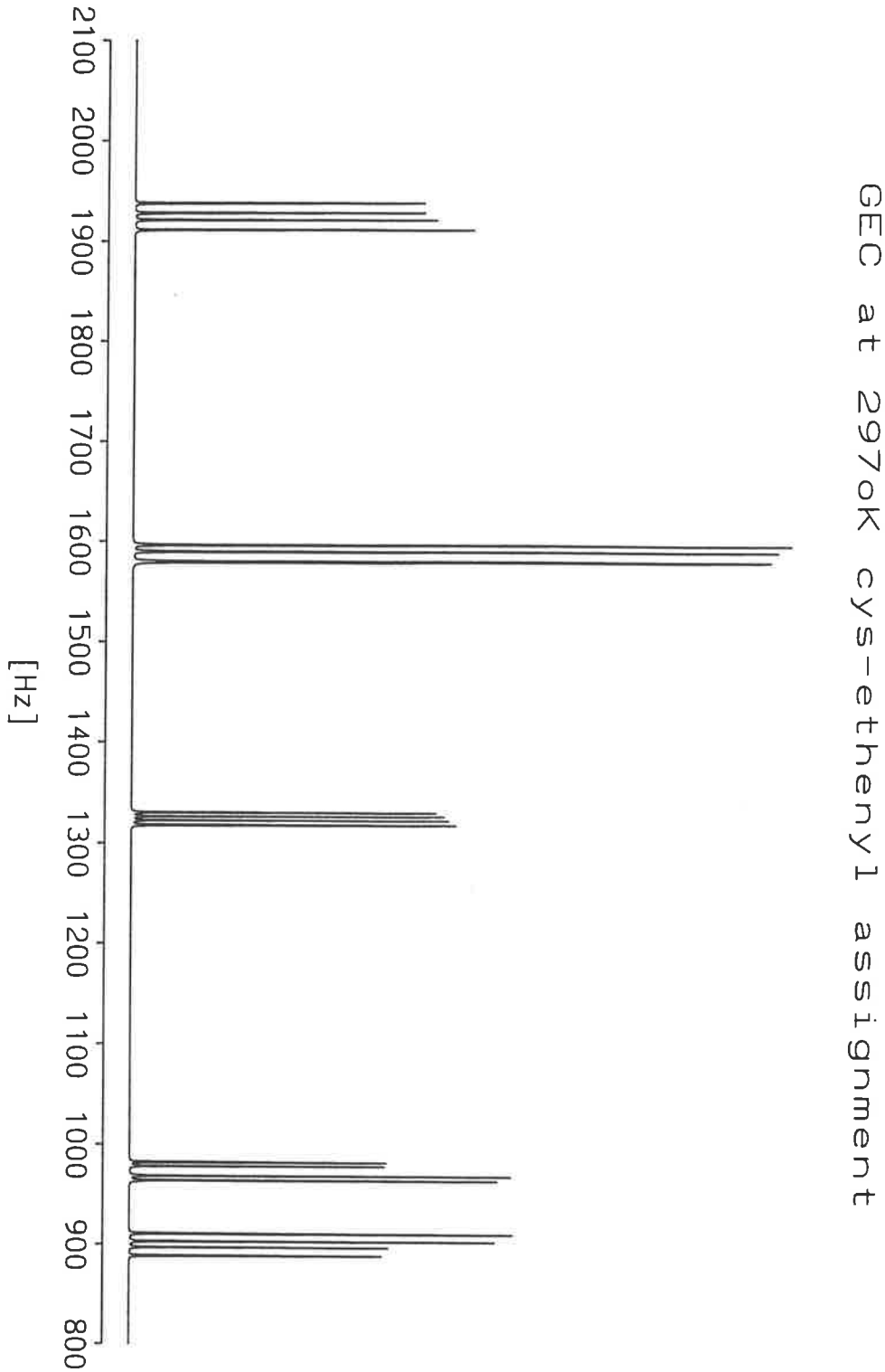
Appendix B21 Proton NMR for a proton decoupling experiment on γ-glutamyl-S-ethenyl-cysteine irradiated at 6.42ppm.



