The toxicity of *Vicia* species and their utilisation as grain legumes

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Veni, Vidi: *Vicia*

Please, insert photograph here

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Summary

The worldwide utilisation of *Vicia* species as forage and green manure crops is well established. Except for *V. faba* L., the utilisation of *Vicia* species as grain legumes is of minor global economic importance and mainly restricted to the Mediterranean region and South-West Asia, where the grain is used primarily as seed and in ruminant diets.

In Australia, the comparatively high seed yields and low production costs for genotypes from species such as *V. narbonensis* L. (narbon bean or moor's pea) and *V. sativa* L. (common vetch) have provided an attractive alternative grain legume option for dryland farming and have thus stimulated an interest in markets for the grain. Monogastric animals (incl. humans) are the major global end-users for grain legume products. Because of the well known toxicity of *Vicia* spp. seeds to mono gastric animals in particular, this thesis has focussed on those major toxic chemical seed components which are perceived as major constraints to the wider utilisation of these promising crops as grain legumes. A thorough examination of current and past practices of vetch cultivation and utilisation was undertaken to complement this approach.

The two major aims were, first to elucidate the nature of the factors responsible for the low palatability of narbon beans to pigs, and second to review the available information about the toxicity of *Vicia* species and their utilisation as grain legumes.

The potent feed inhibitory activity of Namoi vetch (*V. villosa* Roth cv. Namoi) provided a useful model for the initial stage of this study. Its antifeedant activity was shown to be due to the toxic amino acid canavanine. Inclusion of canavanine in pig diets at a concentration equivalent to that found in Namoi vetch seed accounted fully for the feed inhibitory activity of this legume. The novel effect of this well known arginine analogue may well be explicable in terms of the inhibition of the arginine pathway leading to nitric oxide which is now known to be involved in the control of peristalsis.

The experience gained with Namoi vetch in the feed-intake bioassay proved to be invaluable for the isolation of the much less potent γ-glutamyl-S-ethenyl-cysteine feed inhibitor from the narbon bean. A quantitative assessment of this factor's feed inhibitory activity was not permitted due to the untimely death of our veterinary colleague, Dr. Richard Davies. There is, however, a clear correlation between the total S-ethenyl cysteine content of the tested diets and the negative porcine feed intake responses.

An important difference between *V. villosa* and *V. narbonensis* was noted, as demonstrated by the rate at which the pigs reduced their feed intake. It is remarkable, that the effect of canavanine-containing diets becomes evident only after the second meal, whereas the pigs immediately restrict their feed intake when presented with diets containing S-ethenyl-cysteine. Such a clear delineation of feed-intake responses provides a simple and general classification for feed-intake inhibitors, and may be worthy of further detailed physiological studies. The antifeedant effects of these compounds suggest that they have evolved as part of the plants' anti-predator defence strategy.

Particular attention, including a detailed review of its economic botany, has been given to *V. narbonensis*, a relatively unknown but promising grain crop for Australia. With the chemical identity of the unpalatability established, the selection of more palatable genotypes is likely to provide access for the grain to monogastric feed markets. The historical evidence suggests that *V. narbonensis* is a niche crop of particular value for specific agricultural applications, its conversion into a broad acre crop is a challenge for the future.

V. sativa was investigated as a direct consequence of a request to chemically examine the toxin content of the cultivar Blanche Fleur. By the time that investigation commenced, Blanche Fleur, which was originally introduced to Australia as a hay, forage and green manure crop, had already been prematurely promoted and exported as a cheap replacement for red lentils (*Lens culinaris* Med.) in ignorance of this species' well documented content of γ-glutamyl-β-cyanoalanine and the favism toxin, vicine. A 1992 commentary article to Nature on our observations led to a ban on its importation by India and Egypt. Subsequent poultry bioassays established that the cyano-alanine content was substantially altered by cooking to produce some as yet un-identified nitrile component, but the feed inhibitory activity of the cooked grain was undiminished. Acid hydrolysis of Blanche Fleur, however, removed both, the readily detectable nitrile absorbance as well as the poultry feed-intake inhibition. This observation could potentially form the basis for a simple post-harvest detoxification process for *V. sativa* and other feed stuffs containing acid labile antinutritive factors.

Unfortunately, cases of poisoning by *Vicia* species continue to be reported. These can be grouped into those caused by *V. sativa* and its related species (cyanogenic glycoside *Vicia*nine: HCN poisoning; and anti-nutritional effects of β-cyanoalanine) and those caused by canavanine containing species (*V. villosa*, *V. benghalensis*, *V. ervilia* etc.). Farmers need to be made aware of the well documented biochemical distinctions between *Vicia* cultivars to prevent the accidental intoxication of their livestock with seeds containing high

concentrations of canavanine or vicianine.

Finally, an overview of the voluminous and widely dispersed vetch literature, coupled with the observations in this thesis, suggest that the utility and value of each of the three *Vicia* model species examined in this thesis can be markedly enhanced by the following strategies:

1. Provision of sufficient alternative feed sources to allow feed intake to be regulated by palatability, thus minimising toxin ingestion.

2. Adaptation to *Vicia* toxins a) through selection of a digestive flora capable of detoxification (in the case of ruminants) and b) through selection or modification of animal genotypes with improved biochemical tolerance or even resistance to toxicity.

3. Detoxification prior to ingestion (Post-harvest detoxification)

4. Plant selection or genetic modification of specific toxin biosynthetic pathways to provide cultivars with optimum toxin concentration and distribution in strategic tissues (minimisation of toxins in the end product).

The inevitable conclusion from this thesis is that by incrementing our current fundamental knowledge of the biological chemistry of their naturally occurring anti-predator metabolites, we will promote the intelligent usage of *Vicia* species as highly nutritious grains for a sustainable agriculture.

This thesis has resulted in 2 publications in refereed journals

Publications

Tate, M. E.; Enneking, D. (1992). A Mess Of Red Pottage. *Nature* **359**, 357-8

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Tate, M. E.; Enneking, D. (1993) Toxins and Unpalatability factors. Potential for *Vicia* and *Lathyrus* species as new grain legumes and fodder legumes in southern Australia. Proceedings of the *Vicia*/*Lathyrus* workshop, Perth, Western Australia September 22.-23. 1992. (Garlinge, J. R.; Perry, M. W.) (Eds) CLIMA Occasional Publication No.1 pp. 39-46

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Tate, M. E., Delaere, I., Enneking, D., Glatz, P. C., Malaterre, C. (1995). Towards detoxification of *Vicia sativa*. Paper presented at the *Lathyrus* and *Lathyrism* Colloquium, Dec 1993, Dhaka, Bangladesh (Lambein, F.; Haque, A., eds.) (in press)

Abbreviations

µSec	Micro second
δ	Chemical shift
λ	Wavelength
@	Around, at
° C	Degrees Centigrade
µg	Microgram
2HCl	Dihydrochloride
Ac^-	Acetate
Adlys	Added Lysine
ANOVA	Analysis of variance
Arg	Arginine
ATC	Australian Temperate Crop Collection number prefix
Avp	Available protein
BP.	Before present
BW	Body weight
Ca	Calcium
calc.	Calculated
can	Canavanine
cf.	Compare with
CIS	Commonwealth of Independent States (former U.S.S.R.)
conc.	Concentration
convar.	Convarietas
CP	Crude protein
CSIRO	Commonwealth Scientific and Industrial Research Organisation
ctd.	Continued
CTRY	Country
cv(s)	Cultivar(s)
cys	Cysteine
Cys	Cystine
CZE	Capillary Zone Electrophoresis
d	Doublet (NMR signals)
d.o.	Day old (chicks)
DAP	Diamino-propionic-acid
db	Decibel
dd	Double doublet (NMR signals)
dd	Double distilled
DE	Digestible Energy
Dep.	Department
diam.	Diameter
DNA	Deoxy-ribose nucleic acid
DW	Dry weight basis
e.g.	Example given, for example
ed.	Editor
equiv.	Equivalent to
et al.	And others
ETH	Ethiopia
EUR	Europe
ex	From
FAB	Fast Atom Bombardment
FAO	Food and Agriculture Organisation of the United Nations
g	Gram
G6PD	Glucose-6-Phosphate dehydrogenase
GEC	γ-Glutamyl-S-ethenyl-cysteine
glu	Glutamic acid
GRG	Georgia
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
ha	Hectare
Hac^-	Acetic acid
HCl	Hydrochloric acid
HCN	Hydrogen Cyanide
HDO	Deuterated water
His	Histidine
HMG	Homonuclear gated decoupling
HOAc	Acetic acid
HPLC	High Pressure Liquid Chromatography
hr(s)	Hour(s)
HVPE	High Voltage Paper Electrophoresis
IBPGR	International Board for Plant Genetic Resources (now IPGRI)
ICARDA	International Center for Agricultural Research in Dry Areas
ie.	In example
IFVI	International Forage Vicia (ICARDA number prefix)
IgG	Immunoglobulin G (antibodies)
IgM	Immunoglobulin M (antibodies)
Ile	Isoleucine
incl.	Inclusive, including
IND	India
IPGRI	International Plant Genetic Resources Institute (ex IBPGR)
IPK	Institute fuer Pflanzenzuechtungs- und Kulturpflanzenforschung
IR	Infrared spectroscopy
IR	Infrared spectroscopy
IRQ	Iraq
IU	International Units
iv.	Intravenous
j	Coupling constant (NMR)
JAP	Japan
K	Kilo, 1000
keV	Kilo electron Volts
kg	Kilo gram
kJ	Kilo Joule
km	Kilo meter
kV	Kilo Volt
L	Litres
L.	Linnaeus
lbs	Pounds
LD	Lethal dose
leg.	Collected by
Leu	Leucine
LSD	Least significant difference

m	Overlap (NMR signals)
M	Mole, molar
M+H	Mass plus hydrogen (positive ion)
M-H	Mass minus hydrogen (negative ion)
m.	Million
m/z	Mass to charge ratio
mA	Milli Amperes
MAN	Manchuria
MED	Mediterranean region
Medit.	Mediterranean
MENA	Middle East and North Africa
Met	Methionine
mg	Milli gram
MHz	Mega Hertz
min	Minute
MJ	Mega Joule
ml	Milli litre
$M_{O.G.}$	Mobility relative to Orange G
MS	Mass spectroscopy
msec	Milli second
mth(s)	Month(s)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
nm	Nano metre
NMR	Nuclear Magnetic Resonance Spectroscopy
NPAA	Non Protein amino acid
NS	Not significant
ODAP	Oxalyl-diamino-propionic acid
OPA	O-Phthaldialdehyde
P	Phosphorous
p	Probablility
pers. comm.	Personal communication
pers. obs.	Personal observation
pH	-log hydrogen ion concentration
Phe	Phenylalanine
PM	Post mortem
ppm	Parts per million
PRT	Portugal
R.O.	Reverse osmosis
RBC	Red blood cells
ref.	Reference
Rf	Relative mobility
RL	Roland Laurence
S	Sulfur
S.AM	South America
SA	South Australian Department of Agriculture collection prefix
SEC	S-ethenyl-cysteine
SED	Standard error deviation
sens. lat.	Sensu lato (broad sense)
ser.	Series (taxonomic)
SGD	Systemic granulomatous disease
SLE	Systemic Lupus Erysthematosus
sp.	Species
SPA	Spain
spp.	Species (plural)
ssp.	Subspecies
subgen.	Subgenus
syn.	Synonym
syn.	Synonym, synonymous
T	Temperature
t	Triplet (NMR signals)
t BuOH	Tert-butanol
Thr	Threonine
TMS	Trimethylsilane
tmt	Treatment
transl.	Translated by
Try	Tryptophan
TUR	Turkey
Tyr	Tyrosine
US	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
v/v/v	Volume per volume per volume
VA	Vicia acidic amino acid
Val	Valine
var	Variety
Vit	Vitamins
W.A.R.I.	Waite Agricultural Research Institute
w/v	Weight per volume
WA	West Asia
WANA	West Asia and North Africa.
widespr.	Widespread
wk(s)	Week(s)
X	Cross (hybrid)
yr(s)	Year(s)

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Chapter 1

Cultivated Vetches and Their Utilisation as Grain Legumes

Introduction

"Most of the annual vetches suitable for field crops are well adapted to cultivation in the intermediate year between different sorts of grain crops, for the double purpose of ameliorating the land and affording a supply of fodder. It has even been contended that vetches may be made the means of enabling the arable farmer to support as much live stock as the grazier. By a judicious combination of vetches with turnips, clover, and sainfoin, the poor downs, sheep walks and other waste lands, may be rendered from ten to thirty times more valuable than they are at present. Vetch ought to be more generally grown in most situations, in proportion to the extent of the stock that is kept. However, vetch grown for the sake of its seed, or when it is allowed to stand till it approaches ripeness, is one of the most impoverishing of all our commonly cultivated crops. Most kinds of soils in ordinary cultivation are more or less suitable for vetches. Gravelly loams of medium dryness are the most generally suitable; and all other loamy sorts, from kinds bordering on thin gravel to kinds bordering on stiff clay, will do. A soil of inferior description is requisite for the seed produce of a vetch crop, as a rich soil sends the herbage of the plant into such excessive luxuriance as to occasion a deficiency in the yield of seed. In Gloucestershire and Worcestershire, they sow tares as pasturage for horses, and cut them early enough to allow turnips to be sown the same season. In Sussex tares are of such infinite importance that not one-tenth of the stock could be maintained without them: horses, cows, sheep, hogs, all feed on them " (Wilson, 1852).

The agricultural potential of *Vicia* and *Lathyrus*

There is currently a great need for alternative crops in the drier areas of the Southern Australian cereal belt, where cereal/fallow or cereal/pasture rotations are widely practised. The addition of further grain[1] crops, such as oilseed *Brassicas* or grain legumes would permit a diversification of the cropping rotation to give better disease control. Delane *et al.* (1989) have given a detailed review of the role of grain legumes in sustainable dry land cropping systems.

Whilst peas and faba beans have been successful under medium rainfall and lupins on the lighter sandy soils, suitable grain legumes are lacking for the vast tracts of alkaline and often heavier soils in areas of South-Western Australia receiving less than 350 mm annual precipitation, comprising parts of Western Australia, South Australia, Victoria and New South Wales. Perry (1993) estimated that for Western Australia alone, 4 m. ha of alkaline soils are unsuitable for the cultivation of *Lupinus angustifolius.* These areas are potentially suitable for the cultivation of *Vicia* and *Lathyrus* species adapted to low rainfall and alkaline soils. These could reasonably be expected to become important crops for this region, provided that the produce can be utilised profitably.

Zohary (1973) pointed out that the large clusters of genotypes in the *V. sativa-amphicarpa-cordata* , the *V. dasycarpa-villosa, V. narbonensis-serratifolia, V. pannonica* groups represent a wide genetic base, which has remained largely unexploited for agricultural development. In the words of Houérou (1985) "The magnificent work carried out by the Australian agronomists on Mediterranean legumes particularly on *Trifolium subterraneum sens.lat.*, is justifiably famous and sets an example for the development of new legume cultivars." He emphasised that amongst other legumes, the genus *Vicia*, especially the species *V. monantha* (*V. articulata* Hornem.), *V. benghalensis, V. villosa , V. sativa* hold considerable potential for dry areas.

The general concept of replacing bare fallow fields with forage crops (Wilson, 1852; Carter, 1978) has given new impetus to research on legumes, including vetches. Considerable germplasm for species such as *V. sativa, V. villosa, V. narbonensis, V. ervilia, V. pannonica, Lathyrus sativus, L. cicera , L. ciliolatus and L. ochrus* has now been collected and, in part, has been evaluated at the International Center for Agricultural Research in Dry Areas (ICARDA), in Aleppo, Syria since its conception (Saxena *et al.*, 1993). In addition much valuable agricultural research work on *Vicia* and *Lathyrus* has been carried out at ICARDA with respect to grain and forage production and palatability studies (Abd El Moneim *et al.*, 1988; Abd El Moneim *et al.*, 1990a; Abd El

[1] grain is used in the sense of "seed", the seed being the major product of the crop

Moneim *et al.*, 1990b*;* Abd El Moneim, 1992; ICARDA, 1987; Thomson *et al.*, 1986*;* Thomson *et al.*, 1990; Keatinge *et al.*, 1991; Rees *et al.*, 1991; Saxena *et al.*, 1993). This work is a direct continuation of earlier agricultural research work on *Vicia* (Durutan *et al.*, 1990; Pacucci *et al.*, 1984; Pacucci and Troccoli, 1981; Kernick, 1978; Christiansen-Weniger *et al.*, 1978; Corleto, 1976; Radwan and Al-Fakhry, 1975; Radwan *et al.*, 1974; Kuporitskaya, 1970; Corleto and Maisto, 1968; Tarman, 1964; Villax, 1963; Vasconcellos, 1962; Donnelly and Clark, 1962; Mateo-Box, 1961; Kansu, 1961; Janelli, 1960; Hermann, 1960; Lechner, 1959; Foury, 1954; Tarman, 1954; Laumont, 1954; Horn *et al.*, 1943a; Horn *et al.*, 1943b; Barulina, 1930; Muratova, 1931; Tupikova, 1926; Becker-Dillingen, 1929; Hegi and Gams, 1924; Fruwirth, 1921; Pott, 1907; Pott, 1880; and references therein). It should be mentioned at this point that for the purposes of this thesis much of the Eastern-European (e.g. Aralov, 1984; Aralov and Peretyazkho, 1989; Avadeni and Tarytsa, 1987; Avadeni, 1989; Izmalkov 1987; Klisha *et al.*, 1985; Leokone *et al.*, 1982 etc) and Spanish literature (e.g. Gil *et al.*, 1987; Gil and Martin, 1988) on *Vicia* has not been consulted in detail, due to the difficulties of language and inaccessibility of many reports, but nevertheless these should not be ignored by subsequent studies.

Vetch cultivation in Mediterranean agriculture is currently going through a renaissance, which could well lead to a revolution in the current ley farming and other cropping systems. The Australian version of the ley farming philosophy (Puckridge and French, 1983) can be extended in its practical scope by the inclusion of vetches (*Vicia* and *Lathyrus*) as additional legume species in cereal and cereal/pasture rotations. Vetches are multi-purpose crops allowing for either fodder conservation or immediate cash returns through hay or grain production, while at the same time providing a green manure and grazing option. In addition they are beneficial with respect to weed control as crops prior to pastures. The versatility of vetches complements the high production potential of medics and clovers, and together with other legumes such as *Onobrychis*, *Ornithopus*, *Hedysarum*, *Astragalus*, *Trigonella*, *Melilotus* and the pulses, they could increase the biodiversity of the legume component in Mediterranean agricultural systems. This will inevitably lead to increased animal feed production which according to Oram and Belaid (1990) is urgently needed in many areas.

It is also important to note, that in some situations, biomass production is considered more important than grain yield, and in order to improve the sustainability and productivity of dry land regions in developing countries, forage-livestock systems should be given as much priority as those for grain production (Steiner *et al.*, 1988). *Vicia* and *Lathyrus* species are ideally suited for this purpose and represent an enormous pool of genetic diversity and adaptation for agriculture in temperate to sub-tropical regions.

The importance of economic botany for plant introduction and utilisation of genetic resources

One important aspect of this study was to collect the widely scattered literature on vetches. The introduction of new crop plants to cultures unfamiliar with their utilisation and husbandry can only be accomplished successfully if the knowledge of their economic botany accompanies them. In consideration of the relative novelty of vetches to Australia and the long tradition of their utilisation as crops and pastures in North-West Asia, the Mediterranean region and Europe, it was felt necessary to provide an overview of their utility and potential as a basis for the further agricultural development of this economically important genus. As minor crops, the empirical knowledge of their traditional culture, which was gained over millennia, is of special importance as it can provide economic, ethnobotanic and historical data. It should be documented in conjunction with the preservation of their genetic resources before both disappear as a result of the modernisation of agriculture and the associated genetic and cultural erosion.

Taxonomy

The genera *Vicia* and *Lathyrus* respectively comprise 166 and ca. 150 species of annual and perennial herbaceous legumes of temperate and sub-tropical geographic distribution (Allkin *et al.*, 1983*;* Allkin *et al.*, 1985). Taxonomically, both *Vicia* and *Lathyrus* belong to the tribe *Vicieae* (*Fabaceae*: *Papilionideae*: *Leguminosae*) (Kupicha, 1981). The most recent taxonomic treatment for *Vicia* subgen. *Vicia* is given by Maxted (1993) which complements an earlier detailed review of *Vicia* taxonomy (Maxted, 1991). For a review of the biosystematics of *Vicia* see Hanelt and Mettin (1989). The genus as a whole was revised by Kupicha (1976) who also revised the classification of *Lathyrus* (Kupicha, 1983). An overview over the classification of both genera with a list of all species can be found in Smartt (1990). With respect to the complexities presented by *Vicia* taxonomy, species names and their authorities are used throughout this thesis where this information is available. Species names listed in tables have been checked with Allkin *et al.* (1986) and Maxted (1993) for current accepted taxa and changes have been made where necessary. No attempt has been made to update older names with their current synonyms in the text (The help of Dr Nigel Maxted with the taxonomy is greatly appreciated).

Vicia and *Lathyrus* in Australia

Certain *Vicia* species have been cultivated in Australia for some time (Kloot, 1987). Records for *V. sativa* L. var. *segetalis* Ser. and *Vicia hirsuta* (L.) S. F. Gray in South Australia, date back to the period closely following European settlement (Kloot, 1983). As far back as 1881, von Mueller commented favourably on several *Vicia* and *Lathyrus* species with respect to their suitability for cultivation and introduction to Australia. These included *V. cracca* L., *V. cassubica* L., *V. biennis* L., *V. ervilia* Willd., *V. faba* L., *V. narbonensis* L., *V. sativa* L., *V. cordata* Wulf., *V. globosa* Retz., *V. sepium* L., *V. pannonica* Jacq., *V. sitchensis* Bongard, *V. sylvatica* L., *V. pisiformis* L., *V. dumetorum* L., *V.* Koch, *V. monantha* Koch, *V. hirsuta* Koch, *L. cicera* L., *L. macrorhizus* Wimmer, *L. pratensis* L., *L. sativus* L.

The major development of vetches as Australian crops occurred after the establishment of the CSIRO plant introduction station at Muresk (Western Australia) in 1943, as part of a deliberate search for alternative grain legumes to replace field peas (*Pisum sativum*). The first introductions of *Vicia* and *Lathyrus* were made in 1945 (Bailey, 1952). At this time nine essential characteristics were required of the alternative grain legumes: 1. Ability to be harvested by existing machinery. 2. Growth period of 5- 7 months. 3. Tolerance to red-legged earth mite (*Halotideus destructor*), lucerne flea (*Sminthurus viridis*) and immunity to the pea weevil (*Bruchus pisorum*). 4. Greater tolerance to adverse conditions than peas. 5. Grain and fodder yields at least equal to peas. 6. A seed approximately the size of a pea, or at least large enough to be picked up from the ground by sheep. 7. A residual soil cover after summer grazing. 8. Uniformly maturing and shatter-resistant pods. 9. Ability to tolerate grazing during the growing period (Bailey, 1952). As a direct result of Bailey's pioneering work the *Vicia* cultivars Languedoc, Blanche Fleur, Nyabing, Namoi and Popany were released in Australia (Oram, 1990).

Subsequent research work in South-Western Australia has demonstrated that several *Vicia* and *Lathyrus* species are well adapted to the low fertility soils of the Australian cereal belt with their short winter rainfall dominated growing seasons (Riceman and Powrie, 1952*;* Bailey, 1952*;* Silsbury, 1972*;* Laurence, 1979*;* Georg, 1987a; Georg, 1987b; Georg, 1987b*;* Walton and Trent, 1988*;* Walton, 1992; Siddique and Walton, 1993*;* Davies *et al.*, 1993).

Four species of *Vicia* are currently cultivated in Australia, viz. *V. sativa* cvs. Golden Tares, Languedoc, Blanche Fleur and Nyabing, *V. villosa* cv. Namoi , *V. benghalensis* cv. Popany and *V. articulata* Monantha vetch. *V. hirsuta*, *V. tetrasperma* and *V. villosa* have become naturalised (Jackson and Jacobs, 1985).

Until recently, *Lathyrus* species had not been developed extensively as crops for Australian agriculture due to concerns about their toxicity (Riceman and Powrie, 1952*;* Laurence, 1979). However, in the past, *L. tingitanus* has been grown in Western Australia (Baron Hay and Elliott, 1939), *L. sativus* was considered a useful crop for the North Coast district of New South Wales (Wenholz, 1932) and *L. ochrus* has been recommended for use under irrigation (Giles *et al.*, 1953). *L. odoratus* , valued for its scent and floral beauty, has been a popular ornamental plant in Australia since the 19th century.

Recently a program has been initiated to import germplasm for *L. sativus*, *L. cicera* and *L. ochru*s for initial agronomic evaluation under Australian conditions, with the aim of developing low neurotoxin grain legumes from these species (Siddique and Walton, 1993*;* Davies *et al.*, 1993)

Cultivated *Vicia* species

Several species of *Vicia* have been utilised in agriculture since ancient times (Zohary and Hopf, 1988) while some have been cultivated more recently and others have only ever been cultivated experimentally. Table 1 lists cultivated *Vicia* species, their uses and geographical distribution.

Utilisation of *Vicia* species

The major use of vetches has been in mixtures with cereals as hay, forage and green manure crops (Walton, 1992*;* Bull and Mayfield, 1988*;* Duke, 1981*;* Hermann, 1960*;* Villax, 1963*;* Foury, 1954*;* Laumont, 1954). Recently, great interest has been expressed in their potential utility as pulses (Bull and Mayfield, 1988*;* Garlinge and Perry, 1993). The assumption being, that this would allow for more immediate financial returns to the farming community without the necessity for an extra value-adding process involving livestock production, and would thus constitute an additional end-utilisation option.

Table 1. Cultivated *Vicia* species[2]

Species	synonym	engl. name	Use	Ctry	ref.
Vicia articulata Hornem.	*V.monanthos* (L.) Desf.	One-flowered vetch	G, F	TUR, SPA, MED	1,2,5
V. benghalensis L.	*V.atropurpurea* Desf	Purple vetch	F	IND, MENA	1,8
V. monantha Retz	*V.calcarata* Desf. (Dem)	Bard vetch	G	LIB, MENA	2, 8
V. ciliatula Lipsky	*V.ciliata* Lipsky				7
V. cracca L.	*V. tenuifolia* Gren&Godr	Tufted vetch	F	CHI, JAP	1
ssp. *tenuifolia*(Roth) Gaud	*V. tenuifolia* Roth		F	IRQ	1
V. ervilia Willd	*Ervum ervilia* L.	Bitter vetch	G, F	MENA, WA	1,2,5,8
V. faba L.	*Faba vulgaris* Moench	Broad bean	G	widely	1
V. fulgens Battand.		Scarlet vetch		MENA	8
V. graminea Smith	*V.selloi* Vogel		F, G[3]	S.AM	1,2
V. hirsuta(L.) Gray		Hairy tare	F	IND,EUR,WA,CIS	1,2
V. johannis Taman.			G	TUR	1
V. narbonensis L.		Narbonne vetch	G, F	MENA	1,7,8
V. pannonica Crantz		Hungarian vetch	G	TUR,GRG,MENA	1,2,7,8
V. peregrina		Broad Pod vetch	F		8
V. pisiformis L.		Pale flowered vetch	G	Europe	9
V. sativa L. ssp. *sativa*		Common vetch	G, F	widely	1,7,8
ssp. *amphicarpa*		Subterranean vetch	F		8
V. tetrasperma (L.) Schreb		Smooth vetch	F	White Russia	10
V. unijuga A.Br.	*Orobus lathyroides* L.	Two leaved vetch	F	SIB, MAN, JAP	1,2
V. villosa Roth		Hairy Vetch	F	USA, ETH	1,7,8
ssp. *varia* (Host) Corb	ssp. *dasycarpa* (Ten)Cav	Woolly pod vetch	F	widely	8

V. narbonensis and *V. sativa* have been singled out as the best adapted *Vicia* species for Australian grain legume production under the low rainfall conditions of the drier margins of the cereal belt. This has been ascribed to their relatively high grain yields under adverse conditions. *V. narbonensis* has the additional advantage of an upright growth habit which permits facile mechanical harvesting with existing machinery (Georg, 1987a; Georg, 1987b). Although the need for grain legumes with low toxin levels is well documented, this aspect is conspicuously absent from Bailey's original list of desirable properties, probably because he intended to use *Vicia* species as feed legumes for ruminants, particularly for sheep.

No major toxicity problems are known to occur with feeding grain of *Vicia* species to sheep, provided that they are judiciously used as supplements in conjunction with other feeds and the animals' feed intake behaviour is allowed to operate normally. However with the intended utilisation of these crops as grain feed for mono gastric animals, or even as human food, seed toxicity becomes a highly relevant problem, and one which needs to be investigated thoroughly before further development of these crops for such end-usage can be contemplated. The major focus of this thesis has been to rationalise the utilisation of *Vicia* species as grain legumes, based on a knowledge of their effects on mono gastric animals. Although not a direct feature of this study, informative overlap with *Lathyrus* species has been recorded wherever it is relevant.

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[2]**Abbreviations:** C: Cover crop, G: Grain , F: Forage

CTRY: Country (mainly ex. ref. 1 and 2): CIS: former U.S.S.R.; EUR: Europe; ETH: Ethiopia; IND: India; JAP: Japan; GRG: Georgia; MAN: Manchuria; MED: Mediterranean region; MENA: Middle East and North Africa; PRT: Portugal; SPA: Spain; TUR: Turkey; WA: West Asia, WANA: West Asia and North Africa.

References: 1. Butler (1989) whose sources for her table on cultivated *Vicieae* were (Aykroyd and Doughty, 1982; Davis, 1970; Duke, 1981; Summerfield and Roberts, 1985; Thulin, 1983; Townsend and Guest, 1974; Zhukovsky, 1924) 2. Zeven and de Wet (1982) (sources likely to be based largely on Zhukovsky's work); 3. Duke, (1981) source: Fedchenko, S. (1948) Genus *Vicia* in 'Flora of U.S.S.R. Vol XIII etc. 12 species subgen. *Vicia* 19 species subgen. *Vicilla* as cultivated or semi-cultivated: *V. benghalensis* L., *V. ervilia* (L.) Willd., *V. faba* L., *V. monantha* Retz. (syn. *V. calcarata* Desf.), *V. pannonica* Crantz, *V. sativa* L., *V. sativa* L. ssp. nigra (L.) Ehrh., *V. villosa* Roth (syn. *V. dasycarpa* Ten.); 5. Fischer, (1937); 6. Hermann, (1960); 7. Kupche, (1938); 8. Kernick, (1978); 9. Kunkel (1984); 10. Hegi & Gams, (1924)

[3] cultivated for its seed lectins (Labouriau, 1968; Ottensooser, 1955; Ottensooser, 1958)

The utilisation of *Vicia* species as grain legumes

The use of *Vicia* species as grain legumes requires an understanding of their nutritive value and the potential toxicity of the grain of the different species to the various types of livestock (fish, poultry, pigeons, pigs, horses, cattle, sheep, goats) and humans. This section provides some background on the utilisation of the grain while the following chapters deal with *Vicia* toxicity.

The historical background to the use of *Vicia* species as grain legumes dates back to antiquity, with finds of large hoards of *V. ervilia* dating to 7000 BC., and earliest finds of *V. faba* to 5000 BC. (Zohary and Hopf, 1988). Since chalcolithic times, *Vicia* species have been cultivated, and together with *Lens* spp formed a small but consistent part (2-5%) of the cultivated area at any one time in the Middle-East (Noy-Meir and Seligman, 1979).

In Britain, seed of *V. sativa* has only been produced for re-sowing or for pigeon feed (Loudon, 1880). The proportion of vetch crops sown for fodder increased with the intensification of animal production during the 19th century. In 1900, only ca. 40% of the 240 km^2 of cultivated *V. sativa* in Germany was for seed production (Hegi and Gams, 1924). Of the other *Vicia* species, *V. faba* and to a lesser extent *V. ervilia*, *V. monantha* and *V. narbonensis*, have also been cultivated repeatedly as grain legumes (Fischer, 1937). In some countries and states, such as Germany, Austria, Bulgaria, Lithuania and Spain, fodder vetches (*Vicia* species) have been cultivated as both, grain- and forage crops. In other countries such as Great Britain, Hungary, Yugoslavia, the Netherlands and Sweden, vetches have only been cultivated for use as green forage. In Czechoslovakia *Vicia* species have mainly been cultivated as grain crops (Fischer, 1938). The use of *Vicia* grain in Germany was considerably greater prior to the second world war (Lechner, 1959).

The 19th century agricultural knowledge about vetch utilisation in Britain was summarised by Birnbaum and Werner (1882): Vetches were considered unsuitable as human food, "with the exception of the red summer vetch and the Canadian vetch (*V. sativa alba*) with white seeds whose white flour (ca 10%) has been used to "stretch" wheat flour for bread making in France". For grain production mainly *V. sativa* was used. The seeds of *V. narbonensis* were considered inferior to *V. faba*, this being the reason for its sparse cultivation, although they noted that its flowers had a pleasant scent. "Vetch seeds are a very concentrated, protein rich feed and are especially useful for the production of animal power or meat as a supplement to voluminous feeds, but they are taken by the animals with some reluctance because of their content of bitter substances. Horses still like them best. Detoxification like that used for Lupins has been recommended. Vetches should not be fed in large quantities, because they may otherwise have a detrimental effect on the health of the animals, especially pregnant and young animals. Horses when fed too much vetch without enough work are supposed to have contracted brain damage from the strong and heavy vetch feed." Thus, even a century ago, there was clear recognition of the presence of anti-nutritional components in vetches.

The faba, field or broad bean (*V. faba*) is today still widely used, both as a human food and as livestock feed. Until Columbian times it was the only common bean known in Europe, but since the introduction of the *Phaseolus* beans from the Americas, its nutritional significance has declined. The general paucity of nutritive information about most *Vicia* species contrasts to the very informative literature on *V. faba*, which is the major currently used grain legume in the genus. An excellent treatment of the broad bean as a raw material for food has been compiled by Schmandke (1988).

Besides *V. faba*, the major *Vicia* grain legumes used as feedstuff for farm animals are *V. sativa* L., *V. narbonensis* L., *V. villosa* Roth, *V. monantha* and *V. ervilia* (Fruwirth, 1921; Becker-Dillingen, 1929; Mateo-Box, 1961).

The literature is replete with warnings concerning the use of large amounts of vetch seeds in animal diets. Laumont (1954) advised care with *V. sativa* fodder when it is harvested too late and suggested that the grain should be cracked, soaked and given to farm animals in moderate amounts. Similarly, Kling (1928) recommended that vetches could be used in a similar way to peas, but should be fed in lesser amounts, because their bitter taste ensures that they are not consumed in large quantities by animals. Likewise, Oldershaw (1931) stated that when vetch seed is unsaleable it may then be ground up and fed to cattle and horses instead of peas, but not more than 12.5% of other concentrated feeds.

Table 2. *Vicia* species occasionally used for human consumption[4]

Species	Locality	Parts utilised, comments
V. americana Muhl. ex Willd.	N. America	Young stems boiled or baked
V. amoena Fisch. ex Ser.	E. Asia	Young leaves a pot-herb
V. amurensis Oett.	Manchuria	As above (Tanaka)
V. articulata Hornem.	Medit. Region	Seeds used like lentils
V. cracca L.	Eurasia	Young shoots used as a pot-herb, leaves also used as tea, seeds used ? as food (Hedrick)
V. ervilia (L.) Willd.	Medit. Region	Seeds eaten in soups
Vicia nigricans Hook.		
ssp. *gigantea* (Hook) Lass. & Gunn	California	Seeds edible
V. heptajuga? Nakai	Korea	Young leaves a pot-herb
V. hirsuta (L.) S. F. Gray	Eurasia, N. Africa	Weedy, young leaves and shoots eaten (boiled?), seeds cooked or roasted (Tanaka)
V. hirticalycina Nakai	Korea	Young leaves a pot-herb
V. monantha Retz	Medit. Region	Weedy, seeds used in soups
V. narbonensis L.	S. Europe	"A vegetable" (Tanaka), Hedrick: seeds eaten
V. noena Boiss. and Reut. ex Boiss.	Asia Minor	Seeds edible
V. bakeri Ali	Himalayan Region	Cult, as above? (Hedrick)
V. pisiformis L.	Europe, cult.	Seeds used like lentils
V. pseudo-orobus Fisch. and Mey.	N. E. Asia	Young stems and leaves a vegetable
V. sativa L.	Eurasia, cult.	Seeds ground into flour used in soups and bread, young shoots a pot-herb ; leaves as tea
V. sepium L.	Eurasia	Seeds used as a food (Hedrick)
V. subcuspidata Nakai	Korea	Young stems and leaves a pot-herb
V.cracca L. *ssp. tenuifolia* (Roth) Gaudin	Eurasia	Apparently used as a pot-herb
V. americana Muhlb. var. *sinensis* Gunn	Manchuria	Used as a pot-herb
V. unijuga A. Br.	E. Asia, widespr.	As above
V. venosa (Willd.) Maxim.	E. Asia - Siberia	Used as a pot-herb
V. villosa Roth	Eurasia, weedy	" A vegetable" (Tanaka)

The Use of Vetches as Famine foods

Famines have repeatedly necessitated the utilisation of unorthodox food sources (Bhandari, 1974*;* Salih *et al.*, 1991*;* Salih *et al.*, 1992). In his study of biblical famines Dando (1983) summarised the use of famine foods as follows: " *Famine foods, or non traditional food substitutes used as dietary component replacement, were commonly employed in times of acute food shortages, and knowledge of famine foods was transmitted from one generation to another. Thorough understanding of wild plant and animal food sources, proven methods of food preservation and storage, and dietary flexibility assisted the peoples of the Promised Land through periods of food scarcity. Faced with the threat of food shortages, households hoarded foods, reduced the amount of food consumed and the number of meals each day, and diluted staples with water or mixed staples with unusual substances*". Dando's findings on biblical famines may help to explain the large hoards of *V. ervilia* which have been found in some of the very early Neolithic settlements, documented by Zohary and Hopf (1988). One would expect from this passage that the use of forage and other potentially toxic crops as famine foods would have required the development of techniques to improve palatability. However, from Bhandari's (1974) study of famine foods in the Rajasthan desert it is clear that many of these foods are far from wholesome, with detoxification being mainly used for bitter plants and not for other possibly chronically toxic ones. The potential utility of indigenous plants for human survival during famines is, however, undisputed and this food resource certainly deserves greater attention and further development (Bhandari, 1974).

Knowledge of the safe utilisation of *Vicia* and *Lathyrus* seeds as famine foods is currently not documented. It could reasonably be expected to be found in the folklore of peoples who have had to rely on these crops for food in times of scarcity. This and other economic information should be collected in conjunction with the genetic resources of these species.

[4] **Reference**: Kunkel (1984) pp. 379-380. The annotations used in the table are those of Kunkel (1984). This is very much a compilation from two sources: Hanaka, T. (1976): Tanaka's Encyclopaedia of Edible Plants of the World. Keigaku Publ. Co., Tokyo, 924 pp. ; Hedrick, U. P. (ed.), repr. ed. 1972: Sturtevant's Edible Plants of the World. Dover Publications, New York 686 pp.)

Human consumption of *Vicia* species

This topic is discussed in detail because of the recent controversy about the suitability of *V. sativa* for human consumption (Tate and Enneking, 1992). With regard to the use of *Vicia* species for human consumption, it is conceivable that in the past many of the species would have been eaten by humans at some time or another during evolution. Because toxicity is always dose dependent, the occasional use of these plants for nourishment would probably not pose any great harm. The more toxic seeds also tend to be less palatable. The practice of soaking and leaching seeds would probably have evolved to make them more edible. Most of the low molecular weight toxins in *Vicia* seeds are very water soluble, and can therefore be leached, once the seed coat has been made permeable.

The documented use of toxic plants by hunter gatherer societies from pre-historic times to the present day testifies that humans are capable of rendering almost anything edible. For example the otherwise lethally toxic *Cassava* tuber is prepared by an elaborate process to yield one of the world's staple diets. Similarly, acorns, with their high content of astringent tannins were once the staple diets of humans in the pre-historic Middle East, in Southern Spain and the indigenous people of California.

Reliance on detoxification can be a potential health hazard. Incomplete processing of *Cassava* can lead to endemic goitre and in conjunction with a deficiency of nutritional sulfur it was identified as the cause for "Konzo", an upper motor neuron disease recently described from East Africa in areas where the higher-yielding bitter Cassava varieties are cultivated (Tylleskär *et al.*, 1992*;* Banea, 1993).

Ochse (1931) described the diverse foods of Indonesia, and among the legumes listed, many are quite toxic without adequate soaking, washing or fermenting. Likewise, the Australian Aborigines developed the skills to detoxify the Morton Bay Chestnut (*Castanospermum australe*) and the Cycad nut (*Cycas* species). The laborious leaching procedures involve the cooking or roasting of the plant parts prior to increasing their surface area through slicing or mashing, followed by prolonged leaching in running water. Boiling in several changes of water does not substitute for this age-old practice as far as the effectiveness of detoxification is concerned (Horsfall, 1987). Salih *et al.* (1991) described the debittering processes for the seeds of two uncultivated indigenous woody plants, *Boscia senegalensis* (Pers) Lam ex Poir and *Dobera roxburghi* Planch, which were used during the 1984-85 famine in the Kordofan and Darfur regions of the Sudan.

Although there are some comments in the literature that *Vicia* species have been used as famine foods, there seems to be a paucity of information about this subject. Except for the list of *Vicia* species presented in Table 2 (Kunkel , 1984), no detailed documented study could be found about the appropriate use of these species for human consumption. It is important to note that Table 2 should not be seen as a general culinary recommendation, but rather as a guide to what has been occasionally been consumed in the past. Without a detailed knowledge of preparative and culinary practices used for their consumption "one man's food could well be another person's poison".

Other authors have referred to the use of *Vicia* species for human consumption. The earliest documented evidence in the literature regarding the use of a white seeded vetch for human consumption is given by the 12th century Andalusian agriculturalist Ibn Al Awam who wrote a treatise on Agriculture (Kitab Al-Felahah). With due reference to an earlier agricultural treatise, the "Nabathean Book of Agriculture" (ca. 400 AD.), he described in detail the use of *V. ervilia*[5], noting its harmful effects on pregnant ewes and outlined the preparation of a white-seed vetch (translated uncertainly[6] as *V. sativa alba*) for human consumption. This required the soaking of the seed in several changes of water for some days, followed by decortication achieved by heating of the soaked seed. He recommended that one should not eat the vetch on its own, but it should be used together with the flour of lentils or with washed wheat (gluten?) for bread making. Such bread was then eaten together with fatty meat, butter, oil or milk (Clémont-Mullet, 1866).

Gärtner *et al.* (1801) remarked that the meal of *V. sativa* meal has been used for human consumption during times of want. Baron Ferdinand v. Mueller (1881) considered *V. narbonensis* from South Europe and South-

[5] Interestingly, he also referred to allelopathic effects of the grain when mixed with other plants ("La vesce noire melée aux graines des plantes maraîchères les fait perir")

[6] Gerarde (1636) distinguished between a white and black-seeded types of *V. ervilia*, the latter being better suited for medicinal purposes. His sources were authors of antiquity such as Galen and Hippocrates, which must have also been available to the author(s) of the Nabathean book of agriculture referred to by Ibn Al Awam. This suggests that the white seeded vetch, discussed in the context of *V. ervilia* may have been erroneously identified by Clémont-Mullet as *V. sativa alba*. Therefore, Ibn Al Awam's text may actually refer to the detoxification of *V. ervilia* for use in human consumption.

West Asia to be preferable to *V. faba* for the table because the somewhat smaller seeds were less bitter. Birnbaum and Werner (1882) were of the opinion that vetches were unsuited as human food, with the exception of the red summer vetch and the white-seeded Canadian vetch (*V. sativa alba*) whose white flour (ca 10%) had been used to stretch wheat flour for bread making in France. However, Alefeld (1866) explicitly stated that he did not recommend a single variety of *V. sativa* for human consumption because vetches were much more inferior to peas and lentils in this respect. Bois (1927) stated that according to Ducomet (no ref.), the grain of *V. serratifolia* Jacq., *V. lutea* L., *V. angustifolia* Reichenb., *V. tenuifolia* Roth (species which grew in France) could be mixed with wheat for food, under famine conditions, because they were rich in albumins and carbohydrates. The same author also cited Chesnut[7] who recorded that the young stems and leaves of *V. americana* Muehlenberg, were collected and eaten like a vegetable by various tribes in California. A cottage bread with a beanie flavour made from the seeds of a mixture of wild legumes (mainly *V. johannis* and *Pisum elatius*) was well liked by local farmers near Eskishehir, Turkey (Scheibe, 1934). Gillet noted that the seeds of *V. narbonensis* were eaten as a pulse in a foothill village some 19 km N. E. of Arbil, in Northern Iraq, and the leaves were fed to stock as a fodder (Townsend, 1974). In Eastern Turkey, near Dyarbakir, *V. narbonensis* is apparently eaten as a pulse, after it has been boiled with some salt. The plants are collected from the nearby lentil fields where they grow as weeds (Enneking, pers. observation, Ergani village, 1991)

Clearly, the documented evidence for the occasional human consumption of *Vicia* species, other than *V. faba* is very limited and there seem to be no evidence for their use as staple foods. No information about the longer term effects of such food on human health could be located.

The white seeded vetch or Canadian lentil

In the 19th century, Wilson (1852) reported the human consumption of a white tare which was also called the Canadian lentil or Napoleon pea (*V. sativa alba*). It had white or cream coloured seeds, with an apparently much milder taste than other cultivars. He noted that it had a more dwarfish habit, and produced a much greater quantity of seeds than the other varieties of *V. sativa*. It was cultivated far more extensively in France and in Canada than in Britain, chiefly for the sake of its seeds. The seeds were reported to have been used for human food, both green and ripe, in soups and other dishes, in the same manner as peas; and they were also ground into flour, for intermixture with the wheat flour for bread making.

In 1924 Hegi and Gams reported that *V. sativa* was seldom used as human food and they considered it much more inferior to peas and lentils for this purpose. To remove the bitterness, the seeds were soaked for at least a day in cold water. However, they mention that the yellowish-white seeds of *V. sativa* forma. *leucosperma* (Moench) Ser. (incl. varieta *ochrosperma* Rchb.) to which the "grosse neue Erbslinse" (large new pea-lentil), the " weisse amerikanische Perllinse" (white American pearl-lentil) and the" weisse Linsenwicke" (white lentil-vetch) belong, were used for human consumption along the river Rhine, in Dalmatia, Southern France and Canada. They described an incident in 1916, when sour lentils (*V. sativa* forma *bipunctata* Alef.?) were for a short time sold in Berlin but were later confiscated from the market by police. As a soup or prepared as vegetable these were apparently quite tasty and digestible. A bitter taste, which they suggested was probably related to the prussic acid content present in some common vetches (*V. sativa*) could be removed through boiling in salt water. They also reported that vetch flour (*V. sativa*) had served variously in bread making[8]. From the Toscana, Italy, a flour called "grano vecciato" was known which contained 1/3 vetch flour. *V. sativa* flour was also a major constituent of Revalenta arabica (a fraudulent concoction, sold as medicine in the United States).

A recent report about the use of *V. sativa* for human consumption is given by Kaul (1985) for a black-seeded variety of *V. sativa*, which in Kashmir is locally known as "hemba gassa" and the seeds are collected from the pods and taken as a vegetable.

Dr. Peter Hanelt, IPK, Gatersleben, Germany has obtained a white-seeded variety of *V. sativa*, which reputedly is used even today like lentils for human consumption in the historical province of Ratcha in Western-Georgia (Caucasus)(pers. comm.).

Although, the above information documents the use of *Vicia* species as food, their harmlessness, especially after longer periods of consumption, remains unproven. The detrimental effects of *V. sativa* on pregnant rats, documented early this century (McCollum *et al.*, 1919), should be a warning that the anti-nutritional properties

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[7] Contributions from the United States herbarium, Dep. of Agriculture 7 (3) p.362

[8] The flour of white-seeded grain legumes blends well with wheat flour and can be used to increase the weight of bread.

must be thoroughly evaluated before its consumption as a staple diet can be sanctioned by the scientific community.

Vicia seeds for animal production

V. ervilia

This species is one of the oldest domesticated grain legumes (Zohary and Hopf, 1988). It can cause serious intoxication in pigs and poultry (see chapter 3). The older literature refers to the use of the soaked grain as a fattening cattle feed (Gerarde, 1636). The seeds, whose bitterness can be removed through steaming and leaching with hot water are to some extent used for human consumption. More frequently they are fed to pigs and cattle for fattening and often also to poultry (Fruwirth, 1921).

Cattle and sheep are only affected by its toxicity if they consume large quantities of bitter vetch flour, so rations should not exceed 25% bitter vetch for ruminants (Mateo Box, 1961; cited by Gomez, 1983)

A recent study with laying hens in Turkey by Ergün *et al.* (1991) has found that the inclusion of wild *V. ervilia* seed at 4, 8 or 12% had a negative effect on live weight, egg production, feed conversion efficiency and egg weight. However, egg yolk colour improved with increasing vetch concentrations and other parameters, including shell breaking strength remained normal. *V. ervilia* was clearly not a suitable feed for layers. This is probably due to the presence of canavanine (see chapter 2).

