THE SEARCH FOR A NOVEL TOXICANT IN *GASTROLOBIUM* (FABACEAE: MIRBELIEAE) SEED HISTORICALLY ASSOCIATED WITH TOXIC NATIVE FAUNA

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Summary

The decline and extinction of Australian mammals since the arrival of Europeans has been catastrophic. The reintroduction of mammals of conservation significance to areas of their former range is a valuable strategy to aid the future survival of these species. Unfortunately many of these programs have rapidly failed, with the principal cause often considered to be predation by the introduced red fox (*Vulpes vulpes*) and feral cat (*Felis silvestris catus*). Although baiting with poisoned meat baits will control foxes, feral cats are generally unwilling to consume a bait. This has seen recent reintroduction attempts fail as a result of cat predation, in some cases involving only a few individual animals.

A strategy to deal with this predation would be to provide the reintroduction animals with a chemical defense, making them toxic to the predating fox or cat. Such a chemical defense has been stated to have existed in the fauna of south-west Western Australia, with the toxicity of the bones of bronzewing pigeons (*Phaps spp.*) being a focal account. The source of this anecdotal toxicity is attributed to feeding on *Gastrolobium* plant material. Chapter 1 provides a discussion of failed reintroduction programs, how provision of a chemical defense to the reintroduced fauna could provide a strategy to deal with catastrophic predation, and provides an introduction to the *Gastrolobium* genus of plants.

Chapter 2 describes the search and discovery of over 30 historical accounts to support the 'verbal folklore', with the earliest being from 1840. Located accounts include records of rapid deaths in dogs and cats consuming bronzewing pigeons (*Phaps*

spp.), with particular mention made of toxic pigeon bones and *Gastrolobium* seed as the toxicant source. A study of the anecdotes suggests the seeds of species of *Gastrolobium* may contain toxicants additional to the reported fluoroacetate, being more rapidly toxic and with a propensity for skeletal retention.

As fluoroacetate was the only recognised toxicant in *Gastrolobium* seed, and is not known to be rapidly toxic as some anecdotes are reporting, *Gastrolobium* seed was analysed for the presence of additional toxicants. A review of the literature indicated alkaloids to be compounds which can be both skeletally retained and rapidly toxic. Since alkaloids had been reported from the leaves of species of *Gastrolobium*, a search was made for their presence in *Gastrolobium* seeds. The results of this search are presented in Chapter 3. The analysis of *Gastrolobium* seed found no alkaloids, with the putative alkaloids, being compounds responsive to the alkaloid reagent Dragendorff's, identified as phosphatidyl cholines.

The propensity for fluoride to be retained in skeletal material suggested the possibility that the seeds contain additional organo-fluorine compounds, which could help explain the historical anecdotes. Chapter 4 describes the search for these new fluorinated compounds and the discovery of the presence of many new fluorinated compounds within these seeds. It is expected that almost all of these new compounds, as evidenced by their ^{19}F NMR spectra, are new to science. The spectra observed from the petroleum spirit and chloroform extracts of the *Gastrolobium* seeds are suspected to be fluorinated fatty acids. The presence of ^{19}F NMR singlets and doublets, rather than triplets, suggests these compounds are new to science, as only ω-fluoro fatty acids, giving triplets, have been previously described from *Dichapetalum toxicarium*. None of the

fluorinated compounds present in the petroleum spirit and chloroform extracts could be identified to the compound level. In an examination of the morphological distribution of seed fluoride in *Gastrolobium* seeds, the overwhelming majority is in the seed cotyledons. When the entire seed is extracted, it is the methanol extract which contains the overwhelming majority of seed fluoride. This fluoride was believed to be from fluoroacetate, however an inability to isolate a single fluorinated fraction and fluctuations in fluoride levels in a variety of extraction processes, suggested the presence of mutliple fluorinated compounds. It was eventually discovered that the analysed *Gastrolobium* seeds also contain very labile fluoroacetylated sugars and cyclitols.

Chapter 5 presents the potential discovery of five new classes of fluoroacetylated sugars and cyclitols, comprising at least 30 individual compounds, from the cool methanol extract of the seeds. These sugars and cyclitols were found to have primarily a single monofluoroacetyl moiety, though the presence of difluoroacetyl groups is also described. It is still to be established whether these compounds are naturally occurring or entirely an artifact of the extraction and analysis process.

In parallel with seed analysis, three experiments were undertaken to examine both the retention of seed toxicants and the toxicity of milled seed and seed extracts. Chapter 6 describes the testing of the hypothesis that the reported toxicity of bronzewing pigeon bones may be due to the retention of fluoroacetate or its toxic degradation compound fluorocitrate. Using common bronzewing pigeons (*Phaps chalcoptera*) obtained in South Australia and dosed with fluoroacetate, an analysis of plasma citrate concentrations indicated this toxicant to be rapidly metabolised. The analysis of muscle and bone samples identified the presence of fluoroacetate, however the reporting of the bone

samples from the control birds as also possessing this compound, required these results to only be taken as an indication of the possible bone retention of fluoroacetate. An analysis for fluorocitrate in pigeon bones was unsuccessful.

A similar experiment to that presented in Chapter 6 was undertaken with a mammal model, utilising the laboratory rat, and is outlined in Chapter 7. Having indicated the presence in *Gastrolobium* seed of compounds responsive to the reagent Dragendorff's (recognised as the principal alkaloid-indicating reagent), seeds of *G. bilobum* from Quindanning were fed to treatment rats. The skeletons of the '20 day' dose group were extracted and analysed for the presence of both Dragendorff responsive and fluorinated compounds. The identification of the extracted Dragendorff responsive compounds in *G. bilobum* seed as innocuous phosphatidyl cholines (lecithins) may be an explanation for the Dragendorff responsive compounds extracted from the rat skeletons.

An examination of the toxicity and physiological effect of *Gastrolobium* seeds was undertaken in a pilot study using four domestic cats. Although a proposed replicated study utilising a significant number of cats wasn't approved, the pilot study described in Chapter 8 has provided support for the historical anecdotes. The cessation of respiration in Cat 02 in 82 minutes is similar to that reported. In addition, the *G. parviflorum* seed provided to this cat was found to contain the highest concentrations of fluoroacetylated sugars and cyclitols. The toxicity of these new fluorinated compounds is unknown but they may explain the rapid death of Cat 02. The survival of Cat 04, dosed with the chloroform extract of *G. parviflorum* seed, provides further support as this indicates that the seed methanol extract, which includes these fluoroacetylated sugars, is the location of the toxicants causing the rapid deaths.

Chapter 9 provides an overall discussion of the results obtained in this study in furthering our explanation of the historical anecdotes. Studies reporting the incorporation of fluoroacetylglucosamine into hyaluronic acid suggest the fluoroacetylated sugars identified in this study may behave in a similar way and thus may be the skeletally retained toxicants. Why these compounds would cause the rapid deaths is unknown but possible explanations are proposed. In addition, the implications of these new fluoroacetylated sugars as potential chemical defense compounds for reintroduced fauna is discussed with future areas of research and overall conclusions presented.

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DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying and being lodged as an electronic copy on the University web site.

Signed:

David Eric Peacock

Dated: 29th August 2003

Chapter 1: General Introduction

1.1 Catastrophic predation of reintroduced fauna

The principle of predator-prey interaction is a fundamental ecological concept. Inherent within this principle is the understanding that most of these interactions involve inter-related fluctuations between the numbers of both groups, generally resulting in a dynamic balance. One of the primary causes of instability in this fragile balance is the introduction of an alien species into a biological system. Such introductions have occurred on many occasions to Australia, as they have to other countries such as New Zealand. Two alien predators introduced to the Australian ecosystem are the red fox (*Vulpes vulpes*) and domestic feral cat (*Felis silvestris catus*). The red fox was deliberately released in Victoria in the 1860s for hunting (Rolls, 1969). The origin of the feral cat is believed to be from the European introduction of cats in the 1880's onwards (Abbott, 2002) with additional releases of cats occurring through southern Australia in the 19th century as an attempt at controlling vermin such as rabbits (Anon, 1905; Rolls, 1969).

Since European settlement of Australia the decline and extinction of Australian mammals has been catastrophic. Sixteen species within a critical weight range (CWR) (0.035-5.5 kg) (Burbidge and McKenzie, 1989) are considered to have become extinct since 1878 with many others endangered or threatened (Short and Smith, 1994). Predation by the red fox, and to a lesser extent the cat, has been significantly implicated in this decline and loss (Perry, 1973; Christensen, 1980).

The most conclusive evidence for the predatory impact of the red fox in the decline and extinction of Australian mammals comes with their removal and the subsequent

response of prey populations. The almost complete removal of fox predation through regular baiting programs using the poison sodium monofluoroacetate (1080), and the subsequent increase in population of the CWR species being protected, has been conclusively shown for species such as the black-flanked rock-wallaby, *Petrogale lateralis ssp. lateralis* (Kinnear *et al.*, 1988; Kinnear *et al.*, 1998), brush-tailed bettong, *Bettongia penicillata* (Kinnear, unpublished data, in Saunders *et al.*, 1995) and numbat, *Myrmecobius fasciatus* (Friend, 1990).

One strategy used to overcome the decline of threatened species is their reintroduction, often from islands, to other islands free of predators, and to areas of their former range on mainland Australia. The reintroduction of CWR mammals to mainland Australia generally involves reintroducing a species to a habitat inhabited by cats and with foxes being controlled by a 1080 baiting program. Current reintroduction programs attempt to minimise predation such that a sustainable population size can be achieved. With no control of the fox and cat, reintroduction programs would result in animals being released into a 'predator pit' (May, 1977) from which they would never escape. A limitation of current 1080 baiting programs targeting foxes is the apparent subsequent increase in feral cat abundance (Christensen and Burrows, 1995; Risbey and Calver, 1998). Although both primary and secondary poisoning has been used with varying success to control feral cats, primarily at times of prey shortage (Veitch, 1985; Alterio, 1996; Short *et al.*, 1997; Dowding *et al.*, 1999; Gillies and Pierce, 1999; Heyward and Norbury, 1999; Twyford *et al.*, 2000), the control of feral cats and their predation on declining or reintroduced populations is largely limited to shooting and trapping programs (eg. Burrows and Christensen, 1995) due to the general unwillingness of cats to

consume a poison meat bait. Recent research has been directed at trying to improve the consumption of 1080 baits by cats (Eason *et al.*, 1991; Eason *et al.*, 1992; Clapperton *et al.*, 1994).

The initial period of cohabitation by the cat and the reintroduced species appears to end with eventual recognition by the cat of the reintroduced species as a prey species - ie. establishment of a search image (Type III functional response – Holling, 1965). The size and naiveté of the reintroduced species cause it to be targeted as a profitable prey species. Lack of population size or the presence of neighbouring populations in the reintroduced species often prevents it overcoming this predatory impact through recruitment by births or immigration. This predatory impact is shown in the following examples:

- In September 1992, 40 burrowing bettongs, *Bettongia lesueur*, (19 male and 21 female) were translocated from Barrow Island, Western Australia inland to the Gibson Desert (Burrows and Christensen, 1995). These animals were released into an area largely free of foxes and dingoes (*Canis lupus dingo*), as a result of 1080 baiting. Unfortunately predation of the animals began almost immediately, with Burrows (1995) considering the loss of the bettongs to have resulted from two or possibly three cats discovering the colonial, warren inhabiting animals and then within 60 days predating all the reintroduced individuals.
- In conjunction with the burrowing bettong reintroduction discussed above, 40 golden bandicoots, *Isoodon auratus*, (18 male and 22 female) were also translocated to the Gibson Desert from Barrow Island (Christensen and Burrows, 1995). Released to the same area where foxes and dingoes had been largely removed by 1080 baiting, no

animals were known to be alive after 70 days. Although no carcasses were found, they were believed killed and eaten by cats.

- Two separate attempts were made to reintroduce the rufous hare-wallaby (*Lagorchestes hirsutus*) to the Tanami Desert, Northern Territory (Gibson *et al.*, 1994) and both suffered severe predation. In the first reintroduction in 1990-91, 31 animals were released with at least 13 animals (42%), excluding the associated pouch young, believed predated by feral cat(s). The removal of a large 5.1kg male cat, with hare-wallaby fur in its stomach, resulted in no further predation. The second reintroduction in 1991 of 25 animals, resulted in predation almost identical to the first release. Fourteen hare-wallabies (56%) were confirmed killed by feral cat(s). Again, eventual removal of a large 4.8kg male cat from the vicinity of the dead animals resulted in cessation of this predation.
- From September 1995-May 1996 a total of 102 greater stick-nest rats (*Leporillus conditor*), in 2 groups of 40 and 62 animals, were translocated to Venus Bay, South Australia from Reevesby Island and Monarto Fauna Park. These rats lasted less than 10 months and their predation was attributed, in an area largely free of foxes due to 1080 baiting, to cat predation. Of the 40 animals released first, 15 were radio-collared and alive after 7 months. Over 2-4 weeks after the release of the additional 62 rats, 14 radio-collared animals were found killed, with 1 rat recaptured and returned to Monarto. A further 8 weeks of monitoring found no sign of any rats. Although no carcasses were found, bite marks on collars indicated predation by feral cat(s).

- Between April 1994 and January 1995, 67 brush-tailed bettongs (28 male & 39 female) were also released to Venus Bay (Copley *et al.*, 1999). This program was proceeding well until the period November 1994 to March 1995, when 14 radio-collared brush-tailed bettongs (21% of reintroduced animals) were found killed. Previously unobserved tracks indicated a large cat had moved into the area and carcass remains suggested cat predation (Armstrong pers. com. 2000). Eventual trapping of the suspected cat, a large 5.75kg male, resulted in no additional predation being detected and an associated continued expansion in numbers and range by the bettongs.
- On New Zealand's Stewart Island, kakapo (*Strigops habroptilus*) mortality declined dramatically after the commencement of a cat control program, even with the continued presence of some cats. This was attributed to removal of the few cats which had learnt to predate kakapo (Clout and Craig, 1995; Powlesland *et al.*, 1995).

The above examples describe species of conservation significance being predated by only a limited number of individual cats. This predation has been termed 'catastrophic predation' to describe the apparent impact by often individual cats on reintroduction programs. This predatory impact involves most or all of a reintroduced population of animals being targeted and killed seemingly by only individual, or a few, cats. It is considered a variation of surplus killing as the reintroduced animals that have been killed mostly appear to have been eaten. Surplus killing, especially by foxes (eg. Lundie-Jenkins *et al.*, 1993), is outlined in the paper by Short *et al* (2002) and is generally recorded for individual predators where the killing was not to satisfy immediate

dietary requirements but simply due to the presence of the prey animals themselves. 'Catastrophic' also describes the impact this predation has on the reintroduction program and its successful outcomes.

In a review of 25 macropod reintroductions, Short et al. (1992, p. 200) found predation, primarily by foxes and cats, to be "the most consistent factor limiting the success of reintroductions". This is supported by Burbidge and McKenzie (1989, p. 186), who consider predation to have the most significant impact on species which are "geographically restricted or reduced in population size". Such a definition accurately describes the initial status of a reintroduced population of mammals. Sinclair et al. (1998) examined the reintroductions of 4 marsupial species and concluded that in the initial stages of reintroductions 'the predation should be reduced by at least 90% until a sufficient population has been established'.

Control of this predatory impact was the primary goal of this doctoral research. Was there a way to deal with these predators when current techniques have failed? Even when aware of the predation Burrows and Christensen (1995, pg. 40) state "despite our best efforts, we were unable to trap, poison or kill the culprits". The inability of applying conventional control techniques to remove those animals effecting the predation has also been observed in coyote predation on sheep. Conner et al. (1998) found only a low correlation between the number of coyotes removed and predation of sheep and lambs. They consider an inability to remove the 'problem coyote' to explain this lack of correlation, with the removal of large numbers of coyotes therefore suggested to have involved mainly removing animals that weren't killing sheep. Coyote predation and

attempted control thus parallels the situation in many of the reintroduction programs discussed above.

A solution is then to find a strategy to deal with "temporary specialists" (Aanes and Andersen, 1996, p. 1857) - 'temporary' because of the rapidly exhausted reintroduced population and 'specialist' due to the predatory impact often appearing to be the result of only one or two animals. The 90% reduction in predation proposed as necessary by Sinclair et al. (1998) to enable population growth in the reintroduction programs examined, may have been possible through removal of only a few individual predating animals (cats). The suggestion by Sinclair et al. (1998, p. 572) that "if the net rate of increase is highly negative - as it was for burrowing bettongs and brush-tailed phascogales - then near total removal [of predators] may be required" is not therefore necessarily the requirement. Near total removal of predation, not predators, may be what is required. A 90% reduction in predators does not therefore necessarily equate to a 90% reduction in predation. A strategy to deal with this catastrophic predation by temporary specialist predators who are often difficult to control, is the chemical intoxication of the reintroduced animals – giving the animals a chemical defense by making them toxic. This is a similar strategy to toxic collars to protect sheep from coyote predation, which also only targets the predating animal (Burns *et al.*, 1996), except the strategy proposed in this study involves intoxicating relatively small animals rather than use of a toxic device.

1.2 Chemical intoxication – a strategy against catastrophic predation

> "Accumulating toxic substances from the environment and incorporating them into body tissues is a familiar strategy for preventing predation in insects such as the Monarch butterfly"
> (Shier, 1994 pg. 205)

The use of chemicals as a defence mechanism is well recognised for many insects and a classic example of this is the monarch, or wanderer, butterfly *Danaus plexippus*. The monarch butterfly extracts cardiac glycosides from plants of the *Asclepias* genus during its larval stage, stores them as its chemical defence against predators and passes this chemical defence through metamorphosis to the adult butterflies (Holzinger and Wink, 1996). Predators have learnt to avoid these butterflies because of the toxicity of these cardiac glycosides. In the case of the blue jay, Brower (1968) has shown that eating only a single butterfly is enough to induce future avoidance of the butterflies. Meyer and O'Hagan (1992) provide no data but state the caterpillar moth, *Sindrus albimaculatus*, which feeds on *Dichapetalum cymosum*, can not only detoxify fluoroacetate, but also accumulate it (probably in vacuoles) for use as a defence against predation.

Mammals such as the skunk are recognised for possessing a chemical defense which can be used when the animal is threatened, and species of shrew to having the chemical defense of being distasteful and hence avoided as prey. However the chemical defense strategy being proposed in this study is more akin to the poison-dart frogs of Central America. Species such as *Phyllobates terribilis* ingest toxic alkaloids in their diet that provides the frogs with a chemical defense so toxic that eating a single animal means

certain death to an intolerant predating species. Could it be possible for the reintroduced mammals to be chemically intoxicated, such that being eaten would generally kill the predating cat or fox? Could a toxicant such as 1080 be found, or used, to which these introduced predators are exceedingly more sensitive than native predators? Could this toxicant be skeletally retained, thereby providing a more long-term and physiologically stable site of toxicant retention? A graphical model of this strategy is shown in Figure 1.1 using the naturally occurring toxicant 1080, the native brush-tailed bettong with its' high tolerance and the fox and cat predators with their extreme sensitivity to 1080.

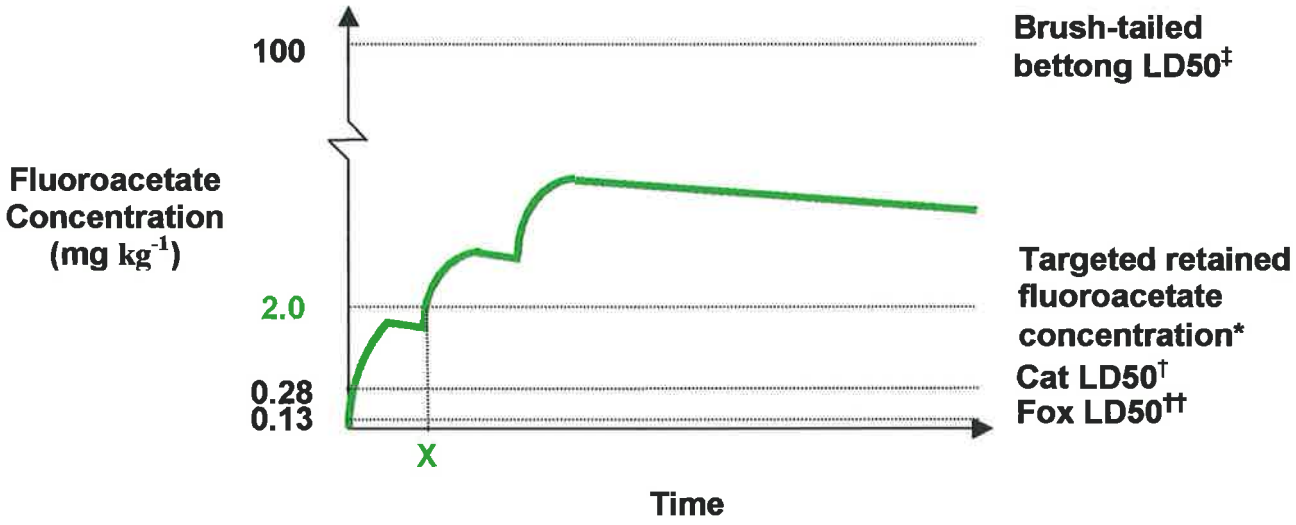


Figure 1.1 Proposed chemical defense using the brush-tailed bettong and fluoroacetate as a model. Bettong should be toxic at, and above, 2mg kg^{-1} fluoroacetate.

X = time to achieve targeted retained fluoroacetate concentration.

* 2mg fluoroacetate will kill most feral cats and foxes (assumes average cat weight 5kgs, average bettong weight 1.3kgs and minimal toxicant loss over time).

‡ Oliver *et al.* (1979)

† Eason and Frampton (1991)

†† McIlroy and King (1990)

This study therefore sought a toxicant targeted primarily at feral cats which answered the above questions. A skeletally retained toxicant, resulting from dietary intake, was proposed as a strategy that would significantly improve the survival of reintroduced CWR mammals. By killing the predating cat(s), or inducing a 'bait-shy' response in those predators that don't consume a lethal dose (eg. Morgan *et al.*, 1996), this strategy was proposed to halt any continuation or escalation of their predatory impact. The search for a toxicant focussed on the *Gastrolobium* genus of plants, with almost all species endemic to south-west Western Australia. This genus is known to be toxic due to the presence of fluoroacetate, and the survival of mammals in the south-west of Western Australia has been partially attributed to the presence of this toxic vegetation. Relict populations of the brush-tailed bettong, western quoll (*Dasyurus geoffroii*), numbat and red-tailed phascogale (*Phascogale calura*) survive in the *Gastrolobium* inhabited woodlands of south-west Western Australia, while they have disappeared from everywhere else in their range (Strahan, 1983). Christensen (1980) proposed that their survival may have been due to the secondary poisoning of predating foxes through eating animals which had been feeding on the toxic *Gastrolobium* vegetation. This poisoning is proposed to have resulted from the direct consumption of *Gastrolobium* vegetative material, or indirectly through consuming animals which had themselves fed on these plants.