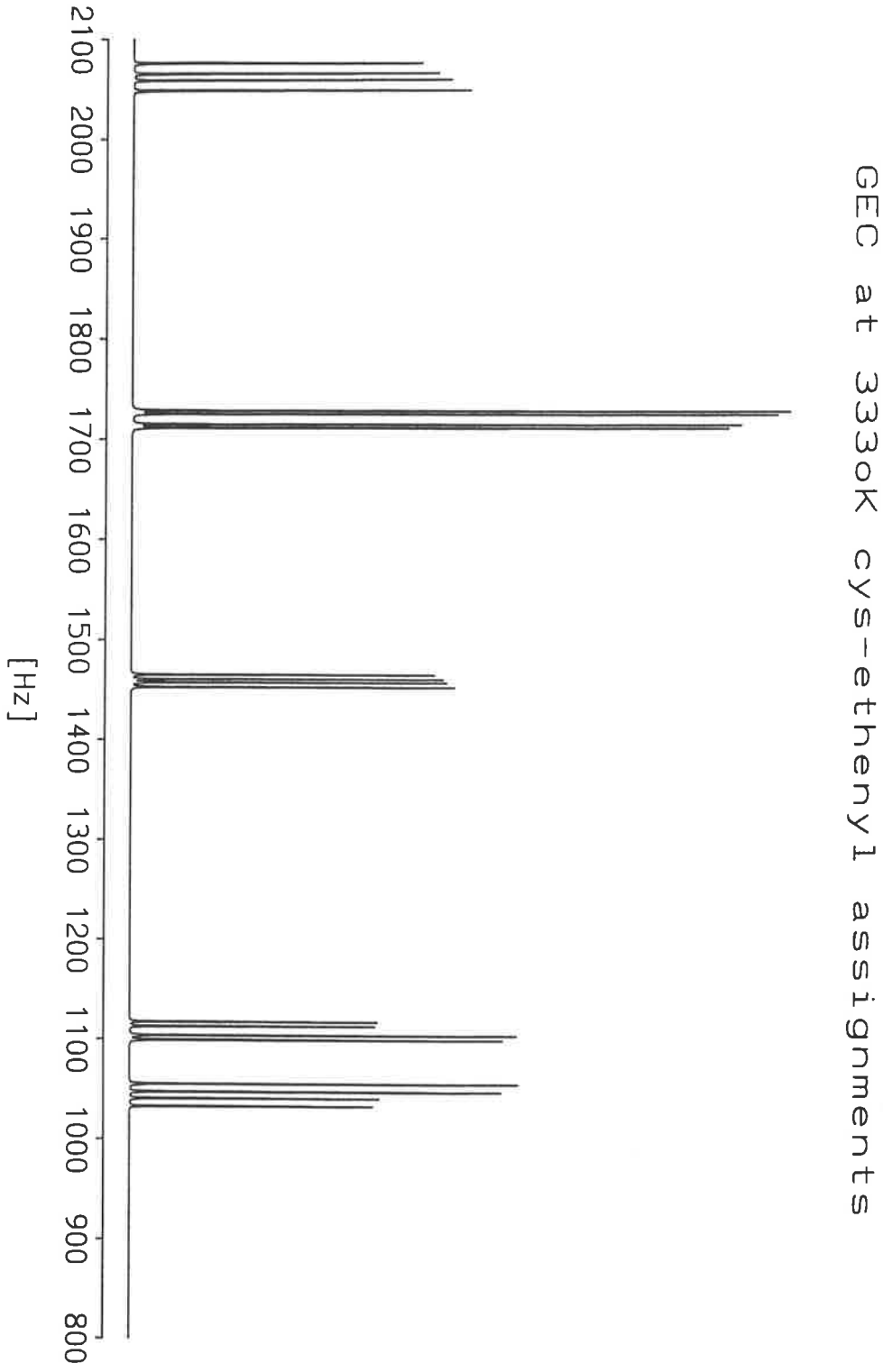
Appendix B22 Proton NMR for a proton decoupling experiment on γ-glutamyl-S-ethenyl-cysteine irradiated at 6.86ppm, at 333°K



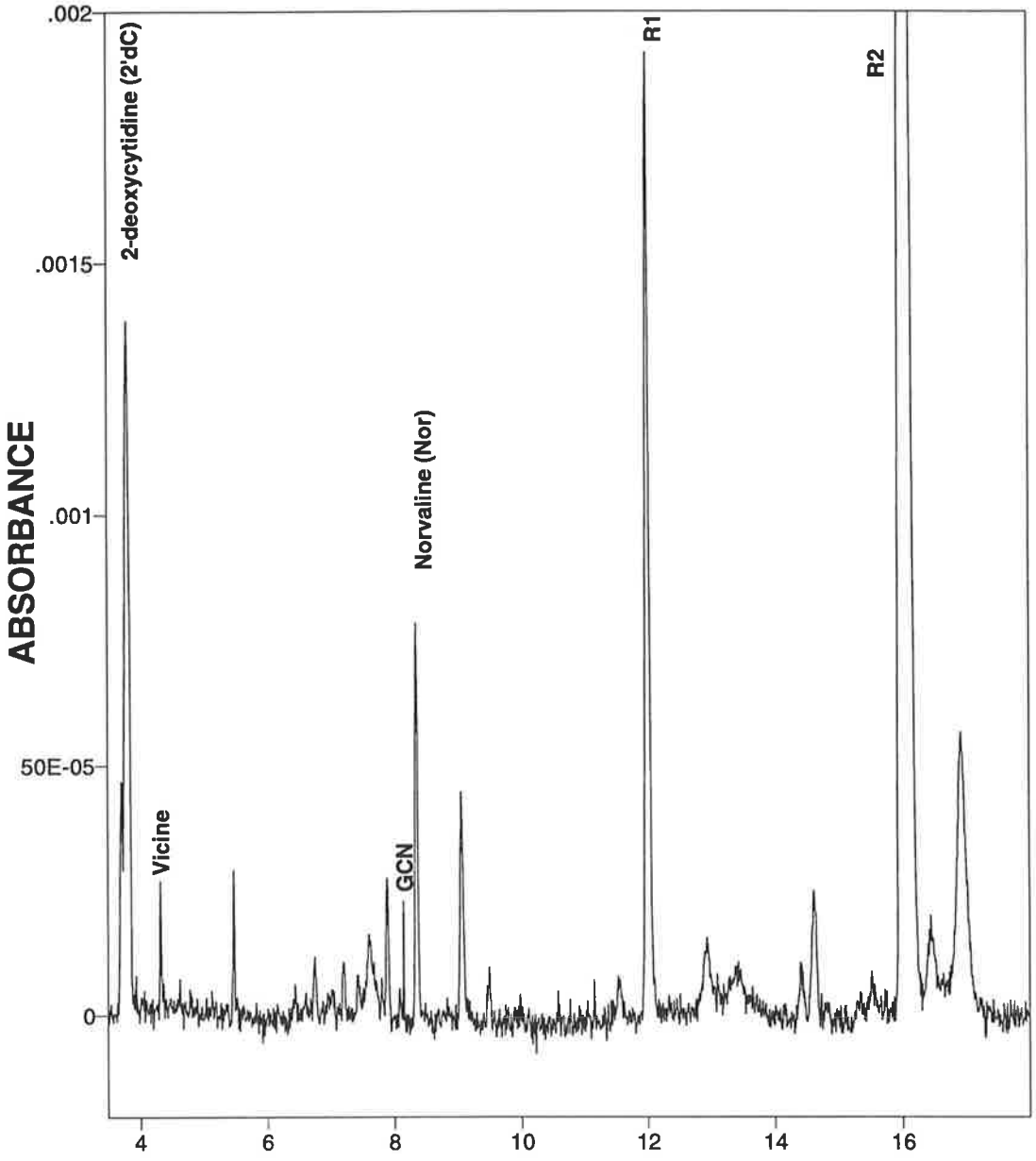
Appendix B23 Proton NMR simulation (Hagele *et al.* 1993) of γ-glutamyl-S-ethenyl-cysteine using the chemical shift and coupling constant data in Table 3.4a for the γ-glutamycysteine residue.