The bitter vetch, *V. ervilia* is a very old crop plant and could reasonably be expected to have been selected for less toxicity[9] during the last 10.000 years. Indeed, compared to other *Vicia* species the content of canavanine in *V. ervilia* seed is rather low (Tschiersch and Hanelt, 1967*;* Garcia and Ferrando, 1989) (more details can be found in Chapter 2). The converse argument, that selection could have favoured bitterness in the presence of predators, such as insects or nematodes applies equally well, and this may explain why (Garcia and Ferrando, 1989) found much higher levels of canavanine in some Spanish *V. ervilia* cultivars from particular areas of cultivation. Individual cultivars therefore differ in their toxicity and it seems that if the grain is first leached it can be fed to mono gastric animals in moderate amounts, and it can apparently be fed in seemingly large quantities to ruminants. In view of a recent study on the metabolism of canavanine in the rumen (Dominguez-Bello and Stewart, 1990), and the relative impermeability of intact seed coats, the soaking of the grain prior to it being fed to ruminants may had the purpose of making it more digestible rather than detoxifying it.

V. sativa

The seed of *V. sativa* has been used to feed horses (Gärtner *et al.*, 1801*;* Hegi and Gams, 1924), but Pott (1907) considered *V. sativa* plants with ripened pods, or the seeds, unsuitable as feed for horses because inclusion of such diets caused brain disease with symptoms of acute brain oedema (Gehirn-wasser-sucht) in these animals. The first scientific digestibility study with *V. sativa* grain was carried out by Gabriel and Gottwald (1887). According to Becker-Dillingen (1929), vetch seed (*V. sativa*) ranks in every relation a little bit higher in terms of nutritional value than field beans (*V. faba*) and can therefore be fed in similar, preferably smaller amounts than these. Cracking and debittering was considered advisable. Mouldy seed had to be boiled prior to feeding. Cracked vetch seed was fed to horses, beef-oxen, pigs and dairy cows. It was also considered good for wethers. Recently vetch seed digestibility in sheep was studied by Round (1988).

According to Mateo Box (1961; cited by Gomez, 1983), the grain can be used for birds (mostly pigeons), or even pigs and cattle, after it has been ground into a flour. However, it may cause constipation and dermatitis in pigs and lathyrism in horses. In cattle and sheep it causes somnolence, muscular paralysis and death by asphyxiation . A level of 400 g/day (20% of the ration) should not be exceeded for fattening pigs, and 10% for piglets (Piccioni, 1970; cited by (Gomez, 1983)

Snook (1947, 1948) found little indication of toxicity for *V. sativa* (Hawkesbury strain) in guinea pigs, chicken and wether bioassays, however he did not pay any attention to the observed feed intake inhibitory effect of the grain on all animals and the duration of the feeding assays was limited by the seed supply. This author has often been cited in the Australian literature with regard to the safe and profitable feeding of vetches to animals,

[9] The author has tasted some crystalline canavanine.2HCL. After an initial sharp taste which is probably due to the HCl, no other immediate taste was detectable, however after ca 5-10 mins, associated with increased salivation, a nauseating taste developed which lingered on despite thorough cleansing of the mouth with water.

however these citations are based upon equivocal evidence to say the least.

Milled *V. sativa* seed (HCN 64.9 mg/kg) was found to be highly palatable during a three months feeding period with 3 adult Kumaoni bulls which had *ad libitum* access to wheat straw. In comparison to the control feeding period on groundnut cake, lower red blood cell counts and haemoglobin levels were found during the feeding of *V. sativa*, but as the values were within the range reported for this breed, it was concluded that milled *V. sativa* could be fed as a concentrate to cattle (Pandey and Pal, 1960).

In Australia, the grain of *V. sativa* varieties "Languedoc" and "Blanche Fleur" is readily marketable as pigfeed (Bull, 1989). This statement was based on the experimental testing of a diet containing 35% *V. sativa* cv. Languedoc (Davies, pers. comm.) for its suitability to pigs. This was followed by unpublished research work carried out in South Australia by a commercial company (Metro Meat) wherein the seeds of *V. sativa* cvs. Blanche Fleur and Languedoc were tested at a 10% feed inclusion level in a commercial operation with 200 animals (Edwards, pers. comm.). Davies (pers. comm.) had found that the live weight gain of pigs feeding on diets containing 35% Languedoc vetch was reduced by 15%, but he could not unequivocally attribute this to any anti-nutritional factors because of errors involved in the metabolisable energy determinations. Edwards (pers. comm.) obtained variable results feeding 10% *V. sativa*, with the last of three trials giving the best weight gains, but in this particular trial the animals had previously received diets containing 5% *V. sativa*, which could have confounded the results through a possible metabolic adaptation to the vetch diet. In fact, metabolic adaptation to the toxicity of *V. sativa* is an exciting possibility, with a similar effect documented in an experiment with layer poults feeding 5% and 10% *V. sativa* (Glatz and Hughes, 1993).

The performance of *V. sativa* in pig diets at the 10% feed inclusion level was considered encouraging, and led to an increased planting of this species, because of the available market in the pig-industry. Upon inquiry into the ongoing practice of feeding *V. sativa* to pigs, it was made clear that it is only commercially attractive if the grain can be bought at 2/3 the price of peas ie. it is only profitable when the price of peas is high and *V. sativa* can be produced cheaper than peas (Edwards, pers. comm.).

The toxic effects caused by diets containing *V. sativa* grain will be discussed in detail in chapters 3 and 7. With respect to its safe utilisation it is worthwhile to point out that the levels of the β-cyanoalanine toxins in the grain vary with the cultivar (Ressler *et al.*, 1969), and in the case of the cyanogenic glycosides, these are reported to increase in the plant after flowering (Akbari, 1965) and also to differ dramatically amongst individual cultivars (Hanelt and Tschiersch, 1967). Therefore, the safe feeding of *V. sativa* grain will depend very much on the individual cultivar in question. Regardless of whether animals are monogastric or ruminant, they should be introduced gradually to this type of feed, because it seems that there is potential for biochemical adaptation to the toxins, however more research is definitely needed to follow the important observations of Edwards (pers. comm.) and Glatz and Hughes (1993).

Vicia sativa consumption and its effect on milk yield and quality

According to von Knieriem (1900), Haubner (1845) was the first to question the suitability of *V. sativa* seed as a feed for dairy cattle because of its negative effect on milk production. However, the findings of the studies by Ouiek (1896), Kühn (1898) and von Knieriem (1900) questioned this belief and produced evidence to support the contrary view. But even in the 1930's the notion was still held that vetch seeds were less suited as a dairy feed, because of their negative influence on milk secretion Kling (1928). Kühn (1898) cautioned not to feed cracked *V. sativa* grain at much more than 2 kg day^{-1} 1000 kg^{-1} live weight for prolonged periods, because he had encountered some initial problems such as indigestion and reduced feed intake during the transition to feeding *V. sativa* grain to some dairy cows. He recommended feeding the grain in cracked form. If the grain was to be soaked it had to be squashed prior to feeding as otherwise a large proportion passed undigested into the faeces. He reported that up to 4.5 kg $head^{-1}$ day^{-1} of cracked seed had been fed as a sole concentrate to dairy cows without negative effects on milk production and taste, but at this level of feeding the consistency of the butter was firmer and more crumbly than usual. Pott (1907) advised that this hardening effect on butter consistency of the vetch grain diets could be overcome by simultaneous feeding of beet roots.

Schwarz and Finzenhagen (1937) reported the presence of 50 mg HCN/kg in *V. sativa* seed, and found that by soaking and steaming the soaked seeds the majority of HCN was removed. Richter and Herbst (1937) evaluated this method more thoroughly. Seeds were soaked in water for 3 hours and took up 25% of their weight in water. The thus pre-treated *V. sativa* seeds were then put into a pre-heated potato steamer where they were exposed to temperatures of 95-105 °C for 10 mins. Repeated tastings led to the result that the seeds which prior to steaming had a bitter taste had acquired a pleasant nutty flavour after steaming. Prussic acid content of the air dry material: Untreated material 0.0863 $^{0}/_{00}$, steamed 0.0557 $^{0}/_{00}$. Thus a 35% reduction in prussic acid content was achieved. They then demonstrated that the feeding 3 kg $head^{-1}$ day^{-1} of either steam debittered or

untreated *V. sativa* seed for ten days to cows (510 - 640 kg body weight) had no negative effects on feed intake, total milk or milk fat yield.

In contrast, Richter (1937) studied in detail the effects of four *V. sativa* treatments on milk, butter and whipped cream quality. The whole and ground grain, either debittered (by steaming) or untreated was fed at 3 kg head^{-1} day^{-1} to dairy cows. He found that the milk in the first period of feeding had a pronounced vetch flavour and had to some extent a bitter taste. However this defect diminished with time. Richter suggested that the animals be gradually accustomed to vetch diets and that the adaptation of the cow be monitored by tasting the milk for the presence of the vetch flavour and bitterness. The author cautioned against the use of milk from vetch-fed cows for infants, without debittering of the vetch. This study should be of interest to milk producers intending to feed *V. sativa* grain to cows, because it gives data on the important milk, butter and cream parameters. In general, the feeding of grain legumes appears to give a harder butter and the time for disappearance of the off-flavour varies with individual animals.

Piccioni (1970; cited by Gomez, 1983) noted that milk is bitter if dairy cows are fed 2 kg of *V. sativa* grain day^{-1}. The taste of vicine and convicine passes into the milk, which renders it unsuitable for both, direct consumption and cheese production. In consideration of Richter's observations on the transience of this phenomenon, the milk obtained from animals unaccustomed to feeding on diets containing *V. sativa* grain should be tested for the presence of vicine as well as ß-cyanoalanine and γ-glutamyl-ß-cyanoalanine and for possible metabolites of these toxins. Bioassay evaluation of such milk for toxicity may also be warranted.

The evidence from the literature suggests that any initial problems with milk quality from vetch grain fed animals, can be easily monitored by taste analysis and may be transitory in nature. At maximum feeding rates of 3 kg head^{-1} day^{-1} this feed may therefore find a profitable use in the dairy industry.

***V. articulata* Hornem.**

Fruwirth (1921) considered this crop which is suitable for lime-deficient light soils to be more important as a green fodder. Besides the tender straw, the cracked grain was considered useful for the fattening of animals in quantities of up to 1/3 of the ration (no species mentioned). Mixed with other feeds it was also fed to dairy cattle and horses. The seed could also be used for human consumption, as it was known as black lentil, however he considered them inferior in taste compared to lentils and they could only rarely be found in markets.

In Spain, the area once sown to this grain crop exceeded that of lentils (Barulina, 1930). The grain is best tolerated by sheep which sometimes reject it because of its bitter taste. Birds, in general, do not consume it, except for pigeons which like it a great deal (Mateo-Box, 1961; cited by Gomez, 1983).

V. narbonensis

Kansu (1961) tested *V. narbonensis* grain in a digestion trial with Akkaraman sheep and found that this feed could be used in concentrated rations for sheep because it contained a high amount of digestible nutrients and he predicted that this species would play an important part as a feed in the near future.

Almost 20 years later in Australia, Allden and Geytenbeek (1980) produced evidence which suggested that *V. narbonensis* (line RL 140001) was unsuitable for sheep (6 months old Suffolk x Merino wethers) because it had a negative effect on wool growth while at the same time their weight gain was normal, compared to sheep feeding on standing crops of *V. sativa*, *V. faba* and other grain legumes.

Recent work at the School of Agriculture and Forestry, University of Melbourne has indicated that the utility of *Vicia narbonensis* as a supplemental feed grain for sheep has been underestimated in Australia (Jacques *et al.*, 1991). No detrimental effect on wool growth was noted after feeding narbon beans as supplements to mature Merino wethers which had *ad libitum* supplies of pasture hay. At twice weekly feeding of either *Pisum sativum* (Dun pea) or *V. narbonensis* (line RL 140004) with *ad libitum* pasture hay available, superior animal performance was recorded for the narbon beans over peas at the lower allocation of grain (1% of live weight/day), compared to the same supplements fed at 2% of live weight/day. The peas offered to the sheep were eaten within half a day, whereas the *V. narbonensis* grain was consumed more slowly over two days (Jacques, 1990). The lower palatability of the narbon beans compared to peas seems to result in a more even consumption over time and hence better utilisation of the grain supplement. Thus the lower palatability of *V. narbonensis* may be of advantage to farmers because it saves repeated feeding and therefore labour costs.

More work on feeding *V. narbonensis* grain to sheep is clearly warranted, and attention should be given to its effect on lamb growth, lactation and the reproductive performance of ewes and rams. Because of the high sulfur content of the narbon beans constant attention should also be given to wool growth and quality. The lack

of any documented negative effects of *V. narbonensis* on sheep in the Mediterranean and the Middle East suggests that either no attention has been given to wool growth performance of sheep on such diets, or that there are other factors involved such as diet composition, sheep breed or rumen biology.

Polymorphism in the levels of reduced glutathione (GSH) in sheep red blood cells (RBC) have been documented (Agar, 1975) and genotypes with low levels have been found to be more susceptible to Heinz body formation in response to kale feeding (Tucker and Kilgour, 1973). A recent *in vitro* study, testing the response of high and low GSH Sheep RBC to oxidant stress found evidence for increased Heinz body formation in the low GSH type RBCs, thus supporting earlier studies (Ikuo Goto *et al.*, 1993). The frequency and nature of the low GSH RBC type varies with individual sheep breeds. In Merino sheep, a breed for which a high frequency (40%) of the low GSH trait was found (Tucker and Kilgour, 1972), the defect was regarded as due to a reduced activity of γ-glutamyl-cysteine synthase, the first enzyme of GSH synthesis (Smith *et al.*, 1973*;* Young and Nimmo, 1975). The finding that higher wool growth capacity was negatively correlated with RBC GSH concentrations, has led to the suggestion that this biochemical trait may prove to be useful for selecting sheep for higher wool production (Board *et al.*, 1974).

The discrepancy between the findings of Allden and Geytenbeek (1980) and Jacques *et al.* (1991) could therefore be due to genetic differences between the sheep (Suffolk x Merino and Merino wethers respectively) employed in these studies. In addition, the latter study included *ad libitum* supplies of hay, while feeding the grain only as a supplement, whereas the former tested the sheep production capacity of pure mature legume stands. Furthermore, the lines of *V. narbonensis* and the age of the sheep used in the two experiments differed. Future feeding studies with *V. narbonensis* may benefit from the inclusion of suitable control treatments for high and low RBC GSH type sheep, different age groups and the testing of different plant genotypes.

V. narbonensis grain has been used for feeding livestock, particularly cattle, which appear to tolerate it better than pigs and sheep, provided that it is given in ground form. It has a taste which the animals come to accept, but a taint may be conferred to the milk. (Mateo-Box, 1961).

Allden and Geytenbeek (1984) noted that cattle feeding on mature stands of grain legume crops (*Lathyrus ochrus*, *Vicia faba*, *Lupinus angustifolius*, *Pisum sativum*), gained weight only on *V. faba* which had large seeds and non-shattering pods. Their weight gain on Lupins was adequate as long as the seeds were retained. Sheep gained weight on all crops. This study showed that the performance of sheep feeding on mature grain legume crops may bear no relationship to the performance of cattle under similar conditions, due to differences in feeding behaviour with sheep being grazers and cattle browsers.

In the European literature, the grain of *V. narbonensis* has been identified as a suitable ruminant feed, especially for cattle (Mateo Box, 1961*;* van der Veen, 1960) thus the crop holds particular promise for increased beef production, while for milk production there may be some off-flavour problems, however if mature crops are to be effectively utilised, attention should be given to the selection or breeding of cultivars with improved shatter resistance. It may also to be important to assess the flavour of meat produced from *V. narbonensis* fed animals.

Davies (1987) found that *V. narbonensis* grain (line RL 140001) depressed feed intake when fed as a major component (35%) of pig diets. Blood samples taken from animals which had been feeding for two weeks on this diet were found to have raised bilirubin levels. A post-mortem on a single pig revealed a mild interstitial nephritis and "oxalate" type crystals in the kidneys.

V. narbonensis does not seem to be an appropriate feed for poultry which have great difficulty eating it (Mateo-Box, 1961). This was partly confirmed by Eason *et al.* (1990) who tested the nutritive value of *V. narbonensis* (line RL 140004, Castleman, pers. comm.) at the 5 and 10% inclusion level in the diets of day old broiler chicken fed to 21 days of age. Performance was compared with *Pisum sativum*, *Lupinus angustifolius* , *Glycine max* and a meat meal based diet (4 replicates of 12 birds/treatment). Chemical composition of the grain of *V. narbonensis* was found to be similar to that of the peas used in the experiment and the values found agreed with those given by Gomez (1983). The results indicated, as shown earlier (Eason *et al.*, 1987) that narbon beans could be included up to 10% in broiler starter diets without showing any adverse effects on growth performance, however, by comparison with soybeans, the feed intake on the 10% narbon diet was reduced by 4% and the 21 day live weight by 2.6%. Feeding peas at the same level resulted in a 2.7% live weight reduction. In a larger scale experiment with 136 mixed sex chicken which were fed to 42 days of age, all Narbon bean treatments (8%, 14%, 20% dietary inclusion level) had a negative effect on feed intake and resulted in lower weight gains (Johnson and Eason, 1990). Pancreas and liver weights were within normal values, and no trends were detected in the data (Eason, pers. comm.). There is clearly some potential for the utilisation in poultry diets of lines of this crop with low levels of anti-nutritional factors. So far, no information is available for its utility in layer diets and as a feed for other bird species.

Conclusion

The utilisation of *Vicia* species as grain legumes dates back to pre-historic times and there seems to be sufficient potential for the wider utilisation of the minor *Vicia* grain legumes, viz. *V. ervilia*, *V. sativa* and *V. narbonensis*. These grains are well suited as supplements for ruminant production. The maximum dietary inclusion levels of individual varieties and the effect of such diets on end-product quality require further delineation. Development of these crops as feeds for mono gastric animals is still in its early stages and it is hoped that advances can be made through the selection of suitable varieties and possibly through the selection of better adapted animals. There may also be scope for the inclusion of small amounts of *Vicia* grain in current rations in order to improve feed utilisation and reduce waste output.

Chapter 2

Vicia toxins and their biological activity

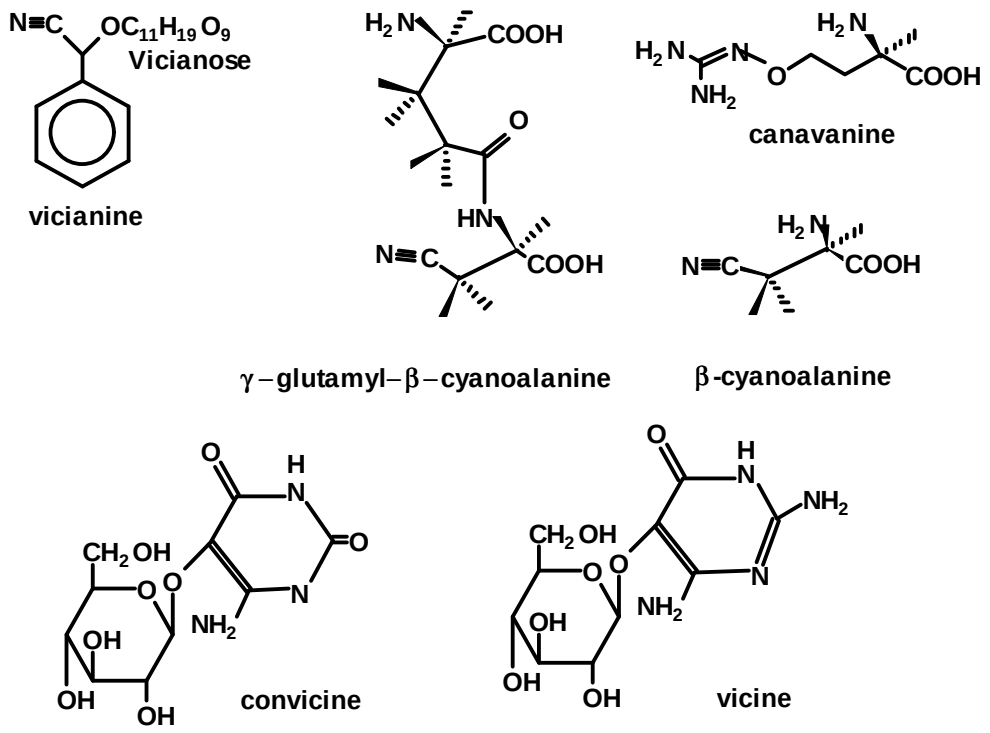
Introduction

Vetch seeds differ widely in their secondary metabolite composition and *Vicia* toxicity is best understood in this context. This chapter therefore describes the distribution and toxicity of the major known low molecular mass (<1000 daltons) toxins in *Vicia* seeds.

The patterns of unusual amino acids, also called non-protein amino acids (NPAAs) which can be readily isolated from the seed meal, have been used for chemotaxonomic classification (Bell and Tirimanna, 1965; Tschiersch and Hanelt, 1967) because they permit partition of the genus into distinct subgeneric groups (Kupicha, 1976).

Tables 1 and 2 list the distribution of the five major low molecular weight antinutritive factors which have been identified in seeds of the genus *Vicia* so far (Fig 1.).

Fig 1. Chemical structures of *Vicia* toxins



Three major groups of species can be separated by the nature of their seed non-protein amino acids (NPAAs) (Bell and Tirimanna, 1965; Tschiersch and Hanelt, 1967; Bell, 1971). Based on the distribution of the cyanogenic glycoside vicianine and the levels of the pyrimidine glucosides vicine and convicine in some species, two further groups are distinguishable. The latter two compounds have been identified by Griffiths and Ramsay (1992) in all species examined in their study, however, high levels were restricted to *V. bithynica* and *V. faba* (*V. sativa* was not examined).

Species of the subgenus *Vicilla* , amongst them *V. ervilia*, *V. articulata*, *V. villosa*, *V. benghalensis*, *V. cracca* contain the toxic arginine analogue, canavanine (group 1). Within the subgenus *Vicia*, one group (which includes *V. sativa*) is characterised by the presence of ß-cyanoalanine and γ–glutamyl-ß-cyanoalanine (group 2). Other species of this subgenus, including *V. narbonensis*, are distinguishable by the presence of the hitherto unidentified peptide VA3 (group 3) which has now been identified as γ-glutamyl-S-ethenyl-cysteine (Chap 5).

Table 1 Seed distribution of *Vicia* toxins according to species in subgenus *Vicilla*

Abbreviations: CN-AA: Cyanoalanine acids eg ß-cyanoalanine and γ-glutamyl-ß-cyanoalanine. Relative concentrations [+] = approx. 1% Dry Weight (DW) of γ–glutamyl-ß-cyanoalanine are indicated, all species with this compound had similar concentrations of ß-cyanoalanine [+] ex ref. [1], presence of both recorded by ref. [2] Canavanine [g kg^{-1} DW ex ref. [2] , relative concentrations [+] = approx. 1% DW ex ref. [1]]

References: 1. Bell and Tirimanna (1965); 2. Tschiersch and Hanelt (1967)

SPECIES	**CN-AAs**	**Canavanine**	**VA_3**
subgen.*Vicilla*			
V. tetrasperma (L.) Schreb.		0.6-0.8 [++]	[traces]
V. laxiflora Brot.		1.2-1.8[++]	
V. pubescens (DC.) Link		1.9-2.8	
V. articulata Horn.		1.5-2.6[++]	
V. hirsuta (L.) Gray		0.8-2.4 [++]	[+]
V. disperma DC.		3.2-5.1[+++]	[+]
V. ludoviciana Nutt.		2.2-5.4	
V. ervilia (L.) Willd.		0.2-0.4 [+][10]	
V. sylvativa L.		2.4-3.5 [++]	
V. unijuga A. Br.		2.5-3.8	
V. cracca L.		2.1-3.8 [++]	[++]
ssp. *stenophylla* Velen.		5.2 [+++]	[+]
ssp. *tenuifolia* (Roth) Gaudin		2.9-3.8[+++]	[++]
V. neglecta Hanelt and Mettin		2.6	
V. biennis L.		1.6-2.2 [++]	
V. villosa Roth		2.5-7.8 [++]	[+]
ssp. *varia* (Host) Corbiere		1.6-4.0 [+++]	[++]
ssp. *eriocarpa* (Hausskn.) Ball		2.3-2.6	
V. benghalensis L.		1.9-3.4 [+++]	[+]
V. monantha Retz.		1.8-2.4 [++]	[++]
V. graminea Smith		[+++]	[++]
V. cassubica L.		[++]	[+ +]
V. orobus DC.		??[11]	[+ +]
V. venosa (Willd.) Maxim.		??	[+ +]
V. unijuga Braun		??	[+ +]
V. onobrychioides L		??	[+ +]
V. ludoviciana L.??	[+ + +]??		
V. sicula Guss.??	[+]??		

The food legume, *V. faba*, is characterised by the presence of high levels of vicine and convicine.

In the pioneering work by Bell and Tirimanna (1965), two of the major acidic ninhydrin-reacting compounds, present in the seeds of species such as *V. narbonensis* were designated VA_1 and VA_3[12]. They observed that VA_1 may be a breakdown product VA_3 under acidic conditions and they documented the peptide nature of VA_3. Chapter 5 describes the isolation and structural determination of the major acidic ninhydrin reacting low

[10] Cacho *et al.* (1989) measured respectively 1.3 and 0.97 g kg^{-1}canavanine; while Garcia and Ferrando (1989) found respectively 0.5-1.1 g kg^{-1} and 0.1-2.6 g kg^{-1} canavanine in the seeds of red and white seeded *V. ervilia* varieties.

[11] The data for these species, especially the latter two, are questionable and several problems could have arisen 1. canavanine concentration too low for detection 2. misidentification 3. misclassification by Kupicha (1976) 4. breakdown products of other compounds could have been mistaken for the cyanoalanine amino acids (unlikely but possible). Otherwise the chemotaxonomic scheme for the division of the genus on the basis of canavanine distribution would be incorrect.

[12] VA stands for unknown Vicia Acidic (pH 3.6) amino acid

MW compound from the seeds of *V. narbonensis*. Its chemical structure has been identified as γ–glutamyl-S-

Table 2 Seed distribution of *Vicia* toxins according to species in subgenus *Vicia*

Abbreviations: CN-AA: Cyanoalanine acids eg ß-cyanoalanine and γ-glutamyl-ß-cyanoalanine. Relative concentrations [+] = approx. 1% Dry Weight (DW) of γ–glutamyl-ß-cyanoalanine are indicated, all species with this compound had similar concentrations of ß-cyanoalanine [+] ex ref. [1], presence of both recorded by ref. [2]; Vicine/Convicine [g kg^{-1} DW, ref. 3; data for concentration ranges: ref. 5; Vicine: presence identified based on retention time and mass spectrum of TMS derivative, ref. [4]]; Vicianine: [µg HCN g^{-1} DW, ref. [2]]

References: 1. Bell and Tirimanna (1965); 2. Tschiersch and Hanelt (1967); 3. Pitz *et al.* (1980); 4. Yasui *et al.*, (1987); 5. Griffiths and Ramsay (1992) 6. Khattab (1988)

SPECIES	CN-AAs	VA_3	Vicine/Convicine	Vicianine
Subgen. *Vicia*				
V. sepium L.	[+ +](2)		Vicine	
V. grandiflora Scop.	[+ +](2)			140-320 (2)
V. macrocarpa (Moris) Bert.	(2)			220-440 (2)
V. sativa L.	[+ +](2)		0.75/0.08	0-990 (2)
spp. *sativa*	(2)			0-300 (2)
ssp. *amphicarpa* (L.) Batt.	[+ +]			
ssp. *nigra* (L.) Ehrh.	[+](2)			240-990 (2)
ssp. *incisa* (M. Bieb.) Arcang.	(2)			
V. peregrina L.	[+]		0.43/trace	
V. michauxii Sprengel	[+ +]		0.38/0.04	
V. peregrina L.	[+]		0.43/trace	
V. michauxii L.	[+ +]		0.38/0.04	
V. hybrida L.	[+ +]		0.75/0.07	
V. lathyroides L.	[+ +]			
V. sepium L.	[+]			
V. faba L.			4.2-10.8/0.3-5.1	
V. bithynica L.		[+ +]	3.62-4.39/0.81-1.09	
V. narbonensis L.		[+ +]	0.18-0.62/0.05-0.16	
V. johannis Tamansch.		(?)	0.26-0.35/-	
V. serratifolia Roth		(?)(6)	0.48/-	
V. hyaeniscyamus Mout.		(?)(6)	0.51-0.70/-	
V. kalakhensis Khatt., Maxt & Bisby		(?)(6)	0.34/-	
V. galilaea Plitm. & D. Zoh.		(?)	0.24-0.87	
V. oroboides Wulf. in Jacq.			V	
V. melanops Sibth. & Smith			0.43-0.78/-	
V. lutea L.		[+ +]	0.21-0.66/trace	
V. pannonica Crantz.		[+ +]		
V. hyrcanica Fisch. & C. Mey.		[+ +]		
V. fulgens Battand.[13]		[+ +]		

ethenyl-cysteine (GEC). The distribution of GEC in the species which are documented to contain VA_3 has not yet been verified, but considering the taxonomically significant distribution of other NPAAs in the genus *Vicia*, it can reasonably be predicted that this compound is identical with VA_3 and therefore distributed as indicated for VA_3 in Table 1. Birch (1983) documented the presence of VA_1 in the stems and leaves of *V. narbonensis* and *V. johannis*, which suggests, by analogy, that VA_3 is present in the seeds of *V. johannis.* Khattab (1988) studied the NPAAs of the closer relatives of *V. narbonensis* and documented the presence of VA_3 in the seeds of some, but not all, accessions of *V. bithynica*, *V. serratifolia*, *V. hyaeniscyamus*, *V. kalakhensis* and only one accession of *V. narbonensis* var. *affinis*. Khattab's inability to detect VA_3 in the rest of the *V. narbonensis* accessions suggests that this compound may have gone undetected in many of his samples. The possible presence of GEC in the seeds of the species closely related to *V. narbonensis* remains to be evaluated and this is indicated in Table 1 by a question mark.

Tables 1 and 2 show the possible combinations of major known low molecular weight toxins in *Vicia* seeds

[13] This species could not be found in Kupicha (1976), but it is listed as accepted taxon by Allkin *et al.* (1986)

with representative species in parenthesis: 1. canavanine (*V. ervilia*) 2. canavanine and GEC (*V. villosa*, *V. benghalensis*) 3. GEC (*V. narbonensis*) 4. cyanoalanine acids and pyrimidine glycosides (*V. sativa* cultivars ?) 5. cyanoalanine acids, vicianine and pyrimidine glycosides (*V. sativa*, *V. sativa ssp. nigra*) 6. pyrimidine glycosides (*V. faba*).

β-Cyanoalanine and γ-glutamyl-β-cyanoalanine are primary products of HCN assimilation[14] in plants (Tschiersch, 1964a; Tschiersch, 1964b; Fowden and Bell, 1965), therefore group 5 constitutes a subset of group 4 (Hanelt and Tschiersch, 1967).

The relative concentrations of *Vicia* toxins are likely to be a reflection of evolutionary selection pressures and may therefore be related to biological functions. A quantitative survey of the nonprotein amino acids, the pyrimidine glycosides and vicianine in the seeds of the genus *Vicia* combined with an assessment of high and low toxin genotypes for their biological performance may provide further insights into this complex and interesting problem.

Other Anti-Nutritional or Potentially Toxic Factors

The emphasis in this study has been on the identification of factors responsible for feed-intake inhibition in mono gastric animals. Other factors, such as enzyme inhibitors (proteinase inhibitors, amylase inhibitors), lectins, phytates, saponins, phytoalexins, tannins, oligosaccharides are outside the scope of this thesis. However they are very relevant to the ultimate nutritional value of *Vicia* seeds, a topic which deserves further attention. The susceptibility of proteinaceous inhibitors to heat denaturation makes them desirable natural defence factors for food legumes. Genetic substitution of these thermolabile factors for the heat stable *Vicia* seed toxins may facilitate the development of presently under-utilised species for human consumption.

Much of the detailed physical, chemical and nutritional data which has been provided by Schmandke (1988) for the utilisation of *V. faba* as a food grain is also needed for other *Vicia* species if they are ever to be widely utilised as feed and food stuffs.

The phenylalanine derivative, ß-(3,4-dihydroxyphenyl)-L-alanine (L-Dopa) can be found in free form and bound as a glycoside in some *Vicia* species (incl. *V. faba*) and was at one time implicated as a causal agent of Favism, however, it is no longer considered to be important in this disease (Mager *et al.*, 1980).

γ–Hydroxyarginine was isolated from the seeds of *V. sativa* (Bell and Tirimanna, 1965). The IR and NMR of the lactones formed by this compound and the related γ–hydroxyhomoarginine were studied by Hirst and Foster (1964). The distribution of this compound in the genus *Vicia* has also been found to be of taxonomic value (Bell and Tirimanna, 1965).

Cyanogenic glycosides in *Vicia* species

Ritthausen and Kreusler (1870) provided the first clue to one of the causes of *Vicia toxicity* with their observation that the meal made from the seeds of *Vicia sativa* released HCN if moistened with lukewarm water. They suggested that some varieties of *V. sativa* which released high levels of HCN could be potentially toxic due to the presence of cyanogenic glycosides. It is interesting that detectable levels of HCN were also found in a white vetch (Hopetown) (cf. Chapter 1, human consumption of white seeded *V. sativa*). Consequently, attention was paid by researchers to the presence of cyanogenic glycosides in the seeds of *Vicia* species.

The earlier studies on cyanogenic glycosides of *Vicia* species were summarised by Johanson (1948). The most recent work on vicianine has been published in Japanese (Kasai *et al.*, 1981a; Kasai *et al.*, 1981b) but was not available for this review.

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14 Although Tschiersch (1966) provided evidence for vicianine acting as HCN source for β-cyanoalanine formation, a point reiterated by Blumenthal *et al.* (1968), recent studies (Peiser et al., 1984) have demonstrated that HCN in plants is derived primarily from carbon 1 of 1-aminocyclopropane-1-carboxylic acid during ethylene biosynthesis. Therefore, the data provided by Tschiersch (1966) can be re-interpreted as an ability of the plant to remobilise the cyanide nitrogen via conversion to β-cyanoalanine and subsequently to asparagine. However, in seedling extracts of *V. sativa* the formation of γ-glutamyl-β-cyanoalanine would be favoured due to the action γ-glutamyl transpeptidase. An examination of different plant growth stages for the activity of this enzyme and the catabolism of vicianine is indicated.

Akbari (1965) found that seed of early maturing varieties of *V. sativa* from Turkey had high contents of HCN (120-270 mg HCN kg^{-1} DW) whereas the early maturing cv. Bologna had a relatively low content (60-65 mg HCN kg^{-1} DW). Variation was high in the medium-early to medium-late varieties (15-220 mg HCN kg^{-1} DW). The late varieties tested (cv. Luxura Vereduna, cv. Svalöfs Süsswicke, cv. Wirosa) had low contents of cyanogens (10-35 mg HCN kg^{-1} DW). It was noted that the level of HCN was relatively constant for both years of cultivation (1962, 1963) except for the German cultivars "Dreesbachs Rheinische" and "Engelens Weihenstephaner SV", which in 1962 had a higher content of HCN. The author attributed this to mould attack, rotting of the lower stem parts and consequent earlier ripening. The HCN content of the vegetative tissue from 100% flowering to pod formation (eg. at the normal harvesting date for the green fodder of *Vicia sativa*) was found to be very low with all accessions, in contrast to the seed content of these lines. Some late accessions had no HCN at this stage. The Turkish accessions had the highest HCN content (10 mg HCN kg^{-1} DW) of 20 accessions measured. With increasing development (end of flowering to seed formation) an increase of the HCN content was found with all accessions, with an especially rapid rise in the early maturing types, but this may be due to seed formation.

Tschiersch and Hanelt (1967) screened the seeds of the Gatersleben vetch collection for NPAAs and cyanogenesis (Table 1). In a separate paper (Hanelt and Tschiersch, 1967) they provided quantitative data on the intra specific variation of this trait for *V. sativa*, tabulating the cyanogen-free accessions of this species and its cyanogenic cultivars. The majority of the *V. sativa* accessions tested had 10-150µg HCN g^{-1}, if any at all. Of 271 lines tested, 51 were found to be HCN free. The cultivar Languedoc 225 was found to contain 58 µg HCN g^{-1} while cv. Blanche Fleur (*V. sativa* convar. consentini) was found to be HCN free. Almost all accessions with yellowish-white seed coats were found to have a low HCN content. Of 10 accessions 9 contained between 0-46 µg HCN g^{-1} and only one white-seeded accession "Bogorodickaja 800" had 142 µg HCN g^{-1} ! It was of note, that two vetch varieties of *V. cordata* which had been bred recently in Israel contained high amounts of vicianine: cv. "Asor" (grown 1965) contained 620 µg HCN g^{-1} , and cv. Beit Dagan 600 µg HCN g^{-1}. The HCN-free accessions within *V. sativa* were found with the spontaneous, weedy tribe convar. *cosentini* (Guss.) Arcang. and with cultivars of convar. *sativa*. Accessions of the lentil mimic, *V. sativa* convar. *sativa* var. *platysperma* Barul. (North-Greece, several European cultivars and breeding lines, amongst them another line from the DSG Schlanstedt) were found to be free of HCN except for one accession (selection from a sample of the DSG Schlanstedt) which contained a minor amount of HCN (28-56 µg HCN g^{-1}). The majority of tested material from the Balkan and Turkey contained vicianine, whereas the material from Iran was predominantly HCN-free. The authors discussed this finding as an example of biochemically-geographic intra specific differentiation. No parallels for this difference (+/-) in vicianine to morphological characters were found. With respect to environmental influences on HCN content it was found that material grown during cooler summers with more rain (eg. 1958) had a higher concentration of vicianine than that grown during warmer, drier conditions (eg 1960). The environmental difference can amount to half of the HCN content found under the warmer growing conditions. It was suggested that registration of cultivars with HCN-rich seeds (>100-150 µg HCN g^{-1}) should be avoided.

Harborne (1982) reviewed the classical study on the cyanogenesis polymorphism of *Trifolium repens*. The distribution of cyanogenic *Trifolium repens* genotypes was found to correlate with the occurrence of snails which feed on this species. The distribution of cyanogenic genotypes of *V. sativa* is also likely to be the result of biotic selection pressure, and the absence of this trait in the majority of the Iranian accessions suggests that in this particular environment the biotic selection pressure for cyanogenesis was not important. The majority of the Iranian accessions were collected during a FAO collecting trip in 1952-1954 (Prof. Kuckuck), and may have been selected for their domestication characteristics, thus excluding any wild material. This may be an indication for the utilisation of *V. sativa* as a grain legume in Iran. However, if the non-cyanogenic character is consistent in both wild, cultivated and domesticated Iranian material, then it would reflect the absence of predators against which cyanogenesis is an effective defence strategy.

Toxicity of Vicianine and HCN

Anderson *et al.* (1925) tested the toxicity of vicianine by subcutaneous injection and observed no toxicity; presumably due to the lack of HCN release by this route of administration. Akbari (1965) discussed the problem of toxicity caused by cyanogenic glycosides. The toxicity of these compounds is due to the release of HCN which affects cell respiration. Death by HCN is due to asphyxiation, similar to that of CO poisoning. This occurs when a certain critical limit is exceeded in the blood, which lies between 0.5-1 mg HCN per kg body weight. Reaching this level is a function of absorption/detoxification. When the rate of absorption exceeds the rate of detoxification and the critical limit is reached, then death occurs. The lethal dose (LD) is therefore the sum of the total amount of HCN detoxified and the amount of HCN in the body. The rate of

detoxification is dependent 1. on the reaction of the individual 2. strongly dependent on the rate of feed intake and 3. the rate of hydrolysis of the cyanogenic glycoside, which is dependent on bacterial activity and is much more pronounced in a full rumen than in an empty one. In experimental studies with sheep (Coop and Blakely, 1950; cited by Akbari) it was demonstrated that the LD is a function of absorption. It was suggested that because HCN detoxification is dependent on sulfur amino acid metabolism, a depletion of these compounds in animals grazing on cyanogenic pastures were the cause of the so-called chronic intoxications which manifest themselves in metabolic disorders, skin and haircoat lesions and liver problems (Drepper, 1961; cited by Akbari, 1965)

For the aetiology of Vicism eg. the intoxications by *Vicia* species, cyanogenesis needs to be considered in all cases where ß-cyanoalanine containing *Vicia* species are implicated, because the cyanogenic genotypes constitute a subset of this biochemically well defined group.

Canavanine

Canavanine, which acts as an analogue of the protein amino acid arginine is present in many leguminous species (Bell, 1958; Bell, 1960; Birdsong *et al.* 1960; Bell *et al.* 1978). Its toxicology was originally studied by Tschiersch (1962) and more recently, with a view to employing this compound in tumour chemotherapy a detailed study of its pharmacokinetics and toxicology was carried out by Thomas and Rosenthal (1987 a, b). Tschiersch (1962) found that canavanine fed at 2 g kg^{-1} body weight was rapidly metabolised in mouse liver by what he assumed to be arginase and observed a ninhydrin positive, Ehrlich reagent positive compound which had similar chromatographic properties to citrulline and suggested the possibility that this compound may be ureido-homoserine eg. canaline. He further noted a possible effect on the ornithine cycle as evidenced by the change in amino acid composition in the liver of rats feeding on canavanine containing diets, but presumed that the major toxicity of canavanine would be due to its antagonism to arginine because he was able to alleviate the toxic effects of canavanine by the simultaneous feeding of arginine at a tenfold higher dose.

Thomas and Rosenthal (1987a) established that the toxicity in rats of 2g kg^{-1} L-canavanine (free base) administered either orally or subcutaneously leads to alopecia, loss of appetite and weight loss. The spleen was the most severely affected organ after daily administrations of 2g kg^{-1}canavanine for one week. At higher doses (4g $kg^{-1)}$ a shortlived peak of ammonia was detected by HPLC analysis of blood samples. Oral administration of canavanine resulted in ca. 20% of canavanine to remain in the gut, with very few pellets of faeces being excreted. It was concluded that there was a deficiency of knowledge about the major canavanine breakdown product, canaline, and further toxicology studies of this structural analogue of ornithine were considered necessary to provide a thorough understanding of canavanine toxicity (Thomas and Rosenthal, 1987b). Studies by Hollander *et al.* (1989) have provided *in vitro* evidence for the formation of hydroxyguanidine and vinylglyoxalate (2-oxo-3-butenoate) from canavanine, thus providing the possibility for an additional toxic principle derived from canavanine.

An inhibitory effect of both, canavanine and deaminocanavanine on histamine induced contractions of guinea pig ileum was observed *in vitro* by Ackermann and Wasmuth (1939). This and Thomas and Rosenthal's observation of canavanine's retention in the gut support the later hypothesis (Enneking *et al.*, 1993) that peristalsis may be affected by canavanine through an nitric oxide mediated mechanism (Iyengar *et al.*, 1987; Schmidt *et al.*, 1988a; Schmidt *et al.*, 1988b; Schmidt *et al.*, 1989; Schmidt *et al.*, 1990a; Schmidt *et al.*, 1990b).

Malinow *et al.* (1982) used primates as a model for the study of Systemic Lupus Erythematosus (SLE) induced by 1-2% dietary levels of L-canavanine sulfate. This model is characterised by an anaemia that results from the occurrence of antibodies to red blood cells, by lowered complement components in the serum, by antibodies to nuclear antigens, antibodies to double stranded DNA, lupus erythematosus cells in peripheral blood smears, and the deposition of immunoglobulin and complement in the kidneys and the skin.

Prete (1985) also found that L-Canavanine (free base) induces an autoimmune response in mice when administered at 0.725% of the diet. Effects on feed intake were not recorded. Double stranded DNA antibodies were found in normal mice on canavanine diets. Canavanine also induced significant renal pathology. Glomerular deposition for IgG and IgM increased in the normal mice at 24 weeks. This increase in autoantibody production was correlated with the increase in histologic score and immunoglobulin deposition. Prete concluded that the action of canavanine was primarily due to a disordering of B-lymphocyte function. Thus canavanine can affect B-lymphocyte function and can cause the induction antibody mediated autoimmune phenomena in normal mice.

Prete (1986) isolated a sub-population of autoimmune B-cells exhibiting impaired B-cell function in response to

L-canavanine. This study provided the first evidence that alterations in charged membrane properties of B cells are linked to abnormal immune responses such as diet related lupus phenomena

Morimoto *et al.* (1990) extended these studies and found that L-Canavanine acted on suppressor-inducer T cells to regulate antibody synthesis and that lymphocytes of systemic lupus erythematosus patients are specifically unresponsive to L-canavanine. They established that canavanine acts mainly on CD8(-)Leu8(+)cells, and that the lymphocyte response to L-canavanine depended primarily on the presence of functional types of these cells. From this study it seems that suppressor-inducer T-cells, responsive to canavanine, are especially deficient in cases of SLE patients.

The Toxicity of Canavanine to Ruminants

Very little information about the toxicity of canavanine to ruminants is available, and most of it is circumstantial, based on the occasional poisoning of cattle by canavanine containing plant material (Claughton and Claughton, 1955; Shone, 1961; and references in Chapter 3). The effects of feeding *Canavalia ensiformis* to sheep has recently been studied by researchers at the Rowett Institute (Dominguez-Bello and Stewart, 1990). These authors observed a marked shift towards gram-negative bacteria in rumen fluid samples of *Canavalia* fed sheep. They studied this phenomenon by examining the effect of canavanine on isolated strains of rumen bacteria but did not find a consistent effect of this compound on gram positive species. However, due to the use of a colorimetric method for the detection of canavanine which is specific for the guanidino group of this compound, their procedure would not have monitored whether or not canaline was responsible for the observed rumen changes. The presence of arginase is a prerequisite for the breakdown of canavanine into canaline, the latter is extremely toxic due to its ability to form oximes with aldehydes (eg. Vit B6) and keto-acids (Rosenthal, 1991; and references therein).

Although it appears that in contrast to mono gastric animals, the adaptation of ruminants is probably a function of their rumen flora, the details of canavanine detoxification require further study and there may well be circumstances under which this compound or its breakdown product canaline, can exert toxic effects in ruminants.

Biochemistry of ß-Cyano-Alanine and its γ–Glutamyl Derivative

The neurotoxin ß-cyanoalanine (Ressler, 1962) and its γ–glutamyl peptide were first isolated from the seeds of *V. sativa* (Ressler *et al.* 1963) and more recently also from the toxic fungus *Clitocybe acromelalga* (Fushiya *et al.*, 1993). Variation in the concentration of these compounds amongst different cultivars of *V. sativa* has been documented (Ressler *et al.*, 1969).

ß-Cyanoalanine formed from cyanide and cysteine is a byproduct of ethylene biosynthesis and is therefore of ubiquitous occurrence in the plant kingdom (Peiser *et al.*, 1984). However, in most plants it is rapidly metabolised to asparagine (Tschiersch, 1963; Blumenthal-Goldschmidt *et al.*, 1963; Fowden and Bell, 1965; Blumenthal *et al.*, 1968). Some *Vicia* species have the biosynthetic ability to sequester and accumulate ß-cyanoalanine into γ–glutamyl-ß-cyanoalanine (Table 1). Since these two compounds were metabolised to asparagine in *V. villosa*, *L. odoratus* and *L. sylvestris*, Ressler *et al*. (1963b) suggested that this biosynthetic step is blocked in *V. sativa*.

Extracts from seedlings of *V. sativa* had a high activity of a γ–glutamyl transferase[15] , an enzyme which can catalyse the formation of γ–glutamyl-ß-cyanoalanine from ß-cyanoalanine in the presence of glutathione *in vitro*. Extracts from seedlings of *L. odoratus* showed a high level of asparaginase and little of the transferase activity (Fowden and Bell, 1965).

The toxicity of β-cyanoalanine and its γ-glutamyl peptide have been reviewed (Ressler, 1964; Ressler, 1975). The metabolism and toxicity of these compounds were studied in detail (Pfeffer and Ressler, 1967; Ressler *et al.*, 1967; Sasaoka *et al.*, 1968). Symptoms in chicks fed or injected with β-cyanoalanine were likened to those observed with thiamine deficiency in pigeons, or the strychnine-like convulsive state with opisthotonus in Vit B_6-deficient turkey poults. Rats fed on diets containing subacute levels (0.5%) of β-cyanoalanine for two months showed retardation of growth, high levels of mortality and cystathionine excretion (Ressler *et al.*, 1967). Confirmation of the lethality of a diet of 30% *V. sativa* seed or crude preparation of ß-cyanoalanine

[15] The pH optimum for this enzyme isolated from 6 d.o. *V. sativa* seedlings was pH 8.1-8.2

equivalent to 90% of this level to 4 week old chickens was reported by Arscott and Harper (1963). Feinstein *et al.* (1962) reported that the effects of β-cyanoalanine could not be countered by anti convulsive agents with activity on the central nervous system, but could be alleviated by barbiturates and mephenesin, during anaesthesia. Tests on isolated crayfish intestine suggested a mode of action different from picrotoxin. The same authors also found that β-cyanoalanine, diamino butyric acid and trimethylenediamine blocked nicotine contractions. These findings made it unlikely that β-cyanoalanine acts as any known type of central nervous system inhibitor (Ressler, 1975). The compound showed also no activity on feline spinal interneurones in contrast to the high activity of β-N-oxalyl-L-α,β-diaminopropionic acid (Watkins *et al.*, 1966). Another study demonstrated that pyridoxal.HCL could alleviate β-cyanoalanine toxicity symptoms and would improve survival of rats co-injected with this co-factor and the toxin; it was also observed that high levels of cystathionine were excreted in the urine of animals dosed with the toxin (Ressler *et al.*, 1964). The inhibitory effect of ß-cyanoalanine on cystathionase activity was subsequently established; its potent inhibitory effect on the transulfuration pathway being due to substrate competition and the γ–glutamyl-peptide having no effect on purified cystathionase activity (Pfeffer and Ressler, 1967). Ressler *et al.* (1967) found no evidence for an antagonistic effect of β-cyanoalanine against Vit B_6 but considered it a useful antidote in cases of acute vetch poisoning. Administered ß-cyanoalanine is found in all tissues of the rat and chick, except for the kidney and occurs in form of the dipeptide γ–glutamyl-ß-cyanoalanine and the glutathione analogue γ–glutamyl-ß-cyanoalanine-glycine. Kidney and urine were found to contain free β-cyanoalanine which is the predominant excreted form of the toxin. Further details about γ–glutamyl-ß-cyanoalanine-glycine were reported by Sasaoka *et al.* (1968).