1.3 *Gastrolobium* (Fabaceae: Mirbelieae) – a scourge of settlers and a wildlife resource

Being a legume of the pea family Fabaceae, *Gastrolobium* plants are considered a nutritious food source. They are prolific in their production of seed, which are generally 1-2mm long with a hard seed-coat and known to germinate in profusion after a hot fire. Direct feeding observations are restricted largely to bronzewing pigeons and seed, with one significant scat study and a few other more minor studies – see Table 1.1. Consumption of *Gastrolobium* plants, or animals which had themselves fed on these plants, is however generally assumed to have resulted in the tolerance disparity between the fauna of south-west Western Australia compared to species in eastern Australia, or those introduced to Australia (eg. King *et al.*, 1978; 1981; McIlroy, 1986; Twigg and King, 1991).

European settlement and its agricultural and pastoral expansion into south-west Western Australia during the last half of the nineteenth century and the first 1-2 decades of the twentieth century, was an era when the settlers were exceedingly involved with, and reliant upon, the natural environment. This resulted in the use of the native vegetation as stock feed and the native fauna as human and animal food. The European settlers of south-west Western Australia recognised as early as the late 1830's that toxic plants were present in the environment and were the cause of substantial stock deaths. The first colonial botanist, Mr James Drummond, was involved in numerous discussions, experiments and expeditions with the aim of trying to determine which plants were the cause of these deaths (Drummond, 1840a; b). These experiments included the drenching of sheep and goats with the suspected plants and observing the symptoms noted in

poisoned stock. As the years progressed and additional poisoning cases were recorded, the list of plants believed responsible for the stock deaths was compiled, with most of these plants described as members of the genus' *Gastrolobium* or *Oxylobium*. The 3 species, which appear to have caused most of the stock and domestic animal deaths, were York Road poison (*G. calycinum* Benth.), box poison (*G. parviflorum* (Benth.) Crisp) and heart-leaf poison (*G. bilobum* R.Br.). The significance of the poison plant issue is highlighted by the establishment of a board of inquiry, the report of which was presented to the Minister for Lands and was published in The Western Mail on November 25, 1911(Anon, 1911).

The species causing the significant stock losses were formerly described in the genus' *Gastrolobium* and *Oxylobium* (eg. Aplin, 1971b). The genus *Gastrolobium* has been recently reviewed and the revised genus now comprises 109 species, including these toxic *Oxylobium* species, with all but 2 of these species endemic to south-west Western Australia (Chandler *et al.*, 2002) – see Figure 1.2.

Table 1.1 Species recorded consuming fluoroacetate producing vegetation.

Species	*Gastrolobium* Vegetative Material	Sample Frequency	Literature Source
Common bronzewing pigeon (*Phaps chalcoptera*)	seeds	N/A	(Webb, 1885; Mann, 1906 citing a Mr E. R. Parker; Greig, 1907; Le Souëf, 1907; Anon, 1917; 1921; Foulds, 1921; Wansbrough and Anon, 1922; Serventy and Whittell, 1976; Johnstone and Storr, 1998); Twigg personal communication 2000).
Brush bronzewing pigeon (*Phaps elegans*)			
Western grey kangaroo (*Macropus fuliginosus*)	*G. bilobum* leaves	67% scats	Shepherd *et al.* (1997)
	G. spinosum leaves	N/A	Mead *et al.* (1985)
Black-gloved wallaby (*Macropus irma*)	*G. bilobum* leaves	43% scats	Shepherd *et al.* (1997)
Tammar wallaby (*Macropus eugenii*)	*G. bilobum* leaves	50% scats	Shepherd *et al.* (1997)
Brush-tailed possum (*Trichosurus vulpecula*)	*G. bilobum* leaves	40% scats	Shepherd *et al.* (1997)
	G. grandiflorum leaves	5.7% of leaf fragments in scats at single Uluru site	J. Foulkes pers. com. (2003)
Bag moths (*Ochrogaster lunifer*)	*G. microcarpum* leaves	N/A	Twigg (1994)
Seed weevils (Bruchidae)	seeds	N/A	Twigg (1986)
Seed harvesting ants	seeds	N/A	Twigg *et al.* (1983)

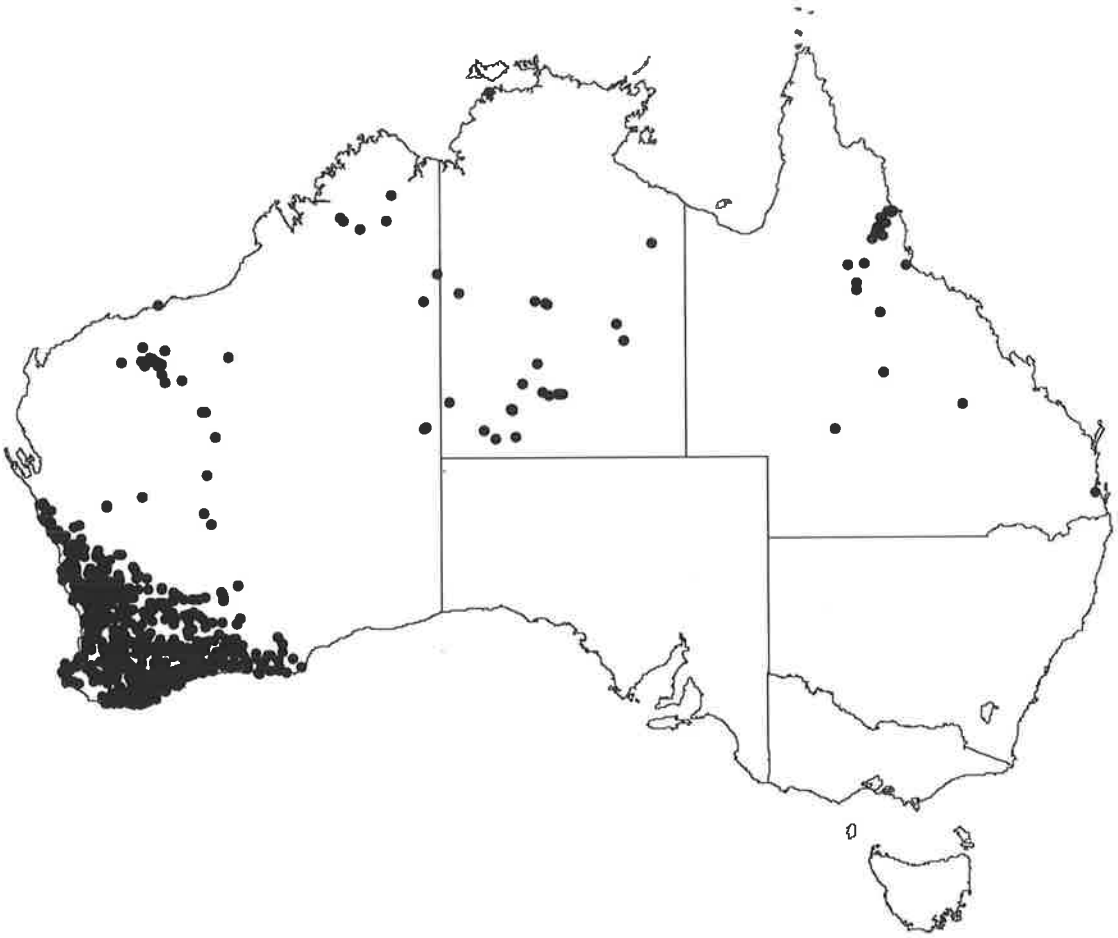


Figure 1.2 Distribution of *Gastrolobium sens. lat.*
(From the data of Chandler *et al.*, 2002)

1.4 *Gastrolobium* toxicity

The discovery of MFA (monofluoroacetic acid or monofluoroacetate) in the seeds of *Acacia georginae* (Oelrichs and McEwan, 1962) and the leaves of *G. grandiflorum* (McEwan, 1964) served to focus the subsequent analysis of *Gastrolobium* toxicity on the presence of MFA. Of the 109 species in the revised *Gastrolobium* genus, Gardner and Bennets (1956) state 38 of these species to be toxic to stock. Of these, 30 species have been shown to contain quantifiable concentrations of MFA, with the other 8 either yet to be tested, providing only a 'positive result', or providing a negative result but still considered to be toxic species (Gardner, 1964; Aplin, 1971a; b; Twigg *et al.*, 1996a; b; Chandler *et al.*, 2002). In addition, *Gastrolobium grandiflorum* F.Muell. from northern Australia (McEwan, 1964) and *Gastrolobium brevipes* Crisp from central Australia (Twigg *et al.*, 1999) are also considered to be toxic. The toxicity of all these species is generally attributed to the presence of an organofluorine compound, interchangeably described as fluoroacetic acid (eg. Mead and Segal, 1972), monofluoroacetic acid (eg. Aplin, 1967), fluoroacetate (eg. Twigg *et al.*, 1999) or sodium monofluoroacetate (eg. Chandler *et al.*, 2001). In most cases, the plant tissue examined for toxicity was leaf tissue and analysis was specifically directed at qualifying and quantifying the presence of MFA. These results are presented in Table 1.2, together with the results for *Acacia georginae*, the other Australian species known to contain toxic concentrations of fluoroacetate.

The large majority of *Gastrolobium* fluoroacetate analyses are attributed to the botanist T. E. H. Aplin from a series of papers beginning in 1967 (Aplin, 1967), with the summary paper published in 1971 (Aplin, 1971b) most regularly cited. In reviewing the

data attributed to T. E. H. Aplin during this study, it was discovered that an analytical method is never described. In the first paper in the series no method is reported for the analysis of *Gastrolobium* leaves, however the paper states: "Chemical analyses conducted by the Government Chemical Laboratories" (Aplin, 1967 p. 51). An enquiry to the Western Australian Government Chemical Laboratories sourced the archival records relating to these analyses. It was established that the following analytical process determined the *Gastrolobium* fluoroacetate concentrations stated by Aplin. *Gastrolobium* leaves were extracted using xylene and analysed by a rapid screening test (Ramsey and Clifford, 1949; Ramsey and Patterson, 1951). This is a colour intensity test producing thioindigo and the assumption was made using this analysis that the only mono-halogen-acetate present was monofluoroacetate. Results of these analyses were reviewed and confirmed by gas chromatographic and infra-red absorption spectroscopy as described by Oelrichs and McEwan (1962).

The thioindigo method was also used to obtain a result of at least 6500ppm fluoroacetate for the seeds of *G. bilobum* from Araluen, Western Australia (Mead, *pers. com.*). The interference of short-chain fatty acids, also present in plant tissue, to this method is described in the associated doctoral research (Mead, 1980).

Later analyses have used ^{19}F nuclear magnetic resonance (NMR) (Baron *et al.*, 1987) or gas-chromatography mass-spectrometry (Twigg *et al.*, 1996a; b; Twigg *et al.*, 1999) to confirm the presence of fluoroacetate in some of the species analysed in the earlier work and also in some newly analysed species.

The discovery and quantification of fluoroacetate, often at extremely high concentrations, was used to explain the toxicity of these plants. However upon review of

the historical anecdotes toxicants additional to MFA seem to be indicated. This possibility was supported by other literature as shown below.

Early chemical analyses proposed alkaloids as the *Gastrolobium* toxicants, prior to awareness of any organo-fluorine compounds. Mann and Ince (1905; 1906) analysed the leaves of the 2 principal toxic species, York Road poison (*G. calycinum*) and box poison (*G. parviflorum*) and described 2 new alkaloids, respectively cygnine and lobine. Upon testing, these alkaloids caused death in guines pigs and sheep very quickly (details given in Chapter 3). Thus the original explanation for the toxicity of *Gastrolobium* species was consistent with the presence of these, or similar, alkaloids. Permanganate of potash was proposed as a remedy for stock poisoning by authors of the study above, and the reported success of this remedy (eg. Anon, 1920) supported the toxic agent being an organic compound. Subsequent research states significant concentrations of alkaloids, suggested by indicator reaction precipitate, in leaves of wallflower poison (*G. grandiflorum*) (Webb, 1949) and the identification of 4 alkaloids in rock poison (*G. callistachys*) leaves (Cannon and Williams, 1982).

Even after the discovery of fluoroacetate in *Gastrolobium*, research has suggested other organo-fluorine compounds as likely additional toxicants:

- An indirect analysis of *Gastrolobium* vegetative material was undertaken by Hall (1972). In this study, although Hall was anticipating the presence of organic fluorine in the form of fluoroacetate, a different analytical method was undertaken to that used previously and in subsequent studies. Rather than using a technique specifically designed at qualifying and quantifying the presence of fluoroacetate, Hall used different solvents in conjunction with alkali-fusion and a fluoride selective electrode. One of the

resultant observations was the presence of organic fluorine in different solvents with a subsequent conclusion being the possible "... presence of a fluoro-carbohydrate or a fluorinated amino acid" (Hall, 1972 p. 863).

- Analysing *G. parviflorum* seed using ^{19}F NMR, Rogers (1984) reports the presence of a weak triplet in addition to that of fluoroacetate. However this finding could not be replicated in another sample of *G. parviflorum* seed and was omitted from the resultant paper (Baron *et al.*, 1987).

- *Acacia georginae* is recognised as a species which can be toxic due to the production of fluoroacetate (Oelrichs and McEwan, 1961), however 'fluoroacetate and other fluorinated organic compounds appear to be present in the waxy exudate on the surface of the toxic leaves' (Hall, 1972, p. 855). Peters and Shorthouse (1971) identified fluoroacetone, but indicated this would not explain all the observed loss of fluorine from the leaves, with other volatile fluorine compounds probably present.

- The presence of ω-fluoro fatty acids in the seeds of *Dichapetalum toxicarium* (Peters and Hall, 1960; Hamilton and Harper, 1997), a genus in which other species contain fluoroacetate (O'Hagan *et al.*, 1993), suggests fluorinated fatty acids could also be present in *Gastrolobium* seeds.

This literature therefore appears to suggest that species of *Gastrolobium* contain either MFA, or toxic alkaloids or both, with other organo-fluorine compounds of unknown toxicity also possible. 'Verbal folklore' records species of wildlife in south-west Western Australia as having been toxic. Some of this folklore even stated the bronzewing pigeons (see Figure 1.3) in this region as possessing toxic bones. In the search for a toxicant able to provide a chemical defense to reintroduced native fauna, an

investigation was made of this folklore, searching for historical literature that would substantiate and describe this phenomenon. It is the search for and anlaysis of this historical literature which forms the basis of Chapter 2.

Table 1.2 Australian Plants Stated to Produce Significant Concentrations of Fluoroacetate

Scientific Name	Common Name	Fluoroacetate Content (mg/kg)	Distribution	Literature Source
Acacia georginae	Gidyea	Leaves - 25 Leaves - 10-40 young immature seed – 400	Central Australia	Oelrichs and McEwan (1961) Oelrichs and McEwan (1962)
Gastrolobium appressum	Scale-leaf Poison	"Confirmed toxic species"	SW Western Australia	Gardner (1964)
Gastrolobium bennettsianum	Cluster Poison	1300	SW Western Australia	Aplin (1971b)
Gastrolobium bilobum	Heart-leaved Poison	Seed from Araluen, near Perth – at least 6500 2650 Leaf – 190 ± 20 Seeds – 440 ± 40 Mature leaves – 65.33 ± 54.83 Young leaves – 329.6 ± 275.21 Flowers – 433.33 ± 341.01	SW Western Australia	R. Mead pers. com. 2001 Aplin (1971b) Baron *et al.* (1987) Twigg *et al.* (1996a)
Gastrolobium brevipes		Leaves – 52.9 ± 34.7 Pods – 132.4 ± 52.7	Central Australia	Twigg *et al.* (1999)
Gastrolobium brownii		80-260	SW Western Australia	Chandler *et al.* (2002)
Gastrolobium callistachys	Rock Poison	100-1000	SW Western Australia	Aplin (1971b)
Gastrolobium calycinum	York Road Poison	400 Mature leaves - 7 ± 4 Young leaves - 1401 ± 348 Flowers – 1548.5 ± 203.5	SW Western Australia	Aplin (1971b) Twigg *et al.* (1996a)
Gastrolobium crassifolium	Thick-leaved Poison	150	SW Western Australia	Aplin (1971b)
Gastrolobium crispatum		<20*	SW Western Australia	Chandler *et al.* (2002)
Gastrolobium cuneatum	River Poison	1200	SW Western Australia	Aplin (1971b)
Gastrolobium densifolium	Mallet Poison	0 - but considered toxic	SW Western Australia	Aplin (1971b)
Gastrolobium floribundum	Wodjil Poison	1350	SW Western Australia	Aplin (1971b)
Gastrolobium glaucum	Spike Poison	200	SW Western Australia	Aplin (1971b)
Gastrolobium grandiflorum	Wallflower Poison	185	Northern Australia	McEwan (1964)
Gastrolobium graniticum	Granite Poison	Flowers – 1240 Leaf - 900	SW Western Australia	Aplin (1971b)
Gastrolobium hamulosum	Hook-point Poison	100	SW Western Australia	Aplin (1971b)
Gastrolobium heterophyllum	Slender Poison	0 - but considered toxic	SW Western Australia	Aplin (1971b)
Gastrolobium laytonii	Kite-leaved Poison	500	SW Western Australia	Aplin (1971b)

* Chandler (2002) states this result from the Western Australian Department of Mines Chemistry Centre; no method described.

Scientific Name	Common Name	Fluoroacetate Content (mg/kg)	Distribution	Literature Source
Gastrolobium microcarpum	Sandplain Poison	0-600 Leaf – 180 ± 20	SW Western Australia	Aplin (1971b) Baron *et al.* (1987)
Gastrolobium ovalifolium	Runner Poison	0 - but considered toxic	SW Western Australia	Aplin (1971b)
Gastrolobium oxylobioides	Champion Bay Poison	0-1050	SW Western Australia	Aplin (1971b)
Gastrolobium parviflorum	Box Poison	100-2500 Seeds – 400 ± 40	SW Western Australia	Aplin (1971b) Baron *et al.* (1987)
Gastrolobium parvifolium	Berry Poison	300	SW Western Australia	Aplin (1971b)
Gastrolobium polystachyum	Horned Poison	0 - but considered toxic Leaf – 115 ± 10	SW Western Australia	Aplin (1971b) Baron *et al.* (1987)
Gastrolobium propinquum	Hutt River Poison	0 - but considered toxic	SW Western Australia	Aplin (1971b)
Gastrolobium pycnostachyum	Round-leaved Poison	175	SW Western Australia	Aplin (1971b)
Gastrolobium racemosum	Net-leaved Poison	1500 Seeds – 2990 ± 160	SW Western Australia	Aplin (1971b) Baron *et al.* (1987)
Gastrolobium rigidum	Rigid-leaf Poison	+ve – no quantitative result; considered toxic	SW Western Australia	Aplin (1971b)
Gastrolobium rotundifolium	Gilbernine Poison	150	SW Western Australia	Aplin (1971b)
Gastrolobium spathulatum (was *Nemcia spathulata*)		Young leaves – 40 and 80	SW Western Australia	Twigg *et al.* (1996b)
Gastrolobium spectabile	Roe's Poison	400	SW Western Australia	Aplin (1971a)
Gastrolobium spinosum	Prickly Poison	0-400 Mature leaves – 0.42 ± 0.15 Young leaves – 0.4 ± 0.2 Flowers – 1.15 ± 0.85	SW Western Australia	Aplin (1971b) Twigg *et al.* (1996a)
Gastrolobium stenophyllum	Narrow-leaved Poison	90 Seeds – 3470 ± 180	SW Western Australia	Aplin (1971b) Baron *et al.* (1987)
Gastrolobium tetragonophyllum	Brother-brother	750 Seeds – 2370 ± 120	SW Western Australia	Aplin (1971b) Baron *et al.* (1987)
Gastrolobium tomentosum	Woolly Poison	+ve – no quantitative result; considered toxic	SW Western Australia	Aplin (1971b)
Gastrolobium trilobum	Bullock Poison	0 - but considered toxic	SW Western Australia	Aplin (1971b)
Gastrolobium velutinum	Stirling Range Poison	300	SW Western Australia	Aplin (1971b)
Gastrolobium villosum	Crinkle-leaved Poison	Seeds – 310 ± 30 Mature leaves - 4 Young leaves - 48 Flowers – 74	SW Western Australia	Baron *et al.* (1987) Twigg *et al.* (1996a)

Figure 1.3 Common bronzewing pigeon, *Phaps chalcoptera*, Latham 1790 (G. Chapman; Readers Digest Complete Book of Australian Birds, 1986). A large pigeon reaching at least 420g (this study), recorded as being in great numbers (Anon, 1912; 1921) and accumulating in great numbers when drinking (200 drinking at soak - cited in Johnstone and Storr, 1998). They would therefore have been an abundant and relatively easily obtained human food source (eg. Anon, 1917).

Chapter 2: Toxic Native Fauna – A Historical Perspective

2.1 Introduction

Chemical intoxication of reintroduced native fauna is considered to be a potential tool to help alleviate the catastrophic predation of this fauna. The hypothesis is that with suitable toxicants the animals would thereby become toxic to any predating cat or fox, and in so doing prevent these predators from becoming catastrophic predators. The search for a toxicant which behaves in this way focussed on the 'verbal folklore' from south-west Western Australia, which reported that the wildlife in this area caused the death of domestic cats and dogs.

A search was made for historical accounts regarding the toxicity of the wildlife of south-west Western Australia in order to substantiate the 'verbal folklore' and also to provide an insight into details of the poisoning occurrences. It was anticipated that these details would guide the search for intoxicating chemical(s).

2.2 Methods

All identified historical accounts of the toxicity of the wildlife of south-west Western Australia, particularly accounts about the toxicity of the bones of the bronzewing pigeons, were sourced. Sourcing the original material cited in a document enabled this material to be located and resulted in the discovery of the correspondence of Mr William Catton Grasby, a columnist for "The Western Mail" newspaper from 1906 to 1927, with farmers of that period. The Battye Library in Perth, Western Australia, possesses a file of this correspondence (call number MN 139) as well as microfilms of "The Western Mail". These microfilms were searched from 11th February 1905 to 28th December 1933 (microfilm reels # 46-123), covering the period 3rd February 1906 to 30th October 1930 when Mr Grasby edited a column variously entitled 'Our Mutual Help', the primary source of poisoning accounts. The 1923-1933 decade of this weekly newspaper contains accounts of stock poisoning with numerous samples of toxic *Gastrolobium* plants submitted for identification. No additional reference is made to toxic native wildlife, with instead the current presence and decline of the small mammals, and the arrival of the rabbit and fox issues of more pressing concern. It is suspected the wildlife was now no longer a primary food source or agricultural concern, and much of the *Gastrolobium* vegetation was now cleared, with its' toxicity and management now well established.