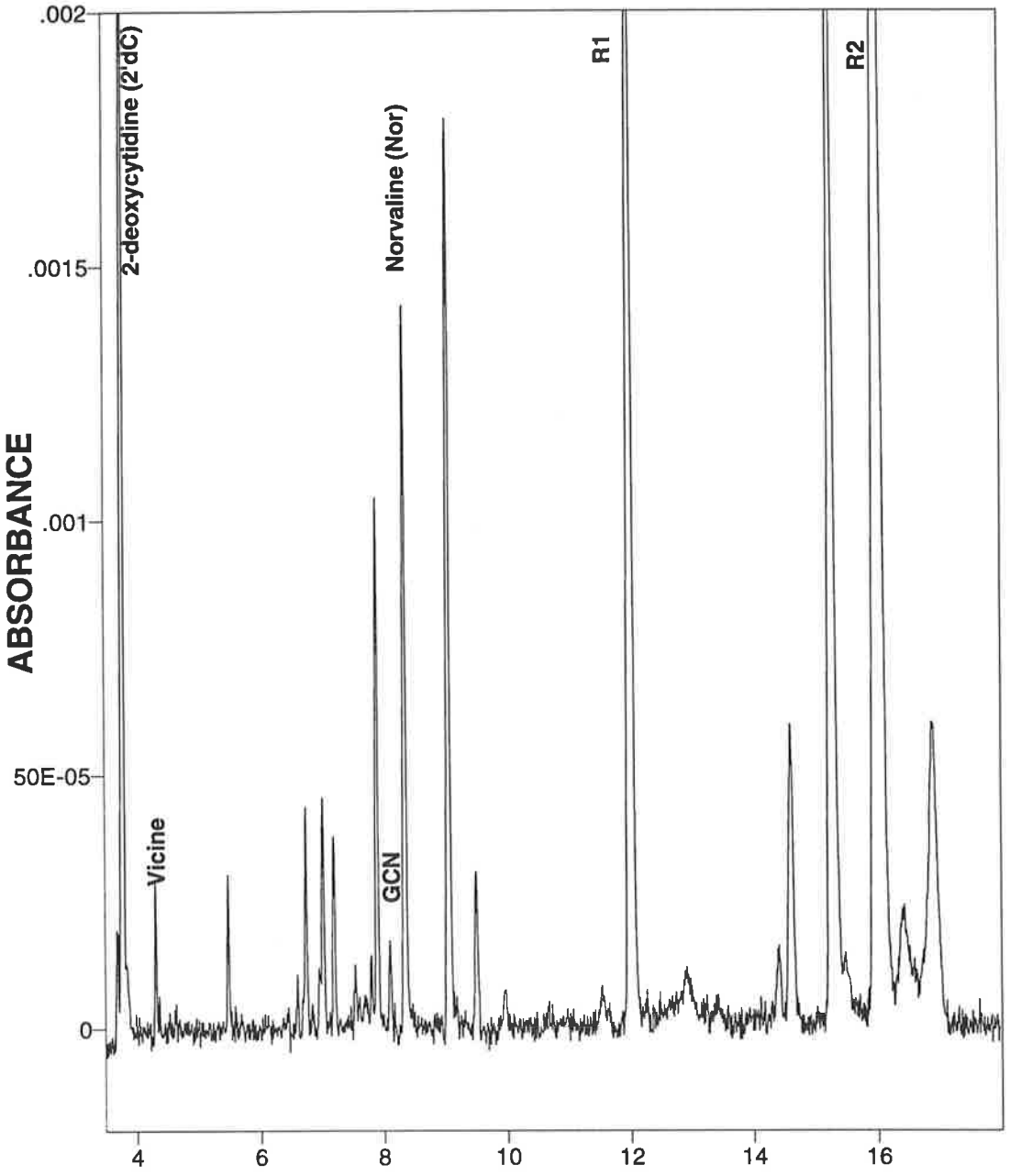
Appendix B24 Proton NMR simulation (Hagele *et al.* 1993) of γ-glutamyl-S-ethenyl-cysteine using the chemical shift and coupling constant data in Table 3.4a for the S-ethenyl-cysteine residue at 297°K.