With regard to detoxification, Giza *et al.* (1963) reported that guinea pig serum asparaginase could slowly hydrolyse ß-cyanoalanine to aspartic acid, thus providing a possible explanation for the observed species differences in toxicity. The discovery of γ–glutamyl-ß-cyanoalanine-glycine (Ressler *et al.*, 1967; Sasaoka *et al.*, 1968) provided an additional mechanism for detoxification ß-cyanoalanine by sequestration. However, the possibility of an interference with glutathione metabolism was also cautioned (Sasaoka *et al.*, 1968).

The available evidence suggests that neurotoxicity is not the major mode for the deleterious effects of ß-cyanoalanine but rather an indirect consequence. Its inhibitory effect on the transulfuration pathway is likely to be of more significance as it reduces the availability of cysteine, and can be expected to be particularly damaging in the absence of dietary sources of cysteine and high demand on existing glutathione levels.

γ–Glutamyl-S-Ethenyl-Cysteine (GEC)

The isolation and characterisation of this compound are described in chapter 5. In view of its unpalatability to pigs and by inference from the unpalatability of *V. narbonensis* to poultry and humans, it should be regarded as potentially toxic. A possible toxicity of GEC is limited by its unpalatability which prevents the ingestion of sufficiently high concentrations of the compound. This may explain why no cases of acute toxicity have been reported for species such as *V. narbonensis*.

The limited information about its toxicity of *V. narbonensis* in pigs suggests that it may cause haemolysis, and kidney damage through the formation of crystalline precipitates (Davies, 1987). Further studies of its chemistry, pharmacology and toxicology are clearly needed.

Pyrimidine glycosides

Vicine and convicine are ß-glycosides of the pyrimidines divicine and isouramil, respectively (Bendich and Clements, 1953; Bien *et al.* 1968) and are hydrolysed by ß-glucosidases (Hérissey and Cheymol, 1931; Mager *et al.*, 1965; McKay, 1992). High levels of vicine are present in the seeds of *V. sativa* and *V. faba* (which also contains significant levels of convicine) (Table 2). The ingestion of meals prepared from the seeds of *V. faba* can trigger the onset of Favism, an acute haemolytic disease which affects individuals lacking sufficient activity of the NADPH producing enzyme glucose-6-P-dehydrogenase (G-6-P-D) in their red blood cells (Mager *et al.*, 1980). Several genetic variants of this enzyme deficiency, which is thought to confer an adaptive advantage against malaria, are known to occur worldwide, with Favism being an extreme manifestation of this trait (Vulliamy *et al.*, 1992). It is particularly prevalent in some Mediterranean and South-West Asian populations (Belsey, 1973). Vicine and convicine have been implicated in Favism (Lin and Ling, 1962a, 1962b, 1963; Mager *et al.*, 1965; Marquardt, 1989) because their hydrolysis products are unstable and form radicals which can cause a depletion of reduced glutathione (GSH) in G6PD deficient red blood cells. Oxidised glutathione (GSSG) can be regenerated through reduction by NADPH. A lack of sufficient NADPH due to G6PD

deficiency impedes GSH replenishment and predisposes the red blood cells to oxidative damage which can, ultimately, result in a haemolytic crisis (Mager *et al.*, 1980; Marquardt, 1989; and references therein).

Conclusion

Vetch seeds differ widely in their secondary metabolite composition and *Vicia* toxicity is best understood in this context. The non-protein amino acids canavanine and β-cyanoalanine, the dipeptides γ-glutamyl-β-cyanoalanine and (γ-glutamyl-S-ethenyl-cysteine), the cyanogenic glycoside vicianine, and the pyrimidine glycosides vicine and convicine are the major presently known low molecular weight seed toxins in the genus. The distribution of the non-protein amino acids allows the division of the genus into canavanine (subgen. *Vicilla*) and non-canavanine containing species (subgen. *Vicia*). Further divisions can be made according to toxin distribution at the subgeneric and even intra specific level.

Chapter 3

Vicism: Intoxication by *Vicia* species (other than *V. faba*)

Introduction

Vicia toxicity has been known since antiquity. Columella, Plinius, Galen and Dioscorides were clearly aware of the toxic effects of *Vicia ervilia* (Gerarde, 1636). The consumption of *V. faba* was avoided by Egyptian priests[16], while Pythagoras, the mathematician and mystic philosopher, who presumably was a sufferer of Favism, forbade his followers to eat beans or even go near a field of them (Becker-Dillingen, 1929; Hanelt, 1972, Grmek, 1980). Other *Vicia* species (*V. sativa, V. villosa , V. dasycarpa, V. benghalensis* etc.) have been reported to cause poisoning in humans and livestock. Kobert (1906) coined the term Vicism, as distinct to Favism, for such poisonings. In the more recent literature intoxications by *Vicia* species, mainly caused by *V. villosa* and *V. benghalensis*, have been referred to as vetch toxicosis or vetch associated disease (Panciera *et al.*, 1966; Panciera *et al.*, 1992).

It appears that there are besides Favism three other types of *Vicia* intoxications: 1. HCN poisoning 2. non-cyanogenic *V. sativa* poisoning 3. Canavanine containing species poisoning (Vetch toxicosis). Table 1 lists documented cases of intoxication for which *Vicia* species have been implicated, together with the most significant observed symptoms. These cases are grouped according to species into two major groups, the β-cyanoalanine producing and the canavanine producing *Vicia* species. The presence of HCN is noted where this information was available.

The composition of vetch screenings

Many reports do not exactly specify the type of *Vicia* sp. implicated in cases of poisoning, and reference is often made to the use of vetch screenings (Trieurwicken, Trieurage, tare vetches). The composition of such screenings varies with the bio-geographic region and with the particular cropping practices and growing conditions.

The examples presented below may suffice for the identification of the most common *Vicia* species to be found in cereal screenings. More detailed information on this topic can be found in certified seed handbooks, in botanic treatments of local Floras and the weed control literature.

Several different Mediterranean *Vicia* and *Lathyrus* species can appear in vetch fields. A. Chaprie 1912 (cited by Hegi and Gams, 1924) found in one vetch field near Malleray (Berner Jura) amongst others the following species: *V. ervilia*, *V. villosa* ssp. *dasycarpa*, *V. peregrina*, *V. pannonica*, *V. lutea*, *V. hybrida*, *V. bithynica*, *V. narbonensis* var. *integrifolia* and *serratifolia*, *L. cicera*, *L. ochrus*, *L. hierosolymitanus* var. *grandiflorus*, further *Trigonella foenum graecum*, *Bifora radians* and other species which indicated a south-eastern origin. According to Kling (1917) *V. hirsuta* Koch, *V. sepium* L., *V. angustifolia*, *V. cracca* can be found frequently in screenings. The seeds of *V. hirsuta* are often separated during cereal cleaning operations and have been used for adulteration of species for which seeds are harder to obtain e.g *V. cracca*, *V. sepium* (Hegi and Gams, 1924). Anderson *et al.* (1925) found *V. sativa* var. *angustifolia* as a contaminant of *Lathyrus sativus* samples. Fischetti (1985) listed the following weedy *Vicia* species for Italy : *V. villosa* Roth, *V. sativa* L., *V. sepium* L., *V. peregrina* L., *V. hirsuta* S. F. Gray, *V. cracca* L., *V. tetrasperma* Schreber.

Table 2 demonstrates that for Bavaria, in the very dry year, 1976, most seed samples tested were conspicuously free of weed seeds, whereas in the very wet year, 1987, above average weed contamination was

[16] Egyptian priests of the sacerdotal order abstained also from onions as unlawful food (Plutarch), although onions and garlic were highly esteemed foods at the time (Täckholm and Drar, 1954; for a detailed discussion and a wealth of references to the historical and archaeological *Allium* literature)

found. Weedy vetches appeared in 3 % of rye and 2% oats samples, and seem to be much more common in

Table 1. *Vicia* toxicity 1. ß-Cyanoalanine containing *Vicia* species

Species	Ingested Parts (amount)	HCN 17	Animal (Age)	Symptoms	Reference
V. sativa	Plants infested with black aphids, honey dew and mildew		Horses	Photosensitisation	Steiner (1843) , Schrebe (1843)
Pea, bean, vetch	Straw			Lupinosis like symptoms	Reinemann and Jansen (1880/81)
V. sativa	Exclusive feed		Horses	Emaciation, complete alopecia, icterus, orange-coloured conjunctiva, throbbing heart beat (60-100 pulses/min), reduction in T. PM: Enlarged orange liver	Stöhr (1892)
V. sativa	50% cracked and 50% whole seeds 7.5 kg/head/day and vetch hay		60 Horses	10 animals died under symptoms of gradual emaciation, alopecia and signs of colic. PM: Inflamed intestines, enlarged dark-brown liver and swollen spleen.	Stöhr (1892)
V. sativa			Oxen	Skin disease similar to malt and potato eczema except for dry necrosis of sore skin section	Stöhr (1892)
V. sativa			80 Pigs	All died as a consequence of inflamed intestines and swollen livers	Stöhr (1892)
V. sativa	Feed		Horses	Weakness and paralysis of hindquarters. Death after a few days	Wenke (1894)
V. sativa			4 Horses	Amaurosis, hoof inflammation, stiffness and tetanic tension of extremities. One horse died after 14 hrs with manifestations of trismus	Mason (1896)
*V. sativa*ssp. *nigra* syn.*V. angustifolia*	Seed screenings of imported grain (1 black, 1 sprinkled var.)	Yes	Horses	Harmful, causing death in some cases	Filter (1915)
Vicia sp.	Fed with pods		8 foals	Exanthema with alopecia	Prussian Veterinary Reports (1919)
*V. sativa*ssp. *nigra* syn.*V. angustifolia*	Seed, 50% of diet	Yes	Ducks	Ducks: Ataxia, walking in circles, convulsions, paresis, writhing contortion of the body, death. PM: in most cases cerebral congestion was the most striking feature. Excess pericardial fluid in abdominal and thoracic organs. Oedema indicative of haemorrhage under the skin of the head. Congested brain, pink in colour, covered all over with dilated vessels.	Anderson *et al.* (1925)
*V. sativa*ssp. *nigra* syn.*V. angustifolia*	Seed 30, 50% of diet	Yes	Monkeys	Monkeys: less active, crouched in cages, unable to sit up, constantly grinding their teeth. Fibrillar twitchings of arm, leg and flank muscles. Violent convulsions of 5-10 min duration, frequent yawning, hyperexcitability, paralysis. Not Lathyrism	Anderson *et al.* (1925)

[17]**Abbreviations:** BW: Body weight, cv.: cultivar, d.o.: day old (chicks), equiv.: equivalent to, HCN: hydrogen cyanide, hr(s): hour(s), iv: intravenous, mth(s): month(s), PM: Post mortem, T: Temperature, var.: variety, wk(s): week(s), X: cross (hybrid), yr(s): year(s)

Table 1. *Vicia* toxicity 1. ß-Cyanoalanine containing *Vicia* species (ctd.)

Species	Ingested Parts (amount)	HCN[18]	Animal (Age)	Symptoms	Reference
V. sativa (and *V. villosa*?)	Oats, peas, vetch grain mixtures of low quality (unripe, fungal infections)		Horses	*Hemoglobinaemia enzootica cum paralysi pharyngis*.	Hobmaier (1926)
V. sativa	Exclusive feed		Horse	Initially eating with appetite and pleasure. After a few days feed refusal. Vetch taste then needs to be masked by other feeds to improve feed intake.	Hobmaier (1926)
V. sativa	Partial feed		Horses (not all equally affected)	Emaciation, dyspnoea, palpitations, hyperhydrosis are most indicative. Other early signs: colic phenomena, indigestion: strong swelling of the hind parts of the body, abdomen appears stretched and under tension, hunger groove is filled out, hyperperistalsis, later the abdomen hangs flabby .	Hobmaier (1926)
V. sativa pure seed	Aqueous seed extract, iv (400 ml), equiv. 4g seed/kg BW	smell after O/N soak	Horse	Muscle twitching, sweating, accelerated heat beat, dyspnoea, collapse, death after 1 hr. PM: Heart with white outer layers ca. 0.5 cm deep, on cross section other parts of heart also white. Brain surface more moist (subjectively). Other organs: minute punctiform lung haemorrhages.	Hobmaier (1926)
V. sativa pure seed	Aqueous seed extract iv (200 ml), equiv. 2g seed/kg BW	ditto	Horse	5 mins after infusion, weak transient muscle twitching at nostrils, *triceps brachii* and croup. After 30 mins, the back hand gives way repeatedly. Defecation frequent with difficulty, moist faeces. 5 mins later lies down, respiratory dyspnoea and palpitations begin, again spread position of hind extremities. Light sweating. Free psyche, dilated pupils. To prevent *exitus letalis* 3 hourly doses of camphor oil (a 35 ml). After second dose, animal rises, strong muscle twitching. Tetanus-like cramps of the jaw. After 3 hrs T: 39.3°C, pulse 70, respiration 40. Excited by noise. Weak, frightened stance. Pupils close slowly. Tetanic cramp of the mouth, tongue hangs out, occasional phantom chewing, pauses while chewing bites of feed. After another dose of camphor, the animal recovers. (early stage of "*Hemoglobinaemia enzootica cum paralysi pharyngis* ?	Hobmaier (1926)
V. sativa and *V. benghalensis* mixture	Seeds, 12.5 % of diet, ex Screenings (Chilean barley)	Yes	80 Pigs (5-6 mths) (100-140 lbs)	A number of pigs fell ill and died suddenly. PM: Gastritis and patchy enteritis	Clough (1931)

[18]**Abbreviations:** BW: Body weight, cv.: cultivar, d.o.: day old (chicks), equiv.: equivalent to, HCN: hydrogen cyanide, hr(s): hour(s), iv: intravenous, mth(s): month(s), PM: Post mortem, T: Temperature, var.: variety, wk(s): week(s), X: cross (hybrid), yr(s): year(s)

V. sativa	Drench with 20g seed	0.8 mg/ 100g	Rabbit	HCN poisoning within 5 mins and death within 1.25 hrs. PM: HCN poisoning. Large amount of HCN detected in the stomach	Steyn (1933)

Table 1. *Vicia* toxicity 1. ß-Cyanoalanine containing *Vicia* species (ctd.)

Species	Ingested Parts (amount)	HCN[19]	Animal (Age)	Symptoms	Reference
V. sativa	On harvested land		Mules and Horses	Stiff gait, paralysis, particularly in fore-quarters, death. PM: Patchy intestinal inflammation	Steyn (1934)
V. sativa	Exclusive feeding for 1 mths of green forage (ca. 75 kg/day)		Horse (12 yrs)	Mild hemoglubinuria accompanied by icterus. Hemoglobinuria, icterus and heaves (broken wind) are the diagnostic triad for *V. sativa* intoxication, reflecting damage to liver and kidneys. These symptoms occur after massive and prolonged feeding of the green forage or hay	Derzelle (1938)
V. angustifolia	Plants	No	4 calves (8 wks)	Animals became ill, 3 died, the other recovered	Hurst (1942)
V. sativa (with some *V. villosa*)	Milled seed, fed at 20, 40,60, 80, 100% of diet		Rats	Average weight gain(4 weeks) -4, -1, 7, 7, 29, 26 g, respectively. One rat (100% diet): left lung had undergone hepatisation. One control animal showed some tumour like growth of the intestines.	Ruby *et al.* (1955)
V. sativa cv. Willamette	Seed meal, 30 % of diet		Turkey poults (d.o.)	70% mortality in 4 weeks, average survival 18.5 days	Harper and Arscott (1962)
V. sativa cv. Willamette	Seed meal, 40% of diet		Turkey poults (1 wk) (BW: 282g)	90% mortality, average survival 6.5 days	Harper and Arscott (1962)
V. sativa cv. Willamette	Seed meal, 40% of diet		Turkey poults (BW: 532g)	65% mortality, average survival 20 days.	Harper and Arscott (1962)
V. sativa cv. Willamette	Seed meal, 40% of diet autoclaved 8 hrs @13 psi		Turkey poults (BW: 532g)	Zero mortality some diminution in weight gain compared to controls. This was insignificant at the 20% feed inclusion level.	Harper and Arscott (1962)
V. sativa	Seed flour, 30% of diet or crude ß-cyanoalanine		chicks (4 wks)	Complete mortality, average survival time 13 days. Crude preparation of ß-cyanoalanine equivalent to 27% *V. sativa* seed meal in the diet	Arscott and Harper (1963)

[19]**Abbreviations:** BW: Body weight, cv.: cultivar, d.o.: day old (chicks), equiv.: equivalent to, HCN: hydrogen cyanide, hr(s): hour(s), iv: intravenous, mth(s): month(s), PM: Post mortem, T: Temperature, var.: variety, wk(s): week(s), X: cross (hybrid), yr(s): year(s)

Table 1. *Vicia* toxicity 2. Canavanine containing *Vicia* species[20]

***Vicia* species**	**Ingested Part (amount)**	**Animal (Age)**	**Symptoms**	**Reference**
V. ervilia	Seeds	Humans	As a pulse for food far from wholesome, apt to produce vomiting, disorder of the bowels, stuff the head and stomach, weaken the knees. Soaking effective in detoxification.	Pliny (Bostock and Riley, 1940)
V. ervilia	Seeds	Humans	Suitable for physical uses, if eaten it was known to annoy the head, trouble the belly and bring out blood by the urine. In soaked and boiled form used to fatten beasts.	Dioscorides (Gunther, 1959)
V. ervilia	Pasture		A crop sown in March (e.g. late) is toxic and makes cattle which feed on it mad (farmer ' s opinion)	Columella (Boyd Ash, 1954-60)
V. ervilia	Seeds	Humans	Galen, Dioscorides and Hippocrates refer to its use in medicine. " By how much it is bitter, by so much it cleaneth, cutteth, and removeth stoppings ... Overmuch eaten or drunke it draweth the bloud by the stoole, with gripings, and also by urine"	Gerarde (1636)
V. ervilia	Seeds	Pigs	Death	Southall (1879)
V. villosa	Pasture infested with large population of *Jassus sexnotatus* (an insect, cycad, which can cause photosensitisation)	36 Cattle	All animals fell ill, 6 died. Urticaria or dermatitis in patches initially on head and neck, coughing, herpetic mouth rash, reddening and cyanosis of mucous membranes, putrid nasal discharge, rattle noise from the lungs, alopecia, anorexia, loss of strength, gasping breath, free sensorium until death 12-15 days later. PM: Haemorrhagic-serous effusions of subcutis corresponding to urticaria patches. Extensive inflammation of the first three stomachs, in a single case catarrhal abomasal inflammation, severe bronchitis, incipient bronchopneumonia, oedematous glottis, punctiform myocardial hemorrhages, yellow-brown liver, isolated peritonitis and nephritis	Röder (1893) cited by Völker and Fröhner (1950)
V. cracca	Seeds, ca. 10-15 kg/head ex. screenings discarded into waterhole	2 Cows	Cow No.1 ill the same day, died the following morning. Cow No.2: Runny, green faeces with some undigested *Vicia* seeds. Hardly able to raise, stands shivering with spread feet, fixed gaze, grinding teeth, salivation, faint heart beat. T: 38.6°C. Had to be killed. No PM lesions found. Stomach and intestines contained a large amount of undigested seed	Günther (1914)
V. ervilia	seed flour or soup	Pigs	2-6 hrs after ingestion of *V. ervilia* flour or soup, gastrointestinal pain, nausea, vomiting , sweat and cold shivers. These symptoms prevailed for more than twelve hrs.	Wilczek and Tsumi (1919)
V. ervilia	Seeds (even small amounts suffice)	Pigs in several piggeries	The majority of pigs had died under conditions of sleep, muscle cramps and asphyxia	Werner (1934)

[20]**Abbreviations:** BW: Body weight, cv.: cultivar, d.o.: day old (chicks), equiv.: equivalent to, hr(s): hour(s), iv: intravenous, mth(s): month(s), PM: Post mortem, T: Temperature, var.: variety, wk(s): week(s), X: cross (hybrid), yr(s): year(s)

Table 1. *Vicia* toxicity 2. Canavanine containing *Vicia* species[21]

***Vicia* species**	**Ingested Part (amount)**	**Animal (Age)**	**Symptoms**	**Reference**
V. ervilia	Seed flour	Pigs (sows, piglets)	Single meals led to complete inappetence followed by death in some cases. *Cornevin* (1887) is cited for symptoms: somnolence passing into coma, interrupted by muscular trembles. Respiratory centre affected, haematosis is main cause of death. Vomiting can prevent toxicity. No lesions except for asphyxiation	Jean-Blain (1949)
V. villosa	Seed	6 Cattle	5 cattle died. Restless appearance, pain, convulsions when handled. Symptoms were similar to rabies	Claughton and Claughton (1955)
V. sativa with some, *V. villosa*	Milled seed, fed at 20, 40, 60, 80, 100% of diet	Rats	Average weight gain (4 weeks) -4, -1, 7, 7, 29, 26 g, respectively One rat (100% diet): left lung had undergone hepatisation One control showed some tumour like growth on intestines.	Ruby *et al.* (1955)
V. villosa	Seed, 93.5% of diet	Chicks (10d)	100% mortality after 3 weeks Autoclaving (2 hrs, 10 psi) reduced mortality by 17%	Kienholz *et al.* (1962)
V. villosa var. *glabrescens*	Seed meal, 30% of diet	Turkey poults (d.o.)	Reduced weight gain was apparent after 4 weeks	Arscott and Harper (1964)
V. villosa var. *glabrescens*		Turkey poults (4 wk)	Less sensitive than the d.o. poults. 713g weight gain (953g control)	Arscott and Harper (1964)
V. villosa var. *glabrescens*	Seed meal, 84.8% of diet	Turkey poults (d.o.)	100% mortality. Average Survival 10.8 days. Chicks appeared to have starved death.	Arscott and Harper (1964)
V. villosa	Forage, pasture, grazing	Cattle	Dermatitis, conjunctivitis and diarrhoea. Loss of appetite in the more severe cases. PM: intensive infiltration of many organs by lymphoreticular cells, plasma cells, multinucleated giant cells and eosinophils.	Panciera *et al.* (1966)
V. villosa	Seed mixture with rye	5 dairy cows	Within several hrs: Bellowing, sexual excitement, locomotor difficulty, convulsions	Panciera (1978)
V. villosa	Forage, pasture, Grazing	23 dairy cattle herds Friesland, Angus	Rough coat, necrotic lesions on pigmented and non-pigmented skin, conjunctivitis, severe diarrhoea associated with progressive weight loss, ca 50 % mortality with clinically ill cattle. Associated were loss of appetite and red pigments in the urine in some cases.	Panciera (1978)
V. villosa	Pasture	-1 Angus, 1 Angus X in herd of 26 cows	Emaciation, one cow with conjunctivitis, one with diarrhoea. Death 48 hrs after first signs noted. Dermatitis initially around tail and neck, then spreading over trunk and legs. Anorexia.	Kerr and Edwards (1982)

[21]**Abbreviations:** BW: Body weight, cv.: cultivar, d.o.: day old (chicks), equiv.: equivalent to, hr(s): hour(s), mth(s): month(s), PM: Post mortem, T: Temperature, var.: variety, wk(s): week(s), X: cross (hybrid), yr(s): year(s)

Table 1. *Vicia* toxicity 2. Canavanine containing *Vicia* species[22]

***Vicia* species**	**Ingested Part (amount)**	**Animal (Age)**	**Symptoms**	**Reference**
V. villosa	Pasture	30 Holstein cows	4 cows developed skin lesions	Kerr and Edwards (1982)
V. villosa	Pasture (7 mths grazing)	7 Horses	One animal with swelling on lips which quickly spread to rest of body. T 41.6°C, emaciation, bilateral full-thickness corneal ulcerations. PM: Dilatation and thrombosis of cranial mesenteric artery, thrombo-embolism in the lateral caecal artery. Slightly enlarged, white mesenteric lymph nodes, pale kidney cortices, bilateral ulcerative keratitis. Thickened, flooded choroid, filling most of posterior chamber of the eye. Multifocal and diffuse granulomatous inflammation by macrophages, lymphocytes (often with giant cells) of heart, kidney, lungs, dermis, various lymph nodes, ileum, colon, skeletal muscles and most pronounced in the choroid which was congested, oedematous and massively infiltrated. Lesions most prominent perivascularly. Superficial choroid vessels occasionally thrombosed. Retina replaced by thin layer of fibrinonecrotic debris, with degenerating neutrophils. In skeletal muscle, degenerating and mineralised fibres present in areas of inflammation.	Anderson and Divers (1983)
V. villosa X *V. dasycarpa*	Lush pasture (with some rye grass) of which large quantities were consumed	Friesland, Friesland X (3 yrs and older more affected than younger animals)	First Outbreak Sep. 1981 Dermatitis, pruritus most prominent. Diarrhoea in more severe cases, sometimes haemorrhagic. Often fatal. 2 outbreak Dec. 1982 Blood clots in faeces of 2 animals. Conjunctivitis, salivation and mucopurulent nasal discharge. Laboured breathing and coughing in all animals. Good appetite, anorexia only 2-3 days before death. Symptoms milder and survival rates higher in younger animals	Burroughs *et al.* (1983)
V. villosa ssp. *dasycarpa* cv. Namoi	Lush pasture	90 Angus cows with 8 mths old calves at foot	One calf died after one mth. Several animals sluggish while being moved. 4 cows and another calf died subsequently. Other sick cattle showed emaciation, alopecia and elevated respiratory rates. PM: Marked jaundice, areas of haemorrhage in omasum, abomasum, small intestine, gall bladder, on serosal surfaces and in skeletal muscles. Emphysematous lungs, swollen and yellow liver. Severe necrotising granulomatous eosinophilic myocarditis, nephritis and hepatitis all characterised by infiltration with multinucleated giant cells, plasma/lymphoid cells, macrophages, eosinophils	Peet and Gardner (1986)
V. benghalensis	Pasture	Friesland cow	Suspected poisoning	Green and Kleynhans (1989)
V. villosa	Almost pure, lush, pasture 20-29 days grazing	33 Aberdeen Angus bulls	Progressive dermatitis, alopecia, emaciation in 8 bulls. 4 animals died. Detailed PM findings agree with systemic granulomatous inflammation. Weather conditions favoured pure *V. villosa* stands	Odriozola *et al.* (1991)

[22]**Abbreviations:** BW: Body weight, cv.: cultivar, d.o.: day old (chicks), equiv.: equivalent to, hr(s): hour(s), iv: intravenous, mth(s): month(s), PM: Post mortem, T: Temperature, var.: variety, wk(s): week(s), X: cross (hybrid), yr(s): year(s)

Table 1. *Vicia* toxicity 2. Canavanine containing *Vicia* species[23]

***Vicia* species**	**Ingested Part (amount)**	**Animal (Age)**	**Symptoms**	**Reference**
V. villosa	Pasture	Cattle	Systemic granulomatous disease	Johnson *et al.* (1991)
V. benghalensis	Pasture	Horse (10 yr)	Emaciation, conjunctivitis, blepharitis, severe alopecia. Histopathologic lesions are compatible with systemic granulomatous equine disease	Woods *et al.* (1992)
V. villosa	Pasture	Cattle	Systemic granulomatous disease	Johnson *et al.* (1992)
V. villosa	Pasture	Cattle	Experimental induction of vetch toxicosis	Panciera *et al.* (1992)

Table 2. Frequency of *Vicia* sp. in Bavarian Cereal Samples (Fuchs and Voit, 1992)

	W. barley	**W. wheat**	**W. rye**	**S. barley**	**S. wheat**	**S. oats**
V. angustifolia	0.2%	0.15%	1.5%	0.2%	0.3%	0.3%
V. cracca	-%	-%	-%	-%	0.3%	0.3%
V. hirsuta	0.2%	<0.1%	1.7%	0.1%	0.1%	0.7%
V. tetrasperma	0.1%	<0.1%	0.3%	0.1%	-%	0.4%

key: Winter (W) / Summer (S)

[23]**Abbreviations:** BW: Body weight, cv.: cultivar, d.o.: day old (chicks), equiv.: equivalent to, hr(s): hour(s), iv: intravenous, mth(s): month(s), PM: Post mortem, T: Temperature, var.: variety, wk(s): week(s), X: cross (hybrid), yr(s): year(s)

these cereals. Of the identified species, *Vicia cracca*, *Vicia tetrasperma*, *Vicia angustifolia*, *Vicia hirsuta* , only the last two were found in all cereals (Fuchs and Voit, 1992)

The available evidence suggests that the majority of weedy vetches found in European screenings were of the canavanine type. Cyanogenic genotypes of *V. sativa* and *V. angustifolia* seeds are also quite common and represent a second source of potential toxicity in cereal screenings.

With respect to *Vicia* toxicity, the canavanine containing species and those containing vicianine are of most concern, because the utilisation of *Vicia* seed for stockfeed is usually based on experience with a-cyanogenic *V. sativa* cultivars, thus a higher toxicity is unexpected. *V. ervilia* and *V. articulata* which both contain canavanine, are routinely fed to ruminants in the Mediterranean area (Chapter 1), however, it appears that the canavanine level in cultivated *Vicia* grain legumes material is much lower than in forage or weedy genotypes (Tschiersch and Hanelt, 1967; see Chapter 2 Table 1).

The role of unseasonal weather conditions in *Vicia* toxicity

Some of the reported cases of *Vicia* toxicity have been linked to unseasonal weather conditions. Wet seasons tend to favour the growth of *Vicia* species e.g. *V. cracca* (Günther, 1914) , but such weather can also favour the growth of saprophytic fungi. Wilson (1852) noted that lodged crops of tare (*V. sativa*) which had become rotten on the ground often proved prejudicial to horses.

After a wet summer which had favoured powdery mildew build-up, *V. sativa* plants which were almost leafless and in addition infested with a large population of black aphids, caused photosensitisation in horses (Schrebe, 1843). According to Reinemann and Jansen (Preuss. Mittheilungen 1880/81, p. 26, cited by Friedberger and Fröhner (1889) a condition similar to lupinosis could be produced by pea, bean and vetch straw under certain circumstances. Vetch hay infested with rust caused paralysis of the pharynx (Mitteilungen der Deutschen Landwirtschafts Gesellschaft 1895, p. 560; cited by Fruwirth (1921).

Kühn (1898) alerted to the importance of the cereal component as a structural support for vetches. If sown in sufficient quantities the cereal helped to avoid fungal saprophytic infections of the vetch crop by keeping the plants well off the ground.

Unseasonal weather conditions leading to almost pure and lush stands of *V. villosa* which then caused intoxications in cattle were reported by Odriozola *et al.* (1991).

Increased cultivation of *Vicia* species

Hobmaier (1926) pointed out that intoxication of horses by vetches were correlated with the increased cultivation of vetches, the production of which had only increased since ca. 1900 in conjunction with increased dairy production, and that in years when the harvest failed, farmers were forced to feed the vetches which were normally only used to feed cattle, to their horses. This point was re-iterated by Derzelle (1938).

V. sativa toxicity

The numerous cases of intoxication in which *V. sativa* has been implicated are listed in Table 1. A detailed discussion of the pathology is outside the scope of this chapter, but it is clear from the reported cases that severe physiological damage, especially in monogastric animals, can be caused by excessive consumption of either *V. sativa* grain or hay. The role of HCN is not always clear and this toxin may be implicated in many of the reported cases. There is always also a possibility for the presence of other plant species in populations of *V. sativa*, so that even when the grain is thought to be pure seed, there may still be some other *Vicia* genotypes (cyanogenic, canavanine type etc.) present. The *V. sativa* cv. Blanche Fleur is probably one of the least toxic *Vicia sativa* accessions, but due to its high content of γ-glutamyl-β-cyanoalanine, the use of its grain as a pulse without appropriate detoxification needs to be questioned (Tate and Enneking, 1992). The toxin β-cyanoalanine ingested orally can have neurotoxic activity in young chicks; in young rats causes growth reduction and mortality (Ressler, 1962). As already discussed in the previous chapter, β-cyanoalanine inhibits the cystathionase pathway, so its presence in the diet can lead to a depletion of cysteine unless this sulfur amino acid is furnished from other sources. Therefore, any assessment of the safety of a-cyanogenic *V. sativa* cultivars for human consumption needs to take into account the indirect toxic effect on sulfur amino acid metabolism. It is also noteworthy that the sole poultry survivor on a 30% *V. sativa* diet gained 124g weight over the 28 day period (Arscott and Harper, 1964). Adaptation of laying hens to diets containing *V. sativa* has also been reported by Glatz and Hughes (1993).

Vicia ervilia toxicity

Gil (1974) isolated a crystalline substance (ca 3 % of seed DW) from the seeds of *V. ervilia* by the method of Dasler (1954) which proved to be toxic to rabbits, chicks and rats when injected subcutaneously. The chemical structure of the crystals was not investigated and subcutaneous administration of a substance isolated by a method involving lead acetate and barium hydroxide precipitation does not preclude the creation of toxicological artifacts. The study did not consider the presence of canavanine (0.1-2.6 g kg^{-1}; Garcia and Ferrando, 1989) in the seeds of *V. ervilia* (Bell & Tirimanna, 1965; Tschiersch and Hanelt, 1967). The observed neurotoxic symptoms following the ingestion of *V. ervilia* seed meal by mono gastric animals, incl. humans, could possibly be explained by the findings of Thomas and Rosenthal (1987b) who observed a strong peak for ammonia, following the administration of a high dose (4g/kg bodyweight) to rats. The time course of this ammonia peak was accompanied by nervous symptoms. The known presence of L-canavanine in the seeds of *V. ervilia* suggests the hypothesis that this arginine analogue and its metabolites, such as canaline, are additional causal factors for the toxicity of this species.

Vetch associated disease

V. villosa is a species with good cold and drought adaptation. In contrast to other vetches it is able to grow in sandy soils. It was first cultivated in Germany near Magdeburg in the year 1857 and later recommended as a fodder crop (Wittmarck, 1922; cited by Fischer, 1938) which aided its spread. In the U.S. it is cultivated extensively and used as pasturage, harvested as hay and silage or utilized as a cover crop (Henson and Schotch, 1968; cited by Panciera, 1978).

The consumption of the seed of *V. villosa* causes growth depression in chicks and turkey poults and at high levels can result in death (Thayer and Heller, 1945; Arscott and Harper, 1964; Kienholz *et al.*, 1962).

Pastures of Hairy vetch (*V. villosa* Roth) have been implicated in poisoning cattle (Panciera *et al.*, 1966; Panciera, 1978; Kerr and Edwards, 1982; Anderson and Divers, 1983; Burroughs *et al.*, 1983; Peet and Gardner, 1986; Green and Kleynhans, 1989; Odriozola *et al.*, 1991; Woods *et al.*, 1992; Johnson *et al.*, 1992; Panciera *et al.*, 1992).

Clinical signs in cattle grazing vetch include dermatitis, conjunctivitis with oedema of the eyelids, and diarrhoea. The disorder occurs sporadically, with a mortality rate of about 50% of affected animals. The morbidity in affected herds was 6-8%. Hairy vetch is also toxic to horses (Anderson and Divers, 1983), leading to systemic granulomatous inflammation. Oedema, particularly around the lips and eyes, were noted. Conjunctivitis and corneal ulceration occur. The causative agent of hairy vetch toxicity to grazing animals has not been identified. Burroughs *et al.* (1983) described outbreaks of vetch poisoning in cattle in South Africa. These were characterised by a severe dermatitis, high morbidity, and mortality of older cows. Diarrhoea was usually observed. The condition was virtually identical to the outbreaks in Oklahoma described by Panciera (1978).

Panciera suggested that three different syndromes were associated with *V. villosa* intoxications: a) acute illness and death in 5 of 6 cattle that ate vetch from a sack; restless appearance, pain, convulsions when handled (Claughton and Claughton, 1955), b) skin lesions, cough and respiratory distress, general weakness, followed by death in some animals after 2 weeks (Röder, 1893; cited by Völker, 1950), c) rough coat, necrotic lesions on both pigmented and non-pigmented skin, but otherwise similar to photosensitisation reaction, soreness of the eyes (conjunctivitis) and severe diarrhoea associated with progressive weight loss and 50% mortality, recorded with clinically ill cattle in 23 cattle herds (U.S.A.). This condition was also associated with loss of appetite and red pigments in the urine in some cases (Panciera, 1978). Woods *et al.* (1992) noted in a horse similarities in histopathologic lesions between vetch associated disease and equine systemic granulomatous disease (SGD) which is characterised by generalised cutaneous crusting, scaling, and alopecia and multisystemic granulomatous inflammation[24].

[24] Scott, D. 1988, Immunologic diseases. In: Large animal dermatology, pp. 326-328. W. B. Saunders Co., Philadelphia, PA./Stannard, A. A. Generalised granulomatous disease. In: Current therapy in equine medicine, ed. Robinson, N. E. 2nd ed., pp. 645-646. W.B. Saunders Co. Philadelphia, PA (cited by Woods et al., 1992)

Disease progression

In the case of syndrome c) which has also been given the name vetch associated disease (Panciera, 1978), the disease did not develop until at least 2 weeks after cattle had access to pastures containing vetch (*V. villosa*). In most instances, 6 or more weeks transpired before signs of illness were recognised in 1 or more animals. Illness was not observed in 1 cow which eventually died of the disease until about 2 weeks after she was removed from the pasture containing vetch; however, the first sign noticed in this cow was alopecia, which suggested that she had been ill at least 1 to 2 weeks earlier (Panciera, 1978)

Age

The disease was prevalent and most severe in cattle aged 3 years and older, but mild cases were observed in yearlings (Panciera, 1978). Animals three years or older were also more affected than younger ones, which showed milder skin lesions and higher survival rates in the cases described by Burroughs *et al.* (1983).

Breed

Eight of the 13 herds involved consisted of Holstein-Friesians and 5 of Aberdeen Angus (Panciera, 1978). Friesland or Friesland cross animals were the only cattle affected despite a high percentage of non-Friesland cattle in the herd which remained unaffected (Burroughs *et al.*, 1983). Both Angus and Friesland are dairy cattle.

Growth stage of *V. villosa* in relation to toxicity

Major growth of *V. villosa* in Oklahoma occurs in mid June, May and June and maturation and seed production take place in mid June and July. In more northern climates, maturity is reached later in the summer. Most cases of poisoning seem to have occurred in mid-to late spring when the vetch is at the zenith of its growth and approaching maturity. It is likely that the relative intake of vetch is greatest during this period (Panciera *et al.* 1966; Panciera, 1978). A horse which had been grazing *V. villosa* containing pasture since the previous October did not become ill until early May while other horses which grazed the same pasture were unaffected (Kerr and Edwards, 1982). Both outbreaks of suspected *Vicia* species poisoning described by Burroughs *et al.* (1983) occurred during spring when the pasture was well grown, lush, apparently healthy and free of aphids as well as other parasites.

Experimental induction of the disease

Vetch plants collected from summer pasture in Western Australia in late December were fed *ad libitum* to a 6 months-old Friesian heifer for 4 weeks with no apparent effects (Peet and Gardner, 1986). Recently, Panciera *et al.* (1992) were able to induce the symptoms of vetch associated disease by feeding *V. villosa* plant material to a cow which had previously suffered from the intoxication.

Hypothesis: Canavanine is a factor in Vetch associated disease

There are many reports of toxic effects from the feeding of seeds which contain canavanine (see chapter 5 for further details). The cause for the intoxication of cattle and horses grazing on *V. villosa* and *V. benghalensis* pastures is still unresolved. Both plant species produce canavanine, a factor which has not been considered in the context of this grazing problem. The toxicity of *V. villosa* seed for cattle has been well established (Claughton and Claughton, 1955). It is reasonable to suggest that its seeds which contain 2- 3 % canavanine can give rise to acute canavanine toxicity, whereas the SGD cases induced by pasture are examples of chronic toxicity in which lower levels of canavanine and/or its metabolites may play a role.

The acute toxicity of canavanine containing seeds to cattle seems to depend on the initial dose ingested with the first meal, as the cases described by Günther (1914) and Claughton and Claughton (1954) (see table 1) suggest. It therefore appears that a sufficiently toxic dose of canavnine could be ingested on first exposure to the pasture. Feed intake after first exposure is likely to decline as has been observed with pigs feeding on *V. ervilia* (Jean-Blain, 1949), and similarly with pigs feeding on *V. villosa* seed and canavanine containing diets (Enneking *et al.* 1993). Initial high intake of canavanine containing material which varies with individual animals could then explain why some animals are more susceptible than others.

All the reported cases of the SGD syndrome in cattle have involved canavanine containing species e.g. *V. villosa* and *V. benghalensis*. It remains to be established whether or not the cases of equine SGD referred to by Woods *et al.* (1992) are due to exposure to canavanine (or other amino acid structural mimics?). It is well known that canavanine is synthesised by a range of other legumes such as clovers, medics etc. (Bell *et al.*, 1978), however very little quantitative information about the concentrations of this toxin in vegetative plant

tissues is available except for the seedling and reproductive stages in *Canavalia ensiformis* and *Medicago sativa* (Williams and Hunt, 1967; Rosenthal, 1970; Rosenthal, 1972b; Shqueir *et al.*, 1989; Miersch *et al.*, 1992; and references therein)

Przybylska and Rymowicz-Dabrowska (1970) found that the canavanine content of *V. villosa* increased dramatically with the onset of pod formation and suggested that the plant may then be quite toxic to livestock.

Any possible toxicity of canavanine to ruminants needs to take into account the results of a recent study by Dominguez-Bello and Stewart (1990) who fed 20% and 40% *Canavalia ensiformis* diets to sheep. No effects of the *Canavalia* diets on feed intake were observed. The authors observed a shift towards gram negative rods in the rumen bacterial population but this effect could not be reproduced *in vitro* through the addition of canavanine.

Earlier studies by (Addison, 1957; cited by Tschiersch, 1962) observed no toxic effects after feeding young oxen with *Canavalia* seed meal that was fed together with silage and maize straw. The same result came from experiments with Jersey cows (Addison, 1958; cited by Tschiersch, 1962).

Shone (1961) administered *Canavalia* seed meal (39g seeds/kg bodyweight) suspension to Friesland cattle by stomach tube over 3 days with lethal results. Chief symptoms were diarrhoea, weakness, inability to drink or eat, and stiffness of the hindquarters and the major post mortem lesions were dehydration, severe mucoid enteritis, nephritis and emphysema of the lungs. Animals offered pods and leaves ad libitum exhibited reduced feed intake which could be improved by spraying the feed with molasses. These animals showed no toxic symptoms, probably because the amount ingested was too low. Shone's study demonstrates that cattle can get poisoned by a canavanine containing feed but under normal circumstances would limit their intake to avoid intoxication.

All the *Vicia* species implicated in vetch associated disease can produce canavanine (*Vicia* subgenus *Vicilla*). Several symptoms of canavanine intoxication such as autoantibody formation, nephritis and alopecia (Prete, 1985; Thomas and Rosenthal, 1987a) bear resemblance to those observed with that disease (Table 1. Part 2). The systemic infiltration of tissues with granulomatous cells could be rationalised as an immune response to canavanyl proteins. The available evidence on vetch associated disease suggests that an assessment of canavanine and its metabolites, which have not yet been considered in this context, may provide a fruitful avenue for further research.

Conclusion

Vicia toxicity is due to several chemically distinct factors which depend on the particular *Vicia* species that are implicated in individual cases of poisoning. Cases of poisoning can be grouped into those caused by *V. sativa* and its related species (HCN poisoning and anti-nutritional effects of β-cyanoalanine) and those caused by canavanine containing species (*V. villosa*, *V. benghalensis*, *V. ervilia* etc.). It is hypothesised that canavanine and/or its metabolites are causal agents for the toxicity of *V. ervilia* seeds and for the intoxications of cattle and horses by *V. villosa* and *V. benghalensis* forage.

Chapter 5

Chemical isolation of the pig feed intake inhibitor L-Canavanine from the seeds of *V. villosa*

Introduction

Experiments 1-5 in this chapter describe the use of a pig bioassay for the identification of the anti-feedant factor present in the seeds of Namoi vetch (*Vicia villosa* ssp. *dasycarpa*). The seeds of this vetch were found to adversely affect the productive performance of pigs. The late Dr. Richard Davies of the South Australian Department of Agriculture, Pig Research Unit, Northfield, conducted a trial with pigs using the grains of Namoi vetch, and observed that replacement of as little as 4% of Namoi vetch for soybeans in a standard porcine grower diet had a dramatic negative effect on feed intake. An aqueous extraction of the meal from the vetch removed much of the activity and established that the active principle was water soluble. He further went on to show that acid hydrolysis reduced the biological activity significantly (Davies, pers. comm.). This result with crude extracts incorrectly suggested that the factor might be acid labile and it was only in the latter stages of purification, that this biological effect was rationalised as being due to a masking effect by sugars released during hydrolysis.

Dr. Davies' experimental system was attractive because of the high and consistent biological activity of Namoi vetch, which required only 1.5 kg of test diet at the 8% feed replacement level and was amenable to laboratory scale fractionation.

Lepkovsky (1948) noted that "the most frequent defence used by animals against toxic compounds is to limit their food intake so that the ingestion of the compound is reduced to non-toxic levels". We have used this concept in the form of a porcine feed-intake bioassay. The results of this investigation have been published (Enneking *et al.*, 1993).

Materials and Methods

Extractions

30 % aqueous ethanol extracts of vetch flour (Ressler *et al.*, 1961) were found to give the highest yield of ninhydrin reacting material. Standard extractions of vetch flour/30% ethanol (1:5 w/v) were used for all preparations**.**

For the isolation of canavanine, vetch flour/ 0.1M HCl (1:10 w/v) was used. The technique (Ressler *et al.*, 1961) was adapted as follows: hammer-milled vetch seed (*Vicia villosa* ssp. *dasycarpa* cv. Namoi, 10 kg) was extracted with 30% v/v ethanol/water (90 L) in 100 L polypropylene containers at ambient temperature (20-25°C) with intermittent stirring, settling overnight and syphoning of the supernatant extract. The container was twice more filled with 30% ethanol/water, sedimented and syphoned. The combined supernatants were filtered and the dry weight of an aliquot indicated a yield of 162 g kg^{-1} of vetch meal.

Analytical procedures

High Voltage Paper Electrophoresis (HVPE)

HVPE as described by Tate (1968, 1981). Standard buffers used: 1.1 M Formic / 0.25 M Acetic acid (pH 1.7) (56.8 ml formic acid/118.4 ml acetic acid per 2 L) 2. 0.1M Citrate (pH 3.5) (0.1 M citric acid, pH adjusted by solid NaOH) 3. 0.1 M Ammonium bicarbonate (pH 9.2) (32.8 g $(NH_4)HCO_3$ per 2 L, pH adjusted with 30 % NH_4OH) 4. Borate (pH 9.4) (0.2 M boric acid, pH adjusted with 30 % NH_4OH)

Two dimensional HVPE-Paper chromatography

After Efron (1960). HVPE pH 1.7. followed by paper chromatography: Samples were applied as a single spot, 2 cm from the edge in the middle (28.5 cm) of the Whatman No.1 paper (15 x 57 cm). After a 15 min run by HVPE formic/acetic, pH 1.7, 3500 V in the horizontal direction the paper was dried, and after measuring the position of the acidic markers it was cut to size (15 x 20 cm). For development in perpendicular direction to the HVPE separation it was placed in a TLC tank which had been equilibrated with butanol: acetic acid: water (12 : 3: 5) so that the edge was 1 cm deep in the solvent.

Detection Methods

UV absorption 254 and 300nm detection. Paper dip reagents: 1. Amino acids: 1.25% ninhydrin in acetone, heated 3 - 5 mins at 110° C, 2. Carbohydrates: 0.2 % Silver nitrate in aqueous acetone (4g Silver nitrate dissolved in 40 ml water, made up to 2 L with acetone) H_20/NaOH ethanol (Trevelyan *et al.*, 1950) 3. Guanidines: 90% (0.2 M phenanthrene quinone in ethanol, 10% (5 M NaOH), dried and viewed under UV 254 nm for yellow fluorescence (Yamada and Itano, 1966), 4. Pauli reagent 5. Tetrazolium stain (Trevelyan *et al.*, 1950)

Ultraviolet Spectroscopy

UV spectra were recorded on a Perkin Elmer λ 5 Spectrophotometer in ddH_20 at 60 nm/s.

Infrared spectroscopy

Spectra were obtained from samples in the solid state as KCl embedded slotted discs

(0.5% sample/30mg KCl) with a Perkin Elmer 983G infrared spectrometer.