Additional microfilms searched were:

- "The Western Mail" newspaper's earliest issue, from 19th December 1885 to Saturday January 30th 1886 (microfilm reel # 1);

- "The Albany Advertiser" newspaper's earliest issue, from 20st February 1897 to April 1897 (microfilm reel # 1);
- "The Albany Mail & King George's Sound Advertiser" newspaper's earliest issue, from Monday 1st January 1883 to 21st August 1883 (microfilm reel # 1);
- "The West Australian" newspaper, issue Monday April 2nd, 1906 (microfilm reel # 117);
- "The Great Southern Herald" newspaper's earliest issue, from 5th October 1901 to 16th November 1901 (microfilm reel # 1).

2.3 Results

The literature which discusses the toxicity of the wildlife of south-west Western Australia can be approximately divided into the 'modern' and the 'historical'. The 'modern' literature encompasses papers such as Per Christensen's 'A sad day for native fauna' (Christensen, 1980), one of the first to suggest that the mammals feeding on *Gastrolobium* plants may be secondarily poisoning predating foxes. Others discuss the toxicity of the bronzewing pigeons to cats and dogs, citing verbal or other published accounts (Whittell, 1938; 1942; Dumbacher and Pruett-Jones, 1996; Higgins and Davies, 1996) or present the toxicity of the pigeons as established knowledge (Serventy and Whittell, 1976). The books 'Ambition's Fire. The Agricultural Colonisation of Pre-Convict Western Australia' (Cameron, 1981) and 'The Drummonds of Hawthornden' (Erickson, 1969) both contain comprehensive discussions of the toxicity of the *Gastrolobium* in colonial Western Australia. Erickson also mentions the toxicity of the bronzewing pigeon bones as an observation of the first colonial botanist, Mr James Drummond. Sourcing the article of Drummond (1840, p. 2) determined that the account is of toxic bronzewing pigeon "stomachs and guts", rather than bones. Toxic bones were apparently mentioned by "two turn of the century farmers of the Cranbrook district" (Erickson *pers. com.* 1999).

'Modern' literature occasionally states the toxicity of native vertebrates in south-west Western Australia and when stated it is for the bronzewing pigeons *Phaps chalcoptera* and *P. elegans* (eg. Serventy and Whittell, 1976). However, these 'modern' accounts appear to derive from other less modern accounts. For example, the records of Whittell (1942) and Serventy and Whittell (1976) of bronzewing pigeon entrails and bones poisoning dogs and

cats appear derived from Herbert (1921, p. 11), summarising observations collected by Mr. W. C. Grasby, of the "Western Mail".

Although the Battye Library in Perth possesses numerous diaries and correspondence of Mr. W. C. Grasby, none of the original correspondence he received regarding *Gastrolobium* toxicity exists in this collection. These may have been lost in a house (being a "hessian and wood humpy") fire after the death of Mr Grasby in 1930 (John Grasby *pers. com.* 2001). The micofilms of "The Western Mail" do however contain many issues with published letters discussing the authors' accounts of wildlife and *Gastrolobium* related poisonings. These accounts are largely first hand observations of *Gastrolobium* and/or wildlife toxicity and are summarised in Table 2.1. The geographical locations of these anecdotes are presented in Table 2.2, with a map of their nearest modern locations shown in Figure 2.1.

Table 2.1 Historical anecdotes regarding toxic native animals and related issues

Source	Anecdote Date	Anecdote Title	Topic	Author and Location
The Perth Gazette and Western Australian Journal	1840	Report on the botanical productions of the country from York District to King George's Sound	State many believe bronzewing pigeons to feed on the seeds of the unidentified leguminous shrub, from a grove at King George's Sound, and the pigeons' stomachs and guts cause dogs to die with symptoms similar to dogs that feed on poisoned sheep	Mr J. Drummond, Perth
The Albany Mail and King George's Sound Advertiser	July 28, 1885	'Untitled letter'	Cooked bronezewing pigeon; dog ate breastbone and within 10 minutes was running about and yelping and acting poisoned; dog made to vomit, but went mad and died within another 15 minutes; toxicity believed due to local pigeons eating almost entirely *G. bilobum* seed	Webb, W., Albany
Journal of Agriculture (W. A.) vol. 13(1): 486-490	1906	Examination of the Western Australian poison plants	Cites a Mr E. R. Parker as stating that cats and dogs have been killed by the meat of pigeons which have been feeding on the seeds of box poison	E. A. Mann
The Western Mail	September 1, 1906	To poison boodies and parrots	States boodies bury wheat and poison seed; observed approximately 30 York Road seedlings in one place from this caching	H. J. McD., Narrogin
Wild Life in Australia	1907	Western Australia	Cites WA sportsman whose retriever ate the insides, full of York Road poison seeds, of shot bronzewing pigeon and was dead within 20 minutes; also heard of several cases of people being poisoned by eating the seeds	W. H. Dudley Le Souëf, Melbourne Zoological Gardens
The Western Mail	September 28, 1907	Is opossum meat injurious to dogs	Fed dog on cleaned and cooked opossum bodies for about a fortnight before the dog had a fit of madness and died	"Enquirer", Wickepin Road, Pingelly

Source	Anecdote Date	Anecdote Title	Topic	Author and Location
The Western Mail	October 5, 1907	Opossum meat killing dog and cat	Opossum shot in country with box poison in flower was gutted, skinned and cooked; hindquarters fed to sheep dog pup and forequarters to the cat at about 7pm; at about 9pm the cat ran away meowing loudly and disappeared; the dog soon afterwards began yelping and straining at its lead, went mad and soon died	C. G. C.
The Western Mail	October 19, 1907	York Road poison and opossums	Dog poisoned by eating a little opossum insides; 4 cats poisoned by eating 1 boody, plus several other cases; cats poisoned by bronzewing pigeons, which live largely on box and heart-leaf seeds; sheep dog poisoned from eating sheep poisoned by York Road, heart-leaf or both; dog licked up kangaroo blood, soon acted poisoned, but vomited the blood and survived; known dogs to feed on opossums for weeks, but eventually they get a poisoned one	Mr Jas. A. Greig, Williams
The Western Mail	October 19, 1907	Opossum meat poisoning dogs	Opossums in poison country will poison dogs; opossums eat little box or York Road foliage, mainly seeds; known dogs poisoned from eating sheep poisoned by box or York Road plants	Mr F. C. Clinch, Burnerbinma Station, via Yalgoo
The Western Mail	October 26, 1907	Opossum meat and dogs in poison country	Never heard of poisonous opossum meat; states aborigines and their dogs to mostly live on opossum meat	Mr J. Bert, Taylor, Kulyaling
The Western Mail	November 2, 1907	Opossums, poison and dogs	Killed opossums in York Road, box and berry poison country and never lost a dog; known dogs go mad and die from kangaroo rat insides	"Ex-Nor-Wester", Pingelly
The Western Mail	November 23, 1907	Opossum meat and dogs	While at Brookton and Pingelly in 1906, kangaroo dogs went mad and died from opossum entrails	"Question", York
The Western Mail	December 14, 1907	Opossum poisoning	Considers only the opossum's nails and insides to be poisonous to dogs	"Opossum Snapper", Tarwonga Siding

Source	Anecdote Date	Anecdote Title	Topic	Author and Location
The Western Mail	February 6, 1909	Salt for a poisoned dog	Cured staghound dog which had eaten boodie or opossum which had been feeding on York Road poison; gave liberal amount of dry salt also a piece of bluestone; cleaned the dog out but don't let the dog have water for 6 hours or it will kill him	Mr J. Cairns, Glencairn, near Barton, G. S. R.
The Western Mail	August 27, 1910	Box poison	States sheep which eat box poison will run until they die and if a dog eats some of the carcass it will also go mad and will bite anything	W. H. H. Brown, Arthur River
The Western Mail	March 16, 1912	Mysterious death of cats	Questions whether bronzewing pigeons eating poisonous berries in summer could be poisonous to cats	"Settler's Wife"
The Western Mail	March 23, 1912	Death of cats	Old timers around Albany consider bronzewing pigeon bones, that is bone marrow, to be poisonous	"Flywheel", Albany
The Western Mail	April 6, 1912	Cats, pigeons and poison plant seeds	3 cats dead in <1hr from bronzewing pigeon insides when poison seeds are ripe	W. C. Knight, Lilydale, Greenbushes
The Western Mail	April 20, 1912	Cats and dogs killed by eating bones of wild pigeons	In Broome lost cat then dog on separate days to scraps which included bronzewing pigeon and parrot bones	H. D. R., Talgomine, via Merredin
The Western Mail	May 11, 1912	Bronzewing pigeons and poison plants	Bronzewing pigeons are numerous in bush and often killed by flying into wires and their toxicity, agreed with by the above 3 replies/anecdotes, is accepted as an explanation of her earlier question	"Settler's Wife"
The Western Mail	June 1, 1917	Poison bronzewing pigeons	Heard many times of someone losing a cat or dog from eating bronzewing pigeon bones. March 1916 shot pigeons at waterhole and removed skin, feathers and crop before returning. Stewed birds and threw out bones which dog ate and died before nightfall.	"Bronzewing"

Source	Anecdote Date	Anecdote Title	Topic	Author and Location
The Western Mail	June 15, 1917	Bronzewing pigeons and poison	Around 50 years ago ate lots of common bronzewing pigeons, but burnt bones, insides and crop; bronzewings very fond of box poison seed; dogs and cats would die from the insides, not meat, of kangaroo rats; often seen dogs go mad and die from the insides of kangaroo rats; dogs often found dead in pools of water	"Ex-Nor-Wester", Brookton
The Western Mail	June 17, 1920	Poison plants and antidote	8 years of experience with narrow-leaf poison; gives Condy's in preference to the prepared tablets; no success with antidotes on sheep but given early (half tablespoon Condy's in a bottle of water) never lost a horse; must be given early as after digestion any fluid only hastens death	"Overcharged"
The Western Mail	June 2, 1921	Kangaroos, pigeons and poison	Heard kangaroo insides will send dog mad and bronzewing pigeon bones will kill dogs; has often seen dogs and chickens eat the insides of kangaroos without any ill effect	Arthur Foulds, Katanning
The Western Mail	June 7, 1921	Poison plants, pigeons and cats	Used to mainly eat tammar wallabies, finding poison leaves in their stomach. Animals and chickens eating the insides were fine. State that for years they were unable to keep a cat. Always in late summer when the poison seeds were ripe the cats would become sick, vomiting up bronzewing pigeon feathers before they died. They observed no loss of cats during the rest of the year. They were able to save their cats if they gave the antidote (presumably permanganate of potash) with the first symptoms appearing. "Sometimes the cat simply disappeared. Once we found two, one morning, dead outside the house with pigeon feathers nearby." … "We ate the pigeons ourselves, being careful to burn bones and insides, and never lost a dog. But the cats were nightly hunters and at that time the thick timber around harboured the bronzewings in great numbers."	C. H., Kojonup

Source	Anecdote Date	Anecdote Title	Topic	Author and Location
The Western Mail	June 16, 1921	Kangaroo eating poison	Around 1901 was grubbing York Road poison on 'Yattah Block', a few miles out of Chidlow Wells; little pet kangaroo would eat York Road without any ill effect; kitten ate kangaroo meat cooked and raw without any ill effect; sheep in district were eating the poison bush and dying	"Pet Kangaroo", Three Springs
The Western Mail	January 19, 1922	Why did my dogs die?	Three kangaroo dogs well fed with kangaroo meat, mostly cooked, some raw, went mad, ran around and then into the bush, or was shot	J. T., Corrigin
The Western Mail	February 2, 1922	Possible cause of death of dogs	Has seen dogs go mad and run away, believed due to them eating sheep poisoned by box poison or York Road poison	G. D. T., Bunbury
The Western Mail	February 2, 1922	Dead dogs	States "it is well known that dogs and cats go mad and run about after eating the inside of pigeons and kangaroos that had eaten poison plants"	"Economic", Dudinnin
The Western Mail	February 2, 1922	Dogs dying from eating kangaroo	Believes the toxicity of kangaroo and sheep meat is due to the blood containing the poison from the poison plants, with meat from kangaroos and sheep bled immediately safe for dogs. Lost 2 dogs, which ran around, began barking and went mad from eating the flesh of a poisoned sheep. States it may take 7-8 hours for a dog to die; dogs and sheep can be saved by bleeding (by cutting the ears or, if that doesn't bleed freely, from just above the eyes); dogs and cats can be poisoned by the insides of native animals which eat poison, particularly when the plants are flowering and seeding; cattle die more quickly than horses	Mr Tom Coff, Perth, formerly of Nyabing
The Western Mail	February 9, 1922	Poison plants, marsupials and dogs	Lost dog with symptoms same as "J. T.", believed due to dog eating brush bronzewing pigeon which had been feeding on box poison seeds; knew of kangaroo dog poisoned by an opossum which had been feeding on box poison flowers	J. J. Wansborough, Taylor's Well

Source	Anecdote Date	Anecdote Title	Topic	Author and Location
The Western Mail	February 9, 1922	Poison plants, marsupials and dogs	Dog began howling and running around in a large circle before it ran off and died; believed due to dog eating opossum insides; also heard of fox-terrier dying from eating a sub-adult bronzewing pigeon	"Border Leicester"
The Western Mail	January 12, 1933	A poisoned dog	Believed dog died from eating a rabbit which had been killed by eating narrow-leaf poison. Died morning after eating rabbit, with about 3 hours from first symptoms to death.	"A. A.", Katanning

Table 2.2 Coordinates of toxicity related anecdotes where a geographical location is stated

Anecdote Theme	*Gastrolobium* species if stated	Anecdote Localities*	Latitude†	Longitude†
Toxic bronzewing bones	*bilobum*	Albany	35° 01' S	117° 53' E
Toxic bronzewing bones/bone marrow		**Albany**	35° 01' S	117° 53' E
Toxic bronzewing insides		'Lilydale', Greenbushes	33° 42' S	115° 48' E
Toxic bronzewing & parrot bones		**Broome**	17° 57' S	122° 14' E
Toxic possum guts and nails		Tarwonga Siding (now Culbin)	33° 11' S	116° 56' E
Toxic possum guts		**Brookton**	32° 22' S	117° 02' E
Toxic possum guts		**Pingelly**	32° 32' S	117° 05' E
Toxic kangaroo rat guts		Pingelly	32° 32' S	117° 05' E
Not heard of toxic possum meat		Kulyaling	32° 27' S	117° 03' E
Toxic possums	*parviflorum* and *calycinum*	'Burnerbinma Station', via Yalgoo	28° 47' S	117° 21' E
Toxic possum, boodie and bronzewing pigeons	*bilobum*, *parviflorum* and *calycinum*	Williams	33° 00' S	116° 53' E
Toxic possums		Pingelly	32° 32' S	117° 05' E
Toxic sheep carcasses	*parviflorum*	Arthur River	33° 20' S	117° 02' E
Toxic possum or boodie	*calycinum*	'Glencairn', near Barton (now Piesseville)	33° 12' S	117° 17' E
Non-toxic kangaroo meat and poisoned sheep	*calycinum*	**Chidlow Wells (now Chidlow)**	31° 52' S	116° 15' E
Toxic bronzewing bones and kangaroo guts		Katanning	33° 41' S	117° 33' E
Toxic bronzewings		Kojonup	33° 50' S	117° 09' E
Boodies caching seed	*calycinum*	Narrogin	32° 56' S	117° 10' E
Toxic kangaroo rat guts	*parviflorum*	Brookton	32° 22' S	117° 02' E
Toxic kangaroo meat		Corrigin	32° 19' S	117° 52' E
Toxic bronzewings and possum	*parviflorum*	Taylor's Well	32° 32' S	116° 52' E
Toxic sheep carcasses	*parviflorum* and *calycinum*	Bunbury	33° 20' S	115° 38' E
Toxic bronzewing and kangaroo insides		Dudinnin (now Dudinin)	31° 52' S	117° 54' E
Toxic kangaroo and sheep meat		Nyabing	33° 32' S	118° 08' E

* Location of author is assumed to represent the location of the anecdotal account. Where the actual location of the account is stated it is listed in bold text.
† Latitude and longitude of nearest identifiable feature used. Obtained from Geoscience Australia website (http://www.ga.gov.au/map/names) or W. A. Dept. of Land Administration

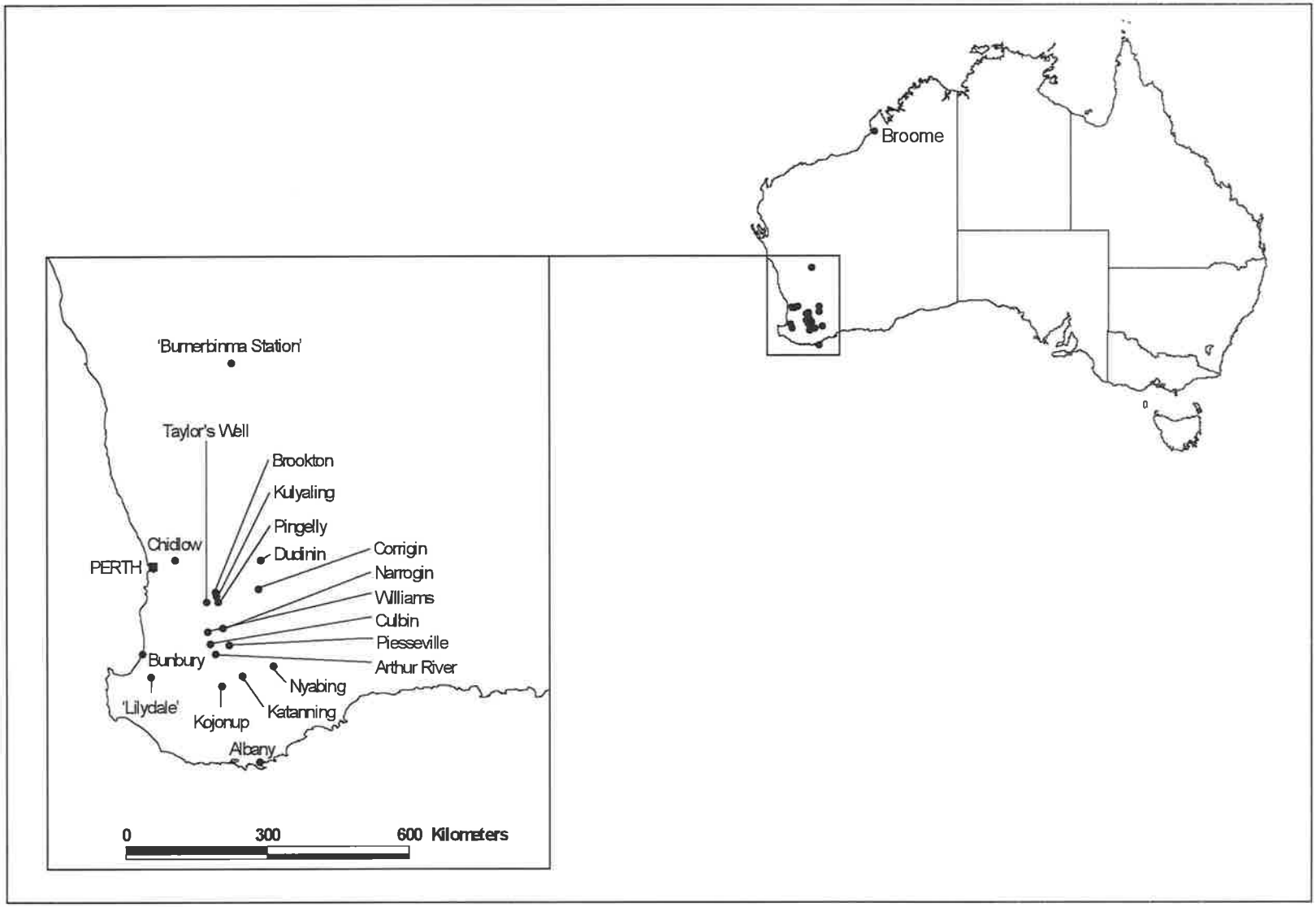


Figure 2.1 Nearest modern locations of toxicity related historical anecdotes in Western Australia

The following is a summary of the located records of cat and/or dog deaths reportedly due to the consumption of native animals:

- Bronzewing pigeons reported killing cats or dogs (Webb, 1885a; Mann, 1906 citing a Mr E. R. Parker; Le Souëf, 1907; Anon, 1912a; b; c; Knight, 1912; Anon, 1921a; Wansbrough and Anon, 1922; Serventy and Whittell, 1976).
- Bronzewing pigeon bones specifically reported killing cats or dogs (Webb, 1885; Anon, 1912b; c; 1917b; Wansbrough and Anon, 1922).
- Cats or dogs reported dying after eating remains of opossums [sic], boodies [sic] or kangaroo rats [sic] (Anon, 1907a; b; c; d; e; Clinch, 1907; Greig, 1907; Cairns, 1909; Anon, 1922c; Wansbrough and Anon, 1922).

Other points of common agreement in the literature, and some personal accounts, include the following:

- That bronzewing pigeons will eat *Gastrolobium* seed is stated by many (Webb, 1885; Mann, 1906 citing a Mr E. R. Parker; Greig, 1907; Le Souëf, 1907; Anon, 1917a; 1921; Foulds, 1921; Wansbrough and Anon, 1922; Serventy and Whittell, 1976; Johnstone and Storr, 1998; Twigg personal communication 2000).
- Opossums [sic] have been reported to eat *Gastrolobium* seed (Clinch, 1907) or plants (Anon, 1907a; Wansbrough and Anon, 1922) and *G. grandiflorum* foliage has been observed in brush-tailed possum scats at Uluru (Jeff Foulkes personal communication).
- Anecdotes where the symptoms of dog, and occasionally cat, poisoning are described of a "mad" nature are (Webb, 1885; Anon, 1907a; b; c; e; Clinch, 1907; Greig, 1907; Brown, 1910; Anon, 1912d; 1917a; 1921a; 1922a; b; c; Coff and Parker, 1922).