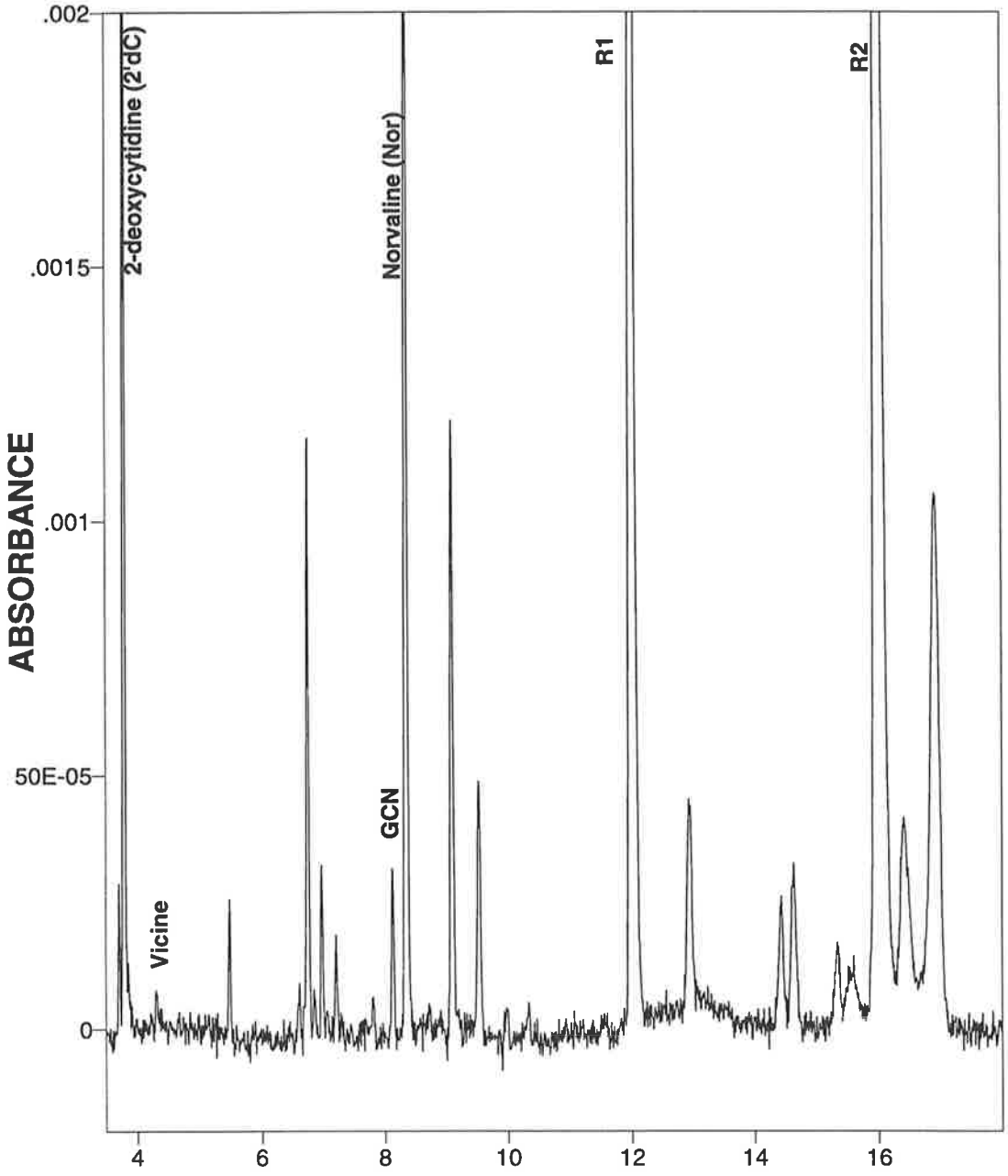
Appendix B25 Proton NMR simulation (Hagele *et al.* 1993) of γ-glutamyl-S-ethenyl-cysteine using the chemical shift and coupling constant data in Table 3.4b for the S-ethenyl-cysteine residue at 333°K.



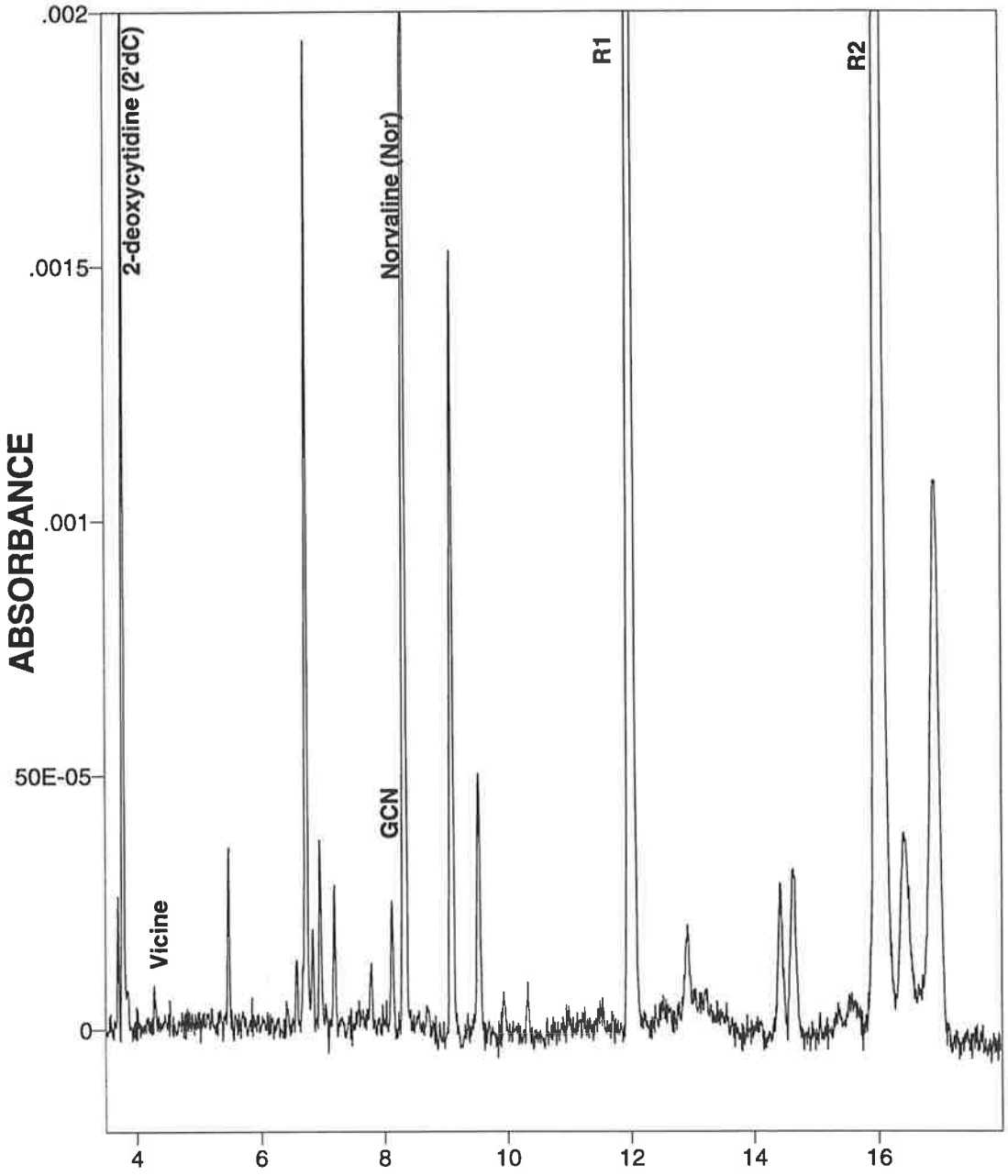
Appendix C.1 The micellar electrokinetic chromatographic separation of IFVI 303, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