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR on FX 90Q in D_2O, C/H probe

a) Trigonelline: HDO (4.70 ppm) as reference, 89.55 MHz, off set 54.5 MHz, ^{2}D internal lock, pulse width 11 µSec, pulse angle 45 ° , pulse delay 4.18 sec, 8K datapoints, no window, 256 pulses, spectral amplitude 45 db, spectral width 1000 Hz, decoupling mode HMG, Temp 24 ° C, 5 mm tube.

b) Deaminocanavanine: t BuOH as reference, sample concentration 25 mg ml^{-1}, 89.55 MHz, off set 54.5 MHz, ^{2}D internal lock, pulse width 15 µSec, pulse angle 245 ° , pulse delay 10 msec, 8K datapoints, no window, 16 pulses, spectral amplitude 45 db, spectral width 1000 Hz, Temp 24 ° C, 5 mm tube.

^{13}C NMR on FX 90Q in D_2O, C/H probe

a) Deamino-canavanine: t BuOH (32.45 ppm) as reference, sample concentration 80 mg ml^{-1}, 22.49 MHz, ^{2}D internal lock, pulse width 11 µSec, pulse angle ~45 ° , pulse delay 2 sec, 8K/4K datapoints, window 20 (1.8 Hz LB), ~22 K pulses, spectral amplitude 78 db, spectral width 5000 Hz, BB decoupling mode,Temp 24 ° C, 5 mm tube.

NMR spectra were kindly provided by Dr. Graham P. Jones (Dep. Horticulture, Viticulture and Oenology, Waite Agricultural Research Institute) whose help and advice during the course of this study have been invaluable.

High Pressure Liquid Chromatography (HPLC)

The reverse phase, pre-column derivatisation method of Jones and Gilligan (1983) was used for the determination of canavanine concentration. The assistance of Holger Gockiawak, Australian Wine Research Institute with the analyses is gratefully acknowledged.

Fast Atom Bombardment Mass Spectrometry (FAB-MS)

Mass spectra were obtained by the Finnigan Mat TSQ 70 triple stage quadrupole mass spectrometer of the Australian Wine Research Institute, tuned for operation under FAB mode with Xenon gas for bombardment. Glycerol, glycerol/water or thioglycerol were used as the matrixes for the positive and glycerol or ethanolamine for the negative ion mode. The help of Drs. Graeme Currie and Vassilios Marinos is gratefully acknowledged.

Separation methods

Preparative HVPE

Samples were applied as a band and separated by HVPE. Detection by reference to markers and stained strips on both sides of the paper. Cut strips of paper with compounds of interest were eluted with water or 30% ethanol. Rolled up they were placed in cut-off Eppendorf tubes which had the bottom perforated. Such a tube was then placed into another intact tube for centrifugation. Elution with a minimal amount of solvent was achieved by repeated wetting of the paper strip with solvent (50-100 µl) followed by centrifugation in a bench top centrifuge. The eluate was then concentrated in a vacuum centrifuge or by passing nitrogen gas over the solution.

Dialysis

Dialysis in 4 Visking tubes (1 m x 8 cm diam) containing an aqueous vetch meal slurry, (0.5 kg + 1.25 L H_2O, per tube). Tubes were knotted tightly (2x) at one end, left loose and sufficiently long at the other end and temporarily supported in a vertical position, to act as an exhaust during autoclaving (20 min/121° C). After autoclaving, the loose ends of the tubes and their sterile contents were tightly knotted (2x) prior to dialysis (4° C, 13 x 17h/17 L dd H_20) in a 28 L container. A final exchange with 95% ethanol (15 L), permitted the sterile contents to be readily filtered, washed with 50% ethanol (2.2 L) and air dried to constant weight for bioassay. The nonsterile dialysates were discarded.

Hollow fibre filtration

Amicon hollow fibre filtration apparatus, fitted with a air driven dairy pump (Amicon cartridge type H10P10, cut off limit 10.000).

Cation exchange chromatography

Cation exchange resin Mitsubishi SK 1B 5-100 mesh (equivalent to Dowex 50 W) in H^+ and NH_4^+ form was used for the isolation of total cations and basic cations, respectively. Adsorbed material was eluted by using appropriate ionic strengths of either HCl or NH_4OH. Usually 1-2 M solutions were used in this study, except when fractionation was desired. For this purpose gravity fed gradients of water with increasing acid or base strengths were prepared by setting up two containers of equal volumes of (1) water and (2) either 2M HCl or 2M NH_4OH. The two containers were connected by a siphon tube. The ion exchange column was fed from container (1) which was fitted with a magnetic stirrer to allow for sufficient mixing of the concentrate entering through the siphon from the slightly higher standing container (2). Further details about cation exchange fractionations of vetch extracts and the isolation of canavanine.2HCl are described below (Experiments 3-5).

Biological experiments with Pigs

Bioassay

From an operational point of view, the major limitation in using a large animal approach, is the scale of the isolation procedure necessary to obtain a clear cut bioassay response. The percentage of the dietary replacement necessary for the ($p<0.01$) level is inversely dependent upon the potency of the feed-intake inhibitory source. It is important in the treatment phase that no significant discontinuity in feed-intake response becomes apparent during the experimental period, due to an excessively large replacement by the negative control (soybean meal, in this work) in the base diet. A single four day 8% dietary replacement treatment for four pigs (each approximately 20 kg) required an extract or residue equivalent to 1.5 kg of the original Namoi meal to elicit a clearcut $p<0.01$ response. The effort necessarily involved with this bioassay is compensated to a considerable extent, because the pig is a relevant enduser as well as a useful model for human nutrition.

In experiments 1-4 , which relate to the fractionation and identification of the active factor, four pigs (Large White) per treatment were used. In experiment 5 the number was increased to eight animals per treatment to examine the effect of analytically pure canavanine hydrochloride on feed intake. Pigs (8-9 weeks old) were grouped according to weight into pens (blocks) and were individually offered two meals per 24h (1st meal 3 pm, 2nd meal 9 am). For each individual pig, the difference between allocated feed and the weight of the leftover food (dried if necessary) gave the absolute daily (24h) intake (kg/day), which was then (for graphical purposes) divided by that individual's mean intake during the pre-treatment phase to obtain its feed-intake

ratio. Individual daily feed allocations employed for experiment 5 (table 1) as well the fractionation experiments (1-4, table 2) were calculated according to the following expression (allocation (kg)= 0.75 x $W^{0.65}$ x M/D; W= live weight (kg), M= maintenance requirement (kJ/kg), D= digestible energy of the diet (kJ/kg)). This quantity of feed was equivalent to 75% *ad libitum* feed intake, but it has the advantage that wastage is strictly controlled. Live weight was measured at the beginning of each phase, ie.: pre-treatment (days 1-4), treatment (days 5-8) and post-treatment (days 9-12). During the treatment phase, 8% of the basal pre-treatment diet was replaced (table 1) with various experimental diets. Soybean and Namoi vetch meals were used respectively as the negative and positive controls.

Details of the general feed formulation are given for experiment 5 in table 1. The corresponding formulations for experiments 1-4 differ only in the replacement (80g kg^{-1}) of the base diet. Solid Namoi vetch residues from dialysis, extraction or chemical treatments were substituted directly for the equivalent 80g kg^{-1}content of vetch ignoring the specific activity alterations due to minor changes in weight. Likewise cation exchange fractions were adsorbed onto soybean meal (80g kg^{-1}) again ignoring the specific activity alterations due to minor changes in weight. At this stage the important question, was simply whether feed intake was inhibited or not.

Fractionation by bioassay

Four porcine feed-intake experiments (1-4), tested various diets containing extracts of Namoi vetch, the associated residues, ion exchange fractions of the extracts as well as physical and chemical treatments of the Namoi vetch meal.

The final experiment (5) was designed to establish whether crystalline L-canavanine dihydrochloride incorporated in the soybean meal at 38% and 100% of the measured canavanine content of Namoi vetch was dose dependent and at the higher level was sufficient to account for the observed feed inhibition induced by Namoi vetch. In addition measurements were made after the first feeding to more precisely determine the onset of feed-intake inhibition.

Table 1. Diet formulations for Experiment 5 (kg t^{-1})

Component	**Base** [25]	**Soy**	**Namoi**	**+38% can**[26]*****	**+100% can**[27]*****	**+lysine**[28] *****	**+lysine** [29] **+38% can***
Wheat	790	727	727	727	727	727	727
Fishmeal	170	156	156	156	156	156	156
Soy meal	---	80	---	80	80	80	80
Namoi	---	---	80	---	---	---	---
Can HCl	---	---	---	1.23	3.25	---	1.23
Lys HCl	---	---	---	---	---	2.86	1.08
Dicalcium phosphate	5.0	4.6	4.6	4.6	4.6	4.6	4.6
Limestone	3.0	2.8	2.8	2.8	2.8	2.8	2.8
Tallow	25.0	23.0	23.0	23.0	23.0	23.0	23.0
Salt	2.5	2.3	2.3	2.3	2.3	2.3	2.3
Min/Vit/ [30]	2.0	1.8	1.8	1.8	1.8	1.8	1.8
Total	997.5	997.5	997.5	998.7	1000.8	1000.4	999.8

[25] ANALYSIS : (Base diet g kg^{-1}): Fat, 76.0; Fibre, 21.8; CP, 190.6; Arg, 10.4; His, 4.5; Ile, 7.3; Leu, 13.5; Adlys, 10.3; Met, 4.3; Cys, 3.8; Phe 8.1; Tyr, 6.3; Thr, 6.7; Try 2.2; Val 9.4; Ca 9.9; P, 6.2; Avp, 4.9. Other: DE, 15.1 MJ kg^{-1}; Adlys/DE 0.69 g MJ^{-1}.

[26] Final feed concentrations (above base diet) per kg diet: Canavanine 2.HCl 5.12 mmol kg^{-1}

[27] Canavanine 2 HCl 13.04 mmol kg^{-1}

[28] Lysine 2.HCl 13.04 mmol kg^{-1}

[29] Lysine 2.HCl 5.12 mmol kg^{-1} and Canavanine 2.HCl 5.12 mmol kg^{-1}

[30] Provided (per kg diet) : vitamin A, 11 000 IU; D, 2 200 IU; E, 40 IU; K, 2 mg; thiamin, 1.5 mg; riboflavin, 5 mg; pyridoxine 2 mg; calcium pantothenate, 11 mg; niacin, 20 mg; folic acid, 1 mg; biotin, 150 ug; vitamin B_{12}, 20 µg; Cu, 10 mg; Fe, 100 mg; Zn, 150 mg; Mn, 50 mg; I, 0.5 mg; Mo, 0.5 mg; Co, 0.2 mg; Se, 0.13 mg; ethoxyquin, 100 mg.

General procedure for feed preparation

Individual ion exchange fractions or extracts equivalent to 1.5 kg of original Namoi vetch meal were concentrated to dryness at <44° and added to soybean meal as a slurry, which was made up from the minimum amount of water needed to dissolve a given fraction plus enough ethanol to wet the meal evenly. Half the soybean meal was stirred into the liquid to give a moist solid, which was then mixed with the remainder of the meal to yield a friable solid. The mixture was dried, in shallow trays with occasional stirring, in a current of warm air. The dried material (constant weight), was ground and sieved to less than 2mm prior to admixture with the remainder (92%) of the basal wheat and fishmeal diet.

The following alphabetical treatments (table 2) were tested in each experiment (a) was the (-ve) soybean control an (b) the (+ve) Namoi vetch control.

Experiment 1

(c) Autoclaved Namoi vetch meal, (30 min/121° C) (d) Defatted Namoi vetch meal residue (dichloromethane, 17 h Soxhlet extraction), yield (971g kg^{-1}) (e) Concentrated solutes ex 30% Ethanol extractions (3 x 4 L kg^{-1}), yield (162 g kg^{-1}) of defatted Namoi vetch meal residue.

Experiment 2

(c) Dialyzed Namoi vetch meal retentate.

(d) Performic oxidised vetch meal.

Namoi vetch meal (2.2 kg) was suspended in water (5 L). Performic acid was prepared by gradual addition of cold (5°) 300 g L^{-1} hydrogen peroxide (0.5 L) to 98%-100% formic acid (0.5 L) which was allowed to stand for 0.5 h. It was then gradually added with continuous stirring to the vetch seed meal suspension in an ice bath, so that the temperature did not rise above 40°C. Excess peroxide (starch/iodide detection) was destroyed by addition of sodium metabisulfite (200 g). To simplify drying, the deperoxidised slurry was filtered on a Büchner funnel and air dried. The filtrate was concentrated separately *in vacuo* before being added back to the dried filter cake. The bioassay control, for this oxidised vetch, comprised an equivalent amount of sodium sulfate added to Namoi vetch meal.

(e) Cationic fraction: identical to the total cationic treatment (c) in experiment 3 below.

Experiment 3

Six cation exchange fractions (c-h) for bioassay were obtained as follows:

Namoi vetch meal (1.6 kg), was sequentially extracted with 30% ethanol (2 x 6 L), filtered and concentrated at 40° to a dark syrup (296 g ie. a yield of 184 g kg^{-1}). Supernatant extracts were adsorbed on Mitsubishi SK IB sulfonic acid cation exchange resin (H^+). The column (2.5 L) was washed with water (5x bed volume) and the neutral sugars and anionic components of this eluate were discarded. Cations were eluted with 0.5 M L^{-1} NH_4OH (30 L) and all fractions (2 L) were monitored by paper electrophoresis (1.0 M L^{-1} acetic acid/0.75 M L^{-1} formic acid, pH 1.7, 37V/cm, 0.25h, ninhydrin detection). Fractions more cationic than glycine were combined as the basic amino acid fraction and contained the bulk of the canavanine, with small amounts of arginine and lysine. Acidic amino acids were present in those fractions with electrophoretic mobilities equal to or less than glutamic acid and were partitioned according to their major electrophoretic components into three bulked fractions (5-8, 9-12, 13-15). All eluates were concentrated *in vacuo* at 37° C. Yields per kg of Namoi meal were calculated from aliquots as follows: The complete ninhydrin positive eluate (separate elution) was bulked to obtain the total cation fraction (c) (yield 33.5 g kg^{-1}). Individual bulked fractions were: (d) the acidic amino acid fractions 5-8 (yield 2.3 g kg^{-1}), (e) the acidic amino acid fractions 9-12 (yield 3.5 g kg^{-1}), (f) the acidic amino acid fractions 13-15 (yield 0.9 g kg^{-1}), (g) the neutral amino acid fraction (yield: 16.1 g kg^{-1}), (h) the basic amino acid fraction (yield 15.7 g kg^{-1}). For the bioassay the residues from each of these eluates (equivalent to 1.5 kg Namoi vetch) were dissolved in water (400 m L), to which 95% ethanol (400 m L) was added and the mixture gradually added to 1500 g soybean meal. For the treatment phase 80g of each adsorbate was substituted for 80g of soybean meal in the -ve control (table 1).

Experiment 4

Two treatments were chosen to examine whether crude canavanine hydrochloride was active and whether there was any anion (Cl) effect.

(c) Sodium chloride 123.6 mM kg^{-1} soybean meal. (d) Crude (>95%) crystalline canavanine.2HCl 15.4 g kg^{-1}, ie. 61.8 mM kg^{-1}, soybean meal. This concentration is equivalent to 38% of the Namoi vetch seed canavanine concentration (0.163 M kg^{-1}). For isolation of the canavanine dihydrochloride, see purification method prior to recrystallisation.

Experiment 5

For details of this experiment see the diet formulation in Table 1. Analytically pure canavanine.2HCl was added to soybean meal at two concentrations equivalent to 100% and 38% of the canavanine concentration present in Namoi vetch seed (29 g kg^{-1} = 0.163 M kg^{-1}). An additional control of an equimolal concentration of lysine.2HCl (0.163 mol kg^{-1}) on soybean meal, was included to allow for either an anion (Cl^-) or a dibasic amino-acid effect. The sixth treatment comprised an equimolal mixture of canavanine.2HCl (0.064 M kg^{-1}) and lysine.2HCl (0.064 mol kg^{-1}) on soybean meal. In this experiment the initial feed-intake at the first (3pm) feeding of the treatment diet (day 5) was measured separately in order to assess when the effect of canavanine on feed-intake became apparent.

Isolation and purification of L-Canavanine dihydrochloride

Analytically pure, crystalline canavanine.2HCl was isolated from the turbid, aqueous supernatant liquor of a sedimented aqueous extract (200 L) of the Namoi vetch seed (20 kg) by adsorption onto a 6 L sulfonic cation exchange (NH_4^+) column (Mitsubishi SK I B (water purification grade)).

After thorough washing with water (including backflushing[31] to remove particulate matter), and desorption with 2M HCl (30 L), concentration *in vacuuo* at 55° C yielded a yellow solid residue. The residue was suspended in 5 mol L^{-1} HCl (1 L) filtered with a sintered glass (#3) funnel and washed with 5 mol L^{-1} HCL (6 x 100 mL). Paper electrophoresis at pH 1.7, showed the bulk of the ferric salts and ammonium chloride were present in the mother liquor and washings. The crude crystalline canavanine hydrochloride residue was sucked dry and residual HCl was removed by storage in a vacuum dessicator over soda lime to yield crude canavanine hydrochloride (480 g, 59.1% based upon the canavanine content of Namoi). Repeated extraction (6x ,1.5 m L g^{-1}) of this residue, with warm (85°) 5 mol L^{-1} HCl, separated it into an acidic filtrate and a residue (NH_4Cl). Removal of Fe^{3+} by ether extraction of the acidic filtrate, and charcoal decolourization, produced colourless crystals of canavanine.2HCl at 5°C. Two recrystallizations with 5 mol L^{-1} HCl (1.5 m L g^{-1}) produced 148 g (18.2%) of analytically pure canavanine.2HCl, $[a]_D$ 17.4° +/- 0.3°, (H_2O, c=1, l=1, 20°C). Anal.: ($C_5H_{12}N_4O_3$.2HCl, requires: C, 24.1%; H,5.7%; N,22.5%; Cl,28.5%; Found: C, 24.0%; H,6.1%; N,22.3%; Cl,28.6%). HPLC (Jones and Gilligan, 1983) analysis of (0.1M HCl /35°/17h) extracts (Bell 1960) indicated a concentration of 0.163 mmol g^{-1} (29 g kg^{-1} D.W.) in the original Namoi vetch meal.

Statistical design and analysis

Each of the five experiments was laid out as a randomised complete block design with four blocks. Feed-intake was measured on four successive days in each of the pre-treatment, treatment and post-treatment phases. Analysis of variance was performed on the data in each of the experimental phases using the GENSTAT statistical package. The feed intake results for the treatment period are presented together with the standard error of the difference of means (SEDs) (Table 2). In experiment five, linear contrasts were used to further investigate the relationships between the diets.

Feed Intake Ratio

Feed intake is expressed as the dimensionless feed intake ratio parameter, which was calculated by dividing the daily feed intake by the average daily feed intake for the pre-treatment period. The data is presented in a series of graphs showing for each experiment the pre-treatment phase (e.g days 1-4), followed by the treatment (e.g. days 5-8) and post-treatment phases (e.g days 9- 12). In the following figures the treatment phase is indicated by an arrow.

[31] Backflushing of the resin is possible if the glass wool plug at the bottom of the column can be held in position. This was achieved by placement of a stainless steel wire mesh grid over the plug. The wire mesh was secured by stretched polyethylene tubing, fastened by the rubber plug at the bottom of the column. Sufficient space at the top of the column is also essential to allow the resin to expand, mix and circulate freely.

Results

Table 2. Summary of pig bioassay experiments

Experiment	Treatment	Feed-intake (kg/day)	Pig weights, max-min; mean (kg) No. animals
1	a) Soybean (-ve control)	0.89	16-22.5; 18.8
	b) Namoi vetch (+ve control)	0.40	20 animals
	c) autoclaving	0.42	
	d) defatting	0.35	
	e) aqueous ethanol extraction	0.66	
	SED	0.075	
2	a) Soybean (-ve control)	0.94	15-20; 16.7
	b) Namoi vetch (+ve control)	0.19	20 animals
	c) dialysis retentate	0.79	
	d) peroxidation+NaHSO3	0.27	
	e) cationic fraction	0.60	
	SED	0.083	
3	a) Soybean (-ve control)	1.02	19-26; 22.8
	b) Namoi vetch (+ve control)	0.30	32 animals
	c) cationic fraction	0.57	
	d) acidic amino acid fns 5-8	1.02	
	e) acidic amino acid fns 9-12	1.07	
	f) acidic amino acid fns13-15	1.04	
	g) neutral amino acid fns 2+3 A	1.07	
	h) basic amino acid fns 2+3 B	0.67	
	SED	0.087	
4	a) Soybean (-ve control)	1.02	18-23; 20.0
	b) Namoi vetch (+ve control)	0.40	16 animals
	c) NaCl	1.08	
	d) 0.043 M Canavanine.2HCl	0.65	
	SED	0.078	
5	a) Soybean (-ve control)	1.13	19-36; 26.6
	b) Namoi vetch (+ve control)	0.45	48 animals
	c) 0.163 M canavanine.2HCl	0.51	
	d) 0.163 M lysine.2HCl	1.14	
	e) 0.062 M canavanine.2HCl	0.90	
	f) 0.062 M canavanine.2HCl+	0.95	
	0.062 M lysine.2HCL		
	SED	0.067	

Table 2 summarises the results of the porcine bioassay experiments expressed as mean feed intakes and SEDs over the experimental treatment periods. The following graphs illustrate these results expressed as feed intake ratios. The treatment period is indicated by an arrow.

Fig. 1. The effects of autoclaving, defatting and aqueous ethanol (30%) extraction on the feed-inhibitory activity of Namoi vetch meal

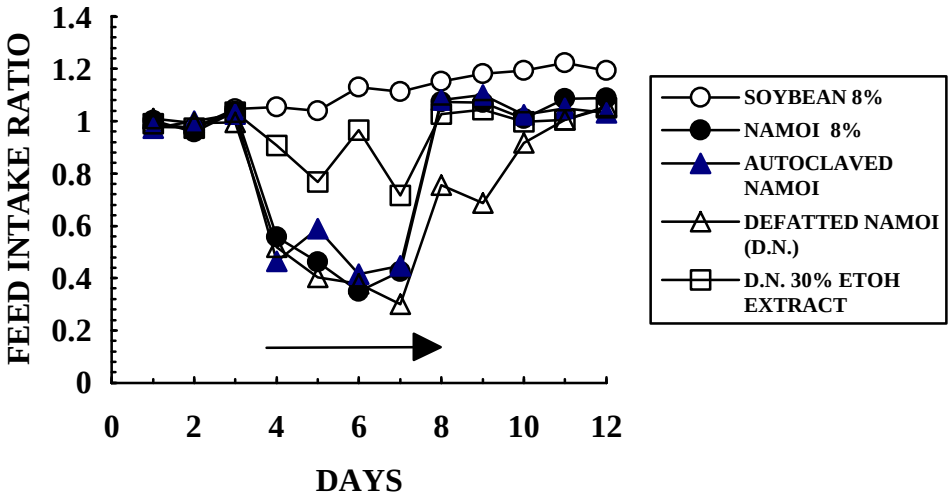


Fig. 1. shows that when the treatment diets were introduced on day 4, that all vetch treatments affected feed intake. It can therefore be concluded that 1. The active principle is thermostable under the conditions used in this experiment (wet autoclaving/30 mins). 2. The active principle is a polar molecule as it is insoluble in non-polar solvents and 3. That it can be extracted with 30% aqueous ethanol. In the next experiment the molecular size, the stability to oxidation and the ionic nature of the active principle were studied.

Fig. 2. Evaluation of size, charge and performic stability of the feed-intake depressant

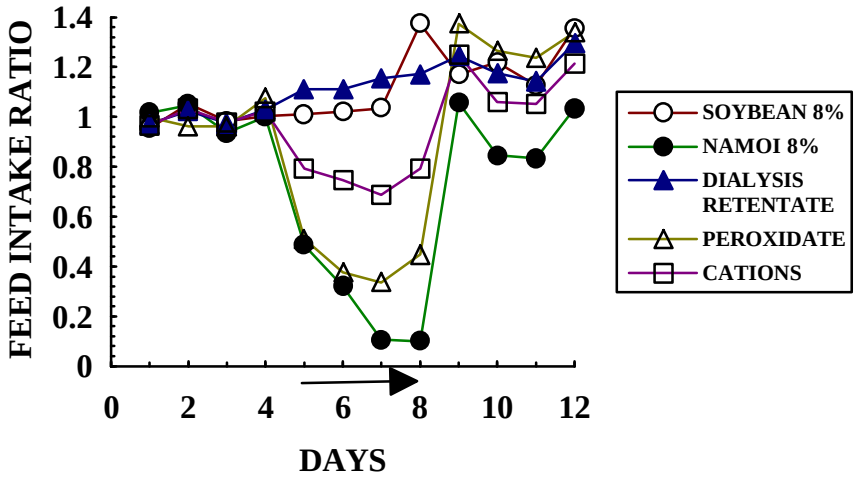


Fig. 2 shows that 1. The sterile Namoi flour dialysis retentate had no feed inhibitory activity indicating that the active principle was of low molecular weight. 2. Peroxidation did not substantially affect the biological activity indicating that the active principle was reasonably stable to oxidation. 3. The cationic fraction eluted with ammonia showed reduced activity which suggested that the active principle is a cationic species which could be unstable in alkali. The acidic and neutral subfractions of this total cationic fraction are shown in Fig. 3.

Fig. 3. The acidic and neutral cationic fractions of an aqueous 30% ethanol extract from Namoi vetch meal show no feed-inhibitory activity

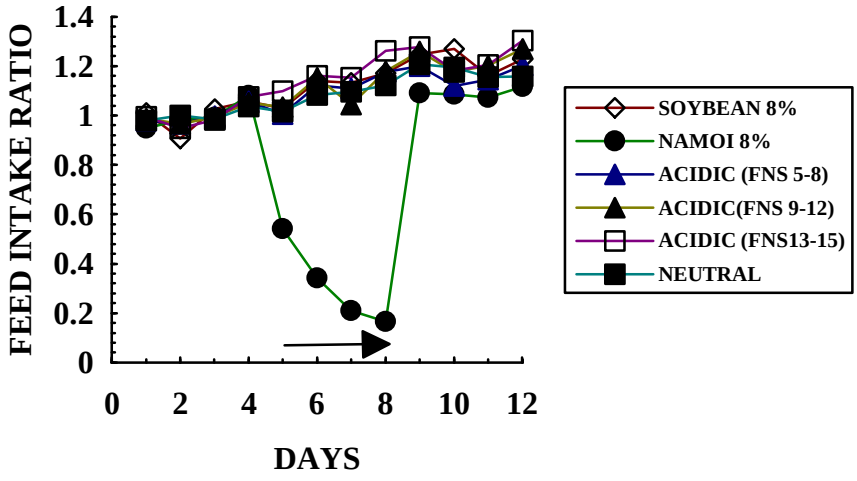
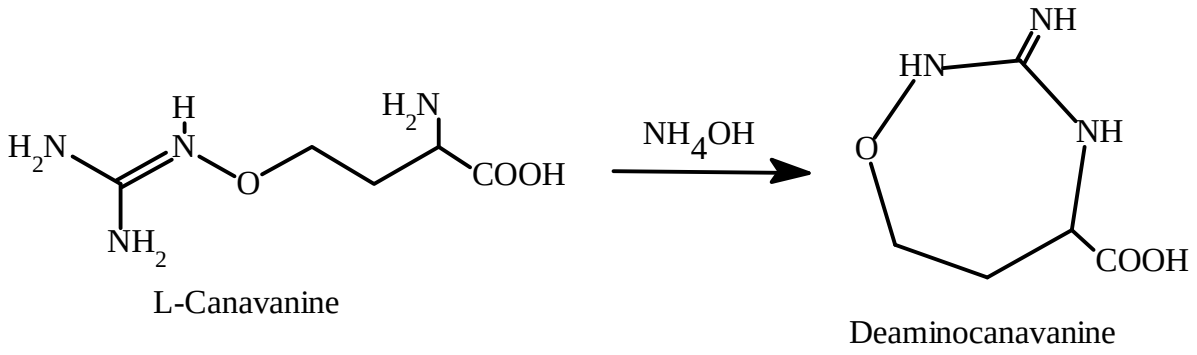


Fig. 3 shows that all the acidic and neutral cationic fractions of Namoi vetch were inactive in the feed intake bioassay. The neutral cationic fraction (with electrophoretic mobilities similar in range to those of glycine to serine at pH 1.7) contained some trigonelline (UV absorbing) and a large amount of a ninhydrin negative (guanidine positive, silver nitrate/sodium hydroxide: grey reacting) compound. Observed spectrometric properties of this compound included: fast atom bombardment mass data $M\text{-}H^+$, 157, $M+H^+$, 159 and ^{13}C NMR data (ppm): 179.5 (COOH), 162.7 (C guanidine), 76.5 (CH_2O), 59.6 (CHNH), 33.2 (CH_2). These data are consistent with deaminocanavanine, and the compound was found to be indistinguishable in all measured properties from an authentic sample of deaminocanavanine prepared by the alkaline degradation of canavanine (Rosenthal 1972a).

Fig 4. Chemical Degradation of Canavanine to Deaminocanavanine



L-Canavanine

Deaminocanavanine

Fig. 5 The effect of the basic cationic fraction of an aqueous 30% ethanol extract isolated from Namoi vetch seed meal on pig feed intake

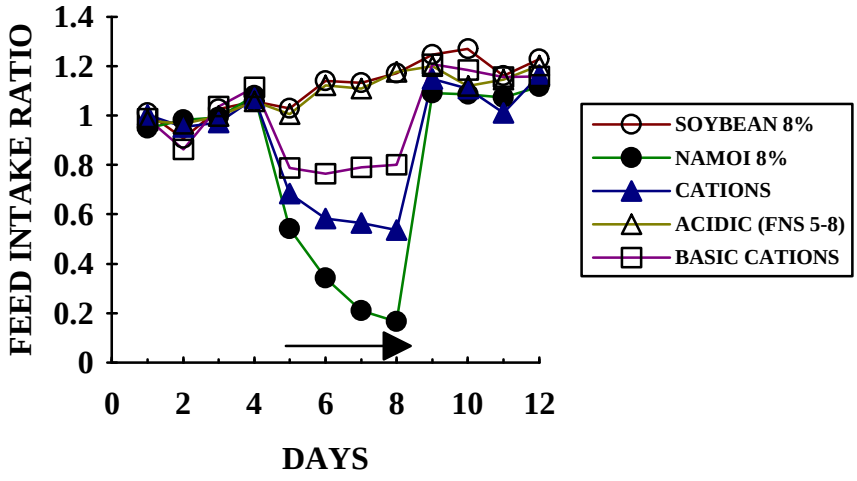


Fig. 5 shows that the feed inhibitory activity of Namoi vetch seed is associated with both the total cation fraction and its basic cationic amino acid subfraction. The major component in the basic cationic fraction of 30% aqueous ethanol extracts of Namoi vetch was found to be L-canavanine, a guanidinoxy-analogue of arginine, which was subsequently isolated as the crystalline hydrochloride salt for examination of the feed-inhibitory activity of the analytically pure salt.

Fig. 6. The effect 4.3 mM Canavanine dihydrochloride on pig-feed intake

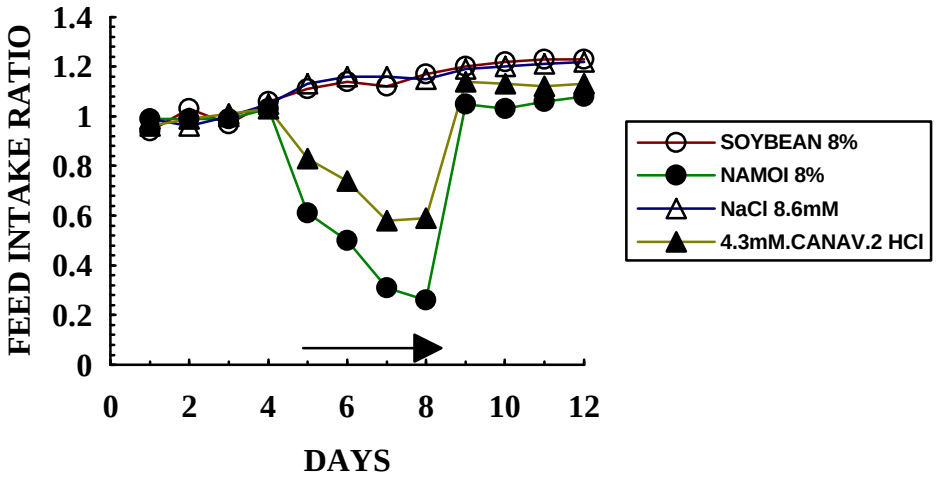


Fig. 6 shows that 1. NaCl, the negative control for the canavanine hydrochloride salt had no effect on pig feed intake at the concentration tested (8.6 mM). 2. Canavanine.2HCl affected pig feed intake negatively. The arbitrarily chosen concentration of 4.3mM of canavanine was determined by the available quantity of purified canavanine.2HCl. Because this level was insufficient to account for the total feed inhibitory activity of the Namoi vetch positive control, the canavanine concentration in the seeds of *V. villosa* cv. Namoi was measured accurately by HPLC and determined as 0.163 mmol g^{-1} (29 g kg^{-1}).

Fig. 7. Canavanine accounts for the feed intake inhibitory activity of Namoi vetch seed

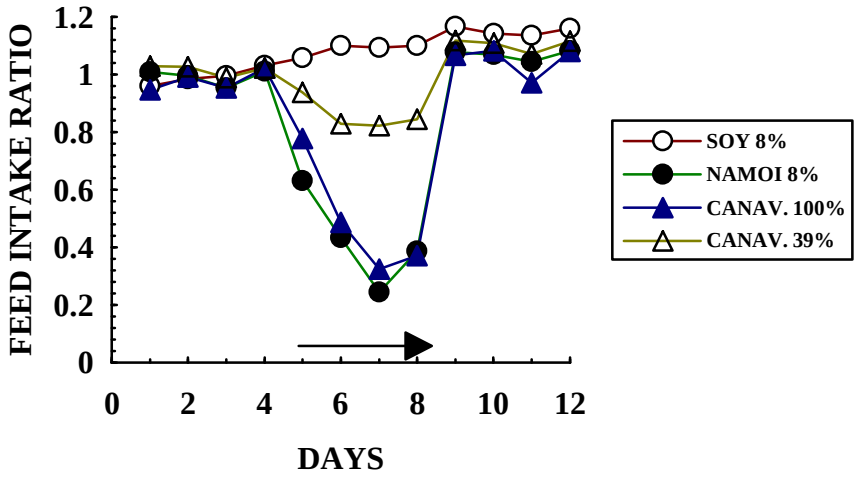


Fig. 7. Feed-intake ratio data from experiment 5, for 20kg pigs fed wheat and fishmeal diets at approximately 75% of their *ad libitum* intake. During the treatment days (5-8), an 8% dietary replacement (c.f. Table 1) with one of the 4 treatments: (i) (-)ve control of soybean meal, (ii) - (+)ve control of Namoi (29 g kg^{-1} = 0.163 mol kg^{-1} canavanine) (iii) soybean + 29 g kg^{-1} (0.163 mol kg^{-1}) canavanine, (iv) soybean +11.3 g kg^{-1} (0.064 mol kg^{-1}) canavanine. SED: (day 1-4: 0.04, day 5-8 :0.06, day 9-12: 0.05). Fig. 7. shows in graphic form that the feed inhibitory activity of canavanine when added to soybean flour in an amount equivalent to the concentration found in Namoi vetch seed is statistically indistinguishable (P< 0.01) from the latter.

Fig 8. Lysine does not affect porcine feed intake

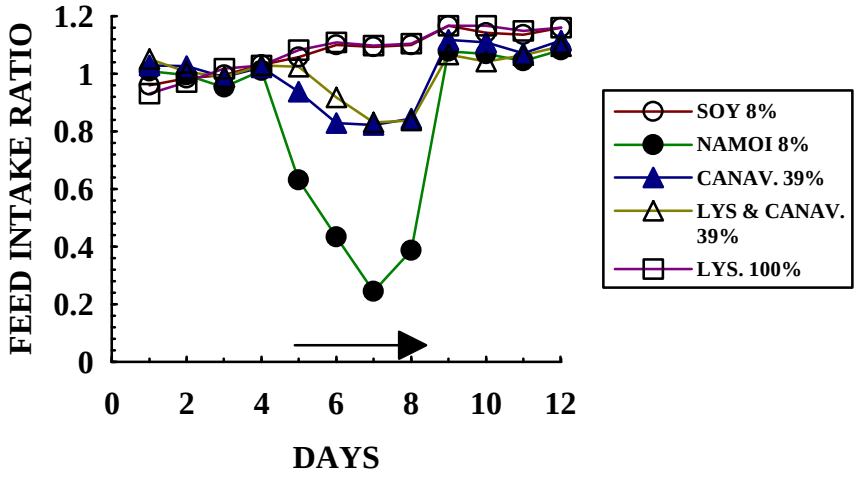


Fig 8. Feed-intake ratio data from experiment 5, for 20kg pigs fed wheat and fishmeal diets at approximately 75% of their *ad libitum* intake. During the treatment days (5-8), an 8% dietary replacement (c.f. Table 1) with one of the 4 treatments (i) (-) ve control of soybean meal, (ii) (+) ve control of Namoi (29 g kg^{-1} = 0.163 mol kg^{-1} canavanine) (iii) soybean +11.3 g kg^{-1} (0.064 mol kg^{-1}) canavanine, (iv) soybean + (0.064 mol kg^{-1}) canavanine and (0.064 mol kg^{-1}) lysine. (v) soybean + (0.163 mol kg^{-1}) lysine.2HCl. SED: (day 1-4: 0.04, day 5-8 :0.06, day 9-12: 0.05). Fig 8 shows that there was no effect of lysine.2HCl on porcine feed intake when included into the pig diets at concentrations equivalent to the levels of canavanine tested in the same experiment. Thus, 1. a general dibasic amino acid effect does not account for the observed feed intake depression and 2. lysine does not alleviate the effects of canavanine when fed at equimolar concentrations.

Detailed results and discussion of experiment five

During the pre-treatment phase, the mean intake (kg) on days 1-4 was 1.04, 1.04, 1.05 and 1.07 respectively, and differed significantly over the four days ($P<0.001$).

Analysis of variance of the feed-intake in the ensuing treatment period indicated that the interaction between diet and day was statistically significant ($p<0.001$). The mean intakes for the six diets by four days are shown in table 3.

Table 3. Mean Daily Intake (kg) / Pig at 8% Dietary Replacement (Days5-8)[32]

Day	-ve Control	Namoi*	38% Namoi can	100% Namoi can	lysine*	+lysine with 38% Namoi can	SED Means
5	1.10	0.67	0.98	0.82	1.12	1.07	0.064
6	1.14	0.46	0.87	0.48	1.15	0.96	0.064
7	1.13	0.26	0.86	0.34	1.14	0.87	0.064
8	1.14	0.41	0.88	0.39	1.14	0.88	0.064

The following linear contrasts clarify the diet x day interactions.

(i) -ve Control versus modified diets containing canavanine ($p<0.001$). (ii) +Lysine versus treatments containing canavanine ($p<0.001$). (iii) +100% Namoi canavanine concentration on soybean versus +Namoi (ns). (iv) Diets containing 38% Namoi canavanine concentration on soybean versus 100% Namoi canavanine concentration on soybean ($p<0.001$). (v) +38% canavanine concentration on soybean versus +lysine with 38% Namoi canavanine concentration on soybean (ns)

These data show that Namoi vetch or canavanine at either 38% or 100% of the equivalent Namoi levels, notably affects feed-intake in the pig. Lysine.2HCl, was arbitrarily chosen as a non guanidino dibasic amino acid control additive. It was fed at the same molal concentration as the canavanine in Namoi vetch and did not emulate either the 38% or 100% canavanine inhibitory effects on feed-intake. The 100% level of canavanine added to soybean meal compared with Namoi meal resulted in similar feed-intake reductions, but the 38% canavanine-soybean diet produced a significantly smaller reduction in feed-intake compared to the treatment using 100% level of canavanine present in Namoi vetch. There is no significant difference in feed-intake depression by the 38% canavanine treatment and 38% canavanine with an equimolal addition of lysine. Taken together, these results clearly demonstrate that the measured feed-intake inhibition produced in pigs by Namoi vetch, is a function of the L-canavanine concentration in the diet.

Analysis of the post-treatment phase in which canavanine-containing diets were no longer fed and the diet once more contained only wheat and fishmeal indicated, that the pigs' voluntary feed-intake behaviour was quickly and substantially restored as demonstrated by the nonsignificant differences ($P=0.267$) among the six post treatment diets. Both the absolute feed-intake (table 2) and the dimensionless feed-intake ratio data shown in Figs. 1-9 are a function of the weight of the pigs.

In experiment 5 the mean post treatment intake for each of the diets was 1.19, 1.14, 1.15, 1.10, 1.20 & 1.12 (diets in same order as in table3). The mean intakes for the final four days (9-12) of the study were 1.16, 1.15, 1.12 and 1.17. Again there was a significant day effect ($p<0.01$).

Gender effect

Significant gender by day interactions were observed for pretreatment (Days 1-4) males: 0.99, 1.00, 1.01, 1.05 and females: 1.08, 1.08, 1.08, 1.09 ($p<0.05$), treatment (Days 5-8) males: 0.91, 0.83, 0.78, 0.77 and females: 1.01, 0.86, 0.75, 0.84 ($p<0.01$) and post treatment (Days 9-12) males: 1.14, 1.13, 1.07, 1.15 and females: 1.18, 1.18, 1.18, 1.18 ($p<0.01$) phases, with generally lower feed-intake means for boars compared with gilts, but the overall pattern of feed-intake inhibition by canavanine containing diets was unmistakeable. Males were also more sporadic eaters than females in both the pre-treatment and post-treatment phases.

Analysis of variance of the pigs weights showed that the female pigs were significantly heavier than the male pigs in the pre-treatment and phases ($p<0.01$ and $p<0.05$ respectively). The gilts were, on average, 630 grams

[32] canavanine abbreviated to can, *soybean+component(s)

heavier in the pre-treatment phase and 730 grams heavier in the treatment phase of the experiment.

Houseman (1973) noted that boars had a lower feed intake than gilts but converted their feed more efficiently. However, Yen *et al*. (1986) found no difference between the sexes in mean food intake except for diets with high lysine (10.4g/kg diet) and protein (160g CP/kg diet) where boars were superior to gilts and these to castrates.

Post ingestion effect

In experiment 5 only, the feed-intake was measured after the first meal in addition to the total consumption for the day at the start of the treatment period (day 5). These measurements provide the data shown in table 4.

Table 4. Feed-intake (kg) for separate meals on day five[33]

Meal	Control	+Namoi*	+38% can*	+100% can*	+lysine*	+lysine with 38% can	SED
1st	0.52	0.48	0.47	0.49	0.54	0.56	0.043
2nd	0.58	0.19	0.51	0.33	0.58	0.51	0.053
Total	1.10	0.67	0.98	0.82	1.12	1.07	0.052

There was no significant difference among the means for the first meal ($p<0.001$), whereas there is a clear reduction of feed-intake of the 100% canavanine containing diets during the second meal.

Taste aversion is a learned behaviour where the negative ingestional consequence of a meal is associated with a particular taste. It also requires a time period of several hours post-ingestion for such an aversion to develop (Asche and Nachmann, 1980). From the data in table 4 it can be infered that for canavanine containing diets, the effect on feed-intake is mediated by a post-ingestional reaction to canavanine and this behaviour is akin to the feed aversion described for pigs by Houpt *et al.* (1979). In pigs, 2-6 hrs following the ingestion of *V. ervilia* the first symptoms of toxicity appear (Cornevin, 1887, Wilczek and Tschumi, 1919).

Discussion

Fig. 1 Shows the voluntary feed-intake bioassay response in pigs. These bioassay data show that after four days on an acceptable wheat and fish meal diet, replacement of 8% with soybean meal in the control diet does not induce any discontinuity. However, replacement of 8% by Namoi vetch, immediately reduced the voluntary feed-intake ratio. The ratio continued to decline to approximately one quarter of the pretreatment intake. Namoi vetch was removed on the eighth day, whereupon the pre-treatment feed-intake was resumed. Data (R. L. Davies,, unpublished) indicate that feed-intake depression continues as long as the vetch is incorporated into the diet.

With the aid of this bioassay we have found (Experiments 1 and 2), that the active component is insoluble in dichloromethane, thermally stable ($125^{o}/30'$), water soluble, resistant to performic acid oxidation, dialysable, slowly extracted by 30% ethanol, and retained with the basic amino acid fraction by cation exchange resin (NH_4^+).

In experiment 3, elution of the total cationic and basic amino acid fractions from the resin with ammonia (Rosenthal 1977a) gave the active bioassay responses shown in Fig 1 as a function of time. In both cases the recovery of biological activity was poor by comparison with the positive control. There was no detectable biological activity in any of the acidic or neutral amino acid fractions. By electrophoretic and ^{1}H NMR examination of the biologically inactive neutral amino acid fraction it was clear that it contained a considerable amount of deamino-canavanine, a well known alkaline degradation product of canavanine (Rosenthal 1977a).

By contrast, elution of the basic amino acid fraction with hydrochloric acid and fractional crystallisation under acidic conditions to separate the accompanying ammonium chloride, produced a biologically active colourless crystalline solid, whose activity was confirmed in experiment 4 (Table 2).

Electrophoretic (pH 1.7 & pH 9.2), NMR, FAB/MS and HPLC analysis of these crystals, demonstrated that they comprised more than 95% canavanine.2HCL. Recrystallization produced analytically pure L-

[33] canavanine abbreviated to can, *soybean+component(s)

canavanine.2HCl, the L- configuration was inferred from the similarity in sign and magnitude of the molar optical rotation of the hydrochloride salt to the molar rotation of authentic L-canavanine sulfate. The hydrochloride salt readily crystallized from acidic aqueous solutions in marked contrast to the sulfate salt. Although the acidic elution and fractional crystallisation procedure produced a lower unrecrystallised yield (59.1%) than the published low temperature ammonia elution procedure (85%, Rosenthal 1977a), all operations can be carried out at room temperature on a large scale and there is no racemisation, or loss of material by deamination to form the biologically inactive deamino-canavanine. Sacrificial recrystallisation gave final isolated yield of 18.2% of analytically pure material, however recovery can be further enhanced by scavenging the mother liquors.

For verification that the biological activity of canavanine was sufficient to account for all the measured feed-intake inhibition of Namoi vetch, the concentration of pure canavanine required for incorporation into the soybean meal to simulate the Namoi vetch concentration was measured by HPLC (Jones and Gilligan, 1983). Analysis of (0.1 M L^{-1} HCl /35°/17h) extracts (Bell, 1960) indicated a concentration of 0.163 mM kg^{-1} (29 g kg^{-1}DW) in the original Namoi vetch meal. This value is in agreement with the commonly reported range of 20-40 g kg^{-1} for *Papilionoidae* seeds (Rosenthal, 1982).

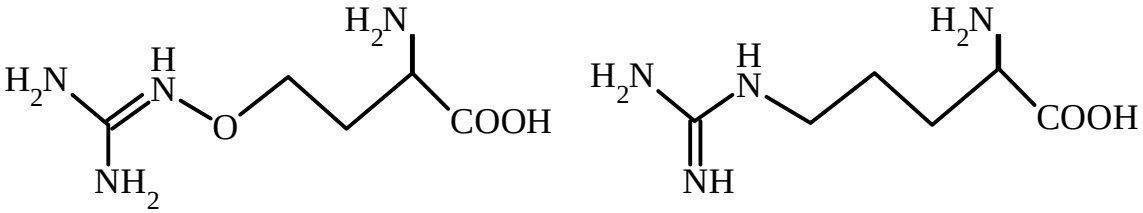
Feed-intake ratios and graphical comparisons

The dimensionless feed-intake ratio is more useful than the absolute intake data for graphically comparing the time dependent patterns of different experiments as depicted in Figs 1 and 2. These feed-intake ratio patterns were an important feature of data assessment for experiments 1-4 during the isolation procedure. The data for experiment five shown in Fig 2, clearly illustrate that canavanine mimics the feed-intake inhibition of Namoi vetch.

Chemical considerations

L-Canavanine (I) is usually described as a guanidinoxy analogue of L-arginine (II). However, the analogy is flawed because the electron distribution of the guanidine portion of canavanine was unequivocally established as (I) by a crystal-structure investigation (Boyar and Marsh 1982). Replacement of the carbon-5 methylene of arginine by an electronegative oxygen alters the guanidine electron distribution as shown (I) and thereby markedly lowers the pKa of the guanidine group from 12.5 to 7.0 whilst leaving the overall dimensions of canavanine virtually unchanged (Boyar and Marsh, 1982).

FIG. 9. Formulae comparing differences in guanidine electron distribution in L-canavanine and L-arginine



L-Canavanine L-Arginine

The resultant cationic charge diminution of canavanine compared to arginine in the physiological pH range near 7, undoubtedly has important biological implications especially when canavanine replaces arginine in proteins or peptides. Most of the well documented toxicity symptoms of canavanine can be explained by its substitution for arginine in various arginine metabolic pathways, proteins (Rosenthal 1977b; Rosenthal 1991) and possibly bio-regulatory peptides. A great variety of peptide hormones in vertebrates and invertebrates contain arginine (De Loof and Schoofs, 1990). Substitution of canavanine for arginine in these bioregulatory molecules is likely to be another factor in the toxicity of this molecule, because the different charge properties of cananvanyl peptides would be expected to alter their binding properties. We can reasonably assume that its effect on pig feed-intake is also arginine related and it should provide an excellent molecule for the future study of feed-intake regulation. The indirect possibility exists, that canavanine may inhibit arginine-related nitric

oxide generation (reviewed by Stuehr and Griffith 1992) which is important in blood pressure control via a mechanism analogous to the relaxation effect of nitric oxide on rat aortic rings (Schmidt *et al.*, 1988a, b). Such an indirect mechanism could conceivably influence feed intake regulation via some as yet unidentified nitric oxide effect on peristalsis, and is worthy of further study.