2.4 Discussion

Drummond (1840, p. 2) was one of the first to record the death of a cat or dog from consuming the remains of a native animal, in this case the " stomachs and guts" of the bronzewing pigeon stated to cause the death of dogs. He states a common belief that bronzewing pigeons feed on the seeds of an unidentified leguminous shrub from a grove at King George's Sound (likely to be *G. bilobum*) and that the pigeons' stomachs and guts cause dogs to die with symptoms similar to dogs that feed on poisoned sheep.

Apart from stock deaths, the deaths of cats and dogs was an issue of major concern to the settlers. The conflict primarily occurred, as with Drummond, when the native animals were obtained as a food source, either for the people or their animals. From 1906-1922, W. C. Grasby's 'Our Mutual Help' column in "The Western Mail" recorded at least 28 letters discussing the issue of toxicity of the native animals. The species of primary discussion were the bronzewing pigeon (*Phaps elegans* and *P. chalcoptera*), boodie (*Bettongia lesueur* or *B. penicillata* as name was used for both species), opossum (an opossum drawing in a co-published advertisement indicating these were probably mostly *Trichosurus vulpecula*) and kangaroo rat (probably *Bettongia penicillata*). These species figure prominently, being a primary food source and/or a source of agricultural conflict. In most cases the animals were shot or trapped, cooked and eaten, with the remains given to the cat(s) or dog(s). A classic example is the letter of the taxidermist Mr W. Webb (1885), a supplier of museum plant and animal specimens, written to the editor of the Albany Mail and King George's Sound Advertiser. In this case the bronzewing pigeon was cooked and eaten by Mr Webb, with his dog eating the breastbone and dying within 25 minutes. The pigeon's toxicity to the dog was attributed to *G. bilobum*, the

plant considered the primary food for the pigeons in that area. Another significant example is the anonymous letter written to "The Western Mail" on July 7, 1921. This person records their inability for years to keep a cat, with their cats annually dying from eating bronzewing pigeons in late summer when the seeds of the poison plants were ripe. Some authors disagree with anecdotes relating toxic species of fauna, not having experienced the reported toxicity (eg. Bert, 1907), however they are in the minority and it seems likely that not all of the animals are, or have remained, toxic. The letter by Greig (1907, p. 11) explains this when he writes: "I have known dogs to be fed on opossums not cleaned for weeks without getting poisoned, but eventually they get a poisoned one."

The numerous accounts of toxic wildlife are therefore considered to substantiate the 'verbal folklore' and could largely be explained by the now established presence of fluoroacetate in *Gastrolobium* plant material. The accounts of rapid cat and dog deaths from eating native animals (Webb, 1885; Le Souëf, 1907; Knight, 1912) do however raise a number of questions. The data of Eason and Frampton (1991) regarding cats orally dosed with 1080 baits, suggests a dose-response relationship for time to death and dose. Doses ranged from 0.4-1.6mg/kg and at the highest dose of 1.6mg 1080, one 3.5kg male cat was recorded dying in approximately 7hrs. The mean for the 8 cats dosed at this level was however 16hrs. Tourtellotte and Coon (1951) found no relationship between time to death and dose for dogs orally dosed with 1080 from 0.05-0.12mg/kg. The historical accounts therefore appear too rapid for fluoroacetate, especially when considering the time required for ingested food to travel through the gastrointestinal tract and be absorbed. They suggest a rapid uptake of toxicant(s) is occurring, with an associated rapid physiological impact causing death. The use of the term "mad" in

numerous accounts suggests symptoms akin with those of fluoroacetate dog poisoning summarised by Egekeze (1979) and Gregory (1991), which include wild running, barking and hyperactivity, but is largely contrary to the fluoroacetate dose response symptoms observed in cats by Eason (1991), described as being primarily lethargy and disorientation.

Another possibility is that the *Gastrolobium* seeds eaten by the pigeons contain a toxicant(s) in addition to the established presence of fluoroacetate. Omega fluorinated long-chain fatty acids have been found in the seeds of the African fluoroacetate producing plant *Dichapetalum toxicarium* (Hamilton and Harper, 1997). However Peters and Hall (1960) found the long-chained fluorinated fatty acid, fluoro-oleic acid, from *D. toxicarium* seeds, caused a sudden death, probably from a heart attack, but not without a significant period of delay. This testing was not on cats or dogs, but suggests that the rapid death anecdotes are not due to a long-chain fatty acid, such as fluoro-oleic acid, being present in the seeds.

A further possibility was that the seeds may contain unrecognised alkaloids, a common legume toxicant. Alkaloids such as strychnine are recognised for their rapid toxicity (Pelletier, 1983) and colchicine and caffeine for their skeletal retention (Panariti, 1996; McIntyre *et al.*, 2000).

The distribution of the historical anecdote localities (Figure 2.1) resembles Figure 22 'The pattern of pastoral expansion in the 1840s' by Cameron (1981, p. 173). This therefore suggests Figure 2.1 simply reflects where the more significant European settlement of the period was in conflict with the resident *Gastrolobium* vegetation and the related toxicity issues.

2.5 Conclusion

These historical anecdotes provide the foundation for this doctoral research. It is recognised that these historical accounts do not equate to scientific data. The vagaries of human recollection and perceptions of the time taken for a death to occur may erode the accuracy of these accounts. However taken collectively, poisonings arising from the native wildlife, and bronzewing pigeons with toxic bones, appear to have been occurring throughout the south-west of Western Australia, with one account also from Broome. The believed source of this toxicity is also commonly attributed to the *Gastrolobium* plants. The assumption of this study was that the anecdotes were likely to be largely valid and science had yet to explain the described phenomena. The parallels that this scenario has with the Pitohui birds of New Guinea being known by the locals as 'rubbish birds', only recently explained by the discovery of toxic alkaloids (Dumbacher *et al.*, 1992), is marked.

The recognised presence and action of fluoroacetate could explain many of these historical accounts, however many, especially the rapid death anecdotes, are similarly not explained. The speed of death in these dog accounts would need to be in error by hours to equate with published fluoroacetate studies. It could then be suggested that fluoroacetate may have unrecognised physiological behaviours, including a propensity for skeletal retention, or may be skeletally retained as its toxic metabolite fluorocitrate as per Peters (1969). Neither of these options explains the rapid death anecdotes. Another more likely explanation of toxic pigeon bones and rapidly toxic *Gastrolobium* seed is that fluoroacetate is not the sole toxicant within *Gastrolobium* seed. The analysis of

Gastrolobium seed for alkaloids and fluorinated toxicants will be discussed in the following chapters.

2.5.1 Research approaches to identify *Gastrolobium* toxicants

Three approaches were considered to attempt identification of the toxicant stated in the anecdotes to be rapidly toxic in cats and dogs, and skeletally retentive in bronzewing pigeons:

1. The first was to adopt the approach of Dumbacher *et al.* (1992; 2000) in investigating the belief of New Guinea people that birds of the *Pitohui* and *Ifrita* genus' were unpalatable. This approach involved collecting the birds from largely pristine habitat, extracting their tissues and looking for retained toxicants. As the south-west of Western Australia has been extensively cleared for agriculture and the toxic *Gastrolobium* plants particularly targeted, the dietary link between *Gastrolobium* seed and bronzewing pigeons was considered to have been largely severed. Also it was unknown whether the retention of skeletal toxicants occurred during bone formation when the nestling pigeons were being fed *Gastrolobium* seed. Thus the collection of adult birds with only recent ingestion of the seed could give a form of Type I error. These issues prevented the obtaining of ethics permission for this approach.

2. The second approach, supported by the CSIRO Plant Toxins Group, would be the most resource efficient approach and was the preferred approach for this study. This strategy involves use of an appropriate bioassay to replicate the anecdotes, that is, using a felid or canid model, successively screen for a *Gastrolobium* seed, then seed fraction and then compound(s) which causes rapid toxicity. The toxicological and

skeletal retentive behaviour of that compound(s) could then be determined. An inability to be able to nominate the seed toxicants being dosed to cats in milled seed prevented the obtaining of ethics permission for this approach - except for a small pilot study (Chapter 8).

3. The third approach, and that primarily undertaken in this study, was to analyse the *Gastrolobium* seed in the search for possible toxicants which could be dosed in a bioassay, could explain the anecdotes and could answer the questions posed in 1.2. With the many hundreds of compounds present in seed this is a very difficult task. With the established presence of alkaloids within *Gastrolobium* leaves and fluoroacetate within both seeds and leaves, focus was given to alkaloid and fluorinated toxicants. The analysis of *Gastrolobium* seed for alkaloid or fluorinated toxicants will be discussed in the following chapters.

Chapter 3: Alkaloids in *Gastrolobium* Seed

3.1 Introduction

A number of the historical reports describing the deaths of cats and dogs from eating the remains of native animals appear much too rapid to be fluoroacetate, a known toxicant within *Gastrolobium* seed. Deaths of cats and dogs associated with the secondary ingestion of *Gastrolobium* material, but too rapid for fluoroacetate, include:

- 3 cats "... died within an hour of eating the inside of the birds [bronzewing pigeons]" (Knight, 1912 p. 5)
- retriever dog dead within 20 minutes after eating bronzewing pigeon intestines full of the seeds of York Road poison (Le Souëf, 1907)
- "Ten minutes after eating it [bronzewing pigeon breast bone] he [dog] was quite mad ... I caught him and poured castor oil down his throat, which caused him to vomit ... this however did not ease him as in a quarter of an hour the poor brute died (Webb, 1885 p. 3).

Direct administration of fluoroacetate results in a longer time to death. Rammell (1978) states symptoms of fluoroacetate poisoning in dogs to arise 4-5 hours after ingestion. Even if a massive dose of fluoroacetate is administered ("10 to 20 times the lethal dose"), this has not been found to produce an immediate effect in the species tested but to reduce the latent period ("seldom less than 2 hours") prior to observable symptoms (Quin and Clark, 1947 p. 78). At the highest dose of 1.6mg '1080', Eason (1991) recorded one 3.5kg male cat dying in approximately 7hrs. The mean for the 8 cats dosed at this level was however 16hrs.

Another aspect of the historical anecdotes are the reports of bronzewing pigeon bones killing cats or dogs (eg. Webb, 1885). Like the speed of death, this is not a recognised physiological behaviour of fluoroacetate. Bone retention has been reported for fluorocitric acid, a byproduct of fluoroacetate metabolism, in fluorosed cattle bones (Peters *et al.*, 1969). Bosakowski (1986) however found adult dogs (7.3-10.6kg) did not exhibit symptoms until 1-2 hours after dosing with 8-32mg/kg fluorocitrate, with death occurring at approximately 4 hours. It therefore seems very unlikely that the rapid death and toxic bone anecdotes outlined above were due to fluoroacetate or fluorocitrate ingestion. Another possibility is however that this response may be due to alkaloids.

Alkaloids are produced by a variety of organisms, are often toxicants and have been recorded in *Gastrolobium* leaves. Early chemical analyses of the leaves of 2 of the then principal toxic species, York Road poison (*G. calycinum*) and box poison (*G. parviflorum*), described 2 new alkaloids, respectively cygnine and lobine (Mann, 1905; 1906). In testing on guinea pigs (mean weight 458g), cygnic acid caused death in a mean of 15 minutes ($n = 2$; SD = 12). In guinea pigs with a mean weight of 276g, lobic acid caused death in a mean of 4 minutes ($n = 2$; SD = 1.2). Additional testing of cygnic acid on 2 sheep caused death in 12 minutes. The original explanation therefore provided for the toxicity of *Gastrolobium* species was the presence of these, or similar, alkaloids. These studies proposed permanganate of potash as a remedy for stock poisoning, and the reported success of this remedy (eg. Anon, 1920) supported the toxic agent being an organic compound. However, Gardner and Bennetts (1956 p. 78) state that "later workers [not cited] using the same chemical methods, however, have been unable to

confirm Mann's work and have suggested that the toxic substances isolated by him were degradation products."

Subsequent research has detected significant concentrations of alkaloids in leaves of wallflower poison (*G. grandiflorum*) using reagents which respond to the presence of alkaloids (Webb, 1949). Cannon and Williams (1982) identified 4 alkaloids in rock poison (*G. callistachys*) leaves. Although they state the concentrations of the alkaloids to be 'non-toxic', no reference is made to which species they consider the recorded concentrations to be non-toxic, and no LD_{50} values are provided.

Research on *Gastrolobium* toxicity has rarely examined seed material. The dry matter of the seeds of Leguminosae and Fabacae plants are known to accumulate up to 10% alkaloids (Stobiecki *et al.*, 1993). This suggests that with 4 alkaloids extracted from the fresh leaves of *G. callistachys*, these or others are likely to be at a much higher concentration in the seeds. They may also be stored in another form. Chang (1998) examined the seeds of *Crotalaria scassellatii* (Fabaceae) and found that this plant stores pyrrolizidine alkaloids as 'lipophilic tertiary amines' in its seeds and as *N*-oxides in its vegetative tissues.

Additional research also supports the hypothesis that alkaloids are potentially the toxicants which explain these historical anecdotes:

- the cat is stated to be one of the most susceptible species to alkaloids (Waller and Nowacki, 1978);
- a major concentration of the alkaloid colchicine was detected in sheep bone marrow after a single oral dose, having earlier been detected unchanged in the milk (Panariti, 1996);

- New Guinea birds of the genus' *Pitohui* (5 of 6 species) and *Ifrita* (1 of 1 species) have been found to retain batrachotoxin alkaloids, primarily in their feathers (Dumbacher *et al.*, 2000). These are the same alkaloids sequestered in the skin of the poison-dart frogs (Daly, 1995) and which can still be detected at least 2 years after ingestion (J. Daly personal communication);
- the alkaloid caffeine was detected in 11 human femoral bone samples (McIntyre *et al.*, 2000);
- two alkaloids were extracted with methanol from the feathers of the Mexican red warbler (*Ergaticus ruber*) and although yet to be identified, upon injection into mice they caused a physiological effect (Escalante and Daly, 1994);
- quail are suspected to have retained an unidentified alkaloid in their meat from *Galeopsis ladanum* seed, causing rhabdomyolysis in 20 people (Aparicio *et al.*, 1999);
- Szentesi and Wink (1991) found quinolizidine alkaloids (QA) to be transported, largely unchanged, through two trophic levels additional to the plant, *Laburnum anagyroides* Medik (Leguminosae) itself;

To summarise, alkaloids were investigated as a possible explanation for the deaths of cats and dogs from bronzewing pigeon bones because of the following factors:

- presence of alkaloids in species of *Gastrolobium*;
- their capacity for skeletal retention;
- their established presence in some bird species; and
- their ability to pass through trophic levels without alteration.

3.2 Methods

3.2.1 Source of *Gastrolobium* seed

Gastrolobium seed was purchased from Nindethana Seed Service Pty. Ltd. (Albany, Australia) except *G. bilobum* (Araluen), collected by Robert Davis from the Western Australian Herbarium. All seed was collected in south-west Western Australia. Seed samples, source localities, Nindethana provenance numbers, collection dates and the reference sample number are described in Table 3.1. Where known, seed localities with their latitude and longitude are described in Table 3.2 and shown mapped in Figure 3.1. The seeds of nine different *Gastrolobium* species are shown in Figure 3.2, with an identifying key.

Seed identity was supplied by Nindethana Seed Service Pty. Ltd. In addition, the following seed samples had the identification of their source plants verified by, and lodged with, the Western Australian herbarium: *G. bilobum* (Araluen) - voucher PERTH 06340512; *G. bilobum* (Quindanning) - voucher PERTH 06455565; *G. bilobum* (Tambellup) - voucher PERTH 06455573; *G. calycinum* (Mundaring) - voucher PERTH 06455700; *G parviflorum* (Jacup) - voucher PERTH 06234658; *G. parviflorum* (Tambellup) - voucher PERTH 06455689 and *G. racemosum* (Jacup) - voucher PERTH 06455581.

Table 3.1 Source of *Gastrolobium* seed

Sample Number	Species	Source	Nindethana Provenance Number	Date Collected
1	*G. bilobum*	Quindanning	NS-19238	January 2000
1a	*G. bilobum*	Manjimup	NS-19413	2000
1b	*G. bilobum*	Tambellup	NS-21465	January 2002
1c	*G. bilobum*	Albany	NS-21726	January 2002
1d	*G. bilobum*	Harvey	NS-21743	December 2001
1e	*G. bilobum*	Araluen	-	February 2003
2	*G. calycinum*	Boddington	NS-19244	December 1999
2a	*G. calycinum*	Mundaring	NS-21193	2001
2b	*G. calycinum*	Chittering	NS-21744	December 2001
2c	*G. calycinum*	Boddington	NS-24628	December 2002
3	*G. crassiflolium*	Tambellup	NS-20475	December 2000
4	*G. cuneatum*	Torbay	NS-16690	January 1998
4a	*G. cuneatum*	Torbay	NS-15674	January 1997
4b	*G. cuneatum*	Torbay	NS-22037	January 2001
4c	*G. cuneatum*	Torbay	NS-14847	January 1996
4d	*G. cuneatum*	Torbay	-	January 2003
5	*G. laytonii*	Paynes Find	NS-10408	January 1993
6	*G. parviflorum*	Cuballing	NS-13971	December 1995
6a	*G. parviflorum*	Borden	NS-20348	December 2000
6b	*G. parviflorum*	Jacup	NS-21714	February 2000
6c	*G. parviflorum*	Tambellup	NS-21860	January 2002
7	*G. parvifolium*	Kukerin	NS-20840	December 2000
8	*G. racemosum*	'unknown'	N/C-02127	1989
8a	*G. racemosum*	Jacup	NS-21715	February 2000
9	*G. spathulatum*	Dwellingup	NS-19382	2000
10	*G. spinosum*	Hyden	NS-16975	January 1998
10a	*G. spinosum*	Woogenilup	NS-15033	January 2000
10b	*G. spinosum*	Mundaring	NS-19381	December 1996
11	*G. stenophyllum*	'unknown'	N/C-00869	1989
12	*G. tetragonophyllum*	'unknown'	NS-8144	1989
13	*G. villosum*	Jarrahdale	NS-14742	January 1996

Table 3.2 Coordinates of *Gastrolobium* seed source localities

Source	Latitude	Longitude
Albany	35° 01' S	117° 53' E
Araluen	32° 07' S	116° 06' E
Boddington	32° 48' S	116° 28' E
Borden	34° 04' S	118° 16' E
Chittering	31° 27' S	116° 05' E
Cuballing	32° 49' S	117° 10' E
Dwellingup	32° 44' S	116° 04' E
Harvey	33° 04' S	115° 53' E
Hyden	32° 27' S	118° 54' E
Jacup	33° 46' S	119° 17' E
Jarrahdale	32° 20' S	116° 03' E
Kukerin	33° 11' S	118° 05' E
Manjimup	34° 14' S	116° 08' E
Mundaring	31° 53' S	116° 10' E
Paynes Find	29° 15' S	117° 41' E
Quindanning	33° 02' S	116° 34' E
Tambellup	34° 02' S	117° 38' E
Torbay	35° 01' S	117° 38' E
Woogenilup	34° 32' S	117° 49' E

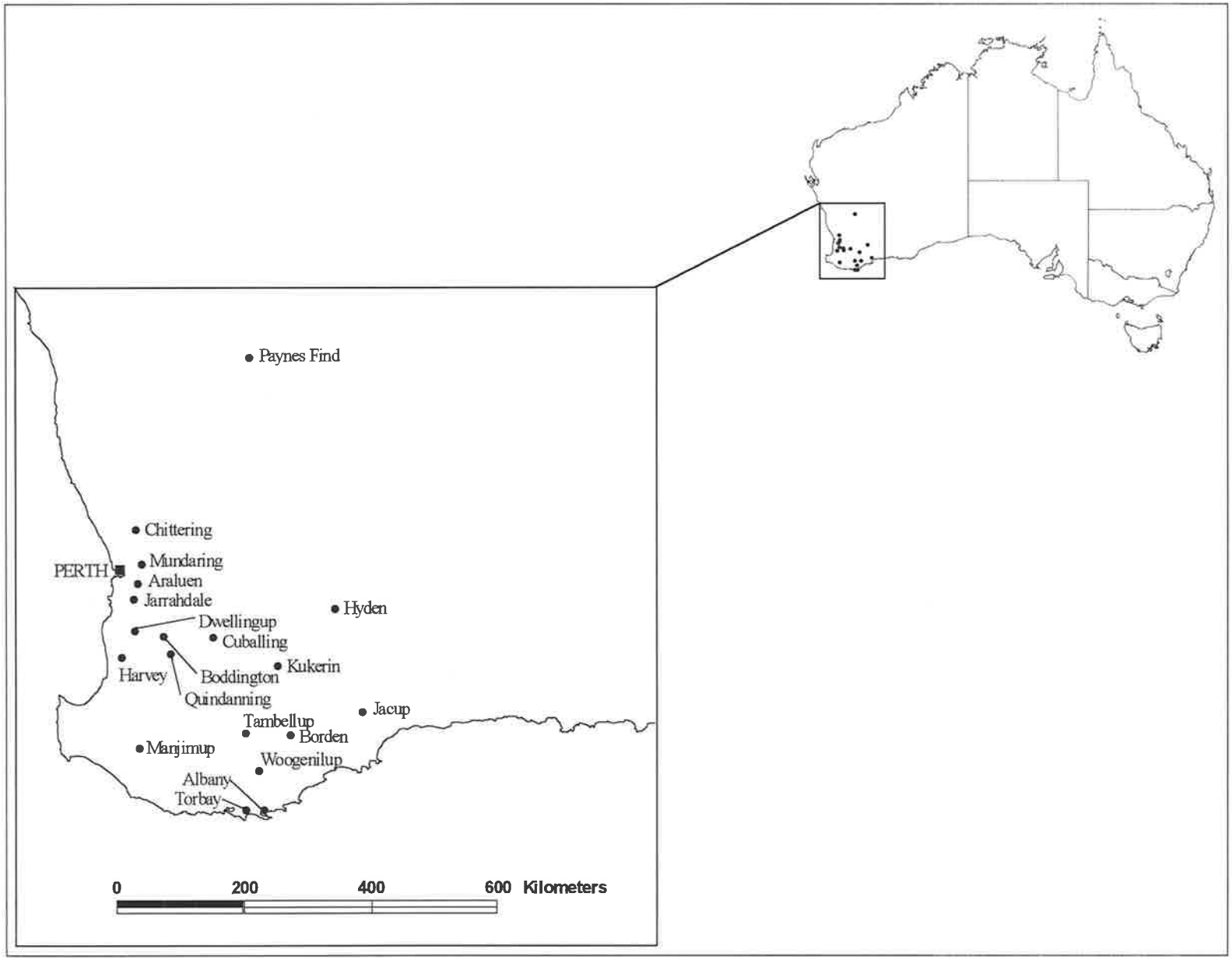
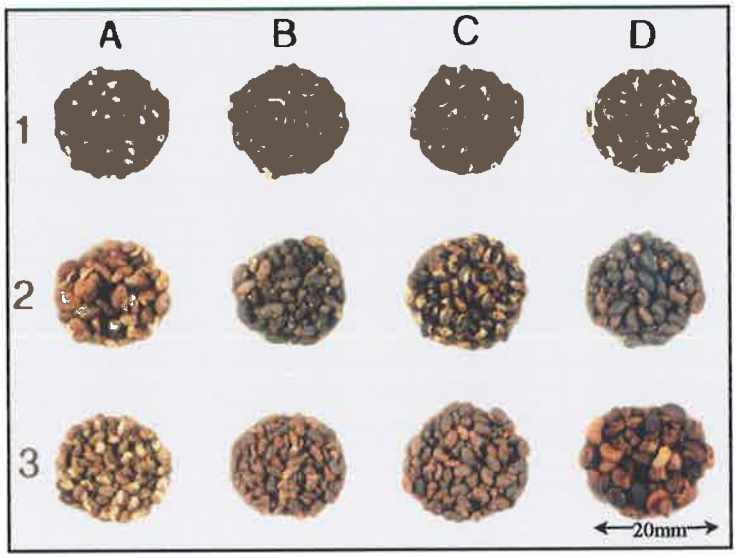


Figure 3.1 Seed collection sites in south-west Western Australia

Figure 3.2 Seeds of nine different *Gastrolobium* species, with an identifying key



Key to *Gastrolobium* Seeds in Figure 3.2

	A	B	C	D
1	***G. bilobum*** (Manjimup, W. A.)	***G. calycinum*** (Mundaring, W. A.)	***G. crassifolium*** (Tambellup, W. A.)	***G. cuneatum*** (Torbay, W. A.)
2	***G. laytonii*** (Paynes Find, W. A.)	***G. parviflorum*** (Borden, W. A.)	***G. parvifolium*** (Kukerin, W. A.)	***G. racemosum*** ('unknown', W. A.)
3	***G. spinosum*** (Hyden, W. A.)	***G. stenophyllum*** ('unknown', W. A.)	***G. tetragonophyllum*** ('unknown', W. A.)	***G. villosum*** (Jarrahdale, W. A.)