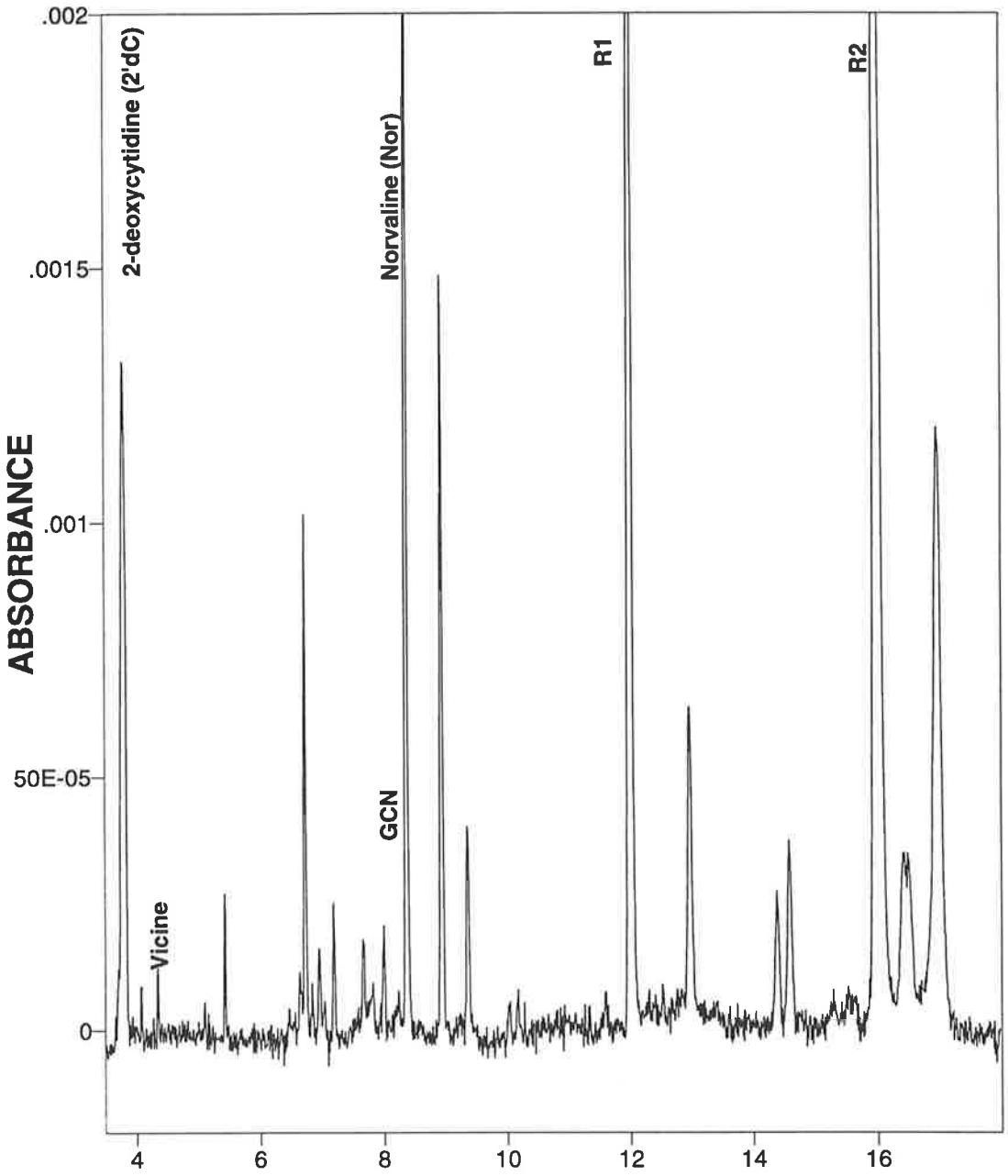
Appendix C.2 The micellar electrokinetic chromatographic separation of IFVI 51, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



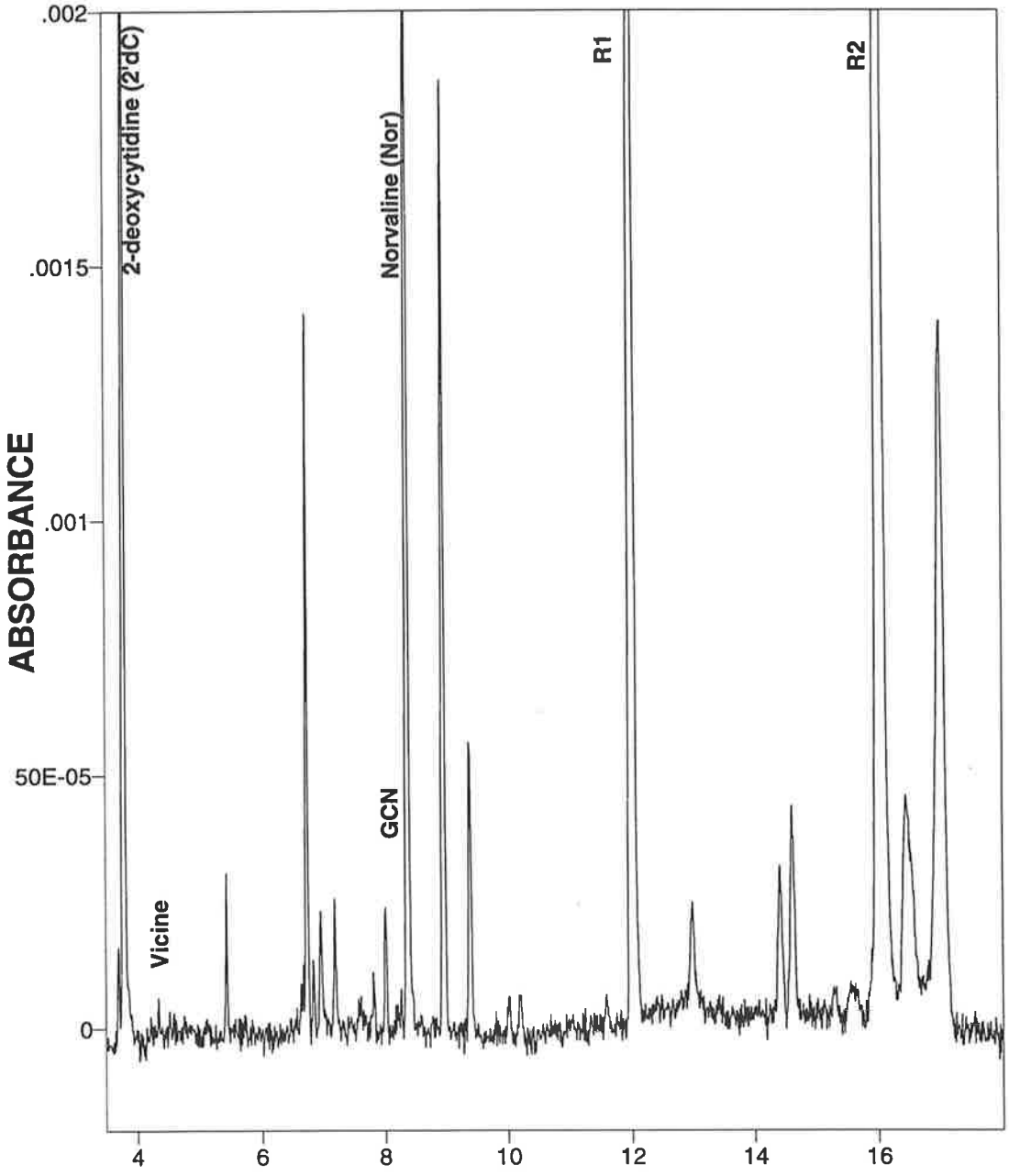
Appendix C.3 The micellar electrokinetic chromatographic separation of IFVI 778, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