Since publication of the above data (Enneking *et al.*, 1993), we have become aware of two important studies concerned with the toxicology and pharmacokinetics of canavanine (Thomas and Rosenthal, 1987a, b). The observed constipation of rats fed with canavanine (Thomas and Rosenthal, 1987b) provides supporting evidence for the hypothesis that the anti-feeding behaviour of canavanine is due to the inhibition of nitric oxide mediated peristalsis. However, the effects of canavanine on rapidly metabolising tissues, such as the digestive enzyme producing pancreas may also contribute to the observed effects on feed intake. The work by Thomas and Rosenthal (1987 a, b) has also highlighted the deficiency in our present knowledge about canaline, a highly toxic metabolite of canavanine.

Biological considerations

There are many reports of toxic effects from the feeding of seeds which contain canavanine to: rodents (Tschiersch, 1962; de Muelenaere, 1965; Wyss and Bickel, 1988), cattle (Claughton and Claughton, 1955; Shone, 1961), birds (Arscott and Harper, 1964, Shqueir *et al.*, 1989, Leon *et al.*, 1990, 1991), pigs (Cornevin, 1887; Wilczek and Tschumi, 1919, Jean-Blain, 1949), monkeys (Malinow *et al.*, 1982) and humans (Roberts and Hayashi, 1983). However, there appear to be only two reports confirming that acute toxicity symptoms can be induced by feeding purified canavanine salts to animals. In one case mice were fed at 2 g kg^{-1} bodyweight (Tschiersch, 1962), and in the other, monkeys were fed diets containing up to 20 g kg^{-1} canavanine as the sulphate salt (Malinow *et al.*, 1982). In neither case was the actual amount of ingested canavanine specified.

As far as sublethal doses of purified canavanine salts are concerned, the only report of aberrant behavioural symptoms induced by low dietary levels appears to be with mice fed 200 mg kg^{-1} day^{-1} (1.14 mM kg^{-1} day^{-1}) bodyweight (Tschiersch, 1962). Prete (1985) reported that L-canavanine fed to a "normal species" of mouse induced autoimmune phenomena and exacerbated these symptoms in a murine systemic lupus erythrematosus model. However, no "substantial" decrease in protein consumption was observed. No details of feed-intake were given in any of these rodent studies. Shqueir *et al.* (1989) supplied feed which contained canavanine sulphate at 867 mg kg^{-1} (= 3.16 mM kg^{-1}) to chickens (initial av. wt. 40g) and found no statistically significant deleterious effects with a feed-intake of 18.33g day^{-1} which equates to an initial canavanine intake per bird of 57.85 µ M day^{-1} or 1.45 m M kg^{-1} day^{-1} bodyweight.

Canavanine and feed-intake

This report represents the first example of voluntary feed-intake inhibition in a monogastric animal by an analytically pure naturally occurring amino-acid analogue. On a porcine bodyweight basis, an initial canavanine intake of less than 43 mg kg^{-1} day^{-1} (= 0.24 mM kg^{-1} day^{-1}) was sufficient to induce an easily measurable feed-intake depression of 20%. Such control of *ad libitum* feed-intake in pigs has been seen as commercially desirable by Fowler (1985)**.**

By contrast, Shqueir *et al.* (1989) found no statistically measurable effects on chickens fed canavanine sulfate at 1.45 mM kg^{-1} day^{-1} bodyweight. Hence on a bodyweight basis, some chickens can apparently tolerate much higher levels of purified canavanine than pigs. Nevertheless, the acute toxicity to poultry of diets which comprised mainly *Vicia villosa* seeds, is well documented (Arscott and Harper, 1964; Kienholz *et al.*, 1962). These seeds can reasonably be expected to contain canavanine approaching the level (163 mM kg^{-1}) found in *V. villosa* ssp. *dasycarpa* in the present study. Hence, the canavanine dosage (mM kg^{-1} day^{-1} bodyweight) for acute toxicity symptoms in poultry has not yet been established. Leon *et al.* (1991) have established that although a short term feed-intake inhibition in chickens fed jack bean *Canavalia ensiformis* (L) DC meal, could be explained in terms of the presence of an uncertain concentration of the thermolabile Concanavaline A. Nevertheless, a thermostable residual feed intake depressant activity for poults and chicks still remained in jack bean meal which had been extruded (135° C) (Leon *et al.*, 1990, 1991). The thermostable residual feed intake depressant activity was tentatively ascribed to canavanine, but the concentration of canavanine in the extruded material was not reported.

The literature records similar feed-intake inhibitory behaviour by diets incorporating seeds of *C. ensiformis* fed to rats (de Muelenaere, 1965) and cattle (Addison, 1958), and of *Vicia villosa* fed to cattle (Panciera *et al.*, 1966). The ingestion of *V. ervilia* can provoke vomiting in pigs (Cornevin, 1887; Wilczek and Tschumi; Jean-Blain, 1949). These seeds are now well known to contain canavanine; for its qualitative distribution see Bell (1960), Bell *et al.* (1978) and for its quantitation see Turner and Harbourne (1967); Rosenthal (1977a); Natelson (1985a) and Cacho *et al.* (1989).

From these diverse reports, virtually all of which directly or indirectly associate this arginine analogue with feed-intake inhibition, it is clear that the effect is not necessarily limited to monogastric animals. Future comparative studies of tolerance and adaptation to canavanine containing diets in different species are clearly warranted**.** In the mean time, considerable care should be exercised in formulating feedstuffs incorporating seeds with a high canavanine content.

In cattle (Addison, 1958), the unpalatability of jack bean meal (Canavanine content 26.5g kg $^{-1}$, Natelson, 1985a) in the diet, was effectively masked by the addition of molasses (~ 18 L t^{-1}). In unpublished work by the late R. L. Davies it was observed that a neutralised hydrochloric acid hydrolysate of a turbid aqueous extract of Namoi vetch seed was less inhibitory to pigs than an equivalent amount of unhydrolysed extract. Examination of the hydrolysate showed the presence of much glucose from starch present in the original turbid extract. From these two reports, and a personal communication from E A Bell, which indicated that rats could be induced to eat canavanine-containing feed when mixed with chocolate spread, it seems that a canavanine induced feed-intake inhibition may be at least partly reversed by including sweet tasting additives in the diet.

Human dietary implications

In view of the reproducible and readily observable feed-intake inhibition induced in both pigs (this work), and rats (de Muelenaere, 1965), by diets incorporating *Vicia villosa* (canavanine, 29 g kg^{-1}) and *Canavalia* (canavanine, 26.5 g kg^{-1}, Natelson, 1985a), the possible deleterious effects to human dietary sources of canavanine needs to be considered. Natelson (1985a, b) has reported the canavanine content of various alfalfa seeds to be in the range 8.33g kg^{-1} to 13.6 g kg^{-1}, and adverse human reactions to alfalfa tablets are documented (Roberts and Hayashi, 1983). Although canavanine is rapidly lost during germination (Bell, 1960) the possibility exists, that hyper-sensitivity in some human individuals to low residual levels of naturally occurring canavanine in alfalfa sprouts could have physiological and dietary implications. Such effects may well be exacerbated in individuals suffering from malnutrition or hepatic disease. The proposed use of this toxin as an anti-tumor agent (Thomas and Rosenthal, 1987a, b) is now questionable because the isolation of canavanine as a potent feed inhibitory substance raises serious doubts about the suitability of canavanine for human therapy.

Nevertheless, for the vast majority of the human population, we support the view expressed by Rosenthal (1978), that although "some canavanine seeds do comprise a dietary nitrogen source in certain human cultures (Shone 1961; Nout and Rombouts 1990; Obizoba and Obiano 1988)" ... " these legumes are (soaked (Obizoba and Obiano 1988); drained (Nout and Rombouts 1990) and) cooked extensively "and in general" none of the commonly consumed table legumes, nor legumes such as soya bean, which are employed as protein supplements contain significant canavanine." The water solubility of canavanine as well as the usage of alkaline conditions for cooking *Canavalia* (Obizoba and Obiano, 1988; D'Mello and Walker 1991), would certainly enhance the destruction of any residual non-extracted canavanine by conversion to deaminocanavanine. In experiment 3, alkaline degradation of canavanine (recovery = 15.7g kg^{-1}, 54%) yielded deaminocanavanine as a major component in the neutral amino acid fraction which eluted with ammonia. Assuming that the unrecovered canavanine was substantially converted to the observed deaminocanavanine we can estimate that this fraction (recovery = 16.1g kg^{-1}) could account for up to nearly half the initial canavanine concentration (= 29g kg^{-1}). However the experiment 3 pig bioassay (Fig 1 and table 2) demonstrated that in contrast to a similar level of recovered canavanine, this neutral amino acid fraction containing deamino-canavanine, showed no significant feed-intake inhibition. The deamination of canavanine under alkaline conditions may therefore be a suitable chemical strategy for its detoxification.

It is evident, that with an understanding of the chemistry of anti-nutritional factors, detoxification strategies can be explained; or newly devised in order to render many potentially toxic leguminous foods safe to eat, which could be a matter of considerable importance in famine situations and for future food resources.

Further research with canavanine

The role of canavanine in producing the symptoms observed in vetch associated disease (chapter 3) has not been clarified, as yet, and experiments designed to test its effect on cattle would establish whether it is involved in the observed toxic effects caused by the forage of *V. villosa* and *V. benghalensis.* The measurement of canavanine concentrations in *V. villosa* ,*V. benghalensis* and *V. ervilia* during different stages of growth, and the effect of desiccation on the content of this toxin in the herbage would allow for a decision to be made, whether animal experiments feeding various levels of canavanine to cattle and sheep are warranted in order to establish the causal agent for the observed symptoms. Because a method for the large-scale isolation of sufficient canavanine to conduct large animal experiments is now available, it should be possible to repeat the pig studies on ruminants and provide definitive data on these animals as well. In these future studies with canavanine, attention should also be given to canaline (Thomas and Rosenthal, 1987b) and other possible

metabolites (Hollander *et al.*, 1989).

It may also be prudent to investigate and document food preparation practices in Moroccan Berber communities where *V. ervilia* is still being used for human consumption (C. M. Francis, pers. comm.) in order to gain a better understanding of utilising canavanine containing species for human nutrition.

Conclusion

The factor present in *V. villosa* ssp. *dasycarpa* which inhibits feed-intake in pigs at a voluntarily ingested dose of < 43 mg kg^{-1} day^{-1} has been identified with the help of a pig feed-intake bioassay as L-canavanine and may be useful in the study of feed intake regulation.

The close structural resemblance of canavanine to arginine suggests that a re-examination of the role of arginine in feed intake regulation could be rewarding. This is especially so, if recently discovered properties such as its role in blood pressure control via the generation of the nitric acid radical (reviewed by Stuehr and Griffith 1992) were to be replicated in other phenomena related to feeding such as peristalsis.

In principle, in a protein deficient world, more leguminous high protein food could be made available for animal and human consumption with a knowledge of the content of undesirable biologically active components such as canavanine. In particular, their stability to heat, hydrolysis, pH, endogenous enzymes and ease of extraction can logically explain or suggest effective post harvest processing methods such as the simple extraction and alkaline heat treatment for jack bean (Obizoba and Obiano 1988, D'Mello and Walker 1991). This post harvest processing can now be seen to be thermally destroying the Concanavaline A content which is already known to affect feed intake in poultry (Leon *et al.*, 1990, 1991) in addition to destroying any residual unextracted canavanine by conversion to the cyclic deamino-canavanine (Rosenthal, 1977b).

Chapter 6

Isolation and Identification of γ-Glutamyl-S-Ethenyl Cysteine as Antifeedant Component from the Seeds of *Vicia narbonensis* L.

Introduction

Isolation of the unpalatability factor from the narbon bean (*Vicia narbonensis* L.) was aided by previous experience with Namoi vetch (*V. villosa*). It was noted that at the 12.5 % and 25% feed-inclusion level of narbon bean flour, the reduction in feed intake was considerably less than that observed with Namoi flour at the 8% level (Fig 3). In order to obtain a clear feed-intake response to the test preparations, the level of narbon beans was increased to a maximum of 35% for the positive control. This level is at the upper limit for the inclusion of *V. faba* cv. Fiord in pig diets (Davies, 1983).

Materials and Methods

Bioassay experiments

Throughout the experiments, unless specified, reverse osmosis water (R.O. H_2O) and ethanol (99% industrial grade) made up to 30 % ethanol were used. Test samples were dried in a forced draft oven (65° C/ 24 hrs) unless otherwise indicated.

V. narbonensis material

The batch of narbon beans (line ATC 60105; RL 140004) used for all experiments was part of a 500 kg shipment obtained during 1990 from the Mallee Research Station, Walpeup, Victoria, whose co-operation in supplying the seed is gratefully acknowledged. Visually the sample comprised a mixture (99.8 %) of *V. narbonensis* with different coloured testa. Distinguishable components (100 g seed) were: 66.9 g brown seeds, 28.0 g green seeds , 5.2 g black, grey and mottled seeds , 0.2 g white angular seeds of an unidentified species.

It should be noted that a sample of this line supplied in 1989 by D. Georg (Dep. Agriculture, South Australia) was more homogeneous and consisted of brown seeds. Electrophoretic comparison (pH 1.7, formic/acetic) of the hulls and cotyledons of the three different coloured *V. narbonensis* seed types revealed no differences in non-protein amino acid pattern.

A separate sample of ATC 60105 (104 seeds) from Walpeup was tested for the presence of pathogens at the Department of Agriculture, Field Crops Pathology Group (testing officer R. Cook, test No. 91-789, 25.2.1991): Visual inspection revealed no discoloured or insect damaged seeds. Two seeds were found to have lesions. Tests for *Ditylenchus dipsaci* (stem nematode), *Aschochyta fabae* (Aschochyta leaf spot), *Botrytis fabae* (chocolate spot) were negative and no other pathogen or damage could be identified. One unknown fungus was found, but it could not be brought to set spores which would have allowed identification.

This material was hammermilled to a flour at the Northfield Piggery and used for the experiments described below. Two further lines of *V. narbonensis*, SA 22669 (ATC 60193; IFVI 1145) and SA 22703 (ATC 60143; IFVI 657) were obtained for varietal comparison in narbon experiment 4, the latter accession exhibiting notable *Heliothis* sp. damage (Easton, pers. comm.). These seeds were ground in a Retsch mill SM1 fitted with a 2mm screen.

Test diet preparations

In contrast to the Namoi experiments (Chapter 2) for which 8% soybean meal was used as the negative control, the higher levels of inclusion necessary to obtain a sufficient effect on feed intake by *V. narbonensis* seed required a different negative control diet. Due to its higher protein content and different amino acid profile, soybean flour was considered to be unsuitable as a negative control at the 35% replacement level. Peas (*Pisum sativum* cv. Dun) are similar in nutritional composition to *V. narbonensis* (Eason *et al.*, 1990) and were used instead.

Experimental design

In previous pig experiments (Chapter 2) it was found that 4 pigs per treatment would give a reliable feed-intake response. The availability of young pigs for our experiments at the Northfield piggery depended on the breeding program which produced litters at three months intervals. Therefore, the required size of young piglets was available approx. every 3 months, and this time interval determined the speed of our biological investigations.

Table 1. Diet formulation (kg t^{-1})

Component	-Base	Pea	Narbon	Test diet
Wheat	790	510	510	510
Fish meal	140	91	91	91
Pea meal	---	350	---	---
Narbon	---	---	350	---
Pea meal & additive	---	---	---	350
Dicalcium	7	4.6	4.6	4.6
Limestone	7	4.6	4.6	4.6
Tallow	50.0	33.0	33.0	33.0
Salt	2.5	1.65	1.65	1.65
Min/Vit/ premix[34]	2.5	1.65	1.65	1.65
Total[35]	999.0	996.5	996.5	996.5

The experimental facility at Northfield allowed for the individual feeding of 32 pigs in 4 blocks, thus permitting eight treatments to be tested at any one time. An additional 32 pigs could be accommodated in trials by consecutive daily feeding sessions. Therefore a maximum number of 64 pigs could be used for feed intake bioassay experiments. For operating convenience it was desirable to design experiments with 32 pigs per experiment. 4 animals per treatment gave statistically robust results in previous trials. In order to utilise the available number of 64 pigs at each three months interval, 2 consecutive experiments were conducted. Each experiment was designed as a randomised complete block with 8 treatments and 4 replicates (2 of each sex). The heavier pigs of the cohort were used in the first experiment and the lighter ones two weeks later. In this way it was possible to test 6 additional treatments within the same experimental period (one every 3 month) giving a total of 12 experimental treatments, and thereby enabled our accelerated progress with the characterisation of the anti-nutritional activity in the seeds of *V. narbonensis*[36].

Pigs were fed a common base diet for one week for acclimatisation to the individual pen feeding system. Their consumption of this diet was then measured for the following four days to give the pre-treatment feed intake. The test diets were then fed for four days (treatment period), followed by a further 4 days on the base diet and the daily feed intake recorded. The animals were weighed before each change of diet and their feed allocation was adjusted to provide a daily energy estimated at 2.9 times maintenance (DE[37]: 14.8 MJ kg^{-1}). The nutritive quality of *V. narbonensis* seed was assumed to be equivalent to that of peas because of their similar chemical composition (Eason *et al.*, 1990).

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[34] Provided (per kg diet) : vitamin A, 11 000 IU; D, 2 200 IU; E, 40 IU; K, 2 mg; thiamin, 1.5 mg; riboflavin, 5 mg; pyridoxine 2 mg; calcium pantothenate, 11 mg; niacin, 20 mg; folic acid, 1 mg; biotin, 150 µg; vitamin B_{12}, 20 µg; Cu, 10 mg; Fe, 100 mg; Zn, 150 mg; Mn, 50 mg; I, 0.5 mg; Mo, 0.5 mg; Co, 0.2 mg; Se, 0.13 mg; ethoxyquin, 100 mg

[35] **ANALYSIS** : (Base diet g kg^{-1}): Fat, 76.0; Fibre, 21.8; CP, 190.6; Arg, 10.4; His, 4.5; Ile, 7.3; Leu, 13.5; Adlys, 10.3; Met, 4.3; Cys, 3.8; Phe 8.1; Tyr, 6.3; Thr, 6.7; Try 2.2; Val 9.4; Ca 9.9; P, 6.5; Avp, 4.9. Other: DE, 15.1 MJ kg^{-1}; Adlys/DE 0.69 g MJ^{-1}.
(Pea diet g kg^{-1}): Fat, 54.1; Fibre, 40.5; CP, 207.0; Arg, 15.4; His, 4.9; Ile, 8.0; Leu, 14.5; Adlys, 12.6; Met, 3.6; Cys, 3.7; Phe 9.1; Tyr, 6.9; Thr, 7.4; Try 2.1; Val 9.8; Ca 6.8; P, 5.5; Avp, 3.6.
Other: DE, 14.8 MJ kg^{-1} ; Adlys/DE 0.85 g MJ^{-1}.

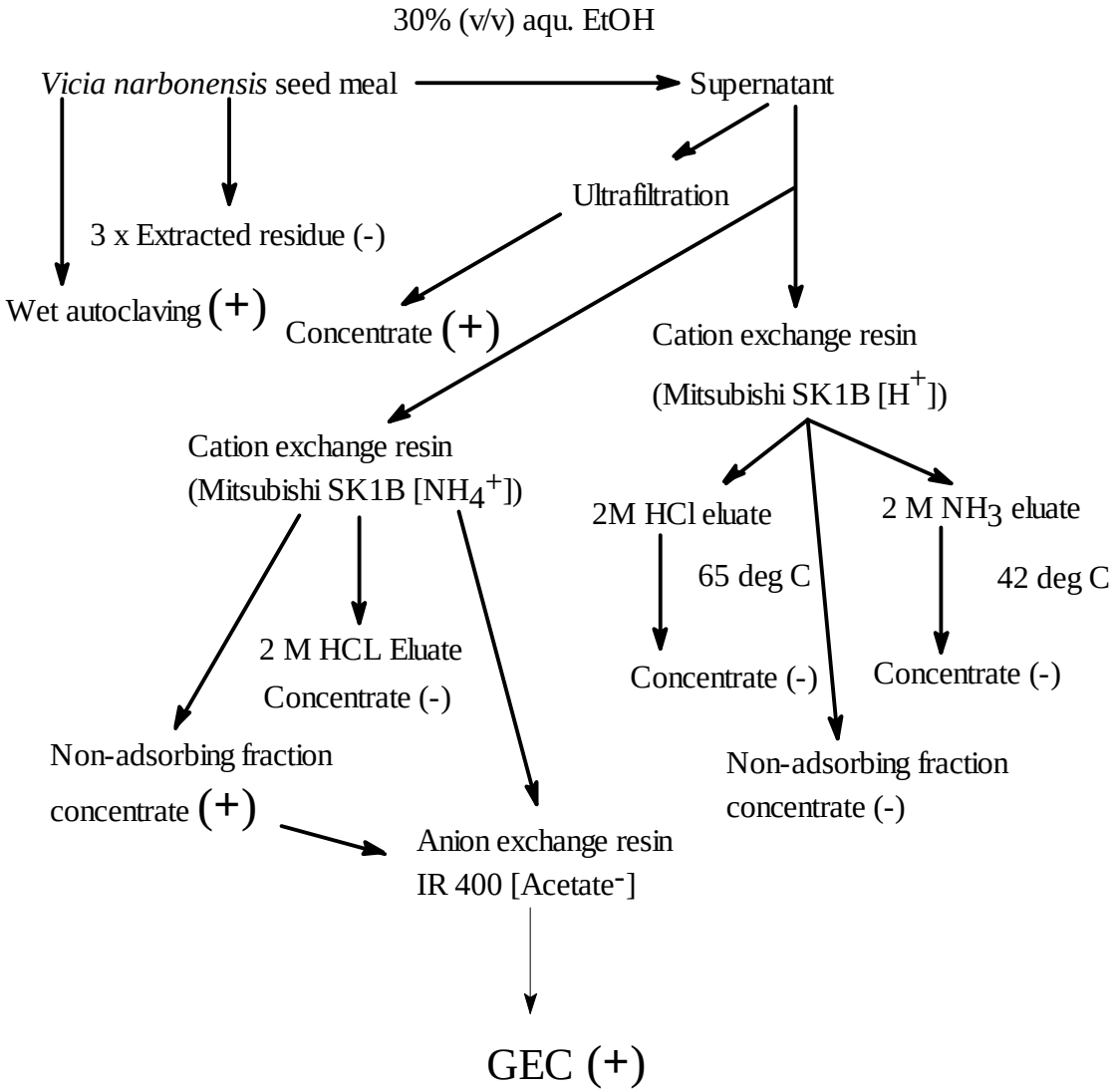
[36] Only treatments giving clear cut results are reported below

[37] DE: Digestible Energy

Statistical analysis

Analysis of variance analysis was calculated for each treatment phase (pre-tmt, tmt, post-tmt). The means and their standard errors were tabulated for the feed intake data (Table 3), while the feed intake ratio (daily intake/average daily pre-tmt intake) was calculated for use in graphical comparisons. The invaluable and patient help of Lynne Giles, statistical consultant, Dep. Plant Science, W.A.R.I. with the analysis of the data is gratefully acknowledged.

Fig 1. *Vicia narbonensis* Fractionation scheme and Bioassay response (+/-)



Narbon Experiment 1

c) Autoclaved Narbon meal

Hammermilled narbon bean (*V. narbonensis* accession ATC 60105) flour (9 kg) was moistened with 1L R.O. H_2O/kg flour and autoclaved (121°C, 1 hr, slow exhaust) in stainless steel containers (water baths) in 3 separate batches. Lumps were broken up prior to drying in a forced draft oven (65°C, 36 hrs) in two filter paper covered galvanised iron trays, filled 10-15 cm thick with layers of autoclaved mash, followed by regrinding in a Retsch mill (2 mm sieve size). Estimated milling losses 0.7%. Final weight: 8.5 kg.

d) Narbon Aqueous 30% Ethanol Extract

Hammermilled narbon beans were extracted 1:5 (w/v) with 30% ethanol. The clear supernatant ca. 50 L from each 20 kg batch was siphoned off to give the prime extract. 50 L of 30% ethanol were then added to remove another 25% of solutes from the remaining half, and these 50 L were used to start the next batch. The combined prime extracts of 60 kg (150 L) were ultrafiltered (Chapter 3). Virtually no residue was left after filtration. The ultrafiltered extract was concentrated (7 L) *in vacuo* at 42° C in a Büchi Rotavapor 185 Ex (50L flask, continuous feed). For the initial experiments concentrations were determined by serial dilution and comparison of these dilutions with extracts of known concentration by HVPE, at pH 1.7, formic/acetic, 4000 V, 10 min (1.25% Ninhydrin in acetone, 110° C, 2 min) The aliquots equivalent to the original 30% ethanol extract were then calculated for the individual fractions. Application of the concentrated 30% ethanol extract (4.8 L, DW: 1353.6 g) to 8 kg pea flour (10% moisture content) gave 8737 g after drying (18 hrs, 65 °C).

e) Narbon Extract Residue

Narbon flour (20 kg) was suspended in 30% ethanol (100 L) and after repeated stirring over two days was left to settle. 50 L of clear supernatant was removed (50%) and replaced by a further 50 L of 30% ethanol. This sequential extraction procedure was repeated twice, removing a further 25% and 12.5 % respectively, thus removing 87.5% of the equilibrated 30% ethanol soluble material. The extent of extraction was monitored by HVPE of each of the individual extract fractions which were concentrated *in vacuo* for storage. The extracted narbon flour residue was dried, reground and used as a replacement for peas in the test diet (e).

f) Narbon Acidic and Neutral Cations + (Acids and Neutral species)

Crude 30% ethanol narbon bean extract (150L), obtained a) as clear supernatant and by centrifugation (Heraeus-Christ Macrofuge, 6 x 2 L polyethylene bottles; Beckmann GP Centrifuge (GH -3.7 Horizontal Rotor) 500 ml Polyethylene bottles) of the remaining sludge, was fractionated by adsorption of its basic cations using a Mitsubishi SK1B Cation-exchange resin (6 L) (NH_4^+ form). The non-adsorbed eluate (150 L) was then concentrated *in vacuo* at 42° C to 6 L. The estimated concentrate (2.5 L, DW: 607 g) equivalent to 8 kg of narbon flour was then adsorbed onto pea flour (8 kg). The mash was dried at 45 ° C in a Büchi Rotavapor 185 Ex (50 L flask) and reground. (total DW 9 kg)[38].

g) Narbon Non-Cationic Fraction (Acids and Neutral species)

This fraction was obtained by adsorbing all cationic material of the narbon 30 % ethanol extract (150 L) onto the cation exchange column (6 L) (H^+ form). The eluant (150L) was adjusted to neutral pH with NaOH and concentrated *in vacuo*. The resulting concentrate (3.1 L) was applied in appropriate concentration (1.55 L, DW: 868.0 g) to 8 kg pea flour and dried in the Büchi Rotavapor (total DW: 9.12 kg).

h) Narbon Basic Cations

The basic cationic fraction was isolated as described for the isolation of canavanine (chapter 2, Enneking *et al.*, 1993), by adsorbing the narbon 30% ethanol extract (150 L) (see f.) to the cation exchange column 6 L (NH_4^+ form), followed by washing using the back flushing method (Chapter 4) and elution with 2M NH_4OH. The eluate was concentrated *in vacuo* (1.4 L) and an aliquot (700 ml, DW: 117.8 g) equivalent to 8 kg narbon flour

[38] The technique is not suitable for this purpose because depending on the moisture level of the flour, spherical clumps may form. Drying of this particular preparation was successful because caked flour had formed baffles which ensured adequate mixing and prevented sphere formation.

was applied to 8 kg pea flour. Final weight 7.95 kg.

Narbon Experiment 2

c) Enriched Narbon Seed Coats

Narbon seed was cracked in a hand driven roller mill. A faba bean seed-cleaning machine fitted with air draft and sieves was used to separate hulls from cotyledons by repeatedly passing partially separated fractions through the sieves. The separation was further improved by hand picking. Approx. 3 kg of the enriched hulls fraction containing 47% cotyledon/embryo grit and 53% hulls (this percentage was determined by hand separation of a sub-sample (100 g) into hulls and cotyledons) were obtained by the above procedure. This material (2 kg) was ground and added to 8 kg pea to give a total of 10 kg equivalent to a w/w content of 10% hulls for the experiment.

d) Narbon Cotyledons

The dehulled cotyledon and embryo fraction (9.7 kg), obtained as already described above for treatment c. was ground and fed at 35% of the diet.

e) Hydrolysed Narbon Cations

The cationic fraction adsorbed to the cation exchange column (H^+ form), was eluted with 2 M HCl and dried *in vacuo* at 55-65 °C. From a total of 1.5 L thus obtained, 1 L (DW: 422.0 g) was adsorbed to 10 kg peas (100 ml/kg) and dried (Final DW: 9.6 kg).

Narbon Experiment 3

In the preceding experiments, the addition of treatments to constant amounts of pea flour resulted in weight differences (> 1 kg). This difference was not taken into account for diet formulation, so in this and the following experiments**,** all treatment diets were added in the same relative proportion to the amount of pea flour used. In addition, all treatments, including the controls were reground in a Retsch mill (2mm mesh screen) to obtain flours of equal particle size, because the hammermilled flour was coarser than the flour obtained after Retsch mill grinding.

c) Narbon Bean Germination

Preliminary experiments had shown that narbon beans (ca. 10% moisture) imbibe approx. 1.25 times their weight in water on a wet weight basis, and exude about 1 % solids during the first soak (24 hrs). Narbon beans (9 kg) were soaked in water (20 L) for 18.5 hrs. The un-imbibed water (9.5 L) contained a total of 0.8 % of the seeds' exuded solids (Table 2). Thus 1.17 x the weight of the grain was imbibed (previous value 1.25). The imbibed seeds were then allowed to aerate by suspending them in a shade cloth, which was placed into a stainless steel wire mesh tray (autoclave tray). The following day the seeds were soaked in 30 L water of which 2 L were imbibed at 25 °C. Most seeds were already germinating as the radicle could be seen emerging. After 5 days most black seeds were germinating with the radicle emerging from the seed. After 8 days most seeds were sprouting, although some black seeds remained ungerminated. At this stage, soaking the sprouts in 30 L of water led to a recovery of 25 L, with 5 L being adsorbed to or absorbed by the sprouts. The sprouts were dried at 45 °C/36 hrs in a forced draft oven and ground to a flour.

Table 2. Soaking of whole seeds

Date	Soaking time	Volume (L)	weight of dried 100 ml aliquot (g)	total solids (g)	% loss
14.3.91	18.5 hrs	9.5	0.722	68.6	
15.3.91	1 hr	28	0.022	0.6	
18.3.91	1 hr	25	0.020	0.5	
				67.9 (total)	0.77

d) Acidic and Neutral Cations + (Acids and Neutral species) Fraction (A/N)

The same active concentrate as used in Narbon experiment 2. This time 2 L were used instead of the arbitrary 2.5 L of the concentrate employed in experiment 2 where the antifeedant activity of 2.5 L was found to be higher than that of the +ve control (Final DW : 8371.8g).

e) Acidic + Neutral Cations

The Acidic and Neutral Cation (NH_4^+) exchange eluate (A/N) prepared as described for experiment 1 was adsorbed onto the cation exchange resin (6 L)(H^+). The unadsorbed uncharged species were discarded. The cations were eluted with 2M NH_3 and concentrated *in vacuo* at 42° C. This treatment had a strong odour akin to H_2S (Final DW: 7895.2 g).

Narbon Experiment 4

In this experiment all treatments preparations were dried at 80°C/12 hrs, except for the pea control (10% moisture). This difference was taken into account during diet formulation.

b) -ve Control (Pea 35%) + Ammonium Acetate

Ammonium acetate (300g) was added to 8.5 kg pea flour to act as a negative control for the presence of ammonium acetate (estimated at 300 g) in the crude peptide fraction (final DW : 7.75 kg)

c)-e) Narbon accessions

Two additional lines of *V. narbonensis* were obtained from Dr. Bill Easton at the MRS, Walpeup, Victoria. c) line RL 140004 (+ ve control in previous experiments) Final DW: 8.09 kg. d) line 22669 final DW: 7.76 kg. e) line 22703 final DW: 7.33 kg. These differences in weight did not influence the final dietary inclusion level since feed allocation is a function of pig weight and the pigs used in this particular experiment were relatively young (table 2).

f) Narbon peptide

2 L (equiv. 100 L extract) of the active A/N fraction diluted to 20 L were loaded onto an Amberlite IR 400 (5 L)[Ac^-]. After elution with 1M ammonium acetate and concentration *in vacuo* (2 L, DW: 473.74 g)), half of this was added in liquid form directly to 8.5 kg pea flour to minimise thermal breakdown of the active component during drying. Final DW: 8.39 kg

g) Non Peptide Fraction

The non-adsorbed part of the diluted A/N fraction (20L) loaded onto the Amberlite IR 400 (5 L)[Ac^-], which contained the rest of the acidic and neutral cations and uncharged species, was concentrated *in vacuo* 42° C (1.25 L) and added to 8 kg pea flour. Final DW: 8.74 kg

h) 2 % Acetic Acid Soaked/Autoclaved Narbon

Whole narbon beans (10 kg) were soaked for two days in an excess (22 L) of 2 % acetic acid, autoclaved (121° C, 15 mins) and dried. Final DW: 8.4 kg

γ-Glutamyl-S-Ethenyl Cysteine (GEC) Chemistry

Elemental analysis was carried out by the Australian Microanalytical Service.

FAB-MS

Analyses were performed with a triple stage quadrupole instrument using Xe as bombardment gas. The established voltage was 7-10 keV, and the ion current, 0.5 mA. Glycerol or acidified glycerol (5% HOAc) were used as liquid matrices in the positive ion mode and triethanolamine in the negative ion mode, with sample conc. ~1 mg ml^{-1}. The help of Dr. Vassilios Marinos is gratefully acknowledged.

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR on FX 90Q in D_2O, C/H probe

GEC: t BuOH (1. 245 ppm) as reference, sample concentration 80 mg ml^{-1}, 89.55 MHz, off set 54.5 MHz, ^{2}D internal lock, pulse width 15 µSec, pulse angle -45 ° , pulse delay 2 sec, 8K/4K data points, window 3 (0.06 Hz), 16 pulses, spectral amplitude 54 db, spectral width 1000 Hz, decoupling mode : HMG cos ω suppression, Temp 24 ° C, 5 mm tube.

^{13}C-NMR on FX 90Q in D_2O, C/H probe

a) GEC: t BuOH (32.45 ppm) as reference, sample concentration 80 mg ml^{-1}, 22.49 MHz, ^{2}D internal lock, pulse width 11 µSec, pulse angle ~45 ° , pulse delay 2 sec, 8K/4K data points, window 20 (1.8 Hz LB), ~ 22 K pulses, spectral amplitude 78 db, spectral width 5000 Hz, BB decoupling mode, Temp 24 ° C, 5 mm tube.

NMR spectra were kindly provided by Dr. Graham P. Jones (Dep. Horticulture, Viticulture and Oenology, Waite Agricultural Research Institute)

Isolation of the active dipeptide fraction, γ-glutamyl-S-ethenyl cysteine

Adapting the method given by Ressler *et al*. (1969) for the isolation of γ-Glutamyl-L-β-cyanoalanine, 20 kg narbon flour were extracted through initial intermittent stirring for two days with 200 L aqueous 30% ethanol. 100 ml NH_4OH (NH_3 25%) were added to raise the pH to 7.5. After settling overnight, the clear supernatant (105 L) was adsorbed to an Amberlite IR 400 [Ac^-] (5 L) and eluted with 1M NH_4Ac^- adjusted to pH 6.5 (10.8 L) in 7 fractions (2, 1.8, 1.7, 1.9, 1.3, 2.1 , 2 L, respectively).

Fractions were monitored by paper electrophoresis at pH 1.7 (1M acetic acid /0.75M formic acid / 10 min./84V cm, $UV_{254\ nm}$/ninhydrin detection). Fractions 2-6 containing the bulk of ninhydrin positive material were combined and concentrated *in vacuo* 42 ° C to a viscous mass (DW 630 g).

Repeated extractions of the residue with warm (50°) methanol removed the ammonium acetate. The oily residue was solidified by trituration with propan-1-ol. Crystallisation was achieved from a minimum volume of water, by addition of propan-1-ol to incipient turbidity. The crystalline product was used for spectroscopic studies and elemental analysis.

Capillary zone electrophoresis (see below) established that the peptide was a major constituent present at 1.4 % DW in *V. narbonensis* (ATC 60105).

Capillary Zone Electrophoresis (CZE)

Attempted quantitative analysis for GEC using HPLC was hindered by the unstable nature of the compound in the presence of deproteination reagents such as salicylic acid and the strongly alkaline conditions used for OPA derivatisation.

Minimum sample preparation was required for analysis by CZE and the pH conditions could be maintained close to neutral to minimise breakdown of GEC, thus making this technique the method of choice for quantitative analysis.

Dried sample flour (0.5g) milled to pass <1 mm screen was extracted by tumbling for 1.5 hrs in 60% (v/v) aqueous ethanol (10 ml) containing hippuric acid (100 ppm) as internal standard. After centrifugation the clarified extracts were analysed by capillary zone electrophoresis under free zone mode (20 mM Na_3PO_4, pH 7.8, 20 kV, λ1 195 nm, λ2 222 nm, 48.5 cm x 50 µ capillary (40 cm effective length). Detection at 195 nm and 222 nm were used for GEC calibration with the internal standard. An authentic sample of S-ethenyl cysteine (SEC) (Thumfort *et al*., 1993) was used to determine its electrophoretic mobility and UV spectral properties. In the absence of sufficient amounts of this compound, the same calibration was used as for the quantitation of γ-glutamyl-S-ethenyl cysteine.

Samples were prepared in duplicate. This set was analysed twice with an 18 hr interval between the two analyses. The data (n=4) for the 195 nm and 222 nm detection are presented in table 5.

The help of Brendan Greirson (Chemistry Centre, Western Australia) with these analyses and the support of Dr. Neil Rothnie for this work is gratefully acknowledged.

Results

Table 3. Summary of *Vicia narbonensis* experiments

Experiment and Treatments	Feed Intake (kg)	Pig Weights
		Min-Max (Mean) (kg)
		No. Of Animals
Namoi Experiment No. 5 (Chapter 2)		19-36 (26.59)
a) -Ve Control (Soybean Flour 8%)	1.13	64 Animals
b) +Ve Control (Namoi Flour 8%)	0.45	
c) 100% Canavanine	0.51	
d) 38% Canavanine	0.90	
e) 100% Lysine	1.14	
f) 38% Lysine + 38% Canavanine	0.95	
g) *Vicia narbonensis* Flour 12.5%	1.1	
h) *Vicia narbonensis* Flour 25%	0.76	
SED	0.067	
Narbon Experiment 1		29-41 (33.44)
a) -Ve Control (Pea 35%)	1.29	32 Animals
b) +Ve Control (Narbon 35%)	0.84	
c) Autoclaved Narbon	0.65	
d) Narbon 30% Ethanol Extract	0.78	
e) Narbon Extract Residue	1.24	
f) Acidic Neutral Cations+(Acids and Neutrals)	0.63	
g) Narbon Acids and Neutrals	1.31	
h) Narbon Basic Cations	1.24	
SED	0.16	
Narbon Experiment 2		27-44 (34.05)
a) -Ve Control (Pea 35%)	1.34	20 Animals
b) +Ve Control (Narbon 35%)	0.92	
c) Narbon Seed Coats 10%	1.3	
d) Narbon Cotyledons	0.58	
e) Hydrolysed Narbon Cations	1.35	
SED	0.09	
Narbon Experiment 3		18-32 (25.42)
a) -Ve Control (Pea 35%)	1.15	20 Animals
b) +Ve Control (Narbon 35%)	0.77	
c) Germinated Narbon Beans, 45°C	0.92	
d)Acidic Neutral Cations+(Acids and Neutrals)	0.94	
e) Acidic + Neutral Cations (ex Cat + [$NH4^+$])	1.1	
SED	0.07	
Narbon Experiment 4		
a) -Ve Control (Pea 35%)	0.99	17.5-26.5 (21.95)
b) -Ve Control (Pea 35%)+ NH_4Ac^-	1.0	32 Animals
c) Narbon Line ATC 60105	0.63	
d) Narbon Line ATC 60193	0.77	
e) Narbon Line ATC 60143	0.56	
f) Narbon Peptide	0.85	
g) Non Peptide Fraction	0.96	
h) Narbon 2 % Ac^-H^+/121 °C/15'	0.87	
SED	0.07	

Fig 2. A comparison of the pig-feed inhibitory activity of diets containing 8% *V. villosa* seed meal or 12.5% and 25% of *V. narbonensis* seed meal

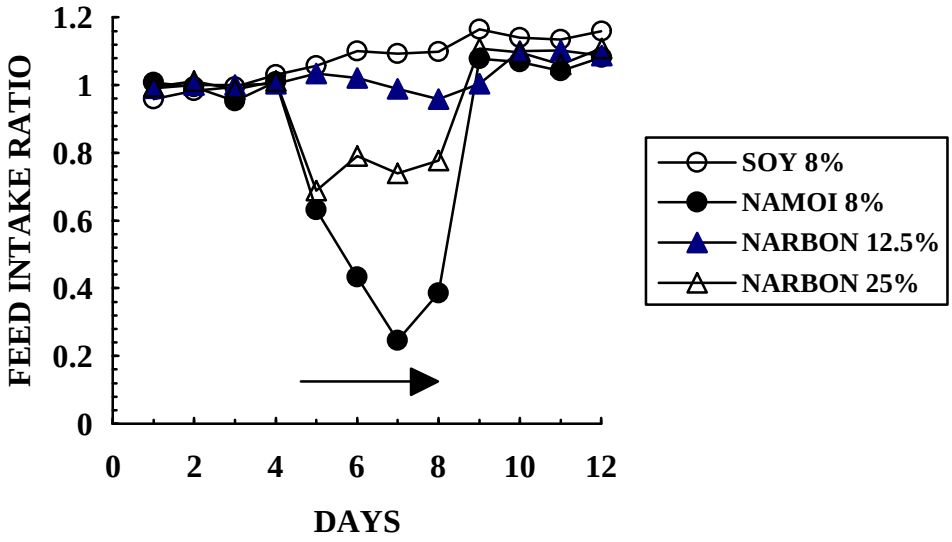


Fig 2. illustrates the much lower feed inhibitory potency of the seed meal of *V. narbonensis* at the 25% feed inclusion level, when compared with that of *V. villosa* at only 8% (treatment period indicated by an arrow). The lower level (12.5%) of *V. narbonensis* had no significant effect on feed intake, however a negative trend is apparent and feed intake declined steadily during the short experimental period.

During this experiment the nature of the anti-feedant effect was studied by measuring the feed-intake response for separate meals as is shown in Table 4:

Table 4 Feed-intake (kg) for separate meals on day five (Fig 2)[39]

Meal	Control	Namoi*	+38% can*	+100% can*	+lysine*	+lysine with 38% can	Narbon 12.5%	Narbon 25%	SED
1st	0.52	0.48	0.47	0.49	0.54	0.56	0.5	0.24	0.05
2nd	0.58	0.19	0.51	0.33	0.58	0.51	0.63	0.47	0.065
Total	1.10	0.67	0.98	0.82	1.12	1.07	1.13	0.71	0.076

Table 5 provides the feed-intake data for the first and second meal of pigs feeding on various treatment diets. These data were obtained during canavanine experiment 5 described in chapter 5. There was a significant treatment effect for each meal ($p<0.001$). In contrast to 100% canavanine and 8% *V. villosa* seed meal, the feed-inhibitory activity of the 25% *V. narbonensis* treatment is apparent after the first meal. These results demonstrate that *V. narbonensis* is unpalatable to pigs upon first exposure while canavanine is not. The latter seems to become unpalatable as a consequence of postingestional effects, whereas the former is likely to exert its effects as a negative chemosensory stimulus.

[39] canavanine abbreviated to can, *soybean+component(s)

Fig. 3. The feed-inhibitory activity in *V. narbonensis* seed is thermostable and is present in the ultrafiltered aqueous 30% ethanol extract (Narbon Exp 1, Table 3)

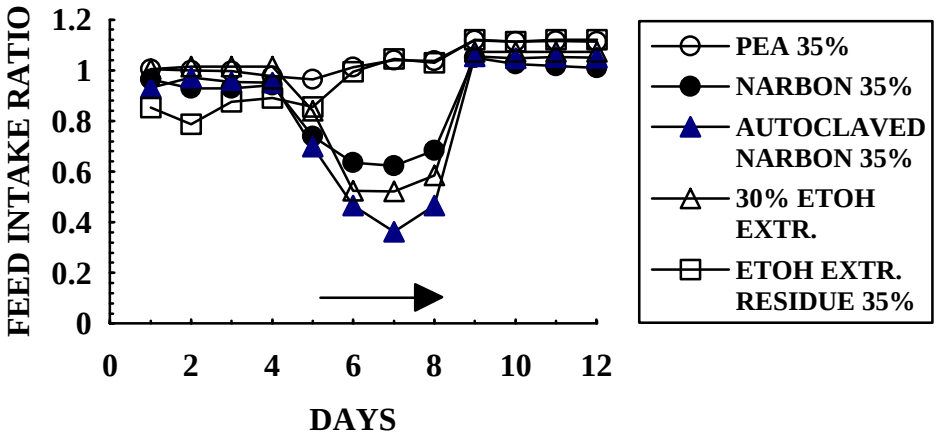


Fig 3. compares the feed-inhibitory activities of autoclaved (wet, 1 hr, 121° C) narbon flour ultrafiltered 30% eth extract, extract residue. The principal conclusions are:

1) The anti-feedant activity was heat stable under the conditions (flour/water 1:1 w/v, 1 hr, 121° C) used. An increase in feed-inhibitory activity following the heat treatment is noticeable but it may be due to breakdown products induced by autoclaving.

2) The activity is extractable in aqueous 30% ethanol. It is present in the extract after ultrafiltration and is therefore of low molecular weight (<10.000). This result suggests that no macromolecular proteinaceous factor is involved in the feed-inhibitory effect.

Fig 4. The feed-inhibitory activity is present in the fraction of the extract which does not adsorb to a cation exchange resin in ammonium form (Narbon Exp 1, Table 3)

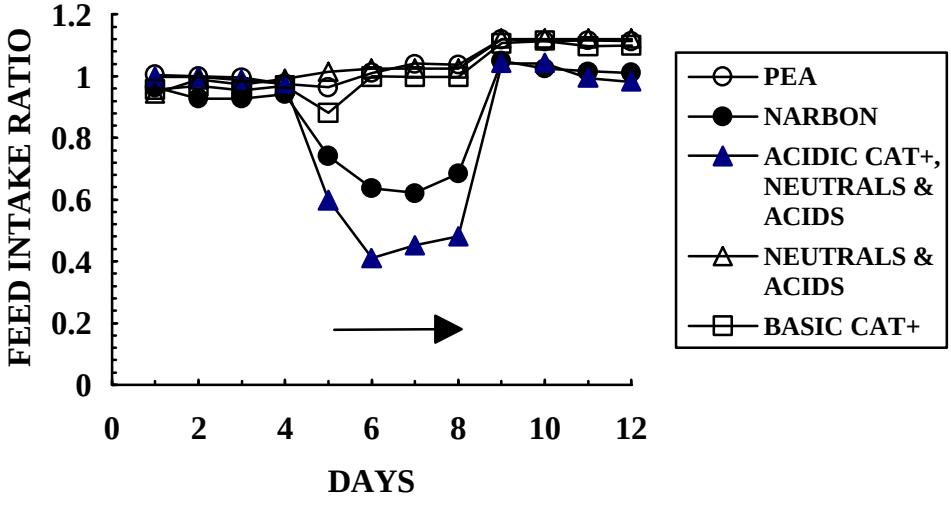


Fig 4. illustrates that a) both the noncationic fraction (neutral and acidic species) and b) the basic cations showed no activity. c) The activity was found in acidic cation and neutral plus the neutral and acidic species. Because the neutral and acidic compounds showed no activity, it can be concluded that the anti-feedant factor must be present in the acidic cationic fraction of aqueous 30% ethanol extracts of *V. narbonensis*.

The major conclusions from Narbon experiment No. 1 are that the feed inhibitory activity is not destroyed by autoclaving, that it is of low molecular weight and that its cation exchange behaviour is similar to an acidic amino acid such as aspartic acid.

Fig 5. High voltage paper electrophoresis of treatments tested by bioassay in narbon experiment No.1

Figure 5. shows the results from paper electrophoresis of 30% ethanol extracts of treatment samples from Narbon Exp No.1. The paper was marked with pencilled circles for UV positive (254 nm) compounds and developed with ninhydrin to detect amino acids and peptides. It illustrates that the feed inhibitory activity in narbon Exp No. 1 was associated with fractions which contained an acidic UV absorbing, ninhydrin positive component.