3.2.2 Extraction of Dragendorff responsive compounds

Gastrolobium bilobum seed (Tambellup) was milled in a water-cooled IKA A10 Analytical Mill. Milled seed (54.5g) was placed in a 500ml conical flask with a magnetic stirrer bar and 400ml petroleum spirit (BDH, AR grade, B.R. 40-60^{0}C). The mixture was extracted for 16.5 hours then filtered with suction through a Whatman No. 2 filter paper with additional petroleum spirit washes. The petroleum spirit was removed under reduced pressure using a Buchi rotary evaporator with the water bath held at 35 ± 1^{0}C. The extract was tested for the presence of alkaloids by analytical TLC.

The seed marc was scraped off the filter paper and extracted for 22 hours with chloroform (BDH, AR grade). The filtration and solvent removal was then repeated and the extract tested for the presence of alkaloids by analytical TLC.

The seed marc was again scraped off the filter paper and extracted for 24 hours with methanol (BDH, HPLC grade). The mixture was filtered and the seed marc re-extracted with another 300mls of methanol for 48 hours. The filtrates were combined and the filtration and solvent removal repeated. The extract was tested for the presence of alkaloids by analytical TLC.

3.2.2.1 Chloroform extract

Based on the results of the TLC analysis (see **3.3**), the chloroform extract was re-dissolved in 20mls chloroform then extracted three times with 5mls of 0.1N HCl. The combined HCl fractions were extracted three times with 5mls chloroform and were added to the HCl extracted chloroform. The chloroform and HCl fractions were tested for the presence of alkaloids by analytical TLC.

10mls of n-hexane (APS, AR grade) was added to the chloroform and the mixture again extracted three times with 5mls of 0.1N HCl. The combined HCl fractions (pH ~1) were neutralised with 38% aqueous ammonia and then extracted three times with ethyl acetate, 10mls (BDH, AR grade). The chloroform and HCl fractions were tested for the presence of alkaloids by analytical TLC.

The chloroform extract was loaded onto a 20cm x 20cm Kieselgel 60 F_{254} silica gel (2mm) on glass preparative TLC plate (Merck) and resolved using a 60:40 chloroform:methanol solvent. The Dragendorff alkaloid reagent was applied to the edge of the plate and the active bands (Rf 0.0; 0.03; 0.17) were scraped off, ground to a fine powder with a mortar and pestle and extracted with 100% methanol. The mixture was sonicated for 5 minutes in a Branson Biz Ultrasonic Cleaner, decanted into centrifuge tubes and centrifuged for 10 minutes at 6000rpm. The supernatant was transferred to plastic disposable 20ml syringes (Terumo) and filtered using 25mm 0.45µm hydrophobic PTFE syringe filters (Millipore). The methanol was removed under reduced pressure using a Buchi rotary evaporator with the water bath held at 35 ± 1^0C.

This methanol extract was loaded onto another 20cm x 20cm Kieselgel 60 F_{254} silica gel (2mm) on glass preparative TLC plate (Merck) and resolved using a 5:2:4 1-butanol:acetic acid:water solvent. The Dragendorff alkaloid reagent was applied to the edge of the plate and the active band (Rf 0.58) was then scraped off, ground to a fine powder with a mortar and pestle and extracted three times with 100% methanol and 10 minutes sonication. The extracts were decanted into centrifuge tubes and centrifuged for 10 minutes at 6000rpm. The methanol was removed under reduced pressure using a Buchi rotary evaporator with the water bath held at 35 ± 1^0C. 10mls distilled water was

added to the methanol extract and the mixture extracted three times with 10mls of ethyl acetate, with 5 minutes sonication. The ethyl acetate extracts were combined and the ethyl acetate removed under reduced pressure using a Buchi rotary evaporator with the water bath held at 35 ± 1^{0}C. Any remaining solvent was removed using an oil pump with liquid nitrogen trap. About 5mg of extract was dissolved in CD_3OD and analysed by NMR.

3.2.3 Analytical thin layer chromatography (TLC)

Extracts were tested for the presence of alkaloids using Kieselgel 60 F_{254} silica gel (0.2mm) on an aluminium thin layer chromatography (TLC) plate (Merck) with a 60:40 chloroform:methanol solvent and Dragendorff alkaloid reagent (Sigma).

3.2.4 Nuclear magnetic resonance (NMR) analysis

NMR analyses were performed using a 600MHz Oxford magnet with a Varian Unity Inova console using Varian VNMR6.1C software. The probe was a 5mm pulsed field gradient probe tuned to ^{19}F (Sfrq = 564.350MHz; Sweep Width = 310, 078Hz; tof = -8846.1; temp. = 25^{0}C). Spectra were referenced to an external reference of either trifluoroacetic acid in D_2O (set to 78.0ppm, in turn referenced to $CFCl_3$ @ 0.0ppm) or sodium monofluoroacetate in D_2O (set to –63.4ppm). Deuterated methanol was from Sigma-Aldrich and Pestanal grade monofluoroacetic acid, sodium salt, from Riedel-deHaën.

3.3 Results

Analytical TLC found no Dragendorff responsive compounds in the petroleum spirit or methanol extracts of *G. bilobum* (Tambellup) seed. Dragendorff responsive compounds were found in the chloroform extract of the seed and remained in this solvent in spite of the addition of HCl and n-hexane. Use of the extraction process described and preparative TLC enabled the extraction of the Dragendorff responsive compounds in sufficient purity to enable analysis by NMR.

Extensive NMR expansions and decoupling experiments identified the putative alkaloids as a mixture of phosphatidyl cholines (lecithins), where the fatty acids were primarily linolenic, oleic and stearic acids. Han *et al.* (1991) describe the NMR analysis of phosphatidyl cholines and this fits this NMR data, including the reported coupling constants. The principal identifying NMR signal was the most upfield singlet (at delta 3.22), being the quaternary ammonium methyl signals of the phosphoryl choline, the N(Me)3 group, attached to the glycerol. This is evident from the ROESY and the NOESY spectra which show cross peaks with both the P-OCH_2 and CH_2-$N(Me)^3$ methylenes. A COSY experiment showed that these two methylenes are coupled with one another.

There are some additional weak peaks that appear to be related to another sort of phosphatidyl choline but this was not analysed further.

3.4 Discussion

Dragendorff reactive compounds were identified in the chloroform extract of *G. bilobum* (Tambellup) and subsequently observed in the similar extracts of *G. bilobum* (Quindanning), *G.* calycinum (Mundaring) and G. *parviflorum* (Jacup). Dragendorff's reagent is well recognised as the established reagent to identify the presence of alkaloids. The approach of this analysis therefore was that if alkaloids were present in *Gastrolobium* seeds, they would have been indicated by this TLC reagent. However the potential alkaloids indicated by Dragendorffs within this experiment were later identified as innocuous phosphatidyl cholines. Dragendorffs is also listed as an indicator of quaternary nitrogen compounds. The report by Webb (1949, p. 8) of "... appreciable amounts of alkaloidal substances ..." in the leaves of *G. grandiflorum*, indicated by the amount of reagent precipitate from using a variety of alkaloid reagents, is probably accurate. Obtaining a positive result with four reagents would be less likely to give the false positive obtained in this study through the use of a single indicator reagent. Although two of the Webb reagents (silicotungstic acid and Mayer's reagent) could be purchased, no method could be located for their preparation and use. Similarly, Cannon and Williams (1982, p. 1497) state they detected alkaloids, and solvent fractions "... gave weakly positive tests for alkaloids", but no method or reagent is identified. Their detailed analysis does describe alkaloids in the leaves of *G. callistachys*. It therefore seems likely that alkaloids are present in *Gastrolobium* plants, but in their leaves and not in their seeds. These compounds, although reported as bone retentive and often rapidly toxic (especially in comparison to the reported action of fluoroacetate) do not explain the reported rapid toxicity of *Gastrolobium* seeds and bronzewing pigeon bones.

3.5 Conclusion

This study was unable to extract and identify any alkaloids in the *Gastrolobium* seeds analysed. Although previous research has identified alkaloids in *Gastrolobium* leaves, with some research stating compounds of rapid toxicity, the Dragendorff responsive compounds extracted in this study were identified as phosphatidyl cholines. It is always possible that some alkaloids are present, but very minor, and were thus overlooked for the more obvious phosphatidyl cholines. This is however considered an unlikely scenario. It is therefore the conclusion of this study that the anecdotes of toxic bronzewing pigeon bones could not be due to their retention of alkaloids through consumption of *Gastrolobium* seeds.

Chapter 4: Organo-Fluorine Compounds in *Gastrolobium* Seed

4.1 Introduction

The presence of organo-fluorine compounds in nature is rare, with only a few natural products described (O'Hagan and Harper, 1999). The most common is fluoroacetate, first described by Marais in 1944 in the African plant *Dichapetalum cymosum* Engl. (Marais, 1944) and then subsequently in the Australian plants *Acacia georginae* F.M.Bailey (Oelrichs and McEwan, 1961), *Gastrolobium grandiflorum* F.Muell. (McEwan, 1964) and additional *Gastrolobium* (Baron *et al.*, 1987; Twigg *et al.*, 1996; Twigg *et al.*, 1999), *Dichapetalum* (O'Hagan *et al.*, 1993) and South American species (Krebs *et al.*, 1994). Additional fluorinated natural products currently identified are the ω-fluorinated fatty acids from the seeds of *Dichapetalum toxicarium* (Peters and Hall, 1960; Hamilton and Harper, 1997) and fluoroacetone in the leaves of *Acacia georginae* (Peters and Shorthouse, 1971; Hall, 1972). Hall (1972) also suggests the likely presence of additional organo-fluorine compounds in *Gastrolobium bilobum* (specifically, a fluorinated carbohydrate or amino acid) and *Acacia georginae*. The most recent fluorinated natural product discovery was 4-fluorothreonine in 1986 (Sanada *et al.*, 1986), although trifluoroacetate can now be considered a natural product (Frank *et al.*, 2002).

The strong possibility that *Gastrolobium* seeds could contain additional fluorinated compounds to the reported fluoroacetate, resulted in adoption of the approach of Hall (1972) for the purposes of this research, utilising alkali-fusion in conjunction with a

fluoride selective electrode to quantify 'total fluorine' (ie. organic fluorine + inorganic fluoride). The successive extraction of *Gastrolobium* seeds with solvents of increasing polarity and analysis for fluorine using a fluoride selective electrode, would enable the presence of additional fluorinated compounds to be detected. The electrode also allows for following of the fluorine and ensuring it is all accounted for in the subsequent solvent fractions. Further analytical methods could then be employed to identify the fluorinated compounds.

It was considered a strong possibility that the derivitisation methods primarily used in fluoroacetate analysis (eg. Okuno *et al.*, 1984; Ozawa and Tsukioka, 1989) could remove the fluorinated functional group (eg CH_2FCOO^-) from the parent molecule (eg. a sugar or amino acid as speculated by Hall (1972)). That is, using a derivitisation method would produce the fluoroacetylated derivative largely regardless of the parent molecule. High-pressure liquid chromatography was therefore used to confirm the presence of fluoroacetate in the seeds without having to use derivitisation. Column chromatography and thin layer chromatography (TLC) were primarily used to fractionate and purify the 'total fluorine' in the solvent extracts. ^{19}F nuclear magnetic resonance (NMR), a non-destructive method of analysis, was primarily used to confirm and monitor the presence of fluorinated compounds. The presence of any ^{19}F NMR signals in addition to a CH_2F triplet from fluoroacetate was considered evidence to reject the null hypothesis: that fluoroacetate is the only organo-fluorine compound in *Gastrolobium* seeds.

4.2 Methods

4.2.1 Source of *Gastrolobium* seed

(See 3.2.1)

4.2.2 Solvent extraction of *Gastrolobium* seed

Seed was firstly hand cleaned of any contaminating material remaining in the purchased seed. It was then milled in a Cyclotec 1093 sample mill (Tecator) and successively extracted with petroleum spirit (AR grade B.R. 60-80^{0}C, BDH), chloroform (AR grade, BDH) and methanol (HPLC grade, BDH) or distilled water. Extractions were primarily by use of soxhlet apparatus, however later extractions were made at room temperature (r.t.) using a shaker or magnetic stirrer. Mixtures were filtered through a Whatman No. 42 ashless filter paper with suction and 2 washes of the seed marc with the appropriate solvent. Solvent extracts were then filtered through a 0.45µm PTFE hydrophobic syringe filter (Millipore) on a Terumo disposable syringe and water extracts through 0.45µm cellulose-nitrate filters (Sartorius). Solvents were removed on a Buchi rotary evaporator with the water bath held at 35 ± 1^{0}C.

4.2.3 Analysis for 'total fluorine'

All batches of seed were analysed for their 'total fluorine' with the results presented in Table 4.2. Solvent extracts were similarly analysed. In addition, to examine the morphological distribution of fluorinated compounds 0.5g samples of *G. bilobum* (Manjimup), *G. calycinum* (Boddington) and *G. parviflorum* (Cuballing) seed was

separated using a scalpel and forceps into the testa + aril and the cotyledons. Separated seed was stored at 4^0C prior to being analysed for their 'total fluorine'.

'Total fluorine' content of the seed and solvent extracts was determined using the 'alkali fusion method for total fluorine analysis' in which all organic fluorine is hydrolysed to release the fluoride ion which is then measured by the electrode. Triplicate samples were analysed where possible. The alkali fusion method used in this study was from Shuqi Ma (1994), who showed its' capacity to deal with both organic fluorine and inorganic fluoride, and was demonstrated by Dr Colin Rix from RMIT University (Victoria, Australia).

Within this method, 5ml 2M NaOH is added to seed or to a solvent extract in a nickel or platinum crucible. The mixture is evaporated to dryness on a hot plate then slowly heated over a burner to ~500^0C for 5 minutes, reducing the mixture to ash. The crucible is allowed to cool and 4ml 0.05M Na_2H_2EDTA added and the weight recorded (W_1). The mixture is then simmered on a hot plate for 10-15 minutes, not allowing it to boil dry. The crucible is cooled and distilled water added to return the weight to (W_1). 4ml 0.8M citric acid is slowly added and the mixture reweighed (W_2). The mixture is simmered on a hot plate for 30 minutes then allowed to cool and distilled water added to return the weight to (W_2). 4ml of the solution is pipetted into a 25ml plastic beaker and 4ml total ionic strength adjustment buffer (TISAB) added then the pH checked to ensure it is in the range of 5-7. The Emf (mV) was measured using a Radiometer 'ISE25F-9 Fluoride Selective Electrode' and an Orion '90-02 Double Junction Reference Electrode' and compared to a standard curve. The electrical potential is proportional to the log of the fluoride ion concentration.

Fluoride standard solutions were prepared by diluting a 1000ppm fluoride standard from AdelLab Scientific (Norwood, Australia) with distilled water. For establishment of the standard curve, standards were prepared by adding 4ml of the fluoride standard solutions to 4ml TISAB.

All samples and standards were measured while being stirred with a small magnetic stirrer bar. The standard curves of Emf (mV) against log[F-] were prepared daily using 1, 5, 10, 20, 50 and 100ppm standards and had an R^2 of ≥ 0.99. Additional 0.1 and 1000ppm standards were used to assess the sensitivity of the electrode and linearity of the curve. The electrode was found to be sensitive to 0.1ppm fluoride and linear to 1000ppm, the highest concentration tested. However attaining a stable EmF reading is slower at the lower concentrations of fluoride. Fluoride standards were replaced when the R^2 fell below 0.98.

An example of a table of standard and sample measurements is shown in Table 4.1.

Table 4.1 Sample table of daily standard and sample measurements

19-Nov-01						
Sample	**Sample (g)**	**log [F-]**	**[F-] in sample (ppm)**	**Emf (mV)**	**Volume (ml)**	**[F-] in seed (ppm)**
Standard		0	1	254	8	
Standard		0.69897	5	223	8	
Standard		1	10	207	8	
Standard		1.30103	20	189	8	
Standard		1.69897	50	166	8	
Standard		2	100	146	8	
G. calycinum seed (Mundaring)	0.5069	1.244867	17.6	190	8	277
G. bilobum seed (Manjimup)	0.5029	1.319522	20.9	186	8	332
G. spinosum seed (Mundaring)	0.5044	-0.92012	0.1	306	8	2
G. spinosum seed (Hyden)	0.5048	-0.6215	0.2	290	8	4

In this example from 19th November 2001, the regression of the fluoride standards gave the equation y = -53.58x + 256.7 with an r^2 of 0.9943 where y is the Emf (mV) and x is the log [F-]. For the *G. calycinum* (Mundaring) seed sample, the Emf (mV) measured 190 millivolts. Thus the log [F-] for this sample is (190-256.7)/-53.58 = 1.245. The **[F-] in sample (ppm)** is therefore $10^{1.245}$ = 17.6ppm and the **[F-] in seed (ppm)** = ((([F-] in sample (ppm)/1000) x **Volume (ml))** x 1/**Sample (g))** x 1000. For the *G. calycinum* (Mundaring) seed sample this gave 277ppm fluoride ('total fluorine') in the intact seed.

4.2.4 Preliminary analytical methods

As fluoroacetate is the only recognised fluorinated compound in *Gastrolobium* seed, preliminary analysis was undertaken to confirm or negate this understanding. Primarily examining the methanol extract, thin-layer chromatography, column chromatography and dialysis membranes were used to fractionate the solvent extracts for 'total fluorine' and further chromatographic analysis. Anion exchange resin was used to determine the proportion of 'total fluorine' in the methanol extract, which would bind to this resin and thereby test whether fluoroacetate accounts for all 'total fluorine' present.

4.2.4.1 Thin layer chromatography (TLC)

The soxhlet methanol extract of *G. calycinum* (Mundaring) seed was applied to 2 Kieselgel 60 F_{254} silica gel (2mm) on 200 x 200mm glass preparative TLC plates (Merck) resolved using a 70:30 chloroform:acetone solvent. The plates were scraped off in 3

bands (0-70mm, 71-140mm and 141-200mm) into separate beakers, 50mls of methanol (HPLC grade, BDH) added, then sonicated for 5 minutes in a Unisonics Ultrasonic Cleaner and filtered through Whatman 1PS filter papers and 0.45µm hydrophobic PTFE syringe filters. The methanol was removed under reduced pressure using a Buchi rotary evaporator with the water bath held at 35 ± 1^0C. A sample of the extracts of each of the 3 bands was analysed for 'total fluorine'. The extract of the first band was 2056ppm 'total fluorine', the second band extract 57ppm and the third band extract 40ppm indicating the soxhlet methanol extract of *G. calycinum* seed contained additional fluorinated compounds to fluoroacetate.

4.2.4.2 Column chromatography

a. The soxhlet methanol extract of *G. calycinum* (Mundaring) seed was transferred using distilled water to a 12.5 x 200mm column of chromatographic silica gel (May & Baker). The column was eluted with 100% methanol (HPLC grade, BDH) with a drop rate of 9 drops min^{-1}. Approximately 5ml fractions were collected, the methanol removed with a gentle stream of nitrogen and a sample of the extracts analysed for 'total fluorine'. The fraction 1 extract was 1665pm 'total fluorine', the fraction 2 extract 540ppm, the fraction 3 extract 296ppm, the fraction 4 extract 1091ppm, the fraction 5 extract 1552ppm, the fraction 6 extract 1556ppm, the fraction 7 extract 2587ppm and the fraction 8 extract 226ppm. These results also indicated the soxhlet methanol extract of *G. calycinum* seed contained fluorinated compounds additional to fluoroacetate.

b. The soxhlet methanol extract of *G. tetragonophyllum* ('unknown') seed was transferred using distilled water and methanol to a 12.5 x 200mm column of LH-20

(Sigma). The column was eluted with 100% methanol (HPLC grade, BDH) with a drop rate of 9 drops min^{-1}. Approximately 5ml fractions were collected and the methanol removed with a gentle stream of nitrogen. Fractions 1 and 2 were combined and a sample of the extracts of this combined fraction and fractions 3, 4 and 5 was analysed for 'total fluorine'. The combined fraction 1 and 2 extract was 1322pm 'total fluorine', the fraction 3 extract 808ppm, the fraction 4 extract 3210ppm and the fraction 5 extract 5811ppm. These results indicated the soxhlet methanol extract of *G. tetragonophyllum* seed also contained additional fluorinated compounds to fluoroacetate.