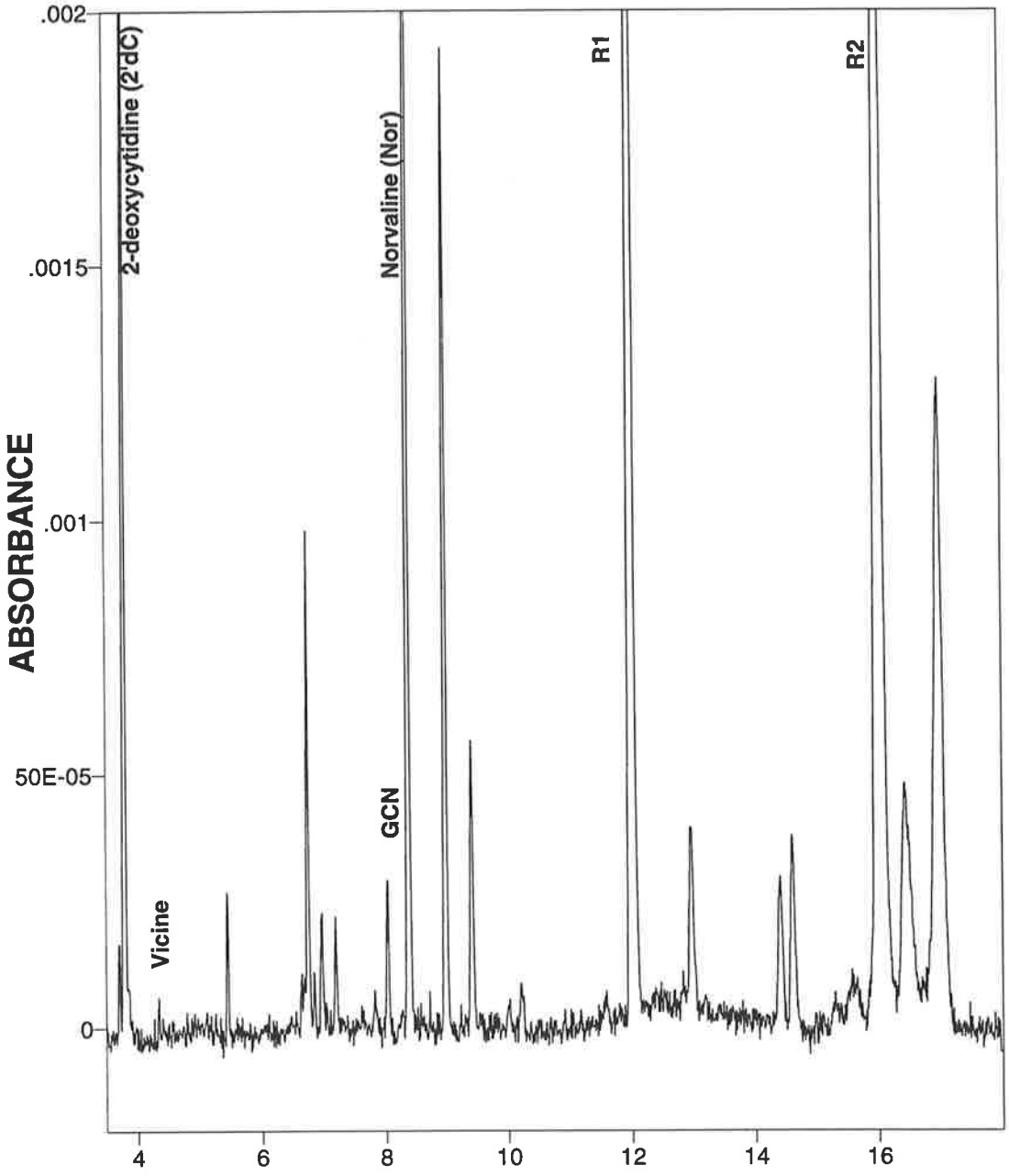
Appendix C.4 The micellar electrokinetic chromatographic separation of IFVI 261, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