Fig 6. The feed-inhibitory activity is present in the cotyledons and can be destroyed by acid hydrolysis (Narbon Exp 2, Table 3)

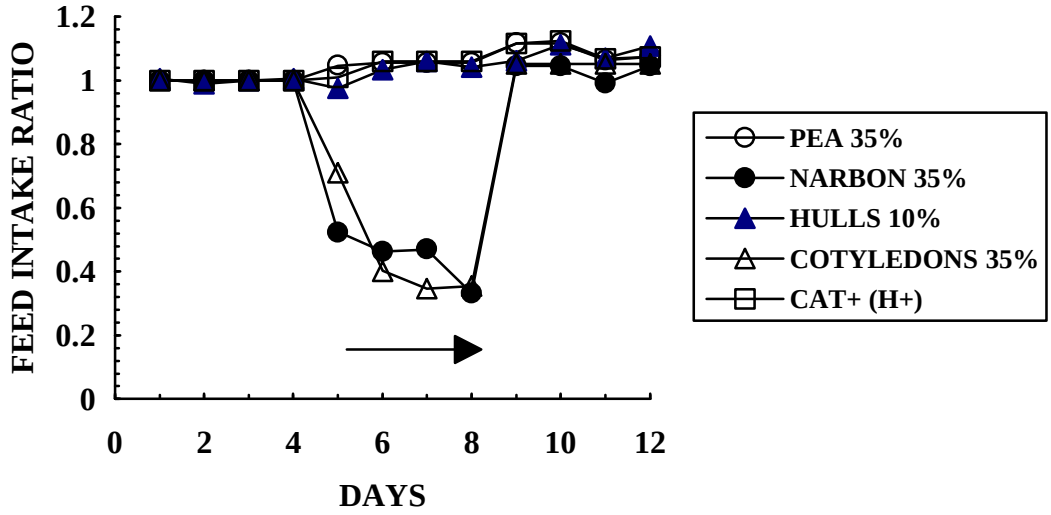


Fig. 6 shows that the feed inhibitory activity was only present in the cotyledons and embryo fraction (treatment period indicated by an arrow). The seed coats had no feed-inhibitory activity, thus eliminating testa components such as tannins. No feed-inhibitory activity was observed for the cationic fraction after it had been eluted from the cation exchange resin [H^+] with 2 M HCl and dried at 55° C, indicating that the feed-inhibitory principle was unstable to acid conditions.

Fig 7. Germination reduces the feed-inhibitory activity of *V. narbonensis* (Narbon Exp 3, table 3)

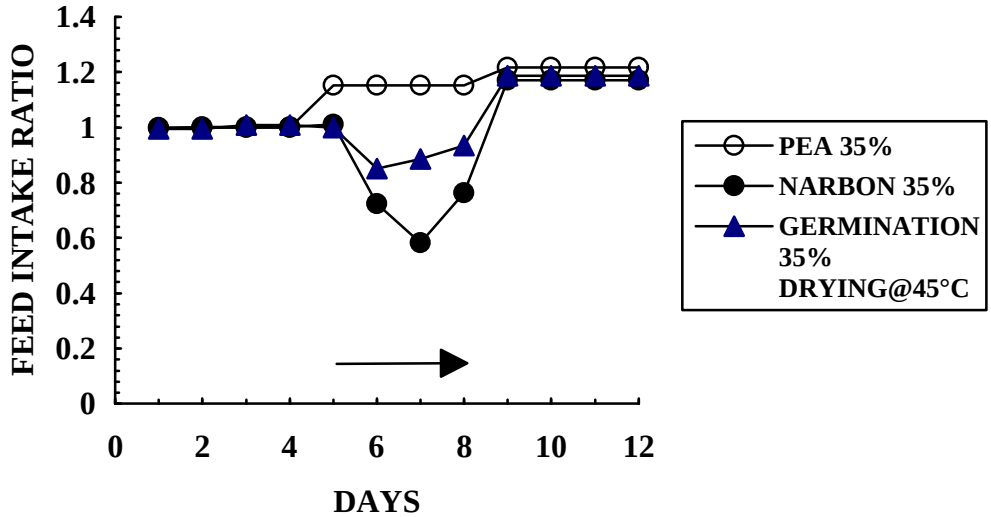


Fig 7. Germination of the narbon beans, followed by drying at a low temperature (@ 45° C) reduced the feed-inhibitory activity significantly (Intake $LSD_{0.05}$ = 0.14; +ve control 0.77; germination 0.92) (treatment period indicated by an arrow). During the preparation of this treatment it was also observed that the seeds release an objectionable sulfurous smell during germination.

Fig 8. The feed-inhibitory activity is destroyed during cation exchange [H^+] chromatography eluting with 2 M NH_3 (Narbon Exp 3, Table 3)

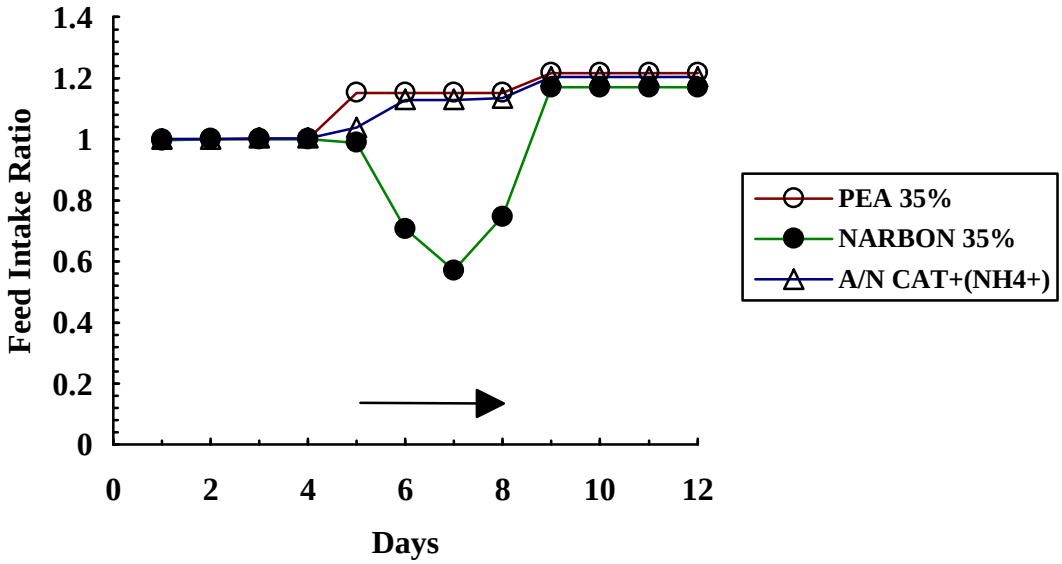


Fig 8. shows that the acidic and neutral cations isolated by adsorption of the active fraction to a strongly acidic cation exchange resin (H+) and elution with 2 M NH_3 had no feed inhibitory activity (treatment period indicated by an arrow). This preparation had a strong sulfurous smell suggesting that breakdown of sulfur containing compounds had occurred during the isolation of this fraction.

At this stage the data suggested that the factor(s) causing the reduction in feed intake was/were of low molecular weight, heat stable and present in the acidic/neutral cation fraction of 30% ethanol narbon extracts. Our attempts to isolate the active components with cation exchange, either by using acid (2 M HCl) or base (2 M NH_3) were unsuccessful since these conditions destroyed the biological activity suggesting that the active principle is labile to acid pH. It was also found that germination (sprouting) decreases the biological activity.

The key observation was the consistent presence of a strong UV absorbing, ninhydrin positive species in 30% ethanol extracts of all fractions showing antifeedant activity (Fig 5).

Further fractionation of the active acidic and neutral cationic fraction aimed at the purification of the putative UV positive component using anion-exchange chromatography.

This major ninhydrin positive fraction of the biologically active acidic and neutral cationic fraction was isolated and its chemical structure determined by Elemental analysis, UV, NMR, MS and degradative methods. Its structure was consistent with γ–glutamyl-S-ethenyl cysteine. The remaining non-peptide fraction of the acidic-and neutral fraction was also included in the experiment in addition to an acid hydrolysis treatment of whole seeds (2 % acetic acid/ 121 °C/15 mins) to verify the observation made earlier that the biological activity could be destroyed by mild acid hydrolysis.

Fig 9. γ-Glutamyl-S-Ethenyl Cysteine Reduces Pig Feed Intake (Narbon Exp 4, Table 3)

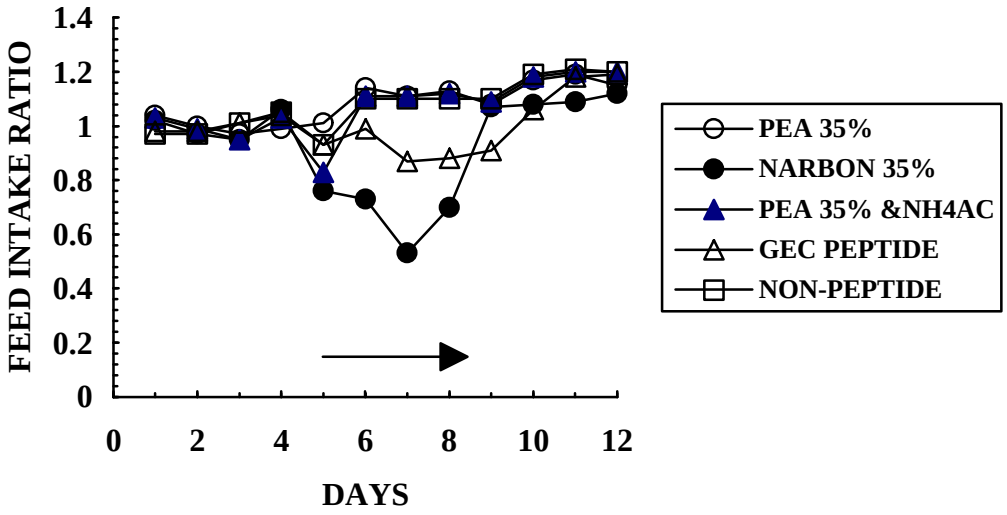


Fig 9. demonstrates that the isolated peptide, γ–glutamyl-S-ethenyl cysteine, had a definite feed-inhibitory activity (treatment period indicated by an arrow). The corresponding non-peptide fraction of the biologically active total acidic and neutral cation (+ neutrals and acids) fraction showed no activity. The control diet containing 300g ammonium acetate showed only a transient effect on feed intake on day one of the treatment period.

Fig 10. Mild Acid Hydrolysis Improves Narbon Bean Palatability (Narbon Exp 4, Table 3)

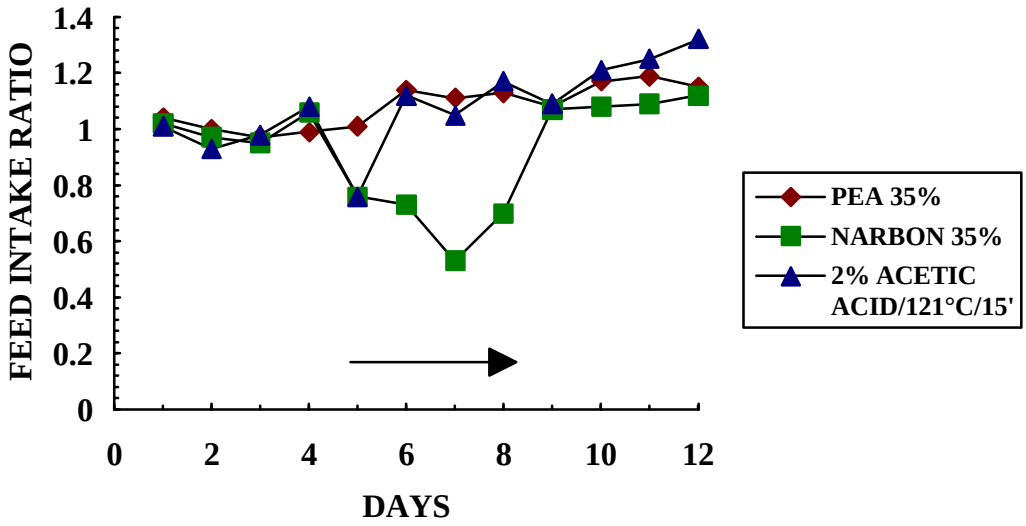
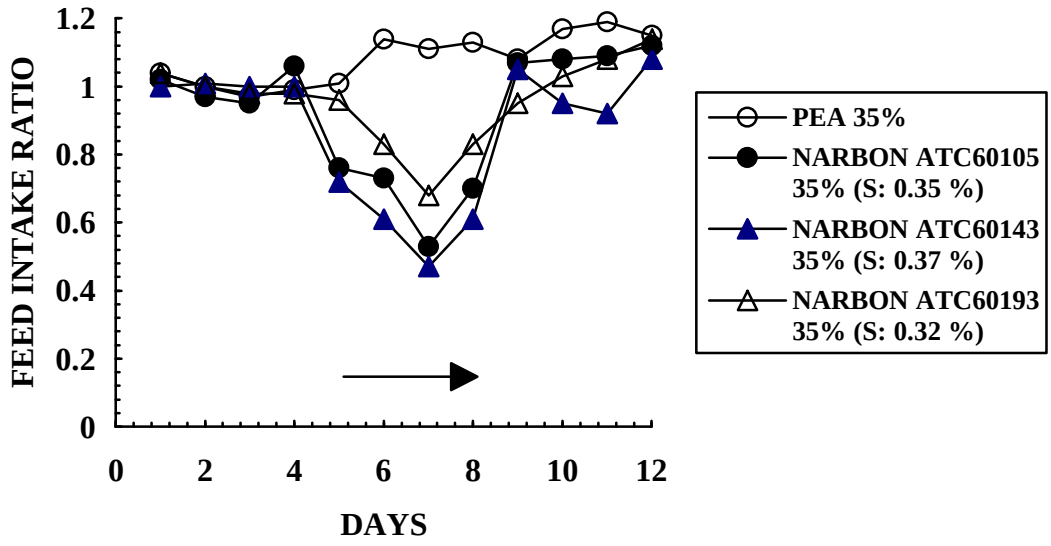


Fig 10. shows that the preparation of whole *V. narbonensis* seeds by soaking for two days in excess 2 % acetic acid followed by autoclaving (121° C, 15 mins) and forced draught drying (17 hrs, 65°C) destroyed the porcine feed-inhibitory activity (treatment period indicated by an arrow).

Fig 11. Evidence for Genetic Variation in Anti-feedant activity between 3 accessions of *Vicia narbonensis* (Narbon Exp 4, Table 3)



In Fig 11. the comparison of the feed inhibitory activity of individual *V. narbonensis* lines is illustrated. Biological activity ranks in the same order as the total sulfur content of these grains.

Fig 12. Seed total sulfur content (%) of different *Vicia* species compared to peas

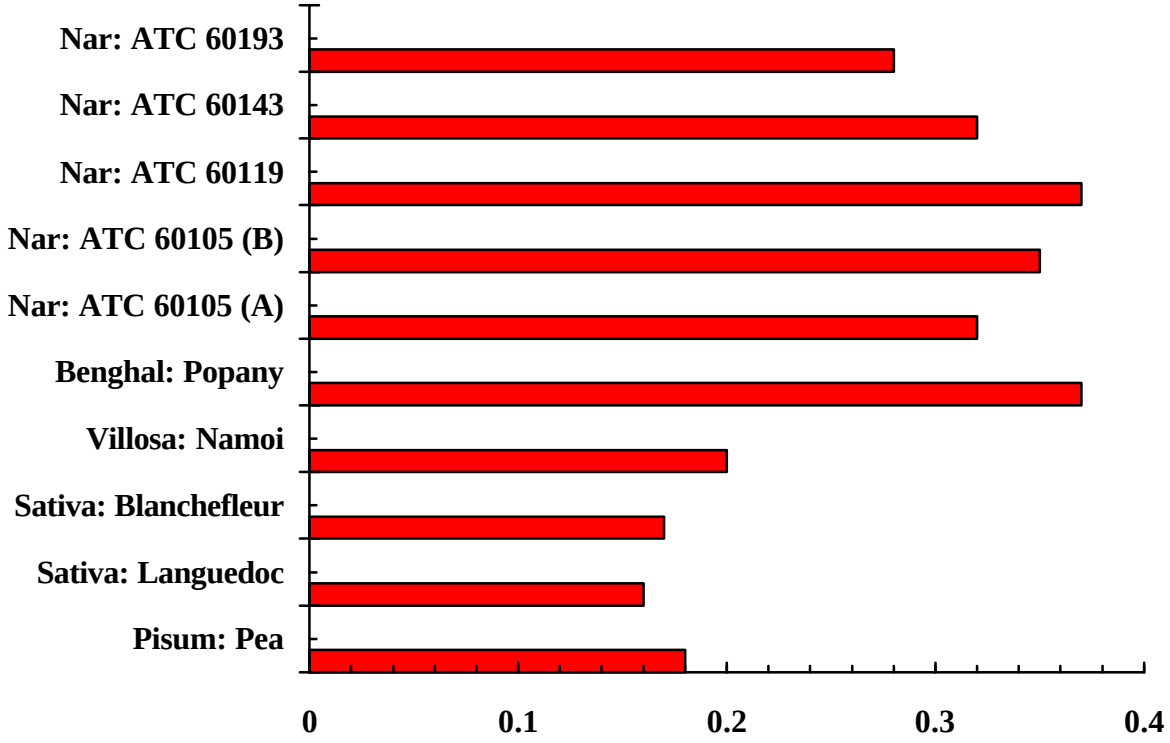


Fig 12. shows that the total seed sulfur content of peas , *V. sativa* cv. Languedoc, *V. sativa* cv. Blanchefleur, *V. villosa* cv. Namoi is much lower than that of *V. benghalensis* cv. Popany and the tested accessions of *V. narbonensis* (ATC 60193, ATC 60143, ATC 60119, ATC 60105 (samples A and B). This result suggests that the presence of GEC with its 11.7% S content in the seeds of the latter two species is reflected in total sulfur content of the seeds, and it establishes that *V. narbonensis* is a grain legume with high sulfur content.

Table 5 shows the concentrations of γ-glutamyl-S-ethenyl cysteine (GEC) and S-ethenyl cysteine (SEC) obtained by Capillary Zone Electrophoresis for the treatment samples tested in the experiments described above. For comparison, calibration was carried out at two UV wavelengths (195 nm, 222 nm). Please note that the concentration of GEC in the autoclaved sample is reduced, while the activity seems to have increased (Fig 3). Some samples contained significant concentrations of SEC. Notably, the acidic and neutral cation + (neutrals and acids), the 30% eth extract, the germinated narbon beans, and the three narbon bean lines tested in Narbon Exp. No.4. Since the samples for experiment No. 4 were dried at 80° C/12 hrs, dry heating may have contributed to the breakdown of GEC. This explains the different GEC concentrations of the two ATC 60105 samples.

The small detectable SEC concentrations in the remaining samples (Table 5) are likely to be an artefact of the extraction. The total S-ethenyl content was therefore calculated as the sum of SEC and GEC.

Fig 13. Correlation between the concentration of GEC and feed intake reduction

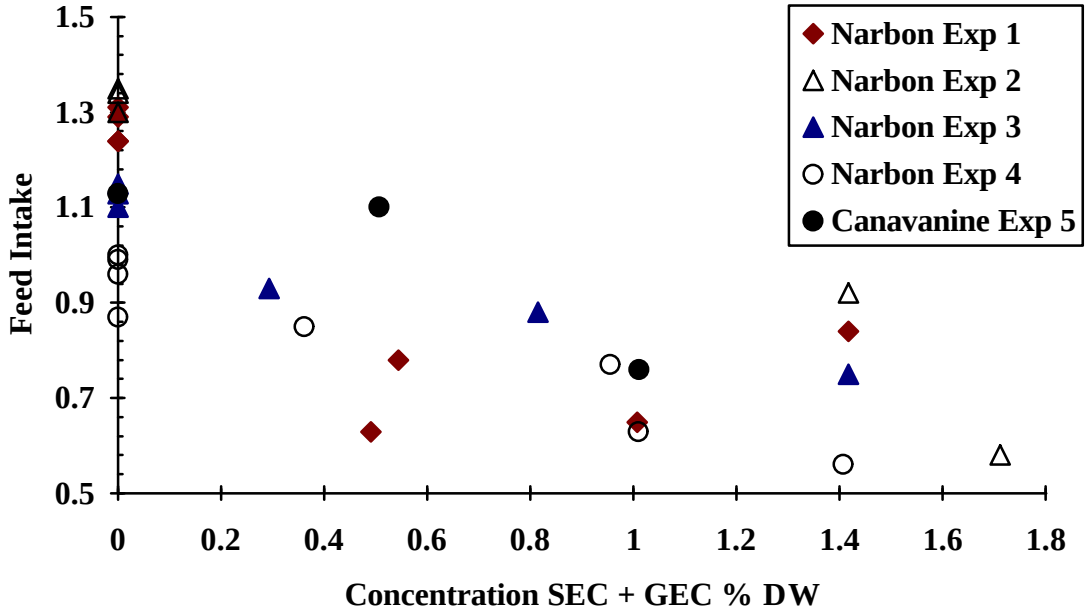


Fig 13 shows the negative relationships between the palatability of different treatments tested (intake means from table 3) and the concentrations of SEC + GEC (222 nm, % DW) listed in table 5. Regression analysis of these data (Table 6) shows that, although the correlation between S-ethenyl cysteine (SEC + GEC) for the total data set is unimpressive, the r^2 values for the individual experiments, except for those from Narbon experiment No. 1, are satisfactory. The correlation coefficients from regressions of GEC concentration against feed intake for Narbon experiments No. 1 and No. 3 are markedly lower than those obtained for the same calculations with the total S-ethenyl cysteine concentration. This is consistent with the significant amounts of S-ethenyl cysteine (Table 5) in the 30% eth extract, acidic and Neutral cation (& neutral and acids) fractions, the germinated narbon beans tested in these two experiments. The data thus indicate that S-ethenyl cysteine, similar to GEC, is also inhibitory to feed intake. Hydrolysis of the peptide into its constituent amino acids does therefore not eliminate the antifeedant activity.

Table 5. Quantitative GEC analysis

Sample	EXP	195 nm , % DW			222 nm, % DW			Feed	
		SEC*	GEC*	Total	SEC*	GEC*	Total	Intake	mM GEC (calc. from total 222nm)
Narbon (ATC 60105) 35%	Exp 5	0.03, 0.06	1.4, 0.09	1.46	0.05, 0.01	1.37, 0.06	1.42		50.7
Narbon (ATC 60105) 12.5%	Exp 5	0.01	0.51	0.52	0.02	0.49	0.51	1.10	18.5
Narbon (ATC 60105) 25%	Exp 5	0.02	1.01	1.04	0.04	0.98	1.01	0.76	36.6
Narbon autoclaved	Exp 1	0.02, 0.02	0.98, 0.05	1.00	0.06, 0.03	0.95, 0.04	1.01	0.65	36.6
30% eth/pea	Exp 1	0.11, 0.13	0.43, 0.13	0.54	0.22, 0.04	0.33, 0.03	0.54	0.78	19.6
Acidic Cations & Neutral	Exp 1	0.12, 0.05	0.24, 0.04	0.36	0.30, 0.02	0.19, 0.00	0.49	0.63	22.8
Narbon Cotyledons	Exp 2	0.02, 0.01	1.70, 0.06	1.72	0.05, 0.01	1.67, 0.08	1.71	0.58	21.0
Germinated Narbon	Exp 3	0.07, 0.09	0.81, 0.10	0.89	0.13, 0.02	0.69, 0.05	0.81	0.92	29.3
Acidic Cations & Neutral	Exp 3	0.11, 0.05	0.14, 0.05	0.24	0.19, 0.02	0.10, 0.05	0.29	0.94	10.5
Narbon Peptide	Exp 4	0.01, 0.03	0.38, 0.07	0.39	0.02, 0.01	0.34, 0.02	0.36	0.85	13.0
ATC 60105**	Exp 4	0.06, 0.05	0.87, 0.05	0.93	0.15, 0.00	0.86, 0.02	1.01	0.63	36.6
ATC 60193	Exp 4	0.09, 0.06	0.79, 0.05	0.89	0.16, 0.01	0.79, 0.05	0.96	0.77	34.8
ATC 60143	Exp 4	0.06, 0.01	1.21, 0.08	1.27	0.19, 0.01	1.22, 0.07	1.41	0.56	51.1

*The error (n=4) has been calculated as 2 standard deviations from the mean and is given together with the mean (mean, error)

** n=3

Table 6. Correlations between the concentration of S-ethenyl cysteine content of treatment diets and their feed inhibitory activities

Experiment	Parameter	r^2	Standard error
all	SEC + GEC (222 nm)	0.60	0.16
all	GEC (222 nm)	0.51	0.17
Narbon 1	SEC + GEC (222 nm)	0.59	0.21
Narbon 1	GEC (222 nm)	0.39	0.25
Narbon 2	SEC + GEC (222 nm)	0.94	0.10
Narbon 2	GEC (222 nm)	0.94	0.09
Narbon 3	SEC + GEC (222 nm)	0.88	0.06
Narbon 3	GEC (222 nm)	0.81	0.08
Narbon 4	SEC + GEC (222 nm)	0.90	0.06
Narbon 4	GEC (222 nm)	0.91	0.05
Canavanine 5	SEC + GEC (222 nm)	0.81	0.13
Canavanine 5	GEC (222 nm)	0.81	0.13

Chemistry of the Biologically Active Peptide, γ-Glutamyl-S-Ethenyl Cysteine

Elemental analysis: $C_{10}H_{16}N_2O_5S$ 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* .

IR

The IR spectrum showed absorption bands (1% threshold) at (cm^{-1} (intensity)):

g-glutamyl-S-ethenyl cysteine.NH_4^+: 3327 (0.54); 2361 (0.45); 2150 (0.45); 1645 (0.53); 1579 (0.53), 1523 (0.52); 1435 (0.50); 1384 (0.50); 1333 (0.50); 1216 (0.47); 1076 (0.44); 963 (0.44); 904 (0.45); 849 (0.45); 776 (0.44); 684 (0.46)

g-glutamyl-b-cyanoalanine.NH_4^+: 3287 (0.89); 3039 (0.9); 2251 (0.69); 2104 (0.69); 1874 (0.68); 1645 (0.89); 1599 (0.9); 1537 (0.89); 1415 (0.89); 1341 (0.81); 1288 (0.74); 1261 (0.76); 1215 (0.73); 1192 (0.69); 1132 (0.73); 1078 (0.71); 934 (0.65); 887 (0.69); 848 (0.71); 804 (0.65); 772 (0.69); 745 (0.65); 713 (0.67); 662 (0.72).

For γ-glutamyl-S-ethenyl cysteine, the 1384 cm^{-1} band may be assigned to a thioether linkage and the 904 cm^{-1} band to ethylenic stretching (Mütsch-Eckner *et al*., 1992). The 1645 cm^{-1} band in both samples is characteristic for the carbonyl stretch of γ-glutamyl bonds (Kasai and Larsen, 1980). Apart from the characteristic nitrile band at 2251 cm^{-1} in the IR spectrum for γ-glutamyl-β-cyanoalanine, the overall similarity of the fingerprint regions for these γ-glutamyl species was apparent.

Fig 14. UV spectrum for γ-glutamyl-S-ethenyl cysteine

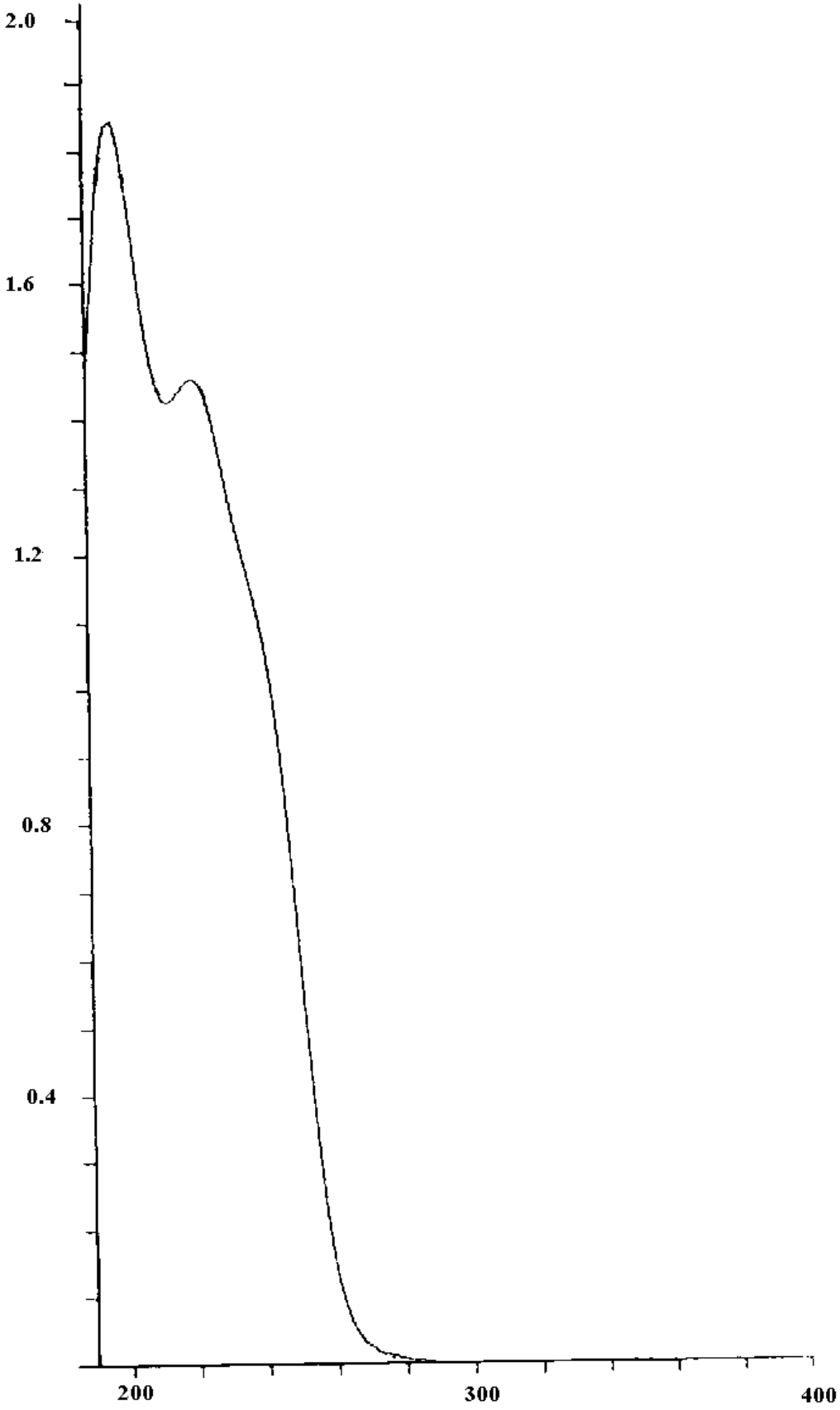


Fig 15. ^{1}H-NMR spectrum of γ-glutamyl-S-ethenyl cysteine

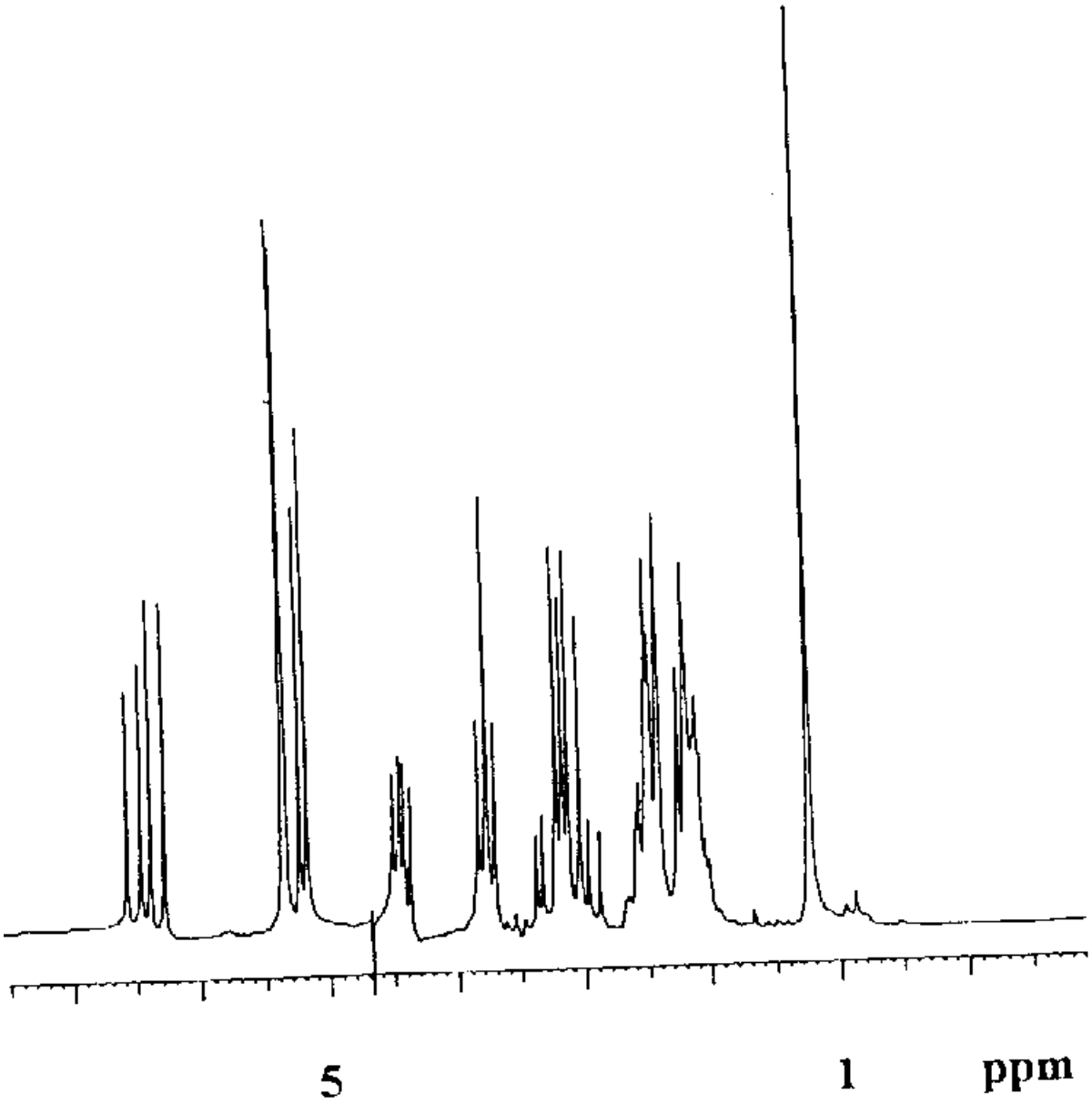


Fig 16. ^{13}C-NMR spectrum of γ-glutamyl-S-ethenyl cysteine

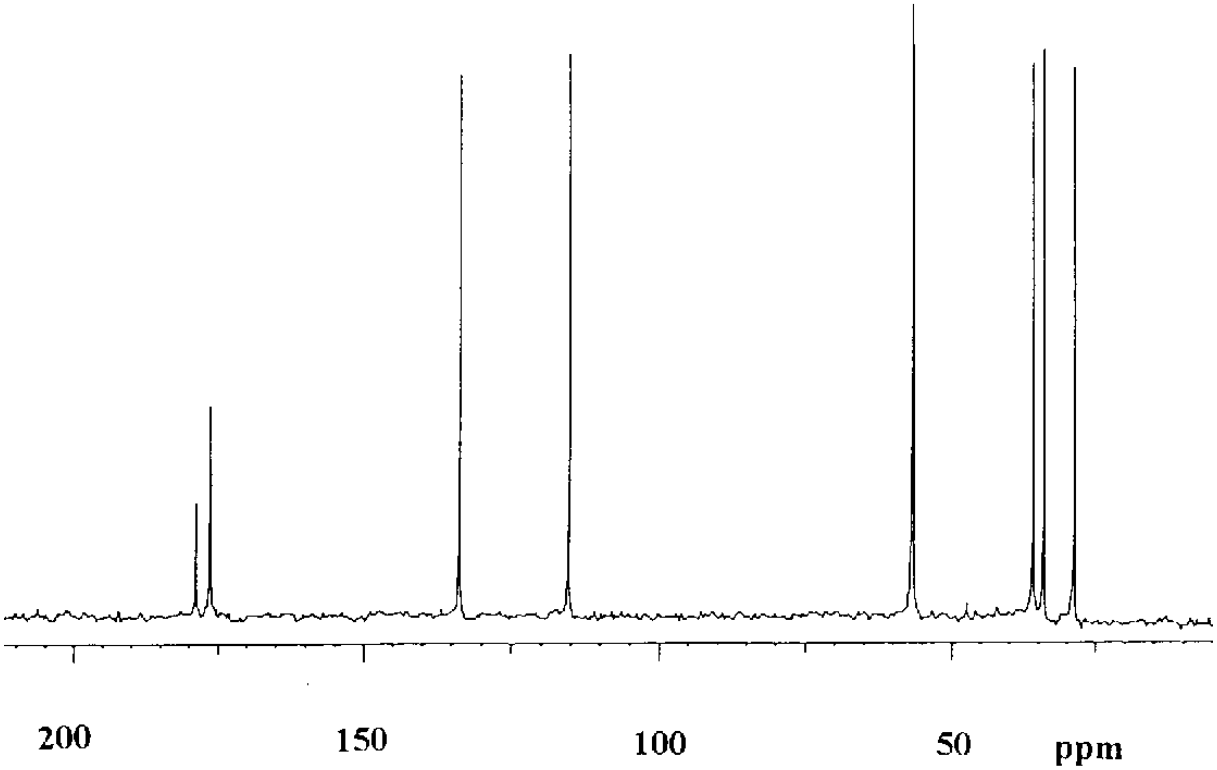


Table 7 Comparison of ^{13}C NMR data for S-ethenyl cysteine (Thumfort *et al.*, 1993) and its γ-glutamyl derivative

γ-glutamyl-S-ethenyl cysteine		S-ethenyl cysteine (Thumfort *et al.*, 1993)	
δ 22.49 MHz D_2O	**Assignment**	**δ 125.8 MHz D_2O**	**Assignment**
29.09	Glu C-3		
34.45	Glu C-4		
36.35	Cys C-3	32.4	Cys C-3
56.99	Glu C-2 (Cys C-2)	53.8	Cys C-2
57.21	Glu C-2 (Cys C-2)		
115.72	Cys C-2'	115.0	Cys C-2'
134.14	Cys C-1'	130.1	Cys C-1'
176.63	Glu C-1		
176.83	Cys C-1	172.9	Cys C-1
179.21	Glu C-5		

Table 8 Comparison of ^{1}H NMR data for S-ethenyl cysteine (Thumfort *et al.*, 1993) and its γ-glutamyl derivative

S-ethenyl cysteine 500 MHz D_20			**γ-glutamyl-S-ethenyl cysteine 500 MHz D_20, t-BuOH std 1.245 ppm (**m: overlap)			
δ ppm	**Integration**	**Multiplicity (j Hz)**	**δ ppm**	**Integration**	**Multiplicity (j Hz)**	**Assignment**
			2.15	2 H	m	Glu H-3a,b
			2.49	2 H	m	Glu H-4a,b
3.16	1 H	dd 14.9, 7.9	3.01	1 H	dd 14.1, 7.9	Cys H-3b
3.34	1 H	dd 14.9, 4.1	3.29	1 H	dd 13.9, 4.6	Cys H-3a
			3.78	1 H	t 6.0	Glu H-2
3.93	1 H	dd 7.9, 4.1	4.44	1 H	dd 7.9, 4.6	Cys H-2
5.36	1 H	br. d 16.8	5.28	1 H	d 16.9	Cys H-2'a
5.36	1 H	br. d 10.0	5.29	1 H	d 10.0	Cys H-2''b
6.39	1 H	dd 16.8, 10.0	6.43	1 H	dd 16.9, 9.8	Cys H-1'

Table 9 Comparison of glutamic acid ^{1}H NMR chemical shift data from the literature with those obtained for γ-glutamyl-S-ethenyl cysteine

Compound	**pH**	**Glu 2H**	**Glu 3Ha**	**Glu 3Hb**	**Glu 4Ha**	**Glu 4 Hb**	**Ref.**
γ-glutamyl isoxazolinone	6.9	3.82	2.18	2.17	2.53	2.53	Lambein *et al*. (1992b)
γ-glutamyl-S-methyl cysteine	?	3.76t	2.2	2.17	2.53m	2.53m	Kasai *et al*.. (1986)
γ-glutamyl-S-ethenyl cysteine	6.85	3.78t	2.15m	2.15m	2.49m	2.49m	

Discussion

The bioassay experiments have shown that the anti-feedant activity in the seeds of *V. narbonensis* is extractable with 30 % aqueous ethanol, of low molecular weight (< 10.000 daltons), cationic, heat stabile, acid labile, present in the cotyledon/embryo fraction and that it is reduced during germination. The biological activity correlated with the presence of a UV absorbing, ninhydrin positive, Ehrlich positive, acidic peptide.

Acid hydrolysis was shown to be effective in improving the palatability of the grain, thus establishing that value-adding can be achieved by simple treatments. This approach to improving the palatability could be diversified, and effective low-technology methods using lactic acid fermentation hold great promise in this respect. This is clearly an area which requires the attention of food technologists.

The Narbon Bean Dipeptide: γ-Glutamyl-S-Ethenyl Cysteine

The major ninhydrin positive component of the biologically active antifeedant fraction obtained by anion exchange chromatography, was crystallised from Propan-1-ol/ water to give a white microcrystalline solid with an elemental composition corresponding to $C_{10}H_{16}N_2O_5S$. The corresponding molar mass integer of 276 was confirmed by fast atom bombardment mass spectrometry in positive and negative modes, to yield strong M+H and M-H ions of integer masses of 277 and 275, respectively.

The product was homogeneous using paper electrophoresis at pH 1.7 ($M_{O.G.}$ = - 0.43), at pH 5.0 ($M_{O.G.}$ = 0.74) and by paper chromatography (Rf 0.22 in Butan-1-ol: HAc^-: H_2O, 12:3:5 v/v/v).

The UV spectrum (Fig 14) showed a distinct peak at 222 nm (ε=5551) and a shoulder at 234 nm characteristic for Vinyl-thio-ethers (Price and Zomlefer, 1950) and was consistent with S-ethenyl cysteine λ_{max}: 223 nm (ε =5279) (Däbritz and Virtanen, 1965).

Acid hydrolysis (1M HCl/ 110°C/6h) yielded cysteine (identified by performic oxidation to cysteic acid) and glutamic acid as the only two ninhydrin positive components detectable by paper electrophoresis at pH 1.7. Detection of these two amino acids, as well as the presence of the Vinyl-thio-ether chromophore and the elemental composition, were sufficient to define the compound as the dipeptide of glutamic acid and S-ethenyl cysteine.

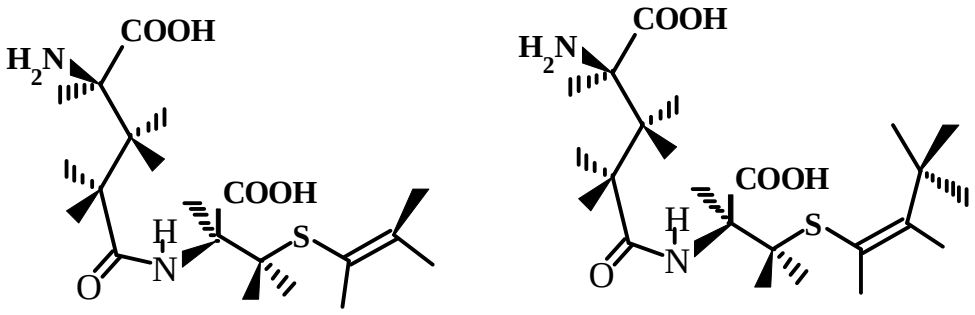
This inference was confirmed by the presence of 10 carbons in the ^{13}C NMR (22.49 MHz, Fig 16 and Table 7). Table 7 compares the ^{13}C signals for the narbon dipeptide with the literature values for S-ethenyl cysteine (Thumfort *et al.*, 1993). There appears to be a relatively constant downfield displacement of 3-4 ppm for the corresponding γ-glutamyl signals when compared to those reported by Thumfort and colleagues. Whether or not this is due to different instrumentation or conditions has not been ascertained.

The most convincing evidence for the proposed structure comes from the ^1H NMR (Fig 15, table 8) and the tabulated comparison data for S-ethenyl cysteine (Thumfort *et al.*, 1993). Table 8 shows the characteristic γ-glutamyl downfield shift (Kasai and Sakamura, 1973) of the cysteine 2-H in S-ethenyl cysteine (δ 3.93 ppm) to δ 4.44 ppm for the corresponding 2-H in the dipeptide from *V. narbonensis*. Table 9 compares literature values of chemical shifts for the γ-glutamyl moiety and these show good agreement. In addition, the 1645 cm^{-1} band in the IR spectrum supports the assigned γ-glutamyl structure (Kasai and Larsen, 1980).

At this stage, the exact stereochemistry of the cysteine and glutamic acid moieties remains to be established, but the abundant natural occurrence of γ-glutamyl dipeptides with both amino acids having the L-configuration makes this the most probable outcome.

The proposed structure for γ-glutamyl S-ethenyl cysteine (GEC) is presented in Fig. 17 together with its higher homologue, γ-glutamyl S-prop-1-en-yl isolated from the seeds of chives (*Allium schoenoprasum*) (Mattikala and Virtanen, 1962).

Fig 17. Chemical formulae for γ-glutamyl-S-ethenyl cysteine and the structurally related flavour precursor from the seeds of chives (*Allium schoenoprasum*)



γ-glutamyl-S-ethenyl-cysteine
(*Vicia narbonensis*)

γ-glutamyl-S-prop-1-en-yl-cysteine
(*Allium schoenoprasum*)

Irradiation with UV light (254 nm) releases a sulfurous odour from paper spotted with GEC after running by HVPE (pH 1.7 formic/acetic). A similar reaction was observed with chive (*Allium schoenoprasum*) seed extracts which released a typical chive flavour upon irradiation. Both GEC and the major ninhydrin and UV (254 nm) positive compound in 30% aqueous ethanol chive seed extracts had similar mobilities at pH 1.7.

It is also noteworthy that the glutamyl moiety is rapidly lost from the dipeptide under acid conditions and could not be isolated from cation exchange resins in the acid form. Even crude 30% ethanol extracts were observed to gradually degrade to produce a second ninhydrin positive, UV absorbing, Ehrlich positive component devoid of glutamic acid. Its paper electrophoretic mobility relative to Orange G was at pH 1.7 ($M_{O.G.}$ = - 0.54), at pH 5.0 ($M_{O.G.}$ = 0.0) and by paper chromatography (Rf 0.32 in Butan-1-ol: HAc^-: H_2O, 12:3:5 v/v/v). It yielded a red colour with Ehrlich's reagent. We now interpret this incompletely characterised artefact to be S-ethenyl cysteine (SEC) previously isolated from the parasitic plant *Olax phyllanthi* by Thumfort *et al.* (1993). Bell and Tirimanna (1965) also reported the presence of a ninhydrin positive species (VA_1) in extracts from *V. narbonensis* seed meals, the electrophoretic properties of which are consistent with S-ethenyl cysteine. The data in Table 5 give an indication of the extent to which hydrolysis of the peptide into its constituent amino acids has occurred in some of the tested feed samples and during the analysis process.

A similar artefactual situation has been observed in these laboratories (I. Delaere, pers. comm.) for 30% ethanol extracts of *V. sativa*, which gradually hydrolyse to form β-cyanoalanine on standing. Diffuse Reflectance Infrared Analysis of the unextracted grain shows a strong signal for the γ-glutamyl nitrile signal at 2251 cm^{-1} but no evidence for the presence of the underivatised β-cyanoalanine nitrile CN stretch signal at 2270 cm^{-1}.

From these observations it seems that particular care must be taken when isolating secondary metabolites to confirm that the isolated product actually is present in the original material and not simply an isolation artefact. In this regard the newly developed and rapid capillary zone electrophoresis technique is preferable to HPLC for sample analysis, however close attention needs to be given to extraction conditions in order to avoid artefacts. Using this method and a reference sample of γ-glutamyl-S-ethenyl cysteine, the content of the narbon bean line ATC 60105 has been found to be 1.4 % DW, which makes this γ-glutamyl dipeptide antifeedant agent for pigs comparable in concentration to the levels γ-glutamyl-β-cyanoalanine, which is present at levels in excess of 1% DW in some cultivars of *V. sativa*.

Quantitative analysis by Capillary Zone Electrophoresis for S-ethenyl cysteine and γ-glutamyl-S-ethenyl cysteine content of the feed samples tested by bioassay (Table 5) showed a linear relationship (Fig 13, Table 6) between feed intake reduction (Table 3) and total S-ethenyl cysteine content. It can therefore be concluded that the isolated dipeptide is the major antifeedant factor present in the seeds of *V. narbonensis*.

Narbon bean toxicity

In Table 4 it is shown that the antifeedant response of the pigs to dietary inclusion of *V. narbonensis* is apparent after the first meal, while that of canavanine is only discernible after the second meal. This suggests that the unpalatability of *V. narbonensis* seeds may be due to an off-flavour principle. However, such an interpretation should be treated with caution.

The study by Davies (1987) which involved the feeding of pigs on a diet containing 35% *V. narbonensis* grain

for a period of two weeks documented the potential harmful effects of this grain. Feed intake declined below maintenance levels so that the trial had to be terminated. Blood analysis of these pigs revealed high levels of bilirubin. The levels of γ–glutamyl-transferase, aspartate transferase were within normal limits, and no significant difference to blood samples taken from the *V. faba* fed controls were found, although a negative trend for these two enzymes was apparent in the narbon fed animals. Upon histo-pathological examination, crystals (? oxalate-type) were found in the kidneys. Symptoms of a mild interstitial nephritis were reported, intestinal villi were smaller than normal, other tissues showed no apparent lesions. The raised bilirubin levels suggest that some blood breakdown had occurred following the prolonged ingestion of *V. narbonensis* meal. It is remarkable that the consumption *V. narbonensis* seeds[40] also affects red blood cells, as is well documented for *V. faba*. The mechanism for red blood cell damage may not require a genetic deficiency for glucose-6-phosphate dehydrogenase as a predisposing factor in this instance.