4.2.4.3 Dialysis membranes

Dialysis membranes specified as 100 molecular weight cut-off (MWCO) Float-A-Lyzer, 500 MWCO Dispodialyzer and 1000MWCO Float-A-Lyzer were purchased from Spectrum Laboratories, California. Spectrum stated the true MWCO for each membrane was probably +100%. The dialysis membranes were floated in 500ml measuring cylinders of distilled water with a magnetic stirrer bar and stirred for up to 7 days with multiple water changes. The contents of the membranes were then transferred to a flask, the water removed under reduced pressure using a Buchi rotary evaporator with the water bath held at 35 ± 1^0C and a single sample of the extracts analysed for 'total fluorine'. Seed methanol extracts with the highest concentrations of 'total fluorine' were utilised. Analysis results can be summarised as follows:

1. 100MWCO membrane with *G. tetragonophyllum* ('unknown') soxhlet methanol extract column fraction of 2533ppm 'total fluorine'. Extract ≤100MWCO 16,550ppm 'total fluorine'; extract ≥100MWCO not analysed.

2. 100MWCO membrane with *G. tetragonophyllum* ('unknown') soxhlet methanol extract of 5831.9 ± 244.1ppm (*n*=3) 'total fluorine'. Extract ≤100MWCO not analysed; extract ≥100MWCO 3,908ppm 'total fluorine'.
3. 500MWCO membrane with *G. calycinum* (Mundaring) soxhlet methanol extract TLC fraction of 8777ppm 'total fluorine'. Extract ≤500MWCO 20,636ppm 'total fluorine'; extract ≥500MWCO 3,379ppm 'total fluorine'.
4. 1000MWCO membrane with *G. calycinum* (Mundaring) soxhlet methanol extract of 1159.6 ± 4.6ppm (*n*=3) 'total fluorine'. Extract ≤1000MWCO 2,372ppm 'total fluorine'; extract ≥1000MWCO 76ppm 'total fluorine'.

These results indicated the soxhlet methanol extracts of *G. calycinum* and *G. tetragonophyllum* seed contained additional fluorinated compounds to fluoroacetate, as 'total fluorine' remained within the dialysis membranes determined as approximately 200MW, 1000MW and 2000MW cut-off. With fluoroacetate (CH_2FCOO^-) having a MW of 77 it should easily pass through each of these membranes.

4.2.4.4 Anion exchange resin

Gastrolobium bilobum seed (Tambellup; 145.6 ± 34.1ug g^{-1} 'total fluorine'; *n*=3) was milled in a Cyclotec Mill. The milled seed (50g) was placed in a 1000ml conical flask and 500ml distilled water added. The mixture (pH~6.5; temperature 23^0C) was put on a shaker and extracted for 17 hours then filtered with suction, with additional water washes, through successive Whatman™ 125mm filter papers up to a No. 42 ashless filter paper. The water extract (600mls) was divided between five 500ml beakers, frozen using liquid nitrogen and the water removed using a freeze-drier. The dried water-extract

(~10g) was combined using distilled water washes, centrifuged at 6000rpm for 10 minutes and then filtered through 0.45µm cellulose nitrate syringe filters (Sartorius). 2mls of filtrate was transferred to a NMR tube for ^{19}F analysis and 2mls transferred to 2 pre-weighed nickel crucibles, dried in a drying oven at 46^0C and tested for 'total fluorine'. The remaining filtrate (~60mls) was transferred to a pre-weighed beaker, and again frozen using liquid nitrogen and the water removed using a freeze-drier. 10g of the dried water-extract (750mg retained for additional analyses) was re-dissolved in 50mls distilled water for anion ion-exchange column analysis.

The seed marc (~22g) was washed with 1600ml distilled water and then an additional 600ml before being dried in an oven at 46^0C, reduced to a uniform fine powder with a mortar and pestle and analysed for 'total fluorine'.

Ion Exchange Column. Dowex 1X8-200 ion exchange resin in the Cl^- form (Sigma) had a stated exchange capacity of 1.2 meq ml^{-1} (equivalent to 120mg ml^{-1} for sodium monofluoroacetate). 40g of resin were converted to the acetate form, being first mixed with 1M NaOAc and transferred to a 160mm x 38mm column. An additional 500ml of 1M NaOAc, 500ml 2M NaOAc and 500ml 4M NaOAc were slowly run through the resin until no precipitate was seen when 1 drop of 1% aqueous $AgNO_3$ was added to a sample of the eluent. The activated resin was then washed with distilled water (1500mls) until the pH was the same as the distilled water (pH ~5.5).

20g of activated resin was transferred to each of two 300mm x 18mm columns (40,715mm^3 resin) and capped with glass wool. Using distilled water the drop rate was set at 20 drops per minute. The following steps were then conducted:

(1) 50 mg of sodium monofluoroacetate (SMFA; Pestanal grade; Riedel-deHaën) was then dissolved in 50mls of distilled water (0.001M) and added to column 1 and the 50mls of aqueous extract of *G. bilobum* added to column 2. The liquids were run level to the top of the columns, then immediately followed by 50ml distilled water, with both solutions collected in a 500ml beaker;

(2) 50ml of 2% NaCl, immediately followed by 50ml distilled water, with both solutions collected in another 500ml beaker;

(3) 100ml of 10% NaCl, immediately followed by 50ml distilled water, with both solutions collected in another 500ml beaker;

(4) 100ml of saturated NaCl, immediately followed by 50ml distilled water, with both solutions collected in another 500ml beaker.

Contents of all 4 beakers for each column were capped with aluminum foil, frozen using liquid nitrogen and the water removed using a freeze-drier. The dried extract from each fraction was transferred to a nickel or platinum crucible and analysed for 'total fluorine'. Results indicated 10% NaCl extracted most 'total fluorine' from the resin in both columns. However, although the saturated NaCl solution removed another 30µg of 'total fluorine' from the seed extract column, the column remained dark from the seed extract. Results indicated approximately 30% of the 'total fluorine' was unaccounted for and was most likely still adsorbed to the column. It is not clear whether this indicates that this 'total fluorine' could not therefore be fluoroacetate but more polar organo-fluorine compounds, or could still be fluoroacetate but caught in the matrix of the seed extract and the resin.

4.2.5 ^{19}F nuclear magnetic resonance (NMR)

NMR analyses were performed using a 600MHz Oxford magnet with a Varian Unity Inova console using Varian VNMR6.1C software. The probe was a 5mm pulsed field gradient probe tuned to ^{19}F (Sfrq = 564.350MHz; Sweep Width = 310, 078Hz; tof = -8846.1). Spectra were referenced to an external reference of either trifluoroacetic acid in D_2O (set to 78.0ppm, in turn referenced to $CFCl_3$ @ 0.0ppm) or sodium monofluoroacetate in D_2O (set to –63.4ppm). Deuterated solvents were from Sigma-Aldrich and Pestanal grade sodium monofluoroacetate from Riedel-deHaën.

4.2.5.1 Solid state ^{19}F nuclear magnetic resonance (NMR)

The most straightforward form of analysis for organo-fluorine compounds in *Gastrolobium* seeds was considered to be solid state ^{19}F NMR. For this analytical method all that was required to prepare the seed sample for analysis was simple milling. It was hoped that this method would confirm the presence of the ^{19}F resonances seen in the liquid ^{19}F NMR and consequently negate their presence being a result of the extraction process or the use of a deuterated solvent for signal locking.

A sample of milled *G. parviflorum* (Jacup) seed, the most fluorinated seed available, was submitted for analysis to Dr Francis Separovic, School of Chemistry, University of Melboune. Pestanal grade sodium monofluoroacetate (SMFA) from Riedel-deHaën and plain flour (Laucke Mill) were provided as a control.

Unfortunately the 300MHz NMR used for this analysis had a large fluorine background. Replacement of the Kel-F caps with Macor caps did not remove this large background peak, possibly arising from teflon within the probe itself. Although a

moderate signal could be seen from the SMFA/flour standard, no resonances were seen in the milled *G. parviflorum* seed.

4.2.6 High pressure liquid chromatography (HPLC)

As preliminary seed and ^{19}F NMR analysis indicated the possible presence of organo-fluorine compounds additional to fluoroacetate, a non-derivitisation method was proposed, to use for their identification, in order to minimise the likelihood of hydrolysis of the parent molecule(s). That is, it was considered very likely that the derivitisation methods primarily used in previous fluoroacetate analyses (eg. Okuno *et al.*, 1984; Ozawa and Tsukioka, 1989), could remove the fluorinated functional group (CH_2FCOO^-) from a parent molecule such as a carbohydrate or amino acid, speculated by Hall (1972) as being present in *G. bilobum* seed. This could result in detection of the fluoroacetyl derivative but mis-identification of the parent compound. HPLC had been used in the past for fluoroacetate analysis (Meyer and Grobbelaar, 1989), with a new method published reporting a much improved detection limit than provided in the previous method (Minnaar *et al.*, 2000a; Minnaar *et al.*, 2000b). The stated limit of detection (LOD) was 12ug L^{-1} and the limit of quantification (LOQ) 40ug L^{-1}. The adjusted method (Minnaar *et al.*, 2000a) was therefore used to confirm the presence of fluoroacetate in the seed methanol extracts using the following materials and conditions:

HPLC: (a) Alltech 425 solvent delivery system

ICI LC1200 UV VIS single wavelength detector

Waters 745 data module (integrator)

(b) Agilent 1100 series HPLC with a diode-array detector

Wavelength: 210nm

Column: Supelcogel C-610H ion exchange column (300mm x 7.8mm)

Supelguard 50mm x 4.6mm guard column

Injection Loop: (a) 20µl; (b) n/a

Eluent: 0.04M H_3PO_4: acetonitrile: water (5.32ml: 20ml: 974.68ml) (pH = 2.0)

Flow Rate: (a) 0.8ml min^{-1}; (b) 0.4ml min^{-1}

Injection: (a) 25µl; (b) 50µl

Run Time: (a) 65 minutes; (a) 30 minutes

Standards (1, 5, 12.5, 25, 50, 100, 200, 400 and 800ppm) were prepared by serial dilution of a 1600ppm standard of SMFA in 0.02M H_3PO_4.

The seed extract analysed by HPLC was the TLC Band 1 extract of *G. calycinum* (Mundaring), which had a 'total fluorine' concentration of 2056ppm (see 4.1.1.1). If the 'total fluorine' was all present as potassium fluoroacetate (KMFA), this would equate to over 12,000ppm. As 45mg of extract was dissolved in 1ml of methanol and filtered through a 0.45µm hydrophobic syringe filter (Millipore), this would have made a solution of approximately 540ppm KMFA. After analysis, this solution was further concentrated under a gentle stream of nitrogen to approximately 250µl and re-analysed.

4.2.7 Gas chromatography-mass spectrometry (GC-MS)

Using head-space sampling of monofluoroacetic acid (MFA) and injecting directly into the GC-MS with no delay in data acquisition, it was found that the MFA came off at approximately 3 minutes. It would therefore not be seen in an underivatised sample, as it would elute in the "solvent front". The MFA was found to respond very weakly in the

GC-MS and to have the primary mass fragments m/z = 33, 45, 61 and 74. It was decided that detection of these ions, seen using ion profiling, at the same retention time should indicate the presence of compounds possessing a fluoroacetyl moiety.

The following GC-MS operating system and conditions were used for the anlaysis of derivatised samples:

GC-MS:	Hewlett Packard 6890 GC-MS
Column:	SGE BPX-5
	30m x 250µm x 0.25µm
Gas:	Helium @ 7.6psi; 1.0ml/min; average velocity 36cm/sec
Injector Temp:	250^0C
Injection:	1µl
Oven:	Initial temperature 50^0C; hold 3 minutes
	Ramp 6^0C min^{-1} for next 300^0C; hold 20 minutes
	Run time 64.67 minutes
Solvent Delay:	5 minutes

Data collections were in the scan or selective ion monitoring (SIM) mode @ 2.39 scans $second^{-1}$ in the mass range 35-350amu.

The following derivitisation steps were then undertaken:

1. A sample of *G. tetragonophyllum* methanol extract from within a 100MWCO dialysis membrane (see 4.1.1.3), with a 'total fluorine' concentration of 16,550ppm, was derivatised with MTBSTFA + 1% TBDMCS (N-methyl-N-(tert-butyldimethyl-silyl)-tri fluoroacetamide + 1% tert-butyldimethylchlorosilane, Pierce Chemical Company).

2. The petroleum spirit or chloroform extracts of *G. bilobum* (Quindanning), *G. calycinum* (Mundaring) and *G. tetragonophyllum* ('unknown') were methylated with diazomethane. This was unsuccessful for sodium monofluoroacetate and although monofluoroacetic acid appeared to react and be methylated, nothing was detected on the GC-MS, suggesting it was still coming off in the solvent front.

3. Derivitisation of monofluoroacetic acid, sodium monfluoroacetate and a sample of *G. tetragonophyllum* methanol extract from within a 100MWCO dialysis membrane was also attempted using N-trimethylsilylimidazole (TMSI) however this was again unsuccessful. There was either no obvious reaction or no deriviatives could be detected.

4.2.8 Inductively coupled plasma – atomic emission spectrometer (ICP-AES) seed elemental analysis

To determine elemental composition and the existence of any significant elemental variation, a selection of provenances of *Gastrolobium* seed were submitted for ICP-AES analysis. Waite Analytical Services, of The University of Adelaide, undertook the analyses, with standard controls, using an ARL 3580B Inductively Coupled Plasma – Atomic Emission Spectrometer. 600mg samples of intact seed were submitted in triplicate for ICP-AES elemental analysis. Samples were oven dried and then digested using a 2-step process with concentrated nitric acid and concentrated hydrochloric acid. This acidified mixture was allowed to cool digest overnight, then was heated with gradual temperature ramping over 5-6 hours to a temperature not exceeding 150^0C, before being diluted to 25ml and analysed.

Seed submitted for analysis were:

- *G. cuneatum* (Torbay) – 3 different seeding seasons (1996-97, 1997-98 and 2000-01) from the same site in order to examine temporal variation within a species. Unfortunately no additional 2002/2003 season seed was available to allow for examination of any elemental variation corresponding to the doubling in seed 'total fluorine' from the 2001/2002 season to the record levels of the 2002/2003 season;
- *G. bilobum* – 5 different provenances (Albany, Harvey, Manjimup, Quindanning and Tambellup) to examine intra-species variation;
- *G. parviflorum* (Jacup) – to examine a species which has very high levels of 'total fluorine' and caused the 'rapid' death of a cat (Chapter 8);
- *G. calycinum* (Mundaring) – to examine a species which did not cause the 'rapid' death of a cat (Chapter 8);
- *G. spinosum* (Mundaring) – to examine a species which contains only trace levels of 'total fluorine'.

4.2.9 Analysis for inorganic fluoride in *Gastrolobium* seed

In order to determine the proportion of seed 'total fluorine' which is organic and which is inorganic, the concentration of inorganic fluoride in the seed was investigated using *G. bilobum* (Quindanning), *G. calycinum* (Boddington) and *G. parviflorum* (Cuballing) seed.

A glass test tube was tared, the seed sample added and the seed weight recorded. 6ml of solvent (2M NaOH or distilled water) were added and the total weight recorded (W_0). The mixture was homogenised using a Heidolph Diax 900, then warmed in a water bath @ 50 ± 1^0C for 2 hours. The mixture was allowed to cool and settle, then it was

reweighed and the appropriate solvent added to adjust the weight to W_0. 2ml of supernatant were pipetted into a 25ml plastic beaker and 2ml TISAB or acetate buffer added. The pH was checked and AR grade glacial acetic acid added to mixtures with the NaOH solvent, to adjust pH to 5-7. The Emf (mV) was measured and compared to the standard curve, as per 4.2.3.

As the *Camellia* plant is recognised for its' propensity to accumulate free fluoride in its' leaves (Shuqi Ma, 1994), these leaves were used as a control. Camellia leaves were collected from a suburban garden in Thorngate, South Australia (Lat. -34^0 53.8'; Long. 138^0 35.82' - GDA). Similar leaves were selected, the central vein excised and the leaf pieces cut into minute pieces with scissors. Leaf pieces were then mixed together for replicated sampling. Leaf samples were analysed as per the seed samples with the mixtures stirred using a Chiltern MT19 Auto Vortex Mixer rather than homogenised using a Heidolph Diax 900. Additional controls were sodium monofluoroacetate (SMFA) and distilled water.

SMFA was analysed, to determine its stability under these conditions and therefore whether any results from the seeds could be from its hydrolysis. A stock solution of SMFA (BDH Chemicals Ltd, Poole England: 86% purity) in 2M NaOH was prepared by adding 150mg SMFA to a 10ml volumetric flask and making it up to the mark with 2M NaOH or distilled water. 200µl SMFA stock solution and 6ml solvent (2M NaOH or distilled water) were added to a pre-weighed test-tube and the solutions mixed using a Chiltern MT19 Auto Vortex Mixer. Samples were then treated and analysed as per the *Camellia* leaves control

4.2.10 Analysis of petroleum spirit and chloroform extracts by saponification

The fluorine containing compounds found in the petroleum spirit and chloroform extracts were hypothesised to be from fluorinated fatty acids as found in the seeds of the African plant *Dichapetalum toxicarium* (Peters and Hall, 1960; Hamilton and Harper, 1997). The absence of triplets in the ^{19}F NMR results (4.3.1) indicates that any fatty acids can not have a fluorine in the omega position as described from *Dichapetalum toxicarium* and would therefore be undescribed. A saponification of these extracts was conducted to test this hypothesis and further purify the samples to assist possible compound identification.

Two 15g samples of *G. tetragonophyllum* seed ('unknown') were milled in a water-cooled IKA A10 Analytical Mill. The samples of milled seed were extracted with chloroform using a soxhlet apparatus (see 4.2.2) and the extract analysed for 'total fluorine'. 2.8g of extract were transferred to a 500ml round-bottom flask, 100ml of 10% potassium hydroxide plus anti-bumping granules added and the solution heated under reflux for 7.25hrs. The mixture following reflux was transferred to a separating funnel with petroleum spirit (M & B, 40^0C-60^0C, AR grade) and distilled water washes and the separating funnel shaken and allowed to separate.

The aqueous layer was transferred to another separating funnel and acidified with 36% HCl to pH ~1. This fraction was then extracted with diethyl ether four times and the combined ether extracts dried with anhydrous sodium sulphate. The ether extracts were then filtered with a Whatman 1PS filter paper into a pre-weighed pear-shaped flask, the

ether removed under a gentle stream of high purity nitrogen and the extract weighed. Samples of the ether extract were then analysed for 'total fluorine'.

The petroleum spirit layer was transferred to a conical flask and dried with anhydrous sodium sulphate. The mixture was filtered with a Whatman 1PS filter paper into a pre-weighed pear-shaped flask, the petroleum spirit removed under a gentle stream of high purity nitrogen and the extract weighed. Samples of this petroleum spirit extract were then analysed for 'total fluorine'.

The ether extracted aqueous layer was transferred to a 500ml conical flask and the water was removed under reduced pressure using a Buchi rotary evaporator with the water bath held at 35 ± 1^0C. Samples of this aqueous extract were then analysed for 'total fluorine'.

4.2.11 Fluoroacetate analysis of *G. bilobum* (Quindanning) seed

Seed was milled in a water-cooled IKA 'A10 Analytical Mill' and two 20g samples, identified only by a number, was analysed for fluoroacetate by the Alan Fletcher Research Station, Department of Natural Resources, Queensland using a gas chromatography – mass spectrometry method based on Ozawa and Tsukioka (1989).

4.3 Results

4.3.1 'Total fluorine' content of intact *Gastrolobium* seed

The 'total fluorine' content of intact *Gastrolobium* seed is presented in Table 4.2 and graphically represented in Figure 4.1. These results indicate that the seeds of *Gastrolobium* species, such as *G. spinosum*, contain almost no fluoride or organo-fluorine compounds (although Hall (1972) recorded 65ppm organic fluorine), while species such as *G. cuneatum* recorded a massive mean 'total fluorine' of 1063.9 ± 77.8ppm (n=3). Significant spatial intra-species variation was found with seed sourced from different provenances (see Figure 4.2) and temporal variation in *G. cuneatum* seed sourced from the same provenance (see Figure 4.3). The results from *G. parviflorum* (Jacup) and *G. racemosum* (Jacup) collected from the same patch of vegetation in February 2000 show inter-species variation can be minimal at a given place and time. This suggests factors such as rainfall, soil type and resident mycorrhizal fungal associations could have a more significant role in seed 'total fluorine' concentrations than perhaps a species' genetic predisposition. The *G. parviflorum* and *G. bilobum* collected from Tambellup in January 2002 were from different patches of vegetation.

As the established view was that fluoroacetate is the sole toxicant within *Gastrolobium* seed, Figure 4.4 illustrates this assumption, with the seed 'total fluorine' presented as the potassium salt, potassium fluoroacetate. The results of the ICP-AES analysis (Appendix 1) found potassium to be the exceedingly dominant cation in the seeds analysed.

'Total fluorine' analysis of distilled water gave only 2.9 + 1.0ppm (mean ± SE; n=3), indicating only minor background levels.