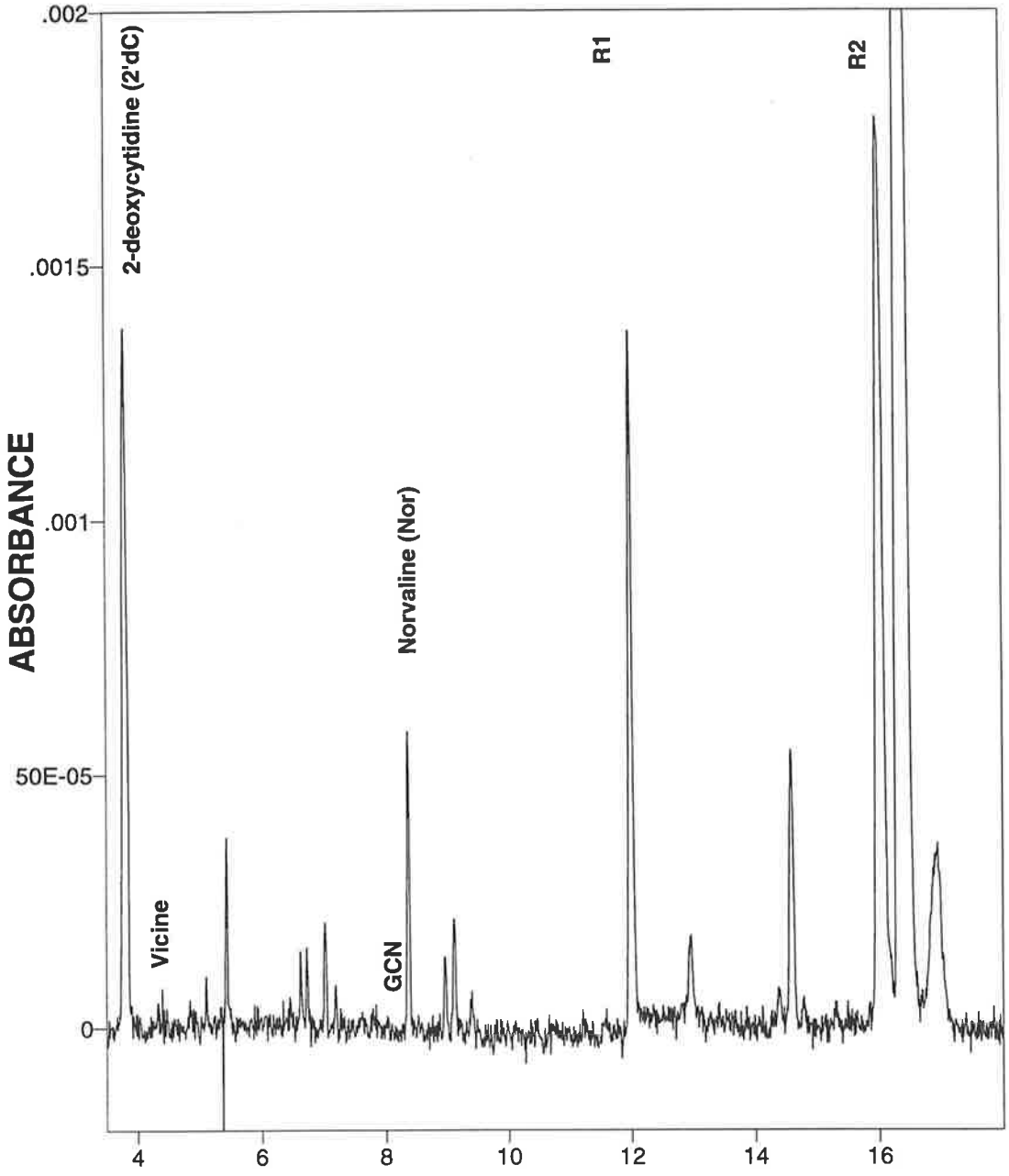
Appendix C.5 The micellar electrokinetic chromatographic separation of IFVI 2847, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



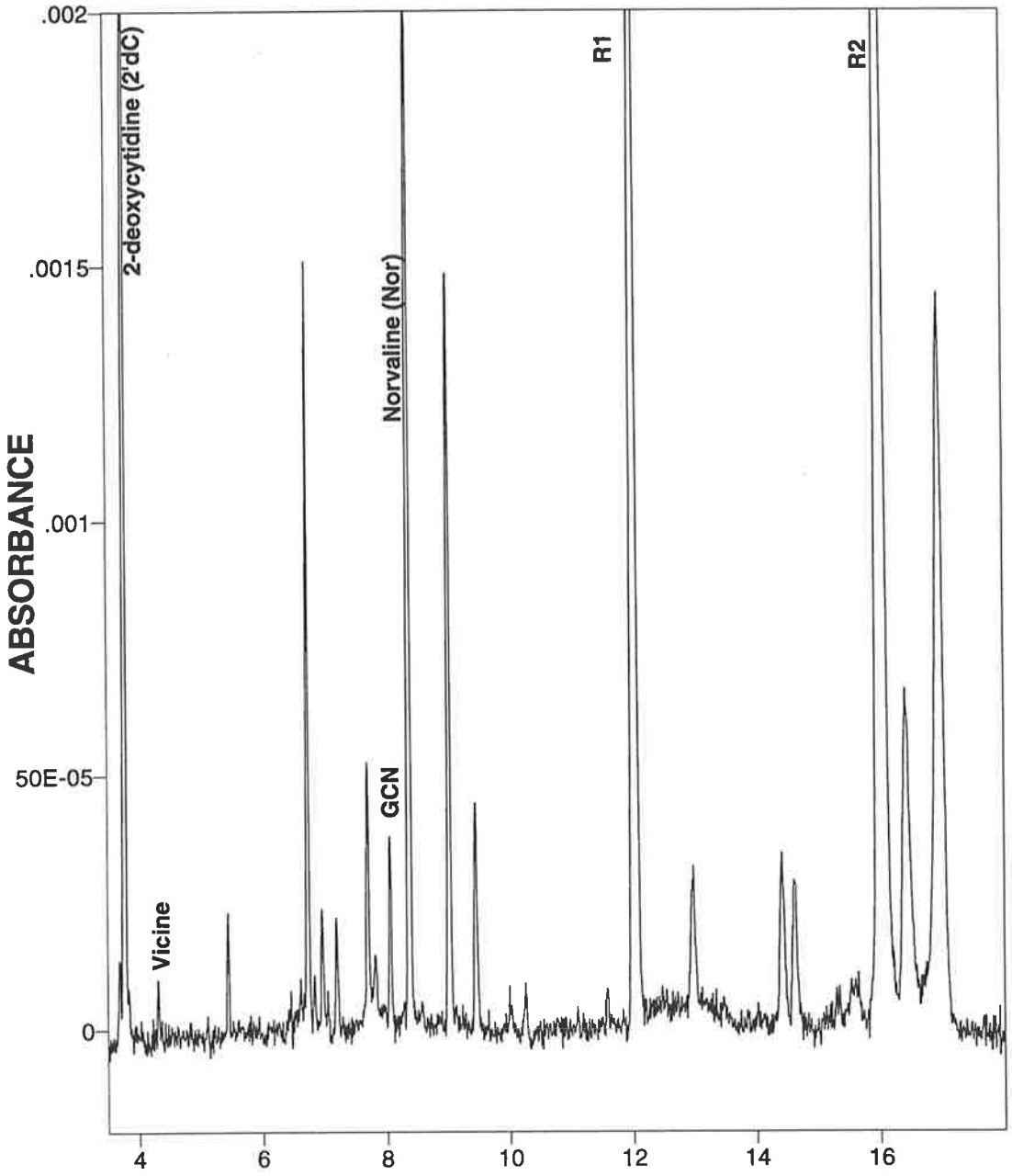
Appendix C.6 The micellar electrokinetic chromatographic separation of IFVI 4334, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