In this context it is noteworthy that several cases of haemolysis in dogs, cattle, sheep due to the ingestion of *Allium* species have been reported (Fenwick and Hanley, 1985; and references therein). Dimethylsulphide has been shown to produce similar hemolytic effects in dogs (Gruhuzit, 1931b) to those caused by onions (Gruhuzit, 1931a). Disulfides have also been shown to affect glucose catabolism in red blood cells, thus diminishing NADPH for glutathione reduction.

The observed symptoms following the prolonged and diminishing ingestion of *V. narbonensis* may, in part, be related to the effects of starvation, however, since there is no information available about the metabolic fate of S-ethenyl cysteine in mono gastric or ruminant animals, the potential toxic effects of *V. narbonensis* ingestion should not be ignored in future studies.

With respect to the observed kidney crystals (Davies 1987) it is interesting that Däbritz and Virtanen (1964, 1965) obtained the higher homologue (cys-S-CH_2-CH_2-S-cys) of djenkolic acid as a major by-product during their synthesis of the cyclised form of S-ethenyl cysteine from cysteine and dibromoethane. Djenkolic acid was first isolated from the Djenkol bean (*Pithecolobium lobatum*) and is known to produce kidney damage due to the deposition of its crystals in the tissues of the kidney following prolonged ingestion of Djenkol beans (Van Veen and Hyman, 1935), which are an esteemed delicacy in Jawa (Ochse, 1931). The observation of crystals in the pig kidney after the feeding on a 35% *V. narbonensis* diet suggests that a metabolite of GEC may be responsible. It is also worth asking whether chive seeds (*Allium schoenoprasum*) which contain an analogue of GEC (Mattikala and Virtanen, 1962) can produce similar effects on feed intake and erythrocytes if fed at 35% in porcine diets. Due to the remarkable chemical similarities, further studies on GEC chemistry and biology are likely to benefit from previous and current work on *Allium* spp. (for excellent reviews see Fenwick and Hanley, 1985; Block, 1992).

The Greeke Beane

In the pre-Linnaean botanical literature *V. narbonensis* is sometimes called *Faba graecorum* or Greek bean (chapter 8) in the belief that this species was the bean referred to by Dioscorides who listed a number of medicinal properties for this plant. The biological properties of GEC distinguish *V. narbonensis* from *V. faba*, thus it should be possible to check Dioscorides' claims and identify the species he actually referred to. The property of a poultice prepared from *faba graecorum* to reduce hair growth may provide a useful bioassay for such a study. However, it should also be noted that H_2S can be generated from a variety of grain legumes during anaerobic fermentation (Mirande, 1921).

γ-Glutamyl-S-ethenyl cysteine and wool growth

The results from the sheep feeding study by Allden and Geytenbeek (1980) (chapter 1) demonstrated that sheep feeding on *Vicia narbonensis* produced less wool than controls while weight gain remained normal. Since sulfur amino acids are important for wool growth, the discovery of the sulfur amino acid analogue, γ-glutamyl-S-ethenyl cysteine provides an new lead for the elucidation of the biochemical basis of this problem.

Grain legume sulfur amino acid content

The palatability of the three lines of *V. narbonensis* and their content of GEC rank negatively with the total seed sulfur content (Fig. 11). Fig 12 shows that the total seed sulfur content of *V. benghalensis* (0.37 %) and *V. narbonensis* (0.28 - 0.37 %) is much higher than that of the other species tested eg. *V. sativa* (0.17 - 0.16 %) , *V. villosa* (0.2 %) and *Pisum sativum*(0.18 %). Comparative total S values were measured by (Bhatty *et al.*, 1977) in different cultivars of *V. faba* (0.21-0.25 %), *Lens culinaris* (0.21-0.30%) and *Pisum sativum* (0.15-

[40] See Chapter 2 for vicine levels

0.24 %). Thus, *V. narbonensis* is clearly a grain legume with a high sulfur content due to its GEC (S: 11.6%) content.

The large differences in total S content between individual lines of *V. narbonensis* suggest that there is genetic variation for this trait. However, the work of Hanelt *et al.* (1978) on the levels of sulfur amino acids in different accessions of *V. faba* has demonstrated that these are also strongly influenced by environmental factors, making a clear distinction between genetic and environmental effects difficult to separate. The effects of different S, and possibly Se, levels on GEC and the biosynthesis of its Selenium analogue warrant further study.

GEC as a possible storage compound for reduced sulfur

The emission of volatile sulfur compounds from plants, especially if these are exposed to excess sulfate, is a well recognised phenomenon but poorly understood in terms of its biological relevance and significance (Rennenberg, 1991). Since the total sulfur concentration in the seeds of *V. narbonensis* is relatively high, compared to *V. faba*, this compound may serve as a storage for reduced sulfur, preventing the uncontrolled emission of volatile sulfur compounds from the plant. If GEC is a sulfur storage compound, then the enzymes or catalytic conditions for its remobilisation must also be present in the plant at some phenological stage. The metabolic conversion of GEC into γ–glutamyl cysteine or glutathione would be required if it is to be utilised as a source of cysteine. This raises the interesting possibility for the existence of an enzyme which converts S-ethenyl cysteine to cysteine and a two carbon unit such as ethylene.

GEC a possible clue to the *V. faba* progenitor

The seeds of *V. faba* contain glutathione (Klapheck, 1988) , and the total S content is approximately half that to be found in the seeds of *V. narbonensis*. Since *V. faba* (2 n=12) is closely related to members of section *Narbonensis* (2n=14) (Maxted *et al.*, 1991), a genetic modification of the biosynthetic pathway leading away from the production of the S-ethenyl moiety must have accompanied speciation in *V. faba*. It is tempting to speculate that the gene responsible for GEC biosynthesis was located on the chromosome arm which is lacking in *V. faba*. In fact, it may have been the lack of this off-flavour which led to a preference for *V. faba* since it can easily be selected for by gustatory analysis[41].

Conclusion

The unpalatability of *V. narbonensis* seed to pigs is due to the presence of up to 1.4 % DW of a high sulfur content (11.7 %) dipeptide, γ-glutamyl-S-ethenyl cysteine (GEC). Characterisation of its chemical and biological properties has provided a sound basis for its quantitation and hence for the further development of *V. narbonensis* as a useful grain legume for animal and possible human consumption.

[41] "The fruit... ...when it is chewed it filleth the mouth full of stinking matter". Dodoens, R.(1583) A NEW HERBALL OR HISTORIE OF PLANTS transl. by Henrie Lyte, London 1595, Edm. Bollifort [Rothamstedt library]

Chapter 7

Evaluation of processed *Vicia sativa* grain by poultry bioassay

Introduction

The recent sale of *V. sativa* for human consumption as a cheap substitute for the red lentil (*Lens culinaris* Med.) (Tate and Enneking, 1992) and the associated controversy (McCallum, 1992; Brown, 1992; Bhat and Raghuram, 1993; Putnam, 1993) has provided the background to this chapter. The crucial question with regard to the safety of *V. sativa* for human consumption and its use as a cheap substitute for red lentils was, whether red lentil cooking methods provide effective detoxification. The presence of the nonprotein amino acids β-cyanoalanine and γ-glutamyl-β-cyanoalanine are likely to pose a potential health threat to the consumer (Tate and Enneking, 1992).

Poultry were chosen as the bioassay for this study because previous work (Harper and Arscott, 1962; Ressler, 1962; Arscott and Harper, 1963, Arscott and Harper, 1964, Ressler *et al.*, 1969) had shown their sensitivity to either the dietary inclusion of *V. sativa*, β-cyanoalanine or γ-glutamyl-β-cyanoalanine, and because recent studies had demonstrated the unpalatability of low levels of Blanche Fleur vetch to this species (Glatz *et al.*, 1992; Glatz and Hughes, 1993).

To assess methods for the preparation of red lentil dhal, a variety of culinary texts dealing with Middle-Eastern cuisine were consulted.

Lentil recipes, survey of culinary texts and nutrition literature

The major use of red lentils (*Lens culinaris* Med.) is for the preparation of red lentil soup or red lentil dhal, which varies in consistency from a thick soup to a slurry, depending on consumer preference. Bailey (1990) illustrated the various types of lentils used in culinary practice, some retaining their shape during cooking, others, such as red and yellow lentil disintegrating easily. Split red lentil is a staple food throughout the Middle East and quickly cooks to a purée . The lentil is the only food that may not need soaking before cooking (Elliot, 1979; Grigson, 1980; Roden, 1987, Bailey, 1990).

Brennan (1984) advised that lentils intended for use in lentil soup should be washed, picked over, cleaned from foreign matter and well drained. For pulses a general requirement for picking over and thorough washing before cooking was indicated; the individual cooking and soaking times varying according to the type of legume to be prepared.

It is clear from the available lentil soup recipes in the culinary literature that no attention is given to the removal of water soluble anti-nutritional factors from the lentil grain by extensive leaching prior to consumption. The perceived purpose for soaking pulses is to make them softer and hence faster to cook, while rinsing is done thoroughly only with those which are notorious for causing flatulence (Elliot, 1979).

Detoxification by leaching

Thermostable non-protein amino acids, such as β-cyanoalanine and γ-glutamyl-β-cyanoalanine are water soluble. Extensive soaking, or cooking in a large volume of water which is then discarded would minimise the ingestion of these toxins. However, such practice inevitably also removes water soluble vitamins and minerals (Tate and Enneking, 1992).

Detoxification by acid hydrolysis

The structures and knowledge gained during the study of the feed-inhibitory activity of anti-nutritional factors from V. *villosa* ssp. *dasycarpa* (Enneking *et al.*, 1993) and *V. narbonensis* seeds (Chapter 5) suggested that toxins, such as ß-Cyanoalanine and γ-glutamyl-S-ethenyl-cysteine could be detoxified by *in situ* acid hydrolysis. Acetic acid (4%) of the same concentration as vinegar was chosen for the acid detoxification treatment for split red vetch because it is readily available and an accepted food ingredient.

Materials and Methods

The aim of the poultry experiment was to simulate as closely as practicable the food preparation method used for the cooking of lentil dhal. Cooked and uncooked red split lentils (*Lens culinaris* Med.) and red split vetch (*V. sativa* L. cv. Blanche Fleur) as well as a control diet were fed to laying hens, in order to assess its effectiveness for detoxification of the vetch. In addition an acid hydrolysis treatment was included to serve 1) as a negative control for the toxicity of β-cyanoalanine and γ-glutamyl-ß-cyanoalanine and 2) to demonstrate the feasibility and ease of value-adding to the vetch grain by using an acid-catalysed detoxification process. Split red vetch was kindly donated by Pea and Grain exporters, Two Wells, South Australia and split red lentils were purchased from a commercial trader (produced in Turkey). The feeding experiment was carried out with the help of Dr. Phil Glatz, South Australian Research and Development Institute, Parafield Poultry Research Centre, South Australia and his staff who are hereby gratefully acknowledged.

Design of the poultry experiment

Six experimental diets were fed at a 10% dietary inclusion level from 26-38 weeks to laying hens (Tegel Super Blacks) housed in single-bird cages in a naturally ventilated shed, with a 16 : 8 hr light : dark program .

The following treatments were tested: 1. normal wheat based diet (-ve control); 2. 10 % split red vetch (+ve control); 3. 10 % split red lentil (-ve control for the uncooked split red vetch); 4. 10% split red vetch cooked as dhal ; 5. 10% split red lentil cooked as dhal (-ve control for the vetch dhal); 6. 10% acid hydrolysed (cooked in 4% vinegar) split red vetch (-ve control for ß-cyanoalanine and γ-glutamyl-ß-cyanoalanine).

The experiment was laid out as a randomised block design with 36 replicates, and commenced when the birds were 18 weeks of age. An eight week pre-treatment phase was used to establish stable conditions. The birds were fed with the experimental diets from 26-38 weeks of age. A post-treatment phase from 26-38 weeks of age followed. For each bird feed intake (every 4 weeks), weight gain (every 4 weeks), egg production (daily), egg weight (3 days/week/each bird) were recorded.

Statistical Analysis

The data for each of the experimental phases was considered separately, and analysis of variance (ANOVA) was used to test for the effects of time, diet and their interaction. Individual analyses were also carried out at each time to assess the effects of the different diets. Missing values were estimated. LSDs are presented with the results.

Preliminary experiments to determine treatment preparation conditions

The quantities of grain required for this experiment (ca. 40 kg/treatment) necessitated the use of larger equipment than used for previous experiments. Dry feed formulation necessitated that all dhal preparations were dried subsequent to the cooking process. Small scale experiments using 10 g samples of split red vetch were employed to determine the volume of moisture required to obtain a thick dhal, and to hydrolyse the non-protein amino acid toxins. It was found that a minimum volume of 3 parts acid solution to 1 part split red vetch were required to obtain effective hydrolysis after 30 mins autoclaving of test samples which were then dried overnight at 110° C/14 hrs, prior to analysis by HVPE. Likewise, a similar ratio 2.5 - 3 : 1 v/w was found to be necessary for the dhal preparation, since otherwise samples were not thoroughly wet. However, during the large scale preparation a ratio of 2 : 1 v/w was found to suffice for the preparation of a thick dhal.

Test diet preparations

Both dhal preparations (treatments 4 and 5) were carried out using a 200L steel drum, filled with 70 L of tap water, to which 40 kg of grain were added. A steam generator was used to heat the mixture to boiling point, which took 35 mins. The dhal was cooked until the cotyledons could be easily squashed between thumb and index finger (55 min for the lentil, 65 min for the vetch).

Acid hydrolysis in 4% acetic acid (distilled water) was carried out in an autoclave (121° C, 30 mins) in stainless steel open containers (water baths), using a ratio of 1:3 (w/v) red split vetch to acetic acid solution, since the conditions for effective hydrolysis had been determined by autoclave. The resulting slurries were dried in a forced draft oven in open galvanised trays (@75°C/72 hrs) and ground.

Table 1. Diet composition (% w/w)

Ingredient	Control[42]	Test diets
Wheat	65.5	64
Meat meal	11.2	10
Peas	9.5	-
Soya meal	4.5	6.2
Test meal		10
Sunflower oil	1.15	1.32
Methionine	0.1	0.1
Marble	7.7	7.7
Dicalcium phosphate	-	0.3
Sodium bicarbonate	0.05	0.07
Sodium chloride	-	0.01
Premix[43]	0.2	0.2
Yolk colorant	0.1	0.1

Amino acid analysis

Quantitative HPLC amino acid analysis for β-cyanoalanine and γ-glutamyl-β-cyanoalanine was carried out by courtesy of the Academy of Grain Technology, Werribee, Victoria on samples of the raw, cooked and hydrolysed red vetch.

Diffuse Reflectance Infrared Fourier Transform (DRIFT) analysis for quantitation of -CN stretching

The three red vetch samples and the raw lentil were also qualitatively examined for their -CN stretching by the Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectrometer at the CSIRO Div. Soils, Glen Osmond, South Australia and the help of Mr. Les Janek is gratefully acknowledged.

[42] Analysis (Control diet): Fat: 4.24; Fibre: 2.83; Ca: 3.65; total P: 0.74; available P: 0.55; Methionine: 0.34; Lysine: 0.8; Protein; 17.8; Na%: 0.15; Cl%: 0.15; ME: 11.42

[43] Premix: 1 part Vitamin Premix (1) and 1 part mineral Premix (2)

1. Vital 400 Vitamin Premix kg^{-1} (Rhône-Poulenc): Retinol (minimum Vit A 0.0 M.I.U.): 2.4 g; Cholecalciferol (min Vit D_3 2.0 M.I.U.): 50 mg; DL-α-tocopherol acetate (Vit E 10.000 I.U.): 10 g; menadione di-methyl pyrimidinole bisulphite (Vit K_3): 2 g; thiamine.HCl (Vit B_1): 1.2 g; riboflavine (minimum Vit B_2): 6.5 g; pyridoxine.HCl (Vit B_6): 1.2 g; cyanocobalmin (Vit B_{12}): 10 mg; Calcium pantothenate: 9 g; nicotinic acid: 15 g; biotin: 20 mg; folic acid: 500 mg; max F: 0.01%
2. Mineral Premix kg^{-1}(Rhône-Poulenc): Choline chloride: 100g; Co: 200 mg; I: 500 mg; Fe^{2+} 30 g; Mn: 60 g; Zn: 50 g; Cu: 4; Mo: 500 mg; Se: 50 mg; max F: 0.01%

Results

Fig 1. HPLC analysis of treatment samples

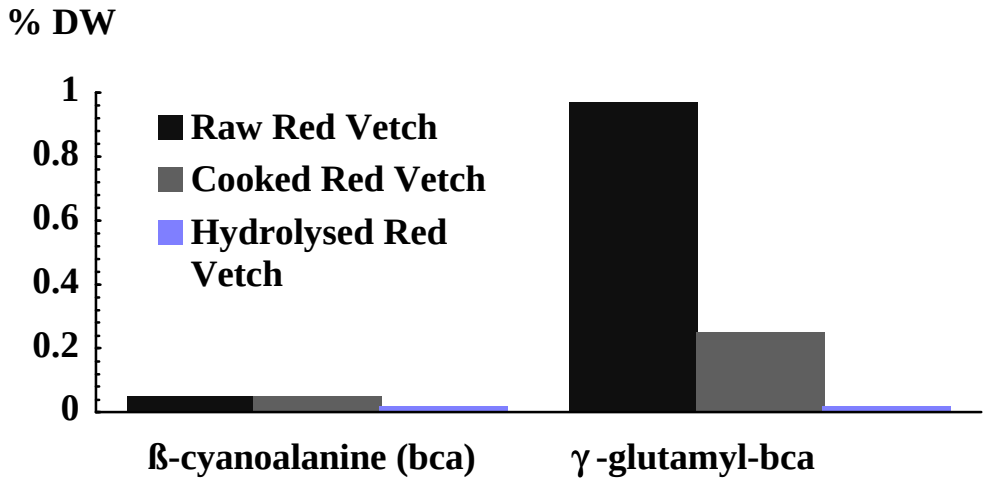


Fig 2. Determination of CN- stretch by DRIFT

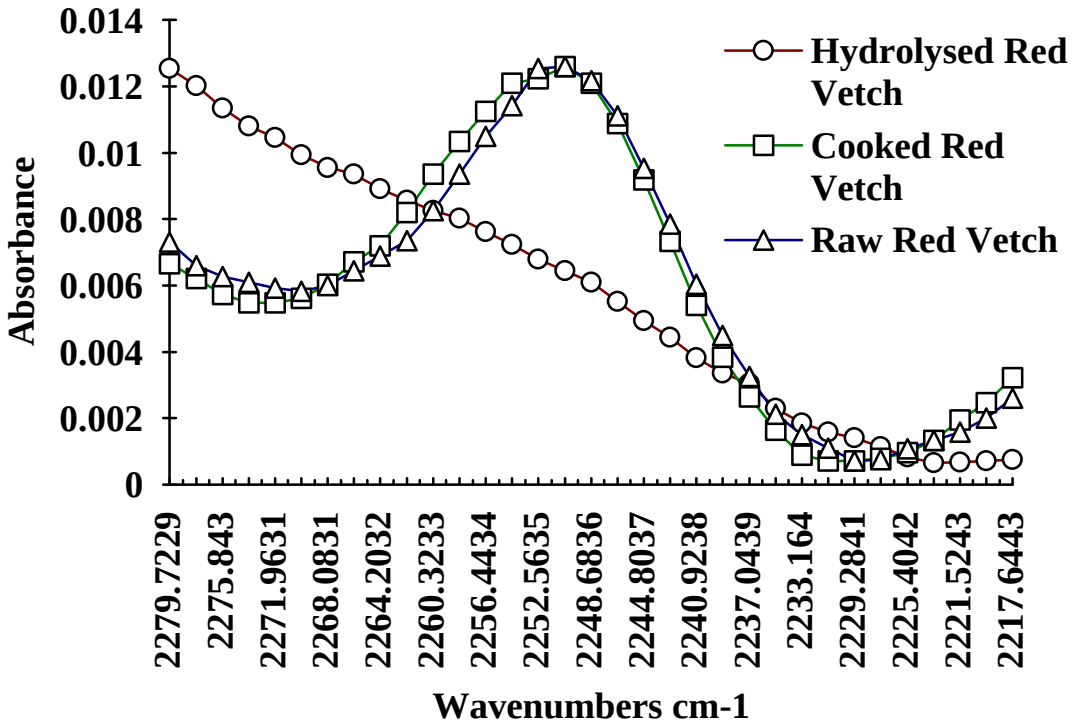


Fig. 2 shows the presence of a CN-stretch in both the raw and the cooked red vetch samples Note the shift of absorption in the CN peak of the cooked red vetch sample, the only detected change observed after cooking. The negligible CN stretch in the hydrolysed red vetch sample is in accord with the almost complete loss of γ-glutamyl-β-cyanoalanine in as indicated in Fig 1.. This figure also suggests that cooking removes 75% of this compound, whereas the CN stretch in Fig 2. shows no change in intensity and a small but detectable increase in the absorption maximum from 2251 to approx. 2253 cm^{-1}.

Table 2. Analysis of Variance (ANOVA) results[44]

AGE (WEEKS)	CONTROL	SPLIT RED VETCH COOKED IN 4% ACETIC ACID/30 MIN	SPLIT RED VETCH	SPLIT RED VETCH DHAL	RED LENTIL	RED LENTIL DHAL	F p	$LSD_{0.05}$
FEED INTAKE (kg/bird/4weeks)								
18	2.6817	2.6197	2.7667	2.6444	2.6461	2.7894	NS	
22	3.04	3.0033	3.0833	3.0517	2.9433	3.1606	NS	
26	3.2094A	3.3372A	3.1794A	3.2317A	3.2467A	3.5628B	tmt <0.001	0.169
30	3.55A	3.3647A	3.1344B	3.26B	3.2483B	3.5272A	tmt <0.001	0.196
34	3.5821A	3.4967A	3.2156B	3.2822B	3.2528B	3.6261A	tmt <0.001	0.162
38	3.5064	3.4089	3.5459	3.6206	3.4972	3.5961	NS	
42	3.8028	3.6578	3.8836	3.8489	3.7561	3.8567	NS	
46	3.6565	3.6111	3.6679	3.745	3.5917	3.7506	NS	
50	3.5828	3.4889	3.5273	3.6222	3.4933	3.6728	NS	
BODY WEIGHT (kg)								
18	1.9722	1.9567	2.005	1.9844	1.9661	1.9606	NS	
22	2.1889	2.1572	2.2072	2.165	2.1367	2.1939	NS	
26	2.225	2.1772	2.2322	2.2278	2.1861	2.2111	NS	
30	2.2883	2.2467	2.2606	2.2567	2.2522	2.3522	NS	
34	2.3974 A	2.3456 A	2.2644 B	2.3033 A	2.3239B	2.4683A	tmt <0.008	0.112
38	2.4562 A	2.425 A	2.2834 B	2.3433 A *	2.3294B	2.5422A	tmt <0.001	0.113
42	2.511	2.475	2.3982	2.4644	2.4394	2.5794	NS	
46	2.5585	2.5194	2.4712	2.5333	2.4889	2.6306	NS	
50	2.6008	2.6033	2.5183	2.6044	2.5317	2.6683	NS	
54	2.6545	2.6394	2.5948	2.64	2.5928	2.6933	NS	

* The difference between Control and Red Vetch Dhal is 0.1129 e.g. (B)

[44] Significant differences are indicated by differering letters. Treatments with the same letter do not differ significantly

Fig 3. Feed intake

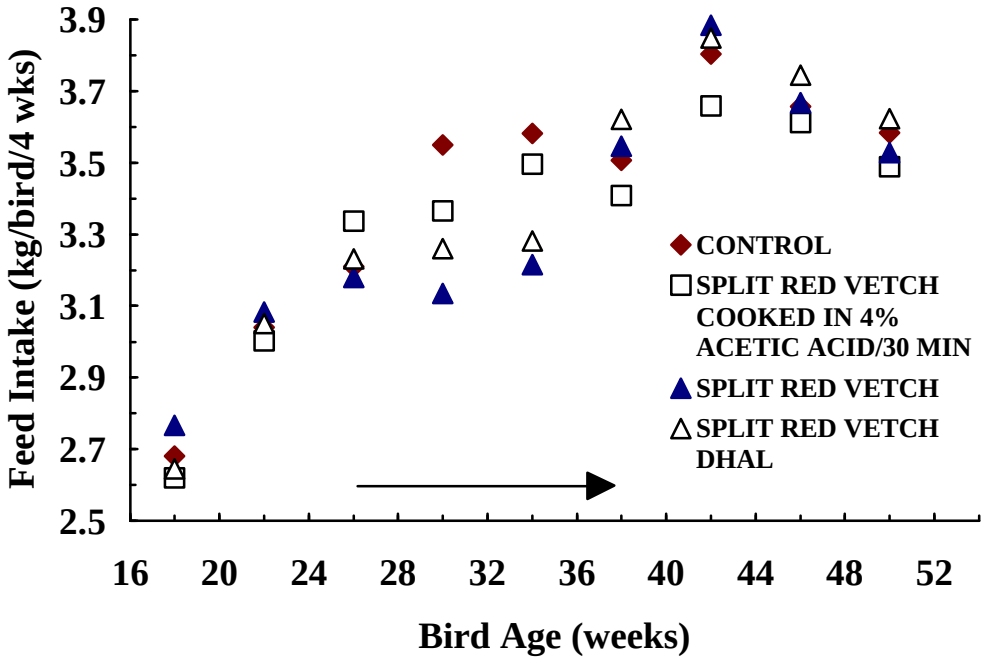
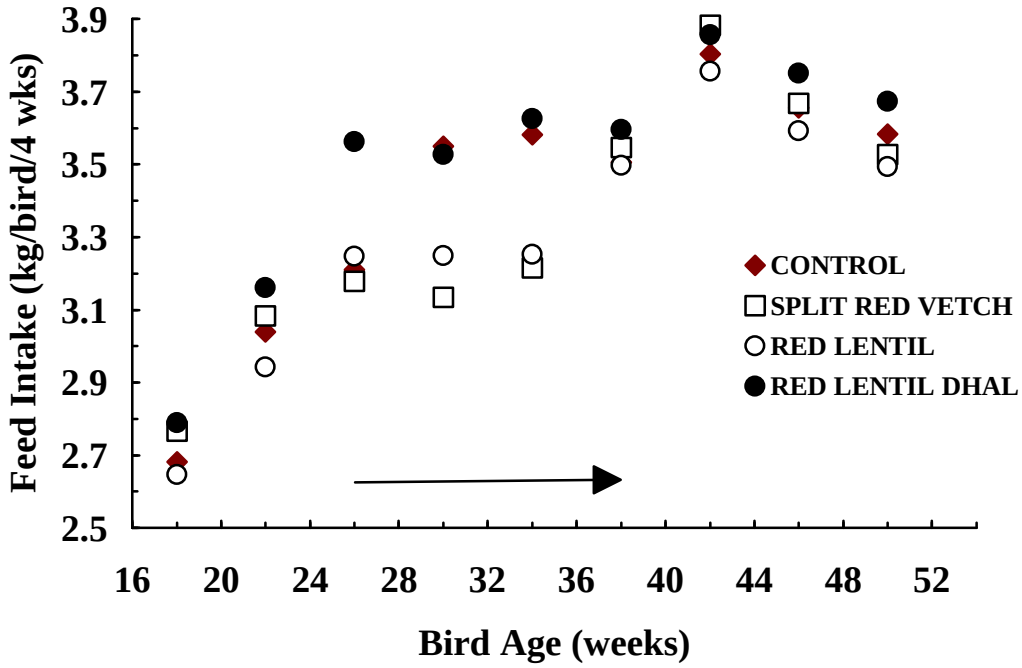


Fig 4. Feed intake



Figs 3 and 4 show the feed intake of the hens fed on different treatment diets. The treatment period is indicated by an arrow(week 26-38). The control diet was fed in the preceding and following periods. There were significant treatment differences ($p<0.001$) in feed intake for the periods 26-29 ($LSD_{0.05}$: 0.169), 30-33 ($LSD_{0.05}$: 0.196) and 34-37 ($LSD_{0.05}$: 0.162) weeks of age.

Fig 5. Body weight

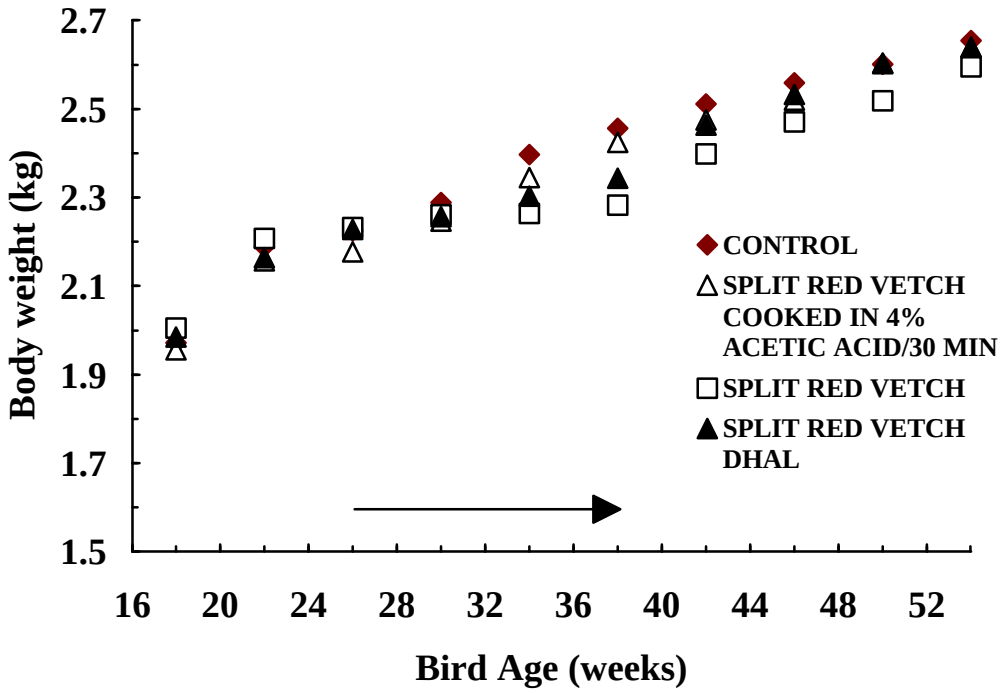
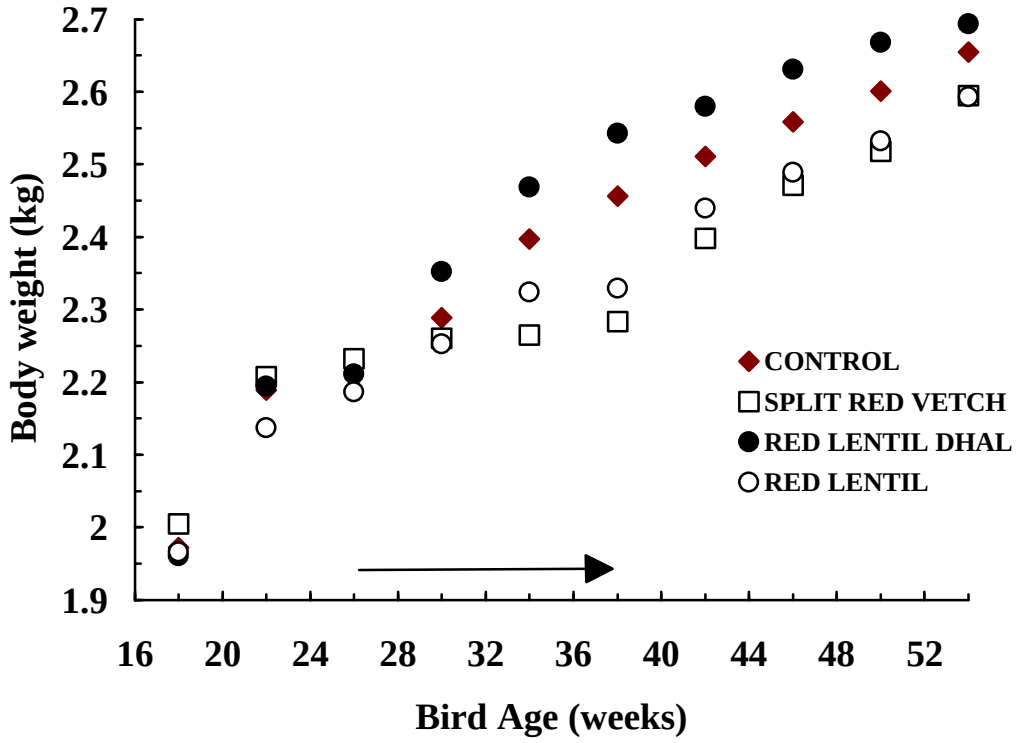


Fig 6. Body weight



Figs 5. and 6. show the effect of the treatment diets on weight gain. The treatment period is indicated by an arrow(week 26-38). The control diet was fed in the preceding and following periods. Significant differences were observed for ages 34 weeks ($p<0.008$, $LSD_{0.05}$: 0.112) and 38 weeks($p< 0.001$, $LSD_{0.05}$: 0.113).

Fig 7. Egg weights

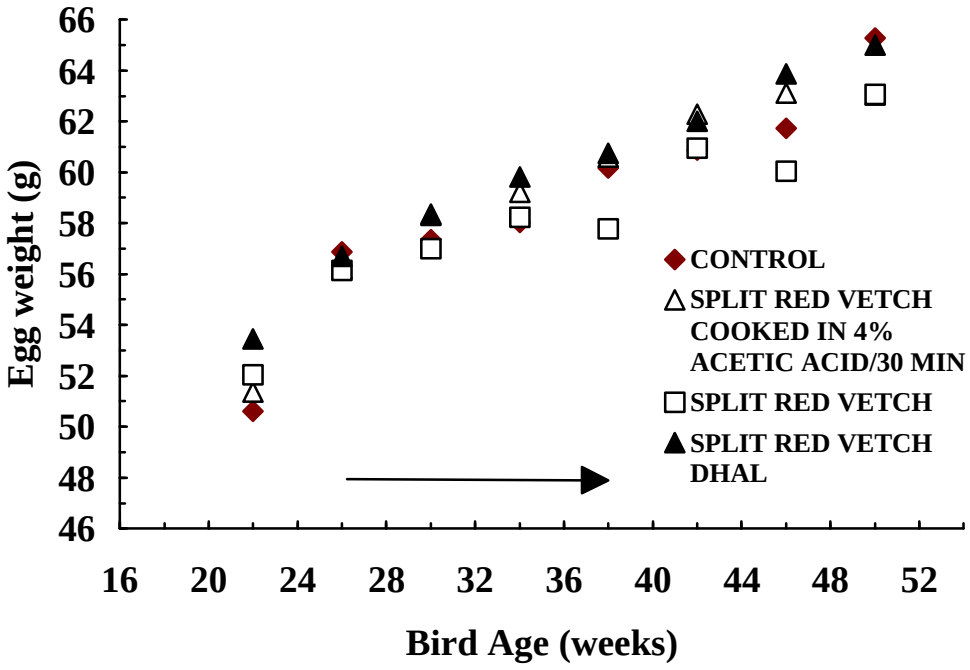
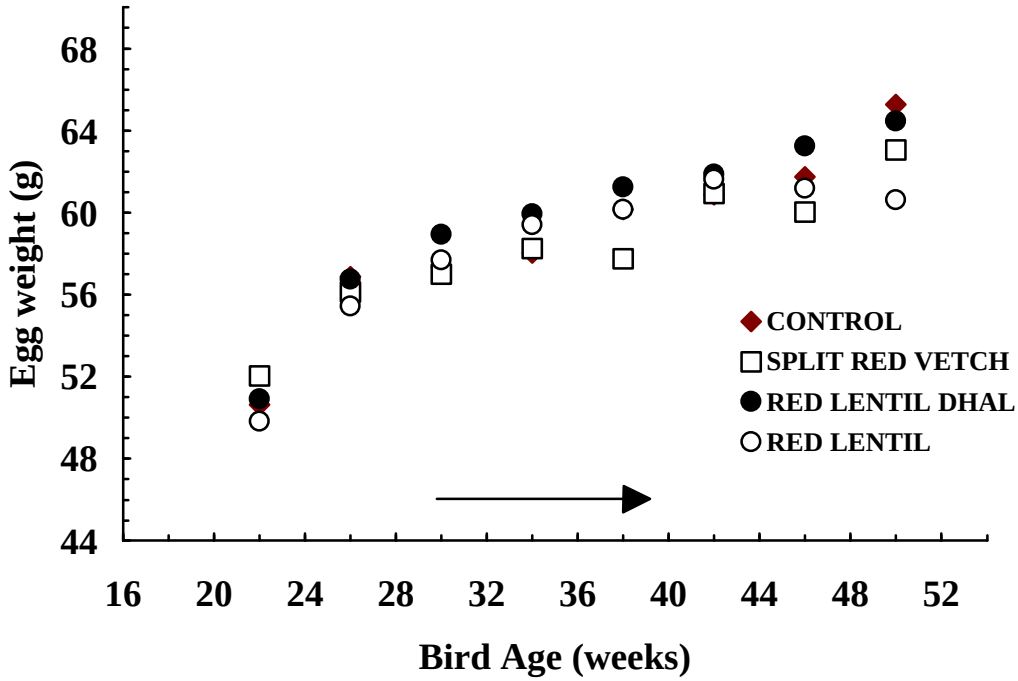


Fig 8. Egg weights



Figs 7. and 8. illustrate the changes in egg weights (total egg weight per bird / number of eggs per bird). The treatment period is indicated by an arrow (week 26-38). The control diet was fed in the preceding and following periods. None of the treatments were significantly different from the control. Please, note the consistently low egg weights of birds feeding on the raw split red vetch.

Discussion

Figs 3-5 show the negative effect of split red vetch on bird feed intake and growth, which is in accordance with earlier observations with the feeding of the same cultivar (Glatz *et al.*, 1992; Glatz and Hughes, 1993). The observed effect is not so pronounced as reported previously by Glatz *et al.* (1992) for whole seeds, which

suggests that the removal of the seed coats eliminated a significant portion of anti-nutritional factors (e.g. tannins).

Figs 3 and 5 summarise the negative effects of the cooked split red vetch (dhal) on feed intake and weight gain, while clearly showing the improvement in feed intake, and thus weight gain, which can be achieved through mild acid catalysed hydrolysis of the grain.

Heat treatment of split red vetch grain is not sufficient to improve bird feed intake sufficiently (Fig 3) and this is reflected in the weight gain at week 38 (Fig 5). The difference between this treatment and the control is just significant at the 0.05 level, while there is no significant difference between the effects of hydrolysed treatment and control on both feed intake and growth.

The interesting effect of the cooked lentil relating to improvement of body weight (Fig 6) is to some extent confounded by the high feed intake in week 26 for the birds randomly selected for this treatment (Table 2), however the results of the subsequent months clearly show that the birds were able to make significant gains in weight on this diet in stark contrast to those fed the raw red lentil and red vetch diets. The nutritional value of lentils can be dramatically improved by heat treatment. The same treatment reasonably improves red vetch but acid catalysed hydrolysis of heat stable factors is required to improve its palatability to poultry.

Since palatability is a good general indicator for anti-nutritional factors (Lepkovsky, 1948), the improved feed intake of birds on the hydrolysed vetch treatment can be ascribed to a loss of toxicity. This is further corroborated by the results from the DRIFT analysis (Fig 1) which show that the CN stretch, ascribable to β-cyanoalanine and γ-glutamyl-β-cyanoalanine, is negligible in the hydrolysed vetch sample, while remaining almost unchanged in the cooked material. A loss of γ-glutamyl-β-cyanoalanine was indicated by HPLC amino acid analysis, thus suggesting the transformation of this compound into a different compound which retains some antifeedant activity. Since the DRIFT quantitation for the presence of the Cyano group in the cooked red vetch is in disagreement with the HPLC data for this sample, HPLC analysis alone for these compounds is not a reliable method for an assessment of the toxicity of the cooked red vetch. The DRIFT technique is suitable for screening of samples for the presence of CN-containing compounds.

The marginally negative effect of the raw red vetch diet was discernible in the egg weight data (Fig 6 and 7) but the treatment effects were not significant (Table 2).

Test diets for the hens used in this experiment were designed to obtain a measurable effect on feed intake while minimising any major detrimental effects on the birds, because previous experiments by Glatz and Hughes (1992) had already demonstrated that diets containing *V. sativa* cv. *Blanche Fleur* at levels of 20 % were unacceptable for poultry and that inclusions levels as low as 5% led to negative effects on feed intake with corresponding reductions in associated parameters such as weight gain and egg production. The results from the experiment were obtained at the lower level of sensitivity for the layer bioassay, and future experimentation with split red vetch are probably better carried out at levels of 15 or 20 % to obtain a better response to the treatment diets.

The aim of the experiment described in this chapter was to assess the toxicity of a typical dhal (lentil soup) prepared from the split and dehulled seeds of *V. sativa* cv. Blanche Fleur in order to obtain an estimate of the potential health hazards which are likely to be encountered if the world wide human consumption of this lentil substitute continues. The results show clearly that unlike red lentils, the anti-nutritional activity of red split vetch is not eliminated by cooking alone.

Evidence for the presence of a new toxin formed from γ-glutamyl-β-cyanoalanine has been obtained through combined DRIFT and HPLC amino acid analysis. DRIFT shows the presence of a CN-stretch in the cooked red vetch material, while this peak is absent in the hydrolysed vetch. HPLC analysis indicates that the presence of γ-glutamyl-β-cyanolanine is reduced by 75%, thus a new compound with a CN moiety must have been formed.

The second aim was to assess the practicality of detoxifying this material by mild acid hydrolysis because the chemical structure of β-cyanolanine and γ-glutamyl-β-cyanolanine suggested that this could be achieved with food grade acids, such as acetic acid. This principle was validated and acid hydrolysis shown to be effective for the inactivation of these toxins.

Conclusion

The formation of an unidentified nitrile containing antinutritional factor following the cooking of *V. sativa* grain has been established. The potential for post-harvest detoxification of *V. sativa* grain through mild acid hydrolysis has been demonstrated and could explored for the development of food and feed products from grain legumes with acid labile anti-nutritive factors.

Chapter 8

The economic botany of the Narbon bean (*Vicia narbonensis* L.)[45]

Introduction

The narbon bean (*V. narbonensis* L.) is a close relative of the faba bean (*V. faba* L.). Following the introduction of germplasm for this species to Australia with a view towards developing alternative grain legume cultivars for dryland agriculture, difficulties with the unpalatability of the grain to monogastric animals were identified as the major constraint to its commercial production (Georg, 1987a; Johnson and Eason, 1990). Being a minor Mediterranean grain and forage crop, little was known about *V. narbonensis* in Australia (Georg, 1987b). This chapter therefore reviews the available agricultural and botanical information in order to provide the basis for the further development of *V. narbonensis* for Mediterranean agriculture; parts of it have already been published (Enneking and Maxted, 1994). A compilation of agronomic data from Australian studies has recently been compiled by Castleman (1994).

Taxonomy

Table 1 provides the classification of *Vicia narbonensis* and its relatives. Taxonomically, *V. narbonensis* has a close relationship with the other large-seeded, robust vetches of *Vicia* section *Narbonensis* (Radzhi) Maxted, with which it forms the *V. narbonensis* complex (Schäfer, 1973; Maxted *et al.*, 1991). Within ser. *Narbonensis* the species are distinguished on the basis of flower colour, leaf and pod shape. The flowers of *V. narbonensis* and *V. serratifolia* are purple, while those of *V. johannis* have a white, sometimes purplish veined standard and red/purple (var. *johannis*) or brown/purple (var. *procumbens*) wing spots. *V. serratifolia* , as the name implies, has serrated leaves. Specimen with leaflet serrations of more than 15 teeth can be consistently distinguished as *V. serratifolia* (Khattab, 1988).

Table 1. *Vicia* L. subgenus *Vicia* section *Narbonensis* (Radzhi) (Maxted, 1992)

Sect. *Narbonensis* (Radzhi) Maxted
Ser. *Rhombocarpae* Maxted
V. eristalioides Maxted
Ser. *Narbonensis* (Radzhi) Maxted
V. kalakhensis Khattab, Maxted & Bisby
V. johannis Tamamschjan in Karyagin
var. *ecirrhosa* (Popov) H. Schäfer
var. *procumbens* H. Schäfer
var. *johannis*
V. galilaea Plitm. & Zoh. in Plitm.
var. *galilaea*
var. *faboidea* (Plitm. & Zoh. in Plitm.) H. Schäfer
V. serratifolia Jacq.
V. narbonensis L.
var. *salmonea* (Mout.) H. Schäfer
var. *jordanica* H. Schäfer
var. *affinis* Kornhuber ex Asch. & Schweinf.
var. *aegyptiaca* Kornhuber ex Asch. & Schweinf.
var. *narbonensis*
V. hyaeniscyamus Mout.

Botanical Varieties of *V. Narbonensis*

V. narbonensis has been classified into five botanical varieties (Schäfer, 1973) on the basis of seed size, hilum colour, presence of an attached funiculus, pod shape and leaf margin serrations (table 2). Traits of

[45] Part of this study was supported by a grant from the Alf Hannaford Bequest Fund, Waite Agricultural Research Institute, University of Adelaide, South Australia

domestication such as gigantism in seeds and leaflets and lack of seed dormancy are found with vars. *aegyptiaca* and *narbonensis*, indicating their anthropogenic origin.

Table 2. Key characters for identification of *V. narbonensis* L. varieties (Schäfer, 1973)

Variety	Characteristics
aegyptiaca Kcke	1-2-(-3) basal shoots; flowers 1-2-(-3); legume 5-7 x 1.1.-1.6 cm, rugose; seed 6-11(-13) mm, central strip of hilum white, funiculus persistent.
narbonensis L.	1-2-(-3) basal shoots; flowers 1-2-(-3); legume 5-7 x 1.1.-1.6 cm, smooth; seed 6-8 mm, central strip of hilum white, funiculus deciduous.
affinis Asch. & Schweinf.	2 basal shoots; flowers 1-2; legume 3.5-5.5 x 0.7-1.1 cm, smooth; seed 4.5-6.0 mm, central strip of hilum beige.
jordanica Schäf.	2-6 basal shoots, basal leaflet entire; flowers 1-2; legume 3.5-5.5 x 0.7.-1.1 cm, smooth; seed 4.5.-6.0 mm-1.1 cm, central strip of hilum beige.
salmonea Mout.	1-2 basal shoots; basal leaflet crenate; flowers 1-2; legume 3.5-5.5 x 0.7-1.1 cm, smooth; seed 4.5-6.0 mm, central strip of hilum beige

Genetic resources

The major active collection of germplasm for section *Narbonensis* is held at ICARDA, Aleppo, Syria, comprising ca 350 accessions (Robinson, pers. comm.). Australia now holds one of the largest collections of *V. narbonensis* totalling ca. 150 accessions. Clearly, the existing collections are relatively small compared to those for other crops and more collecting would be desirable.

Latin synonymy

Table 3 provides a list of Latin names adapted from Maxted (1991). The diversity of names reflects the taxonomic history of the genus, which was discussed by Maxted (1991), and it illustrates some of the complexity which is encountered in the search for meaningful biological information about this species and its relatives. The table is intended as an aid for further studies as it helps in the decoding of biological and agricultural information contained in the literature.

The Latin byname *narbonensis* was given after the city of Narbonne (in ancient times *Narbo Marcius*) in the south of France (Becker-Dillingen, 1926), from where Linnaeus obtained the material for his description. The Latin names such as var. *hortensis*, *genuina*, *culta*, *typic*a are synonymous with *V. narbonensis* vars. *aegyptiaca* and *narbonensis* and indicate their status as cultivated varieties.

Geographical Distribution of *V. narbonensis*

Schäfer (1973) and Maxted (1991) provided maps with the detailed geographical distributions of the individual varieties of *V. narbonensis*. From these data it appears that the centre of origin for the species was probably North-West Asia where the greatest genetic diversity can still be found and not in Southern Georgia (C.I.S.) as suggested by Zeven and de Wet (1988). The species seems to be sparsely distributed in many parts of the Western Mediterranean and North Africa where the large-seeded vars. *aegyptiaca* and *narbonensis* tend to predominate (Schäfer, 1973) and may reflect, as escapees, the extent of the species' former cultivation. More detailed knowledge about the eco-geography of members of section Narbonensis was provided by recent IBPGR plant collecting missions in the Eastern Mediterranean, mainly Syria and Turkey (for detailed references see Maxted *et al.* , 1991)**.**

V. narbonensis was found to be a widespread calcicole species in Syria. Its semi-arid botanical varieties *narbonensis*, *salmonea* and *jordanica* occurred throughout the sites visited, except for var. *jordanica* which could only be found in southern Syria near the Jordanian border (Ehrman and Maxted, 1989). Similar biogeographical information for vars. *affinis* and var. *aegyptiaca* is not available.

The smaller-seeded varieties which are abundant in the Eastern Mediterranean were probably, like other vetches, repeatedly introduced with traded grain to Italy, the Iberian Peninsula and North Africa, from Turkey and the Levant; or they were spread through grazing animals and seed eating birds.