Table 4.2 'Total fluorine' content of intact *Gastrolobium* seed

Sample Number	Species	Sample Weight $\bar{x}$ ± SE (mg)	Fluorine $\bar{x}$ ± SE (ppm)
1	*G. bilobum*	500.1 ± 0.4 (*n*=6)	10.4 ± 3.7 (*n*=6)
1a	*G. bilobum*	502.4 ± 1.2 (*n*=3)	420.6 ± 44.3 (*n*=3)
1b	*G. bilobum*	505.6 ± 2.8 (*n*=3)	145.6 ± 19.7 (*n*=3)
1c	*G. bilobum*	202.2 ± 0.9 (*n*=3)	504.3 ± 25.9 (*n*=3)
1d	*G. bilobum*	504.9 ± 0.9 (*n*=3)	22.8 ± 0.7 (*n*=3)
1e	*G. bilobum*	204.3 ± 1.5 (*n*=4)	421.0 ± 12.3 (*n*=4)
2	*G. calycinum*	474.2 ± 15.4 (*n*=6)	816.0 ± 32.5 (*n*=6)
2a	*G. calycinum*	505.5 ± 1.1 (*n*=3)	250.0 ± 27.1 (*n*=3)
2b	*G. calycinum*	508.4 ± 3.9 (*n*=3)	256.3 ± 38.0 (*n*=3)
2c	*G. calycinum*	507.8 ± 1.0 (*n*=3)	619.9 ± 14.4 (*n*=3)
3	*G. crassifolium*	502.7 ± 1.6 (*n*=3)	668.2 ± 35.9 (*n*=3)
4	*G. cuneatum*	502.6 ± 1.2 (*n*=3)	442.2 ± 14.8 (*n*=3)
4a	*G. cuneatum*	502.4 ± 0.2 (*n*=3)	310.0 ± 6.8 (*n*=3)
4b	*G. cuneatum*	502.4 ± 0.4 (*n*=3)	511.6 ± 17.3 (*n*=3)
4c	*G. cuneatum*	321.0 ± 93.4 (*n*=3)	391.8 ± 12.1 (*n*=3)
4d	*G. cuneatum*	205.1 ± 0.4 (*n*=3)	1063.9 ± 77.8 (*n*=3)
5	*G. laytonii*	508.5 ± 1.3 (*n*=3)	46.3 ± 1.4 (*n*=3)
6	*G. parviflorum*	497.4 ± 4.0 (*n*=7)	548.7 ± 32.2 (*n*=7)
6a	*G. parviflorum*	502.9 ± 1.3 (*n*=3)	118.3 ± 28.4 (*n*=3)
6b	*G. parviflorum*	501.9 ± 0.4 (*n*=3)	975.5 ± 77.7 (*n*=3)
6c	*G. parviflorum*	505.7 ± 1.2 (*n*=3)	433.3 ± 14.3 (*n*=3)
7	*G. parvifolium*	502.5 ± 0.5 (*n*=3)	90.8 ± 6.1 (*n*=3)
8	*G. racemosum*	502.8 ± 1.5 (*n*=3)	713.5 ± 38.7 (*n*=3)
8a	*G. racemosum*	502.9 ± 0.9 (*n*=3)	904.4 ± 55.8 (*n*=3)
9	*G. spathulatum*	503.9 ± 1.4 (*n*=3)	2.8 ± 0.1 (*n*=3)
10	*G. spinosum*	503.0 ± 0.9 (*n*=3)	3.2 ± 0.9 (*n*=3)
10a	*G. spinosum*	502.7 ± 1.2 (*n*=3)	2.3 ± 1.0 (*n*=3)
10b	*G. spinosum*	504.2 ± 1.1 (*n*=3)	1.6 ± 0.3 (*n*=3)
11	*G. stenophyllum*	502.4 ± 0.5 (*n*=3)	673.5 ± 18.6 (*n*=3)
12	*G. tetragonophyllum*	503.2 ± 1.4 (*n*=3)	848.7 ± 46.9 (*n*=3)
13	*G. villosum*	503.0 ± 2.3 (*n*=3)	430.0 ± 33.2 (*n*=3)

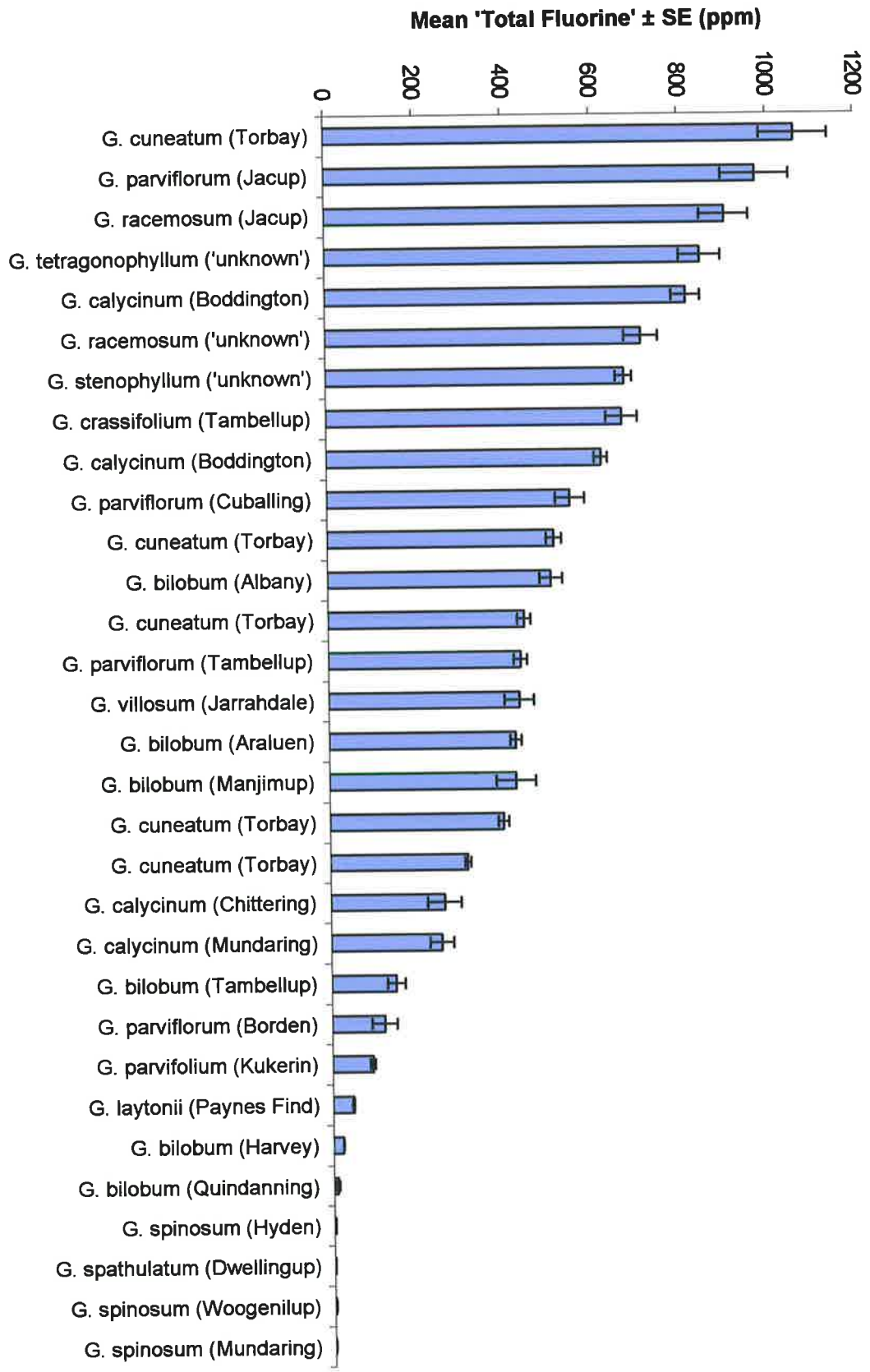


Figure 4.1 'Total fluorine' analysis of intact *Gastrolobium* seed

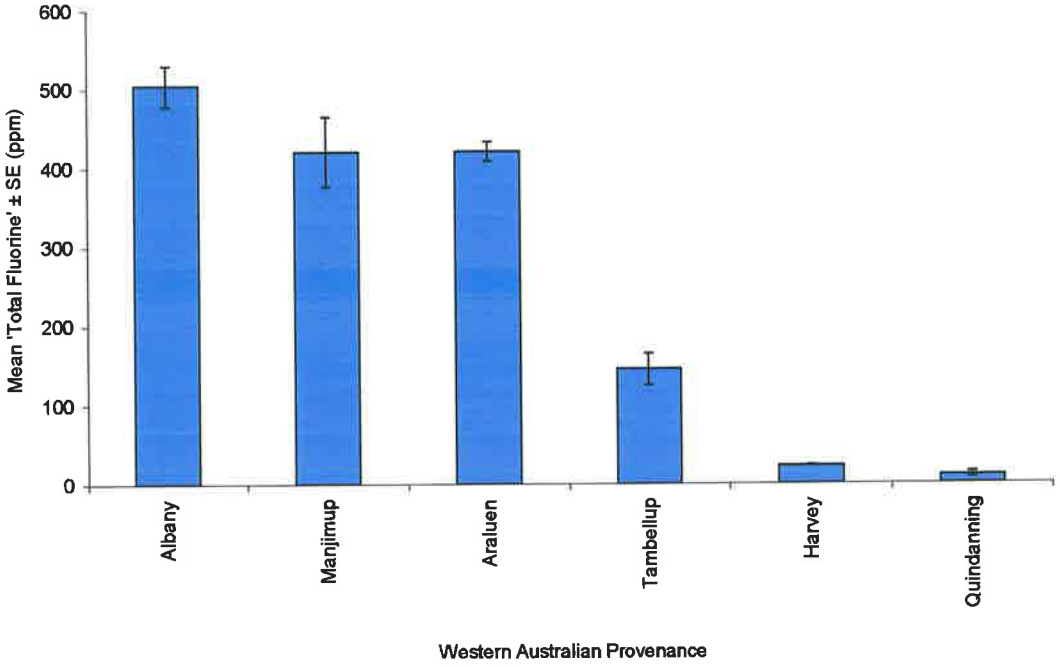


Figure 4.2 Inter-provenance variation in *Gastrolobium bilobum* seed 'total fluorine' concentrations

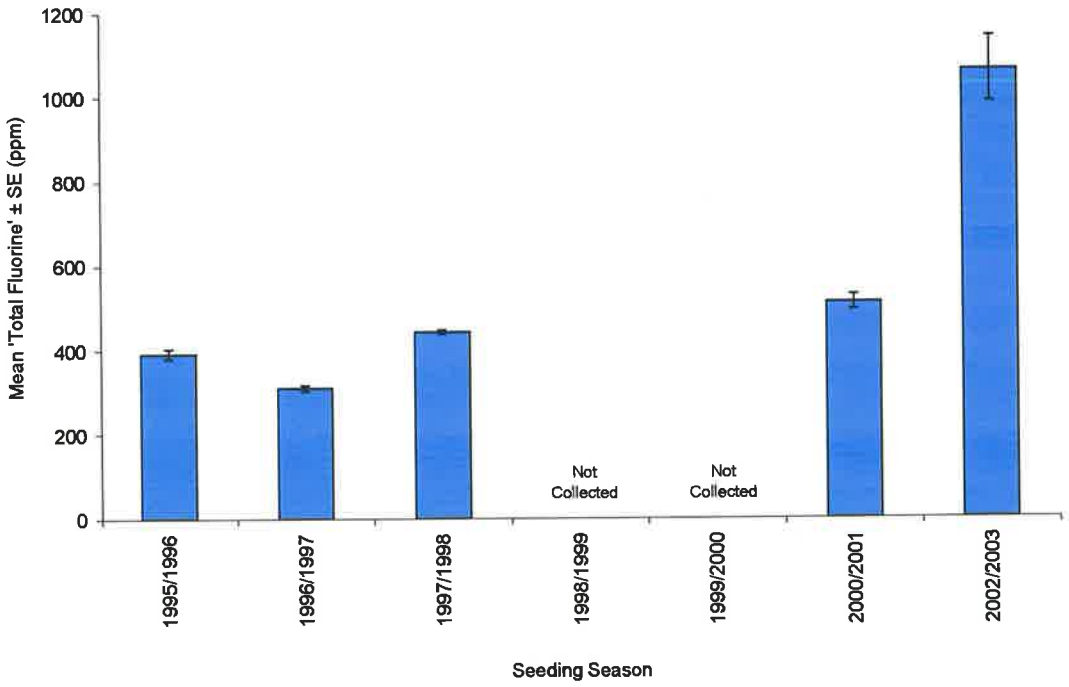


Figure 4.3 Intra-provenance variation in *Gastrolobium cuneatum* seed 'total fluorine' concentrations from 'Southern Comfort', Torbay, Western Australia

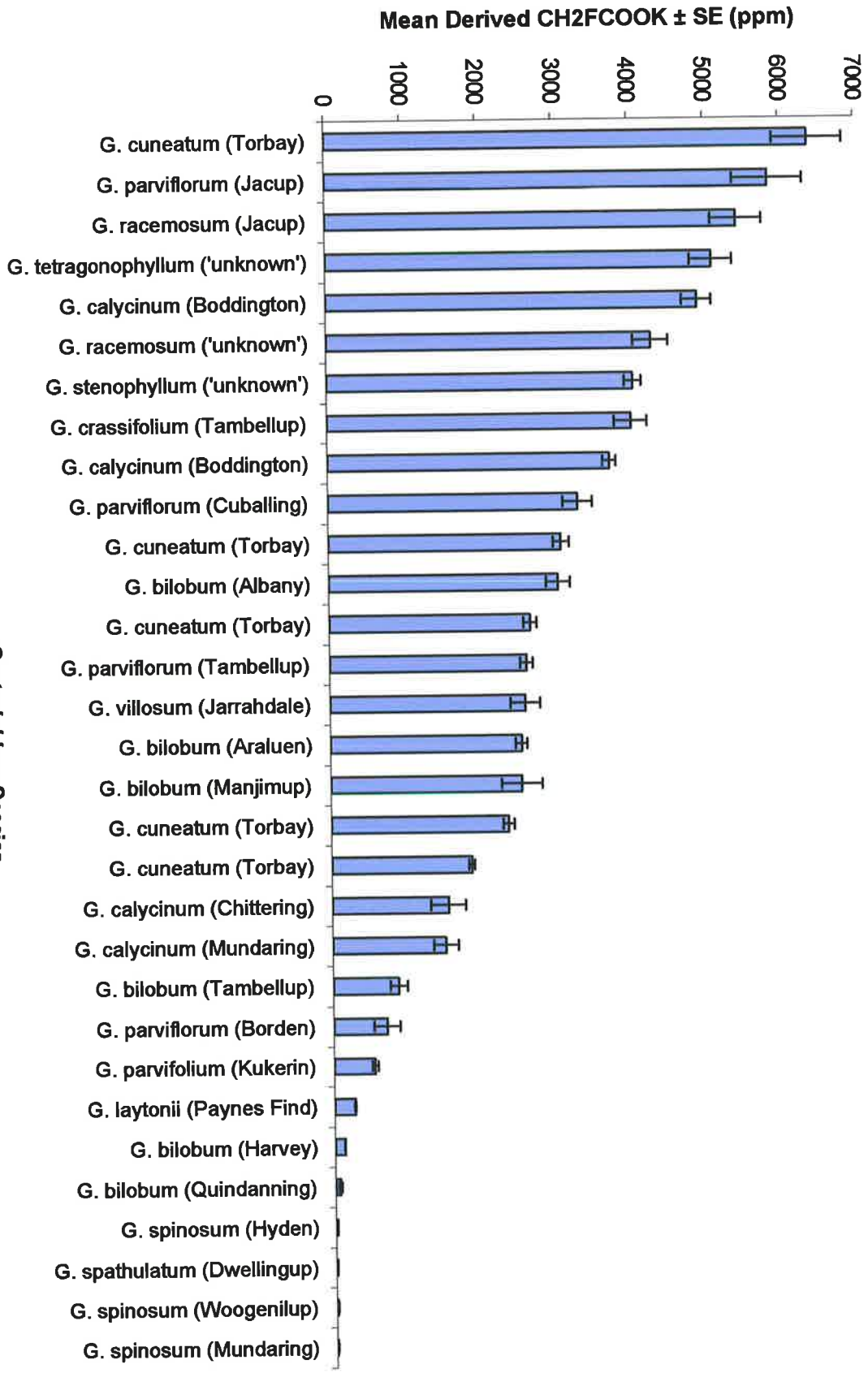


Figure 4.4 Potential potassium fluoroacetate in *Gastrolobium* seed (6 x 'total fluorine'). Assumes fluoroacetate is bound to the most abundant cation.

4.3.2 Analysis for inorganic fluoride in *Gastrolobium* seed

The concentration of inorganic fluoride in *G. bilobum* (Quindanning), *G. calycinum* (Boddington) and *G. parviflorum* (Cuballing) seed is presented in Table 4.3. Little inorganic fluoride was detected in the seed (max. 9.0 ± 4.0; $\bar{x}$ ± SE; $n = 3$), indicating therefore that substantial amounts of organic fluorine is found in the seed. Results for the *Camellia* leaves indicate the hot NaOH successfully extracts inorganic fluoride, though results were significantly less than obtained by the dry weight method of Shuqi Ma (1994). Results for the sodium monofluoroacetate (SMFA) show that only a small percentage of the expected fluoride, (a figure of 61.28ppm should have been obtained if all the fluorine in SMFA was released), was detected. Therefore SMFA is not hydrolysed under these conditions and the results from the seeds do not arise from fluoroacetate hydrolysis. The fluoride detected in the SMFA was probably from NaF contaminating the technical grade SMFA (^{19}F NMR showed the SMFA triplet plus a singlet coincident with the peak from a NaF spike).

4.3.3 Morphological distribution of 'total fluorine' in *Gastrolobium* seed

The *Gastrolobium* seed was divided into the cotyledons, defined as the seed tissue inside of the testa, and the testa + aril, being the seed coat and when present, the attached aril. The percentage of the seed consisting of cotyledons or testa + aril is presented in Table 4.4. The partitioning of the seed 'total fluorine' between the cotyledons and testa + aril is shown in Table 4.5, and summarised as a percentage of the total in Table 4.6. The large majority of 'total fluorine' is found in the cotyledons.

Table 4.3 Analysis for inorganic fluoride in *Gastrolobium* seed

Sample	Extraction With Hot Sodium Hydroxide		Extraction With Hot Water	
	Sample Weight $\bar{x} \pm$ SE (mg)	Fluoride $\bar{x} \pm$ SE (ppm)	Sample Weight $\bar{x} \pm$ SE (mg)	Fluoride $\bar{x} \pm$ SE (ppm)
G. bilobum (Quindanning) Seed	500.3 ± 0.5 (*n*=3)	3.3 ± 0.3 (*n*=3)	502.4 ± 2.8 (*n*=3)	1.8 ± 0.5 (*n*=3)
G. calycinum (Boddington) Seed	506.3 ± 1.7 (*n*=3)	8.3 ± 4.2 (*n*=3)	507.0 ± 0.6 (*n*=3)	6.0 ± 0.7 (*n*=3)
G. parviflorum (Cuballing) Seed	504.5 ± 1.6 (*n*=3)	9.0 ± 4.0 (*n*=3)	504.7 ± 1.3 (*n*=3)	7.7 ± 1.6 (*n*=3)
Camellia Leaves	502.7 ± 1.1 (*n*=5)	485.5 ± 55.5 (*n*=5)	501.3 ± 1.1 (*n*=5)	151.4 ± 1.9 (*n*=5)
Distilled Water	501.1 ± 1.2 (*n*=3)	0 ± 0 (*n*=3)	201.6 ± 0.9 (*n*=5)	0 ± 0 (*n*=5)
Sodium Fluoroacetate	0.49mg fluoride in 0.2ml (*n*=5)	7.1 ± 0.2* (*n*=5)	0.49mg fluoride in 0.2ml (*n*=5)	5.4 ± 0.1* (*n*=5)

* Approximately 2,550ppm fluoride would arise from a complete hydrolysis. These results are believed due to contaminating sodium fluoride.

Table 4.4 Composition of *Gastrolobium* seed

Gastrolobium Species	Cotyledons (Percentage of 0.5g Seed) $\bar{x} \pm$ SE (n=3)	Testa + Aril (Percentage of 0.5g Seed) $\bar{x} \pm$ SE (n=3)
G. bilobum (Manjimup)	55.4 ± 0.4	44.6 ± 0.4
G. calycinum (Boddington)	53.7 ± 0.06	46.3 ± 0.06
G. parviflorum (Cuballing)	55.4 ± 1.04	44.6 ± 1.04

Table 4.5 'Total fluorine' partitioning in dissected seed

Sample	Cotyledons		Testa + Aril	
	Sample Weight $\bar{x} \pm$ SE (mg)	'Total Fluorine' $\bar{x} \pm$ SE (ppm)	Sample Weight $\bar{x} \pm$ SE (mg)	'Total Fluorine' $\bar{x} \pm$ SE (ppm)
G. bilobum (Manjimup)	274.3 ± 2.1 (n=3)	543.1 ± 92.1 (n=3)	220.3 ± 3.0 (n=3)	105.6 ± 11.4 (n=3)
G. calycinum (Boddington)	252.7 ± 13.2 (n=3)	1558.4 ± 58.9 (n=3)	221.3 ± 7.1 (n=3)	188.5 ± 13.5 (n=3)
G. parviflorum (Cuballing)	271.1 ± 9.0 (n=3)	948.8 ± 20.9 (n=3)	218.3 ± 1.7 (n=3)	129.7 ± 8.7 (n=3)

Table 4.6 Summary of mean 'total fluorine' partitioning in dissected seed

Sample	Cotyledons (%)	Testa + Aril (%)
G. bilobum (Manjimup)	84	16
G. calycinum (Boddington)	89	11
G. parviflorum (Cuballing)	88	12

4.3.4 Organo-fluorine compounds in petroleum spirit and chloroform extracts of *Gastrolobium* seed

'Total fluorine' analysis of the petroleum spirit and chloroform extracts discovered the presence of significant but variable concentrations of 'total fluorine', and these results are presented in Tables 4.7 and 4.8.

Utilising ^{19}F NMR an almost identical spectrum of at least seven ^{19}F singlets and doublets were observed in the petroleum spirit and chloroform extracts of *G. bilobum* (Quindanning), *G. calycinum* (Mundaring), and *G. parviflorum* (Cuballing) seeds. Figure 4.5 details the ^{19}F NMR spectrum from 8, 281 scans of the chloroform extract of *G. bilobum* (Quindanning). These resonances are suspected to be from new fluorinated fatty acids, but as they are not triplets, they cannot have a fluorine in the omega position as described from the seeds of the African plant *Dichapetalum toxicarium* (Peters and Hall, 1960; Hamilton and Harper, 1997). They are also not from the extraction materials and glassware, being absent from the solvent extraction of other materials (such as the rat skeletons in 7.2.5).

The methylation of the petroleum spirit or chloroform extracts of *G. bilobum* (Quindanning), *G. calycinum* (Mundaring) and *G. tetragonophyllum* ('unknown') with diazomethane and GC-MS analysis found a range of fatty acids up to arachidic acid ($C_{20}H_{40}O_2$). Although there were numerous other peaks, most were not identifiable by the GC-MS library and in none could the presence of fluorine be positively established. Use of a GC with an electron capture detector (ECD) indicated numerous halogenated compounds were present, however it was unknown if these were solely a response from

fluorine. An inability to compare compound retention times in the data from the 2 gas chromatographs prevented compound identification.