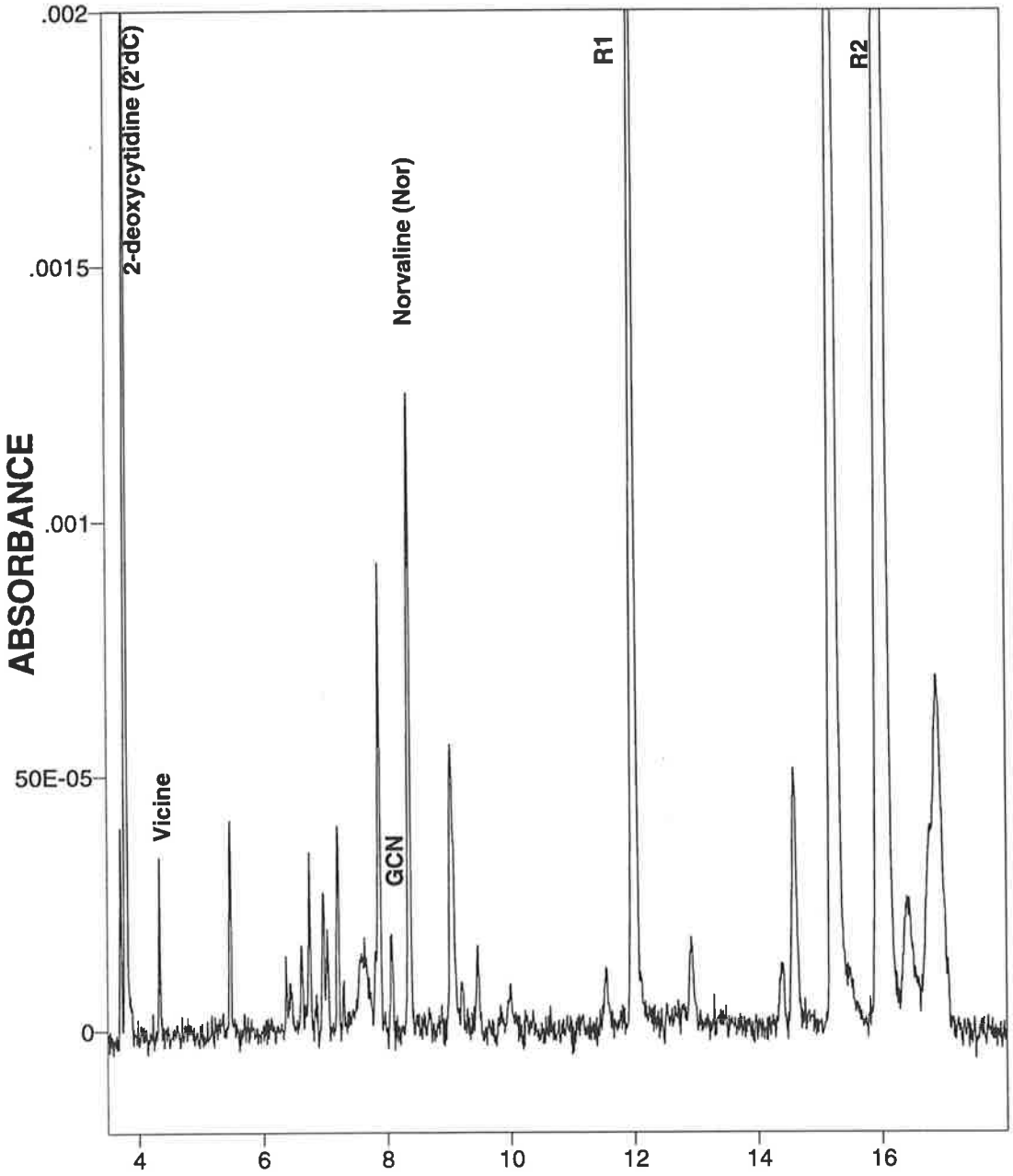
Appendix C.7 The micellar electrokinetic chromatographic separation of IFVI 2863, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



Appendix C.8 The micellar electrokinetic chromatographic separation of IFVI 4152, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



Appendix C.9 The micellar electrokinetic chromatographic separation of IFVI 2859, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.



Appendix C.10 The micellar electrokinetic chromatographic separation of IFVI 4547, an accesssion shown to have <0.2% cyanoalanine concentration as determined by diffuse reflectance using dispersive infrared spectrometry. All authentic *V. sativa* seeds from this accession were shown to have a higher cyanoalanine (>0.7%) concentration.

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