According to Hegi and Gams (1924), *V. narbonensis* and *V. serratifolia* were rarely cultivated in Central Europe. They had been introduced as weeds with foreign cereals or vetches and could be found naturalised in warmer areas such as the Upper Rhine valley, parts of Austria and Switzerland. Earlier establishments had been reported for the Adriatic coast (Istria) and near Basel.

Table 3. Latin synonyms for *Vicia* species of section *Narbonensis* (adapted from Maxted, 1991)

***Vicia narbonensis* L.**	
Faba veterum (Park.)	*V. narbonensis* var. *typica* Fiori & Paol. (1900)
Faba sylvestris graecorum(Park.)	*V. serratifolia* var. *integrifolia* Beck (1903)
Aracus fabaceus(J. Bauhin)	var. *heterophylla* Rouy (=*V. heterophylla* Rohb.)
Faba kairina cui semine minora (J. Bauhin)	var.*serratifolia* (=*V. serratifolia*)
Swarte Boonkens (Dodoens, 1583)	var. *pilosa* Post
Wilde Boonkens (Dodoens, 1583)	var. *laodicena* nov.var. Mouterde
Faba sylvestris fructu rotundo atro (C. Bauhin)	
Faba narbonensis (L.) Schur	**var. *narbonensis***
Faba equina Miller (1755)	*V. platycarpos*
Faba bona Medikus (1787)	*V. narbonensis* ß *hortensis*
Bona narbonensis Medikus (1787)	*V. narbonensis* α *genuina* Gren. et Godr.
Bona speciosa Medikus (1787)	*V. narbonensis* var. *culta*
V. latifolia Moench (1794) (=var. *narbonensis*)	*V. narbonensis* var. *typica*
V. monodelpha Roth (1800)	V.narbonensis var. *integrifolia* Ser. in DC.
V. platycarpos Willd. (1802)	
V. narbonensis var. *hortensis* Lam.(1815)	**var. *salmonea***
V. narbonensis var. *integrifolia* Ser.(1825)	*V. serratifolia* subsp. *salmonea* Mout. (1970)
V. heterophylla (Reich.) Koch (1843)	*V. narbonensis* var. *crenate* Schäfer (1973)
V. narbonensis var. *integrifolia* Koch (1843)	
V. narbonensis var. *genuina* Gren.&Godron (1848)	**var. *jordanica***
V. narbonensis var. *culta* Alef. (1861)	*V. narbonensis* var. *intermedia* Strobl (1887)
V. narbonensis var. *platycarpos* Alef. (1861)	
Faba narbonensis Schur (1877)	**var. *affinis***
V. narbonensis var. *pilosa* Post (1896)	*Aracus fabaceus* J. Bauhin
V. narbonensis var. *heterophylla* Rouy (1899)	*Faba kayrina* J. Bauhin
***V. johannis* Tamamschjan**	
V. narbonensis Roth(1787)	
V. narbonensis var. *platycarpos* Alef. (1861)	**var. *ecirrhosa* (Popov) Schäfer (1973)**
V. narbonensis var. *lutea* Freyn & Sint. (1894)	*V. narbonensis* var. e*cirrhosa* Popov (1927)
V. narbonensis var. *laodicena* Mout. (1966)	*V. turkestanica* Vassilk. (1955)
V. procumbens Schäfer (1973)	
	var. *procumbens* Schäfer (1973)
var. *johannis*	*V. johannis* var. *intermedia* Scheibe (1934)
V. procumbens var. *violacea* Schäfer (1973)	*V. procumbens* Schäfer (1973)
***V. galilaea* Plitm.&Zoh. (1965)**	
V. narbonensis var. *pilosa* Post (1896)	
***V. serratifolia* Jacq. (1778)**	
Faba serratifolia Miller (1755)	*Faba serratifolia* Fuss (1866)
V. monadelpha Roth (1800)	*V. narbonensis* ssp.*serratifolia*(Jacq.)Nyman (1878)
V. serratifolia Willd. (1802)	*Bona serratifolia* (Jacq.) Stankevich (1982)
V. narbonensis var. *serratifolia* Koch (1857)	

Chassagne (1957) reported that *V. narbonensis* had been known at Puy-de-Dôme since the 18th century, and that this species could be found established at abandoned hillsides. The cultivated variety *V. narbonensis* var. *hortensis* (syn. *V. narbonensis* var. *narbonensis*) was first noted in 1925, probably introduced with forage grain from the Black Sea region during the first world war. Pigeons were apparently partial to this rarely cultivated plant which was clearly on its way to naturalisation and had supposedly aided in its spread. Distinct transitional varieties between the wild and cultivated types (var. *heterophylla* Rchb. Rouy) were also noted at some locations.

Cytogenetics

The haploid chromosome number for sect. *Narbonensis* is n=7. They are cytologically uniform, but can be distinguished by minor variations of the short arm lengths of submedian and subterminal chromosomes and in the relative size of satellite chromosomes. Within *V. narbonensis*, Schäfer (1973), distinguished three distinct karyotypes A, B, C. A fourth karyotype D was identified by Raina *et al.* (1989) and they conclude from meiotic pairing properties and non-viable crosses, that genome D is the most distinct and may warrant specific status. However, the single specimen cited for the D genome was obtained from ICARDA and has been identified as *V. serratifolia*. Schäfer (1973) also found a fourth karyotype within section *Narbonensis* for *V. serratifolia*.

Pollination and outcrossing

The Flower construction of *V. narbonensis* is similar to that of *V. faba*. The honey bee (*Apis mellifera*), *Bombus terrestris*, and *B. athophora*, have been observed visiting the flowers. Apparently, geocarpic flowers were said to occur within this species, but these reports required confirmation (Hegi and Gams, 1924)

Although the flowers of sect. *Narbonensis* are well adapted for insect pollination there is a predominance of autogamy. Schäfer (1973) estimated a relatively high outcrossing rate of 5-10%, based on spontaneous hybrids occurring during her study. However, this may be an overestimate (Schäfer, pers. comm.). Variation in outcrossing is worthy of further study as it is important to the breeding system to be used and the stability of individual genotypes.

Interspecific hybridisation

There have been several interspecific hybridisation experiments between the taxa of sect. *Narbonensis*. In general the results indicate that it is relatively easy to cross within a variety, as well as between varieties. There has been some success at crosses between species within section *Narbonensis* but generally no fertile offspring are produced from the F2 (v. Shelhorn, 1940; Schäfer, 1973; Raina *et al.*, 1989). Interspecific embryos (*V. faba* x V. *narbonensis*) usually abort prematurely due to lack of endosperm development (for detailed references see: Maxted *et al.*, 1991; Hanelt and Mettin, 1989; Raina *et al.*, 1989; Lazaridou and Roupakias, 1993).

History and evidence for the cultivation of *V. narbonensis*

The cultivation and subsequent domestication of grain crops in the Middle East and South West Asia seems to have begun, according to current estimates in the 7-8th millennium BC. eg. 9-10.000 years BP.. An assemblage of grain crops comprising Emmer Wheat (*Triticum turgidum* ssp. *dicoccum*), barley (*Hordeum vulgare*), Einkorn wheat (*Triticum monococcum*), Pea (*Pisum sativum*), Lentil (*Lens culinaris*), Chickpea (*Cicer arietinum*), Bitter vetch (*Vicia ervilia*), Broad bean (*V. faba*), Flax (*Linum usitatissimum*) has been found associated with early human settlements (Zohary and Hopf, 1988). Kislev (1985) provided evidence that *V. faba* could also belong to this early crop assemblage.

The areas of the so called "fertile crescent" bounded by the Euphrates - Tigris rivers and adjacent Anatolia contain most of the wild relatives of the early crop assemblage (Vavilov, 1949/50; Zohary and Hopf, 1988) and are therefore thought of as one of the cradles of agriculture. Harlan (1951, 1992) discussed Vavilov's idea's regarding centres of crop plant origin in relation to genetic diversity and proposed a model supporting more diffuse origins of agriculture, based on numerous micro-centres of diversity. With respect to the cultivation and domestication of grain legumes two hypotheses are currently disputed. On the basis of cytogenetic evidence Zohary (1989) proposed single (few) domestication events, while Ladizinsky (1989) in consideration of isozyme variation in lentils argued for polyphyletic (multiple) domestication events. He also suggested that pulses were originally collected as animal fodder. This activity would have favoured the establishment of genotypes possessing reduced seed dormancy in habitats where the feed was transported, thus providing a model for domestication prior to cultivation (Ladizinsky, 1987; Ladizinsky, 1989).

V. narbonensis is difficult to distinguish from *V. faba* in the archaeological record, unless pods or seed coats are present (Zohary and Hopf, 1988). The seeds of the former species may be identified by their round form and shorter hilum, and by the conspicuously short +/- 20µ high hour glass or T cells but these characters are not present in the mostly charred archaeological material (Hopf, 1986). Hence, we have no clear indication of its earliest cultivation or domestication. However, since the total sulfur levels in *V. narbonensis* are almost twice those found in *V. faba*, sulfur concentration in the charred cotyledons may provide an additional marker to separate the two species.

The earliest evidence for the cultivation of faba beans in Jericho dates back to 5000 BC. (Kislev, 1985), and the oldest finds on the Iberian Peninsula can be traced to 3000 BC (Zohary and Hopf, 1988). Large-seededness in the faba bean developed relatively recently, for all archaeological finds from ancient sites belong to var. *minor*.

A find made in Iraq and dated to AD. 1000 is the first archaeological record of larger seeds. However, large-seeded types are known to have been depicted on pottery in China, dated ca. 1000 years earlier (Saxena, ICARDA, pers. comm.). The seed size of the larger-seeded accessions of *V. narbonensis* var. *aegyptiaca* approaches that of the smaller seeded faba beans. Due to the superficial similarities between the two, as well as the frequent presence of the narbon bean in faba bean fields (Schweinfurth, 1891), it seems likely that it was domesticated and has evolved as a secondary crop (Vavilov, 1926) in the shadow of the faba bean. Thus, *V. narbonensis* which in plant habit also resembles faba beans, could be considered its mimic.

The only indubitable find of *V. narbonensis* was described by Laurent-Tåckholm (in Lauer, Laurent-Tåckholm and Åberg, 1951; cited by Schultze-Motel, 1972) and was made in Saqqarah, Egypt and attributed to the III. Dynasty (ca. 2000 BC.). Apparently, another *V. narbonensis* seed was found together with Emmer wheat chaff at the pyramid of Sahuré, Abu Sir (V. dynasty). It was identified by G. Schweinfurth but was lost with the destruction of the Botanic Museum Berlin-Dahlem during the second World War (Schultze-Motel, 1972). These finds indicate the presence of *V. narbonensis* in the Egypt of antiquity, most likely as a weed of cereal crops.

The Nabathean book of Agriculture (ca. 4th century AD., Iraq) cited by the 12th century Andalusian agriculturalist Ibn Al-Awam described a plant resembling faba beans with black odoriferous seeds, which is consistent with a description of *V. narbonensis* or *V. johannis*; and advised that this weed should be removed from the bean fields and used as a manure (Clémont-Mullet, 1866).

Pre-Linnaean botanists were familiar with the otherwise rare *V. narbonensis* from their gardens and grew it for reasons of curiosity and delight in the study of herbs, but no useful properties were ascribed to it. The species (*Faba sylvestris graecorum*, *Pisum nigrum*) was apparently one of the more popular garden plants of the 16th century[46] (Franke, 1594; Zaunick *et al.*, 1930). It appears that the plant material available to most botanists at the time was black-seeded and of unpleasant, sulfurous taste. In Belgium, Dodoens (1583) noted that "if the seed is chewed it filleth the mouth full of stinking matter". However, Camerarius (1586) described the taste of the seed[47] as similar to that of broad beans. This judgement may be based on sampling the ripening seeds which, despite the garlic flavour, have a much sweeter, agreeable taste than when dried, but it also suggests that some varieties of *V. narbonensis* could be more palatable, especially those of var. *aegyptiaca* which appears to be the variety depicted in Camerarius' illustration. At the time, the plants grew conspicuously abundant in some parts of Southern Italy: the promontory of Misenum, near Naples and in the fields of Apulia[48].

According to Gerarde (1636) the "blacke beane" or *Faba sylvestris* was regarded by some botanists to be the true "Physicke Beane of the Ancients", described in the herbal of Dioscorides. They therefore named it *Faba Veterum* and also *Faba Graecorum*, or the "Greeke beane". A comparison of the pharmacological properties of *V. narbonensis* with those of *V. faba* might clarify whether these early herbal remedies made use of the garlic like tasting, sulfurous constituents of the narbon bean. The high total sulfur levels of the seed could also help to distinguish this species from *V. faba* in material from archaeological excavations.

Traditional names for *V. narbonensis sensu lato*

Linguistic evidence, although often discredited because of its uncritical interpretation (Hanelt, 1972) by some authors e.g. (Hehn, 1870), can be used as an indication for the antiquity of certain crop plants, especially if independent names can be identified which predate those of the Indo-European language family. It also provides some interesting details about the perception of a species like *V. narbonensis* by different peoples and indicates end uses. Table 4 lists names from different languages for *V. narbonensis sensu lato* (e.g. in the broad sense). Clearly, not all the collected names presented refer to a single variety of this species, and considering the genetic diversity and distribution of *Vicia* section *Narbonensis*, some names probably refer to

[46]Documented for East-Prussia (Wigand, 1583), Nürnberg (Camerarius, 1588), Silesia (Schwenckfelt, 1601) and the Erzgebirge (Annaberg: Jenisius, 1605) (Zaunick *et al.*, 1930)

[47] The illustration of *V. narbonensis* which accompanies Körber-Grohne's Camerarius citation depicts relatively large seeds of the species with an attached funiculus which are key characters for var. *aegyptiaca*. (Due to their expense, woodcuts from other herbals were in some cases used for new publications, so the exact origin of the var. *aegyptiaca* illustration should be verified to ascertain Camerarius'authorship)

[48] These observations were probably translated by Camerarius from Matthioli, whose herbal was the basis for the german edition. Matthioli also published commentaries to Dioscorides, but his writings were not consulted for this study.

Table 4. Traditional names for *Vicia narbonensis sensu lato*

Name	Language	Translation	Reference
Vesce de Narbonne	French	Narbonne vetch	
Veccia di Narbona	Italian	Narbonne vetch	
Narbonner **Wicke**	German	Narbonne vetch	Hegi & Gams (1924)
Ervilhaca de Narbona	Portuguese	Narbonne vetch	Vasconcellos (1962)
Maus**wicke**	German/Swiss	Mousevetch	Hegi & Gams (1924)
Alberjón	Spanish		Caballero (1940)
Koca **fig**	Turkish	old/aged/famous V.	Plitmann (1970)
Biqia harastaniye	Syria	Harastan vetch	Kernick (1978)
Velika **Grahornia**	Croatian		Ascherson & Gräbner (1909)
Polka Gayana	Kurdish, Iraq		Townsend (1974)
Veccia salvatica	Toscana, Italy	Wild vetch	Penzig (1972)
Vezza	Emilia, Italy		Penzig (1972)
Vezzèl	Emilia (Reggio), Italy		Penzig (1972)
Essa salvadega	Lombardia (Brescia), Italy		Penzig (1972)
Fève des chevaux	French	Horse bean	
Févette	French (Tunisia)	Small bean	Pottier-Alapetite (1979)
Schwarze Acker**bohne**	German	Black field bean	Hegi & Gams (1924)
Französische **Bohne**	German	French bean	Hegi & Gams (1924)
Schwarze **Erbse**	German	Black pea	Hegi & Gams (1924)
Mohren**erbse**	German	Moor's pea	Hegi & Gams (1924)
Ful iblis, fûl-iblîs	Arabic (Palestine, North Africa)	Devil's bean	Löw(1967),Kernick(1978),Post (1932)
Scheer**erbse**	German/Swiss	Mole pea	Hegi & Gams (1924)
Bob clivji	Dalmatian		Visiani (1852)
Fève sauvage	French	Wild bean	
Fava nera	Italian	Black bean	
Bâcher	Nile Delta	Baharian pea	Schweinfurth (1891
Bakhar	Near East		Post (1932)
Bakker,	Morocco		Kernick (1978)
Bakher	Algeria, Egypt		Quezel&Santa(1962),Muschler (1912)
Bâcher(bâkar)	Old semitic		Schweinfurth (1891)
Bazalia	Arabic		Gillet & Rawi 1947 (Schäfer pers.com.)
Balcik	Kurdish Alevite?		Enneking (pers. obs. Tunceli, Turkey)
Yabani baklasi	Turkish	Wild bean	Scheibe (1934)
Bakla balcigi	Kurdish Alevite?		Enneking (Tunceli, 1991)
Haba silvestre	Castilian	Wild bean	(Cienf.) Colmeiro (1886)
Haba loca	Castilian	Crazy bean	(Ouer, Palau) Colmeiro (1886)
Habillas de pájaro	Castilian		(F. Nav.) Colmeiro (1886)
Fabaraca	Asturian		(L. P. Ming.) Colmeiro (1886)
Fabera borda	Catalan	"Bean of the edges"	(?) Colmeiro (1886)
Fava burda, Fá burda	Sardinian		Penzig (1972)
Fava de lovo	Venetia, Italy		Penzig (1972)
Fava salvadega	Venetia (Verona), Italy		Penzig (1972)
Fèva salvadega	Emilia (Romagna), Italy		Penzig (1972)
Fava sarvaggia	Sicilian, Italy		Penzig (1972)
Favi stritti	Sicilian, Italy		Penzig (1972)
Favaccia	Sicilian, Italy		Gussone (1843), Bianca (1859)
Favetta	Sicilian (Catania), Italy		Penzig (1972)
Favera en bañetas	Balearic		(Trias) Colmeiro (1886)
Faveta de Beja	Portuguese	Bean from Beja	Vasconcellos (1962)
Habb Adh-Dhurât	Kurdish, Iraq	Fart seed*	Townsend (1974)
Vederiòla	Emilia		Penzig (1972)
Pisi-pisi de còloras	Sardinian		Penzig (1972)
Moreus	Catalan		(Costa) Colmeiro (1886)
Agriocuç	Cypriot		Economides 1968 (Schäfer,pers. comm.)
Nu`mâni-barri			(Bo) Post (1932)
?Gagus	Kurdish, Iraq		Townsend (1974)
Divlji Bob	Kroatian		Ascherson & Gräbner (1909)
Glen	Kurdish		Gillett & Rawi 1947 (Schäfer pers. comm.)
Berri	Arabic		Boissier (1872)

related species. English names (not listed in the table) include Giant vetch, Broad-leaved vetch, French vetch and Narbonne vetch. In Australia the species has been named narbon bean (Georg, 1987b).

In the context of its history, Hanelt (1972) presented a linguistic analysis for the traditional names of *V. faba*. The Greek name for *V. faba* is *kyamos* and has no related form in any other language. Most faba bean names can be traced to a root like the Semitic *ful*, the Slavic *bob*, the Germanic *bona*, or the Indo-iranic *bakla* which are synonymous with the English word bean. Interestingly, the linguistically isolated Berber name, *ibiu* bears

resemblance to the arabic byname *iblis* (ful iblis = devil's bean[49]) for *V. narbonensis*.

A detailed linguistic analysis of the names for *V. narbonensis* is outside the scope of this study and table 3 is only presented to illustrate the utilisation and different perceptions of this species. The table is divided into names which clearly refer to it as a vetch (e.g. vika, *Vicia*, vesce, veccia, Wicke, vieke, fig, Grahor, grahornia, Ervilhaca, Hegi and Gams, 1924), and those which identify it as a bean or grain legume. It is quite clear from some of the bynames such as sauvage, sylvatica, loca, borda, that in many areas the species *sensu lato* is regarded as a weed or wild plant. The name Habb Adh-Dhurât (fart seed) is an indiscriminate term used for several species e.g *V. sativa, V. narbonensis, V. monantha* in the plains and lowlands of Iraq (Townsend, 1974).

According to Hegi and Gams (1924), the names Mäusebohne (mouse bean), Mäusewicke (mouse vetch) or Scheererbse (mole pea) from the region around Basel and Baden originate from the belief that the plant keeps away field mice and moles (in the North of Switzerland=Scheer). In view of the recurrent mouse plaques in Australia, this lore may have utility once the validity of the implied anti-rodent activity has been evaluated. Because of its black seeds it is called Schwarze Erbse (black pea) or Mohrenerbse (Moor's pea), or according to its origin: Französiche Bohne (French bean) or Römische Wicke (Roman vetch). Whether the *Pisi maurisci* (Moor's peas) mentioned in the *Capitulare de Villis* (ca. 840 A.D.) refer to this species, as was suggested by Dragendorff (1898) appeared questionable because there were no other indications for such an early cultivation (Hegi and Gams, 1924). Fischer-Benzon (1894) identified the moor's peas as black-seeded pea cultivars. Because peas are otherwise not mentioned in the *Capitulare de Villis* but were otherwise quite well established as crops, Fischer-Benzon's interpretation appears plausible (Hanelt, pers. comm.). The question of the true identity of the Moor's pea remains essentially unanswered, especially in the light of a possibly much older culture of *V. narbonensis*.

Recent history

The use of forage and grain legumes for the improvement of soil fertility was advocated in the works of Roman agriculturalists such as Cato, Varro and Columella. Humanism[50] and the development of the printing press facilitated the spread of this agricultural knowledge, for example in the so called "Hausvater" (Home father) literature of the 16th-18th century which aimed at educating farmers to adopt more profitable farming practices (Haushofer, 1985). The innovative use of forage legumes was the cornerstorne of the 16th century agricultural revolution which, facilitated by social changes and non-conformism, was driven by the necessity to improve soil fertility (Stinner *et al.*, 1992). Considering the parallel increase in botanical knowledge, it is thus not surprising that a variety of new legume crops, including *V. narbonensis* were taken into cultivation (Germershausen, 1799).

Definitive agricultural information about *V. narbonensis* is given by Lawson (1836) who reported that it was cultivated in Germany and other parts of the continent as a substitute for common vetch (*V. sativa*). Lawson noted that under Scottish conditions, if sown in autumn, it yielded a close-growing crop of succulent fodder due to its fast growth in the early spring months. The strong beany taste of the leaves was initially not well liked by cattle but in the absence of more palatable feeds such as clover during spring the animals gradually adapted to it.

During the 19th century, both cultivated and wild varieties of *V. narbonensis* were distinguished in the agricultural literature (Alefeld, 1861; Alefeld, 1866). The utilisation of *V. narbonensis* var. *culta* Alefeld was similar to that of common vetch, but for favourable development it was known to demand more warmth, giving in exchange more pods and herbage. The plant was also known as an escapee from cultivation, indicating its potential to naturalise (Alefeld, 1866).

For Australia, the use of *V. narbonensis* from Southern Europe and South-West Asia for human consumption was advocated by the German botanist Baron von (Mueller, 1881), who found it to be "preferable to *V. faba* for the table because the somewhat smaller seeds were less bitter". Cultivars of *V. narbonensis* could be obtained through the seed trade and were commonly listed in catalogues of the major seed merchants (Clos, 1898). However, its use as a forage crop declined towards the end of the 19th century (Wittmarck, 1890).

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[49] Dr. Fazil Düsünceli (pers. comm.). Compare with "devil's dung" a vernacular name for the malodorous resin of *Asa foetida*, the giant fennel (*Ferula foetida* (Bunge) Regel; *Ferula asa-foetida* L.; *Ferula narthex* Boiss.) which is used in Asian medicine for gastrointestinal disturbances, in perfumery and as a spice (Samini and Unger, 1979), a modern substitute for the now extinct ancient spice Silphium (liquamen) (Tannahill, 1988)

[50] Source of many new crops was the pastor's garden (Fischer-Benzon, 1894)

Becker-Dillingen (1926) dealt with *V. narbonensis* as a grain crop citing Fruwirth's (1921) work in Vienna .

The eminent geneticist, Vavilov (1926) used *V. narbonensis* as an example of a secondary crop which had evolved from weed to cultivated plant, referring to the contrast between its weed and crop status in Spain and Italy, respectively.

Mateo-Box (1961) gives a comprehensive account of *V. narbonensis* as a crop plant. He refers to the grain being crushed and used to feed cattle, especially calves, however it imparts its peculiar flavour to the milk if fed to cows. Feed intake of farm animals may initially be reduced but cattle adapt quickly to diets containing the crushed grain and do so much better than sheep, pigs or fowls. As a forage plant, *V. narbonensis* can be utilised in hot, dry as well as mild climates; it is of excellent quality and much appreciated as fodder for all types of cattle. In mixtures with other vetches or with some cereal (barley or oats) it provides a good basis for silage, but only if it is cut at flowering and chopped well. In hot and dry regions it is an excellent legume for green manure. In those places where it is cultivated, the major reason for justifying its use in place of faba beans or common vetch is its resistance to some pests and diseases[51].

These observations clearly suggest that there is a place for *V. narbonensis* in Australian agriculture , however, they should be confirmed by trials under local conditions.

Cultivation of *V. narbonensis*

In conjunction with the further development of *V. narbonensis* as a grain and forage crop its domestication status requires clarification. The following is a summation of the evidence for its cultivation based on the literature, herbarium specimen and available genetic resources of its botanical varieties *aegyptiaca* and *narbonensis*.

Hegi and Gams (1924) thought that *V. narbonensis* cultivation in Southern Europe and North Africa was very recent because the names given to it in the old herbals do not indicate any agricultural utility (table 4). However, this argument does not recognise that herbal authors like the Bauhins and Dodoens were of Northern European descent and therefore unlikely to have directly come into contact with its cultivation, while *V. narbonensis* is a typically Mediterranean crop. In addition, names like *Faba veterum* and *Faba graecorum* which suggest more ancient links were not taken into account.

By contrast, Guinea (1953) referred to *V. narbonensis* as a very ancient crop of the Mediterranean area and remarked on its association with sites of human habitation but provided no supporting evidence.

There is a reasonable possibility that *V. narbonensis* may be an archaeophyte, that is, a plant which marks archaeological sites and which has been associated with human habitation since antiquity. The archaeological line of evidence (Zohary and Hopf, 1988) suggests that mixtures of *V. faba* and *V. narbonensis* were cultivated since pre-historic times. This establishes first of all the status of *V. narbonensis* as a weed of faba beans and may explain the observed domestication traits which could have been unconsciously selected for, similar to the crop mimicry of lentils, vetches and bitter vetches (Vavilov, 1922; Barulina, 1930). Indeed, Vavilov (1926) suggested that *V. narbonensis* had evolved as a secondary crop.

The species has been cultivated on the Iberian Peninsula (Trás-os-Montes, Estremadura) (Colmeiro, 1886), Austria (Südkrain) and southern Germany (Kittel, 1844), Bohemia and Moravia (e.g. near Olmütz) (Hegi and Gams, 1924), at the adriatic coast (*in cultis*: Triest (Biasolletto!), Fiume (Noé!)) (Koch, 1835), Dalmatia (*in cultis* (Visiani !)) (Host, 1831), in Turkey (Plitmann, 1970), Palestine (Löw, 1967), in Syria (Van der Veen 1967; cited by Kernick, 1978) and in Iraq (van der Veen, 1960; Townsend, 1974). In the Flora Iranica the species is mentioned as frequently cultivated (Chrtkova-Zertova, 1979) and others refer to it as cultivated in the Mediterranean basin (Laumont, 1954; Mateo-Box, 1961).

Barbulescu and Ion (1964) refer to its cultivation in Sicily, but as a crop it was only once encountered there in recent times during plant collecting missions (Hammer *et al.*, 1992), however it is recognised as an old fodder crop in Southern Italy (Pignatti, 1971; cited by Hammer *et al.*, 1992). In France seeds were sold at markets whereas in central Europe they were obtainable only (and that rarely) through the seed trade (Hegi and Gams, 1924). The Catalan regions of Roussillon or Cerdagne, in the French Pyrenees are the only French areas of known cultivation for *V. narbonensis* (*V. johannis*?) (Naudin (no ref.) cited by Laumont, 1954). A population of *V. narbonensis* (*V. johannis*?) is presently known in the Atlantic Pyrenees, near Pau, France (Combes, pers.

[51]"En los lugares donde se cultiva, las razones principales que aducen los agricultores para justificar el empleo de esta especie en lugar de las habas o la vez, es la mayor resistencia del alberjón al <<jopo>> y a los pulgones" (p. 147). "Típicamente es un cultivo de secano, incluso para zonas muy secas" (p. 150) (Mateo-Box, 1961)

comm.).

V. narbonensis has apparently also been cultivated in Abyssinia as fodder for live-stock and green manure (Hegi and Gams, 1924; Uphof, 1959), however a recent revision of the Ethiopian flora could not verify older recordsfor the occurrence of the species in this region (Hedberg and Edwards, 1989).

In the sub-montane, rainfed area of Iraq (400-600 mm rainfall), adaptation studies during the 1950's showed that *Vicia narbonensis* and *Lathyrus sativus* were the most promising annual legumes in this area, of *V. narbonensis*. the line 1-25 and the local variety "Shaklava" were most successful (Van der Veen, 1960). It is noteworthy that an accession collected in Northern Iraq (IFVI 67) has consistently out yielded all other lines tested in Syria until recently[52].

A cultivar "Aleppo" (*V. narbonensis*) was in agricultural use in Syria (Van der Veen 1967; cited by Kernick, 1978), a cultivar *V. narbonensis* 229 was selected in Algeria (Barbut, 1954; cited by Kernick, 1978) and in Italy the variety "S. Vincenzo" has been registered by B. Kökeny, Sisforaggera, Bologna (Perrino, pers. comm.). Turkish work has also resulted in the selection of a cultivar which is well suited to the conditions on the Anatolian plateau around Ankara. The line L-1541, collected near Tunceli by Ömer Tarman, was selected as a high yielding forage crop (Munzur, pers. comm.). This particular line had been cultivated under supplemental irrigation as a mixture with *V. faba* on ca. 10-15 ha up until the 1970s in Göktepe village near Tunceli (M. Arslan, pers. comm.).

Recent inquiries to ICARDA have not shed any further light into the whereabouts of the "Aleppo" cultivar mentioned by Kernick (1978). However, the species is currently being cultivated as grain legume in Southern Syria around Daraa and Sweida as a feed for cattle and sheep. Three accessions of cultivated *V. narbonensis* have recently been collected[53] (Maxted, pers. comm.). There are references to the cultivation of *V. narbonensis* under irrigation (herbarium specimen, Kew No 10.436, Erbil, Northern Iraq, leg. Gillet (1948) ; Enneking (pers. obs., 1991) Göktepe village, near Tunceli, Eastern Turkey).

Vavilov (1926) mentioned *V. narbonensis* as a weed in Spain, however, it is curious that Mateo-Box (1961) describes the cultivation of this plant in some detail, and that var. *aegyptiaca* is still growing as an escapee in Andalusia where it has been cultivated in mixtures with *V. faba* as so called "shandy" or "buffer" crops (Cubero, pers. comm.). A cultivated mixture of *V. faba* and *V. narbonensis* var. *aegyptiaca* was obtained by the author in 1991 from local Alevite farmers in Göktepe village, near Tunceli in Eastern Turkey. In Spain var. *aegyptiaca* was found near Miranda and Marisma de los Potros (Hammer and Lehmann, 1978), and samples var. *aegyptiaca* obtained from Dr. A. Martinez, Cordoba had been collected recently in Andalusia[54].

The almost naked (glabrous) *V. narbonensis* var. *integrifolia* Ser. (*V. latifolia* Moench= *V. narbonensis* culta Alef. = var. *typica* Fiori et Paol.) with entire leaflets and stipules was considered to be the true cultivated type (Becker-Dillingen, 1929).

Indications of *V. narbonensis* cultivation are only very rarely encountered in herbarium material. Schäfer (pers. comm.) found the following: -Haußknecht, B., leg. 1864, cult. pr. Artern (Jena herbarium) Halle district. -Fleischer, B., leg. 1883, Bohem. sept. orient. Sloupnice, culta in agris (Jena and Halle herbaria) -Mouterde, Lebanon " seemed to be cultivated" (specimen is var. *salmonea*).

The available data suggest that vars. *narbonensis* and *aegyptiaca* are domesticated forms of *V. narbonensis*. Therefore, the distribution and occurrence of these genotypes may provide an indication of the species' cultivation.

In the Montpellier herbarium (MPU) the present author found one cultivated specimen[55] and several others

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[52] compare with Reeve *et al.* (1985)

[53] *Vicia narbonensis* accessions IFVI 2802, [SOU 867102] and IFVI 2627 [SOU 868054] were found at site 14 in Al Naoora, 37km West of Homs in a village, under the Homs to Tartous road, 1km after site nos.13, in a meadow, near the river where faba beans and cereals were cultivated in strips, altitude: 270m, annual rainfall: 900 mm, soil: Heavy black [basalt]. Another *V. narbonensis* crop IFVI 2627 [SOU 868054] was found at site 131 Suweida, 1km before Dier Labon on Mazraa to Sahba road in stonewalled fields each side of the road before the village, altitude: 860m, annual rainfall: 300 mm, soil: Heavy black [basalt] (Maxted, pers. comm.).

[54] V.N.-61, Villamartin (Cadiz) 1980; V.N.-60, Santa Maria de Transierra (Cordoba) 1984, recogida en la zona de los Baños de Popea, sin cultivar; V.N.-62, Castro del Rio (Cordoba) 1980).

[55] *V. narbonensis*(MPU) Cult. en Transylvania Dr. Schur 30.7.1872 (B & W photo available)

which could be positively identified as *V. narbonensis*[56] while the majority were identified as *V. johannis*.

Several other specimen with gigantic traits (large leaflets etc.) could also be regarded as cultivated types, escapees from cultivation or *V. faba* mimics[57].

Var. *aegyptiaca*

This variety was first described by Körnicke from Egypt. According to Ascherson and Schweinfurth (1889) var *aegyptiaca* was of confined occurrence in the Nile delta in the vicinities of Zaqàzig and Fayoùm.

Schweinfurth (1891) reported that *V. narbonensis* could be found frequently and exclusively in *V. faba* fields, and he took the presence of *V. narbonensis* seed in the pyramids (ca. 2900 B.C.) as evidence for infiltration of Mediterranean weeds into the Egyptian flora.

Schäfer (1973) reasoned that the seed size, upright growth habit and the relatively shatter resistant pods of var. *aegyptiaca* were signs of domestication, so that its status as weed in Egypt (Montasir and Hassib, 1956; cited by Schäfer, 1973) could not be entirely correct. The cited type specimen for this variety were two Turkish lines ((V227), Ankara, Beltsville No 225.732; Eskisehir I-51, leg. Ömer Tarman (V 359)(Schäfer, 1973)).

Schäfer (1973) thought that it would be unlikely to have var. *aegyptiaca* described in Europe,however, the collection of this variety in Spain and Turkey suggests a much wider distribution than previously expected.

The evidence from the literature, herbarium studies and preliminary observations with the Australian *V. narbonensis* collection suggest that var. *narbonensis* and var. *aegyptiaca* are probably the most domesticated of the five *V. narbonensi*s races (large seeds, lack of hardseededness, upright growth habit, reduced shattering of pods). Detailed measurements of seed size, germination percentage, seed coat sickness, seed imbibition, together with other domestication traits such as gigantism, degree of pod shattering, weediness and palatability (GEC concentration) should help in the differentiation of domesticated landraces and truly wild germplasm within existing and future collections.

Production Figures and Extent Of Cultivation

Exact figures for the global cultivation of *V. narbonensis* as a grain or forage crop are unavailable, but for Spain in the 1960's its cropping area was estimated at 7000 ha with a global grain production of 4600 metric tons (Mateo-Box, 1961). In Turkey Durutan *et al*. (1990) and Kurdistan (Northern Iraq and Eastern Turkey) the species is grown on a limited scale under both, irrigation and rainfed conditions. Areas of local landrace cultivation are expanding in the Djebl Druse region of Syria (Maxted (Erskine), pers. comm.). Its economic contribution as a component of natural pastures which provide fodder for nomadic sheep herds (Bhattacharya and Harb, 1973) has not yet been estimated.

Desirable traits in *Vicia narbonensis*

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[56] *V. narbonensis* L. Université d'Alger, Herbier d' l'Afrique du Nord (MPU) cultivé a Rabat (Maroc) de graines du Pays. leg. Miége 1923

V. narbonensis var. *narbonensis*, nice large, almost perfect pods, white hilum on seeds, Dr. M. Maire (pronounced oblong leaflets, seven seeds)

V. narbonensis (B&W photo taken) (MPU) Castille: Bujedo cultivada. 12.7.1911 leg. Harman Elias. 2 pairs of leaflets, max 2.5 pairs (5 leaflets)

V. narbonensis (B&W photo), Algérie 1853

V. narbonensis, Serrian, Hérault (broken specimen)

V. narbonensis (B&W photo), Smindja, Tunisia leg Rille?

V. narbonensis, St. Adrien près Servian (Hérault) Mai 1939

V. narbonensis var. *narbonensis* (B&W photo), St. Adrien près Servian (Hérault), Mai 1939, confirmed/det. Adel Khattab

[57] *V. narbonensis* Libya, Cyrenaica: Wadi Wardanya, between Cyrene and Beda littoria, in Machia on slopes, 6.4.1939 Alt. ca. 550, upper half of keel and wings very dark purplish black., Standard pale mauve veined with purple, leg. N.Y. Sandwith No.2409, N.D. Simpson No. 39372*(*Herbar Kew) Schäfer p.comm.

V. narbonensis L. Université d'Alger, Herbier d' l'Afrique du Nord (MPU) cultivé a Rabat (Maroc) de graines du Pays. leg. Miége 1923 by Dr. M. Maire; pronounced oblong leaflets seven seeds. *V. narbonensis* var. *narbonensis*, nice large, almost perfect pods, white hilum on seeds

V. narbonensis (BandW photo taken) (MPU) Castille: Bujedo cultivada. 12.7.1911 leg. Harman Elias. 2 pairs of leaflets, max 2.5 pairs (5 leaflets)

V. narbonensis has repeatedly attracted the attention of agronomists but as a forage crop it was never really developed much further than the experimental stage (Laumont, 1954). Recent agricultural interest in *Vicia narbonensis* has been generated by the following attributes of this plant: 1) high yields under low rainfall conditions, 2) frost tolerance, 3) disease resistance, 4) insect resistance, 5) Boron tolerance (Georg, 1987a).

High grain yields (1.5-3.5 t/ha) can be obtained from *V. narbonensis* under dry Mediterranean-type winter rainfall conditions (250-500 mm/annum) (Georg, 1987a; Abd El Moneim *et al.*, 1988; Abd el Moneim *et al.*, 1990b; Abd El Moneim, 1992). Similarly, 20 years of research work in Turkey has identified this species as the most promising crop among legumes in rotation with wheat (Durutan *et al.*, 1990).

It therefore appears to have the potential to play a more prominent role in the agriculture of regions with Mediterranean or similar types of environments, particularly in areas where crops other than cereals cannot be grown profitably.

Useful levels of resistance to chocolate spot disease (*Botrytis fabae* and *B. cinerea*) have been noted for *V. narbonensis* (Lowes, 1980; Birch *et al.*, 1985), however, South Australian experience suggests that it may be variety specific, or dependant on environmental conditions. In 1986 serious damage by *Botrytis fabae* (chocolate spot) was reported, and Subterranean clover red leaf virus and Alfalfa Mosaic Virus were identified in trials in South Australia (Georg, 1987b). Recently, due the unseasonal wet weather, a new fungal disease, Clover pepper spot (*Stempophyllum trifolii*) was found to devastate *V. narbonensis* in Victoria and Western Australia (Castleman, pers. comm.)

Birch (1983) found useful levels of partial resistance to the black bean aphid (*Aphis fabae*) in *V. narbonensis* and *V. johannis*, which is influenced by the stage of growth and is found to a greater extent in *V. johannis*. Susceptibility increased from pre-flowering/bud formation to full flowering. It then decreased rapidly during pod formation, filling and maturity. *V. narbonensis* flowered earlier than the slower growing *V. johannis*, thus it was more susceptible to aphids. In addition, *V. johannis* is more densely covered with trichomes on the leaf lamina, veins, stem internodes and pods.

In a detailed and interesting study of stem and bulb nematode (*Ditylenchus dipsaci*) resistance mechanisms in *V. faba*, Gastel (1990) found that root exudates of *V. narbonensis* (IFVI 67) were unattractive to the nematode in contrast to the consistent attractivity of all the *V. faba* lines tested. Nematode multiplication rates were reduced by 80% in *V. narbonensis*, and 70% in the most tolerant *V. faba* line compared to the susceptible *V. faba* cv. Herz Freya. These findings suggested that nematode tolerance in *V. faba* and *V. narbonensis* is based on antibiosis mechanisms. In addition, *V. narbonensis* is unattractive to this most problematic pest. Plant stem extracts (8 hrs acid hydrolysis) analysed for amino acid composition showed double the concentration of cystine for *V. narbonensis*, compared to the *V. faba* lines tested (Gastel, 1990).

In South Australia, recent screening trials for resistance to *Ditylenchus dipsaci* have shown that some *V. narbonensis* genotypes, including IFVI 67 gave good yields despite being infested by the nematode (Scurrah, pers. comm.). Since there seems to be good potential for the selection of nematode tolerant/resistant genotypes, the germplasm of *V. narbonensis* should be included into existing and future screening programs to evaluate their effect on populations levels of the major phytoparasitic nematodes (*Pratylenchus* spp., *Meloidogyne* spp., *Ditylenchus dipsaci*).

Trials in Syria and Iraq have established that crops of *V. narbonensis* are quite resistant to bird damage (Van der Veen, 1960; ICARDA, 1987, Abd El Moneim, 1992) and this may be due to its unpalatability factor (Chapter 5) or tannins. Van der Veen (1960) reported that this resistance to bird damage during its early stages of growth makes this species one of the most bird resistant vetches in northern Iraq.

The species is recognised as an invaluable crop in Turkey where it has been noted to be bruchid resistant[58] and to survive temperatures of -30 deg C (Elçi, 1975; cited by Birch, 1983). The often extreme winters of Northern Iraq require cold tolerant crops. By contrast, in Italy, *V. narbonensis* is known to be susceptible to cold winters, and Mateo-Box (1961) noted that the plant is able to withstand cold conditions in dry soils, but is adversely affected in the presence of too much moisture[59]. Above ground parts of the plant may die off in cold winters, but in spring regrowth from the undamaged root system occurs (Mateo-Box, 1961). There is likely to be considerable ecotypic variation in cold tolerance because the species' distribution extends into cold areas. Reeve *et al.* (1985) obtained striking results (2 t/ ha grain) with a cold resistant *V. narbonensis* var. *narbonensis* in Northern Iraq, while other grain legumes were killed by frosts.

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[58] Bruchid species was not indicated

[59] The species is susceptible to waterlogging (J. Carter, VIDA, Horsham, pers. comm.)

In South Australia, soils with high levels of boron necessitate the use of adapted cultivars. Some genotypes of *V. narbonensis* with tolerance to boron (50ppm) have already been identified, indicating that there is good potential for the development of boron tolerant cultivars from this species (Georg, 1987a; Abdolreza Bagheri, Dep. Plant Science, W.A.R.I., pers. comm.).

Weed potential

Holm *et al.* (1979) documented *V. narbonensis* as a weed in Tunisia (principal weed), Iran, Lebanon (Common weed), Turkey (present as a weed and behaves like a weed but rank of importance unknown) and Israel (part of the flora, confirming evidence for its perception as a weed needed).

According to Robson *et al.* (1991), *V. narbonensis* and *V. serratifolia* are important weeds of irrigated and rainfed cereal crops and are also quite common in orchards. They can be occasionally found in all other crops of the Near East region. As control measures they recommend tillage and the use of herbicides[60].

Clearly, the potential exists for *V. narbonensis* to become a weed and will require attention. A similar argument applies to other *Vicia* and *Lathyrus* species, especially in the Australian context, because Australia is a biological vacuum for most of these species. The widespread occurrence of *V. articulata* in the Flinders Ranges, *V. sativa* in the agricultural areas and *Lathyrus* spp. in the Adelaide Hills of South Australia are testimony to the potential of *Vicia* and *Lathryus* species to naturalise.

Utilisation

The grain and forage can be used as a feed for cattle (Mateo-Box, 1961; van der Veen; 1960; Pott, 1907; Lawson, 1837) and sheep (Jacques, 1990; Jacques *et al*., 1991). However, for utilisation by monogastric animals such as pigs, poultry and possibly as human food, the unpalatability of the seed (Davies, 1987, Johnson and Eason, 1990) caused by the sulfur rich (11.6%) dipeptide γ-glutamyl-S-ethenyl-cysteine (GEC) needs to be overcome. This compound is closely related to similar compounds in chives (*Allium schoenoprasum*) and onions (*A. cepa*). It has been established that this off-flavour precursor can be readily destroyed by acid hydrolysis (chapter 5). Recent success with the introduction into *V. narbonensis* of a gene coding for a sulfur-rich protein (Saalbach *et al.*, 1994) provides a convenient tool (Pickardt *et al.*, 1991) to probe the sulfur biochemistry in this species and to manipulate tissue or compartment specific expression. The fundamental data are now in place for the further development of the narbon bean in Australia as a grain legume and forage crop for low rainfall areas.

Further work with *V. narbonensis*

The grain of *V. narbonensis* needs to be evaluated thoroughly for ruminant production through feeding studies with cattle and sheep. An assessment of meat, wool growth,wool and milk quality in addition to possible effects on reproductive performance of both sexes should be considered.

New end-use option for the grain could possibly be made available through the development of value-added products by acid hydrolysis or fermentation. In addition, the available Australian expertise derived from the work on Lupins will be of benefit for future value-adding ventures with *V. narbonensis* as well as for the more diverse challenges of other *Vicia* and *Lathyrus* grains.

The physiological effects of γ-glutamyl-S-ethenyl-cysteine (GEC) need to be studied more closely in pigs, poultry and ruminants in order to assess whether the compound is toxic or not, and whether exposure to low levels (in "tasty varieties") is going to cause any harm or produce any off-flavours in animal products.

Since the grain of *V. narbonensis* is high in sulfur and selection for improved palatability of the grain is likely to reduce this level, the utilisation of GEC as a sulfur amino acid source by ruminants should be investigated and due consideration be given to the biodiversity offered by the rumen ecosystem. Biochemical research is needed to investigate the biosynthesis of GEC in order to explore ways for tissue specific manipulation and for the retention of the desirable high grain sulfur levels. Environmental effects (climatic, edaphic e.g. S) on GEC production have to be assessed. Mutagenesis breeding should also be initiated to obtain mutants for low levels of GEC and for biochemical studies of its synthesis.

Ethnobotanical research[61] would be desirable on the end-use of narbon beans in the Middle-East e.g. Djebl

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[60] atrazine, diuron, methazole, methazole + napropamide, monuron, noruron (norea), oxadiazon, propyzamide + simazine, simazine, terbacil

[61] For further ethnobotanical research a questionnaire may prove useful and the following questions would be

Druse area in Syria or Northern Iraq (Arbil) and possibly in Turkey where the crop is cultivated for its grain. Ruminants fed with *V. narbonensis* in these areas represent a potential source of adapted rumen inoculum.

Selection of *V. narbonensis* (and members of section *Narbonensis*) for stronger stems, non-shattering and well developed pods, attractive seed colour, fungal, nematode, virus and aphid resistance needs to be carried out. The relative importance of outcrossing in different accessions needs to be determined.

Furthermore, the useful levels of predator and pathogen resistance already identified in some accessions should be further enhanced and studied in relation to grain palatability.

The development of phytosanitary cultivars, possibly with high levels of GEC, is also worthy of consideration for areas where particular pests are prevalent and where cropping can be integrated with ruminant production systems. Alternative economic benefits to be derived from the phytosanitary use of disease and pest resistant genotypes and other end-uses such as ruminant production need to be considered for cultivars which may be unpalatable to monogastric animals.

The existing global collection for *V. narbonensis* and its relatives needs to be expanded to provide a broader genetic basis for crop development. In particular, more *V. narbonensis* needs to be collected from the Western Mediterranean and North Africa (incl. Egypt), Turkey, Iraq, Iran, Afghanistan and Pakistan.

Other species of section *Narbonensis*, especially the widely distributed *Vicia johannis* and *Vicia serratifolia* are worthy of further study to provide information about their adaptation to specific edaphic and environmental conditions and possible development as cultivars in their own right.

Conclusions

V. narbonensis is already a cultivated species. Because of its drought- and cold-tolerance, high seed yields, and the ready ruminant production potential, cultivation of *Vicia narbonensis* is likely to expand into dry areas with alkaline soils where diversification of cereal cropping systems has not yet been achieved due to the lack of a suitable grain legume. It is important that markets for the grain are established. The selection of more palatable genotypes with lower γ-glutamyl-S-ethenyl cysteine levels is likely to provide access for the grain to monogastric feed markets. The historical evidence suggests that *V. narbonensis* is a niche crop of value for specific agricultural applications, its conversion into a broad acre crop is a challenge for the future.

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of interest, not only for the study of *V. narbonensis* and its relatives but in modified form it may also be used for other crops.

Questions:

1.) How is the grain used?

a.) What kind of processing (soaking, boiling, frying, pickling, fermentation) is applied to the grain before its is utilized?

b.) Can the grain be used for human consumption?

c.) How much of the grain can be safely fed to i.) sheep, ii.) cattle, iii.) goats, iv.) mules, v.) horses, vi.) donkeys, vii.) laying hens, chicken, viii.) pigeons, ix.) humans, x.) other?

2.) What other uses are a.) established b.) conceivable for *V. narbonensis* ?

3.)What is known about the relatives of *V. narbonensis* esp. *V. serratifolia* and *V. johannis* and their agricultural utilisation?

4. What is the local name for the crop?

5. Where did it come from?

6. Is it cultivated elsewhere?

etc.

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