Saponification of the chloroform extract of *G. tetragonophyllum* ('unknown') resulted in further fractionation of the 'total fluorine'. The ether fraction (putative fatty acids) gave 12.8 ± 0.1ppm (mean ± SE; n=2); the petroleum spirit fraction (non-saponifiables) gave 11.2ppm (n=1) and the water fraction gave 7.6 ± 0.0ppm (n=2). However 2/3 of the 'total fluorine' appeared to be lost during the process, suggesting volatilisation occurred. Some of this missing 'total fluorine' was found adsorbed to the sodium sulphate used to dry the ether fraction. When analysed by NMR it was found to give one ^{19}F singlet, but to still be a mixture of at least 3 compounds. Analysis of the ^{13}C and proton NMR indicated the presence of aliphatic carbons, a chain of 6-7 carbons, one C=C, a ketone group not directly attached to the C=C and that it was not an ester but most probably a carboxylic acid. This was the closest to an identification achieved for the organo-fluorine compounds present in the petroleum spirit and chloroform extracts, and supportive of the hypothesis of non-omega fluorinated fatty acids. Identification of these organo-fluorine compounds was not pursued further in this study.

4.3.5 Organo-fluorine compounds in the methanol extract of *Gastrolobium* seed

In all seeds extracted the majority of the 'total fluorine' is found in the methanol extract (Table 4.9). Using ^{19}F NMR, a large triplet rapidly appears at -63.1ppm, coincident with a sodium monofluoroacetate spike. With extended scanning however, the cool methanol extracts of *G. bilobum* (Tambellup) and *G. parviflorum* (Jacup) were

observed to have other triplets occurring with the primary triplet (see Figure 4.6). Also additional small triplets were observed downfield of the main fluoroacetate triplet. In *G. parviflorum* (Jacup), after 32K scans in H_2O/D_2O, three overlapping triplets (intensities 1:1.8:2.2) were observed between -76.2 to -76.5ppm, with Js of 50+/- 1 Hz and downfield of another triplet of the same J, centred at -72.7ppm. These signals were 0.2% of the intensity of natural fluoroacetate present at -63.1ppm (see Figure 4.6). In addition, a significant singlet at 78.2ppm was coincident with a spike of trifluoroacetic acid (TFA). The highest recorded natural level is 0.0064ppm from the Dead Sea (Frank *et al.*, 1996), an undetectable concentration using ^{19}F NMR. It is yet to be determined if the result from the seed is due to contamination, however if shown to be from the seed it would be the highest recorded natural level. This may be consistent with its presence in the fluorine hyper-accumulating *Gastrolobium* species. Further confirmation of the natural presence of this compound was not undertaken during this study.

All signals additional to the primary triplet are unassigned and discussed under Figure 4.6. They are not from the extraction materials and glassware, being absent from the solvent extraction of other materials (such as the rat skeletons in 7.2.5).

Table 4.7 'Total fluorine' concentration of the petroleum spirit extract of *Gastrolobium* seed

Extraction Temperature	Sample	Sample Weight $\bar{x}$ ± SE (mg)	'Total Fluorine' $\bar{x}$ ± SE (ppm)
Low	*G. bilobum* (Quindanning)	208.8 ± 6.4 (*n*=3)	82.0 ± 19.7 (*n*=3)
High	*G. bilobum* (Manjimup)	220.2 ± 7.1 (*n*=3)	7.4 ± 0.6 (*n*=3)
High	*G. bilobum* (Tambellup)	220.7 ± 2.5 (*n*=3)	30.5 ± 6.3 (*n*=3)
Low	*G. calycinum* (Boddington)	147.7 ± 3.5 (*n*=3)	115.5 ± 35.4 (*n*=3)
High	*G. calycinum* (Mundaring)	217.4 ± 6.0 (*n*=3)	6.4 ± 1.0 (*n*=3)
Low	*G. parviflorum* (Cuballing)	155.3 ± 2.1 (*n*=3)	101.2 ± 27.8 (*n*=3)
Low	*G. parviflorum* (Tambellup)	231.4 ± 7.9 (*n*=3)	18.7 ± 4.5 (*n*=3)
Low	*G. parviflorum* (Jacup)	250.9 ± 3.4 (*n*=3)	8.1 ± 2.3 (*n*=3)
High	*G. tetragonophyllum* ('unknown')	206.9 ± 3.5 (*n*=3)	13.9 ± 5.1 (*n*=3)

Table 4.8 'Total fluorine' concentration of the chloroform extract of *Gastrolobium* seed

Extraction Temperature	Sample	Sample Weight $\bar{x}$ ± SD (mg)	'Total Fluorine' $\bar{x}$ ± SE (ppm)
High	*G. calycinum* (Mundaring)	147.8 (*n*=1)	25.7* (*n*=1)
Low	*G. calycinum* (Mundaring)	85.0 ± 11.5 (*n*=3)	33.5 ± 19.6* (*n*=3)
Low	*G. parviflorum* (Tambellup)	83.8 ± 18.0 (*n*=3)	17.6 ± 1.0* (*n*=3)
Low	*G. parviflorum* (Jacup)	125.5 ± 2.7 (*n*=3)	60.1 ± 18.4* (*n*=3)
High	*G. parviflorum* (Jacup)	213.3 (*n*=1)	19.1 (*n*=1)
High	*G. tetragonophyllum* ('unknown')	205.6 ± 5.0 (*n*=2)	62.7 ± 3.2* (*n*=2)
High	*G. tetragonophyllum* ('unknown')	224.9 ± 4.5 (*n*=2)	55.1 ± 5.2 (*n*=2)

* Previously extracted with petroleum spirit

Table 4.9 'Total fluorine' concentration of the methanol extract of *Gastrolobium* seed

Extraction Temperature	Sample	Sample Weight $\bar{x}$ ± SE (mg)	'Total Fluorine' $\bar{x}$ ± SE (ppm)
Low	*G. bilobum* (Tambellup)	210.6 ± 6.6 (*n*=3)	2513.8 ± 200.6# (*n*=3)
High	*G. calycinum* (Mundaring)	224.9 ± 10.6 (*n*=3)	1159.6 ± 2.7# (*n*=3)
Low	*G. calycinum* (Mundaring)	203.4 ± 2.5 (*n*=3)	2251.4 ± 22.1# (*n*=3)
Low	*G. parviflorum* (Tambellup)	202.3 ± 1.3 (*n*=3)	4026.1 ± 153.3# (*n*=3)
High	*G. parviflorum* (Jacup)	238.6 (*n*=1)	7147.3* (*n*=1)
Low	*G. parviflorum* (Jacup)	203.2 ± 0.1 (*n*=3)	9788.3 ± 399.8# (*n*=3)
High	*G. tetragonophyllum* ('unknown')	209.8 ± 3.5 (*n*=3)	5831.9 ± 140.9# (*n*=3)

* Previously extracted with chloroform

Previously extracted with petroleum spirit and chloroform

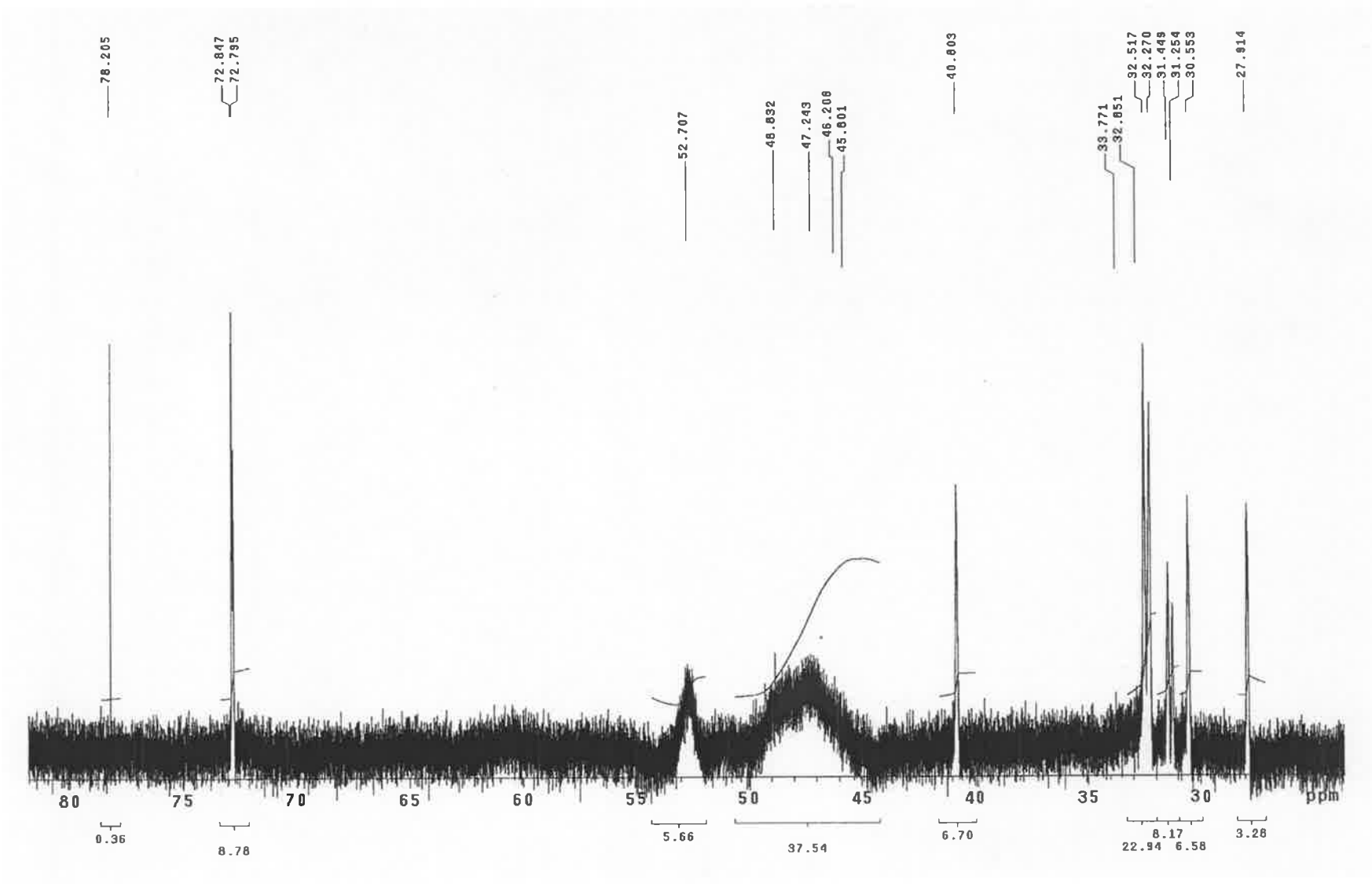


Figure 4.5 ^{19}F NMR resonances from 8, 281 scans of *G. bilobum* (Quindanning) chloroform extract; CD_3OD solvent

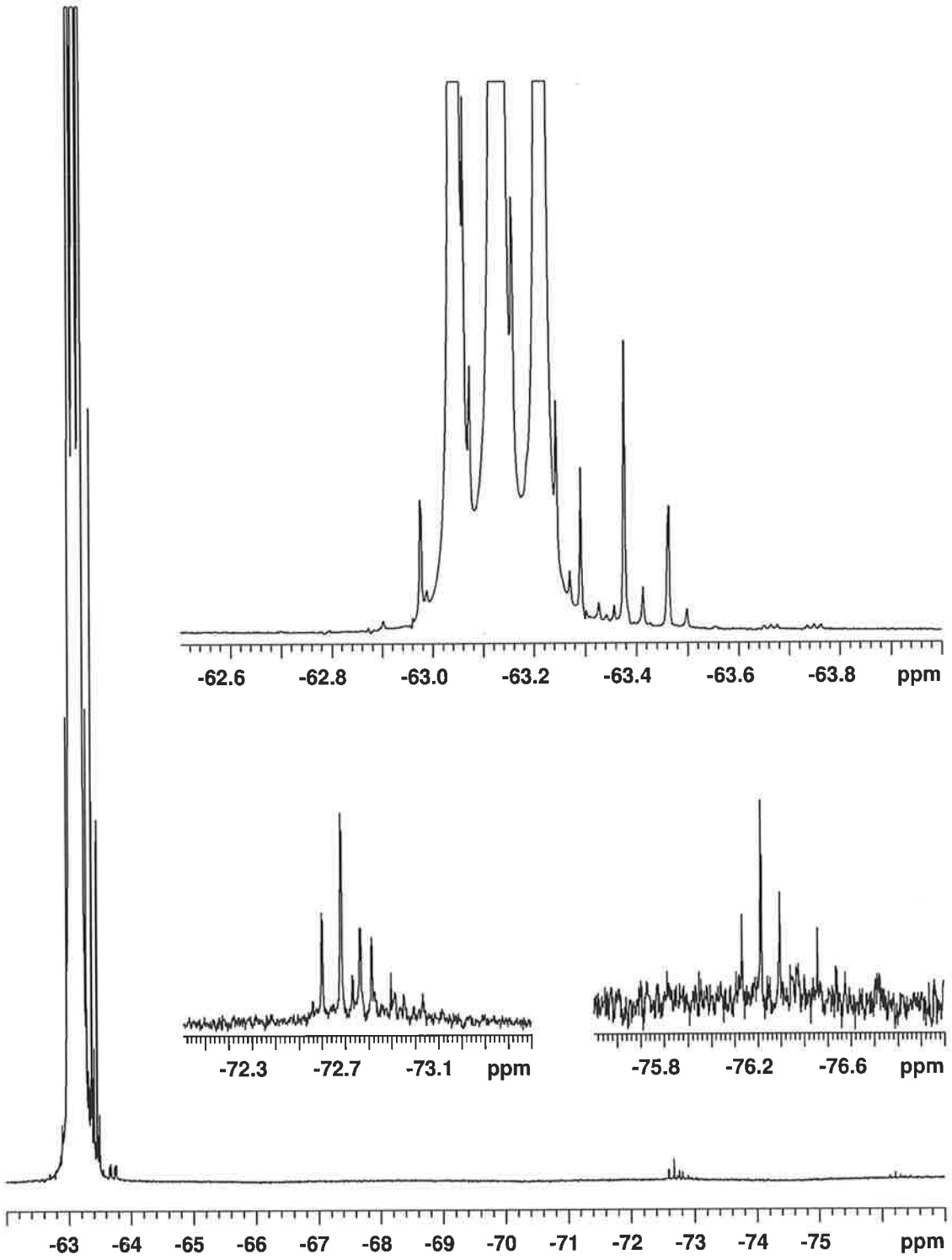


Figure 4.6 ^{19}F resonances from 32, 000 scans of a cool methanol extract of *G. parviflorum* seed. Large triplet at -63.14ppm attributed to fluoroacteate. The additional signals in this region, which appear to be mainly CH_2F triplets, are unassigned. They do not appear to be spinning side bands and should not arise from different fluoroacetate salts. The apparent CH_2F triplets at -72.7ppm are also unassigned. The triplets at -76.2ppm are believed due to fluoroacetylated sugars (see Chapter 5).

4.3.6 High pressure liquid chromatography (HPLC)

The LOD and LOQ reported by Minnaar *et al.* (2000a) could not be replicated in this study. These detection limits were derived from "linear regression analysis", with 25ppm being the lowest concentration of pure monofluoroacetic acid (MFA) which was used to derive these limits. Using the ICI LC1200 UV VIS single wavelength detector, the MFA came off at approximately 12 minutes and 5ppm was the lowest concentration detected, and then only as a very small peak which was difficult to integrate. Using the Agilent 1100 series HPLC with a diode-array detector, the MFA came off at 21.5 minutes with 400ppm the lowest concentration detectable (Figure 4.7). An injection of the *G. calycinum* extract detected a number of unidentified peaks however no peak was observed at the retention time for MFA using the Agilent HPLC (see Figure 4.8) or the single wavelength detector, with their differing sensitivities, for both approximately 540 and also 2060ppm KMFA.

4.3.7 Inductively Coupled Plasma – Atomic Emission Spectrometer (ICP-AES) Seed Elemental Analysis

ICP-AES analysis recorded numerous elements in the analysed *Gastrolobium* seeds with intra- and inter- species variability, primarily in the concentration of trace elements such as manganese and molybdenum. The overwhelmingly most dominant cation present in the analysed *Gastrolobium* seeds is potassium and this was very consistent across samples. The seeds generally contain less than 600ppm sodium, with *G. parviflorum* (Jacup) significantly higher at about 1100ppm. The ICP-AES results suggest seed

fluoroacetate would be most likely present as potassium fluoroacetate. The numerous elements analysed have been graphed and are included as Appendix 1.

4.3.8 Fluoroacetate analysis of *G. bilobum* (Quindanning) seed

Milled seed submitted for analysis had 6.7 ± 0.14ppm ($\bar{x}$ ± SE; n=2) fluoroacetate. As stated in 4.1, it is considered that any fluoroacetyl compound present in the seed would probably be derivitised by this method. However until the presence of additional fluoroacetylated compounds is confirmed, it is proposed to accept this result as a measure of the seed fluoroacetate concentration.

4.3.9 Additional solvent extraction results

Milled *G. bilobum* (Tambellup) and *G. parviflorum* (Jacup) seed was also extracted at room temperature with distilled water and analysed for 'total fluorine'. The *G. bilobum* seed gave a water extract with 944.7 ± 1.0ppm (mean ± SE; n=2) and the *G. parvifolium* seed gave 4076.2 ± 39.7ppm (mean ± SE; n=3). In addition, analysis of the seed residue after extraction gave respectively 8.2 ± 1.4ppm (mean ± SE; n=3) and 180.5 ± 26.9ppm (mean ± SE; n=3) showing an incomplete extraction or the presence of compound(s) unable to be extracted under these conditions.

With the cool successive extractions of *G. parviflorum*, the seed residue after the methanol extraction gave 476.9 ± 8.4ppm (mean ± SE; n=2) and then 285.6 ± 0.4ppm (mean ± SE; n=3) following another methanol extraction together with sonication. This suggests a more extensive extraction process is required, however using this method resulted in extraction of the largest concentration of 'total fluorine' with 9788.3 ±

399.8ppm (mean ± SE; n=3), indicating once again the presence of additional organo-fluorine compounds to the reported fluoroacetate.

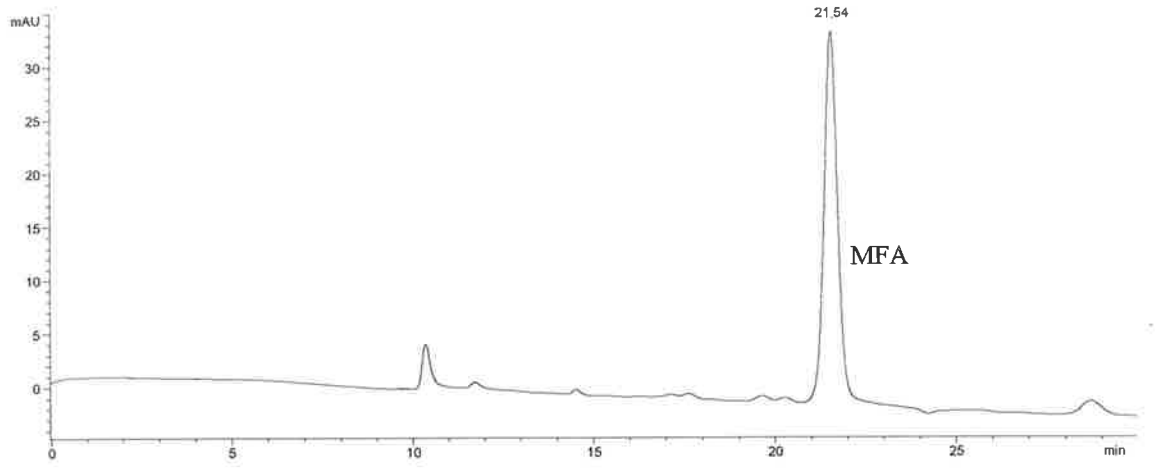


Figure 4.7 400ppm monofluoroacetic acid standard. Agilent 1100 HPLC at 210nm.

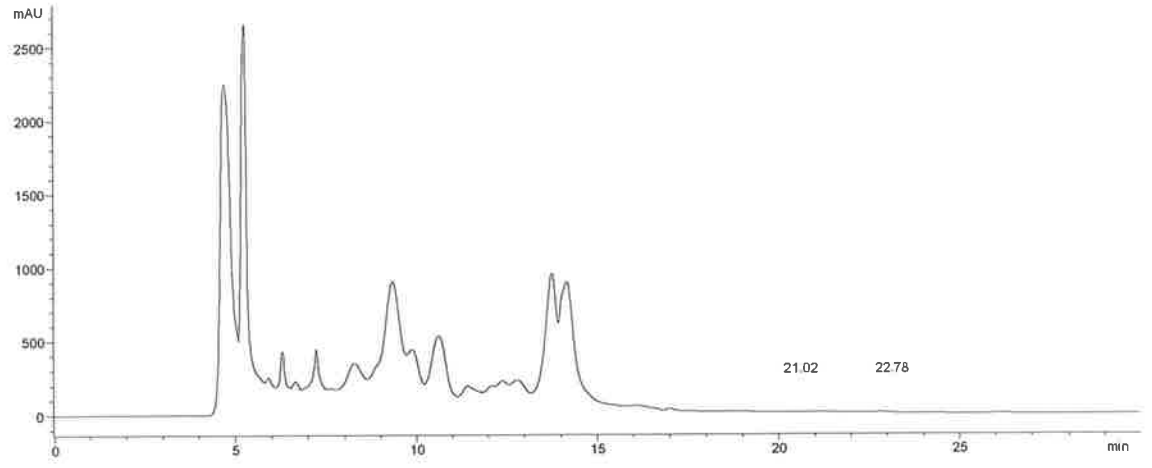


Figure 4.8 *G. calycinum* (Mundaring) TLC Band 1 extract. Extract was 2,056ppm 'total fluorine' giving a 540ppm KMFA solution. Agilent 1100 HPLC at 210nm.

4.4 Discussion

The seeds of *Gastrolobium* were analysed for their 'total fluorine' utilising a process of fusion with sodium hydroxide, with subsequent analysis using a fluoride ion specific electrode. Extreme variation exists between and within species, with species such as *G. spinosum* containing trace levels and *G. cuneatum* (collected from Torbay; January 2003), containing an extremely high concentration of 'total fluorine'. These levels were also found to rapidly increase, with the record concentration in *G. cuneatum* collected from Torbay in 2003 double that recorded in 2002. Almost all of this 'total fluorine' is present as organic fluorine rather than free fluoride. For example, *G. calycinum* from Boddington, Western Australia had a 'total fluorine' concentration of 816.0 ± 79.5 ppm (n=3), with only 8.3 ± 7.2 ppm (n=3) free fluoride. Additionally, this study determined that most of this fluorine is stored within the seed's cotyledons rather than the testa and aril. For the *G. calycinum* seed discussed above, this apportioning was 89% in the cotyledons and 11% in the testa + aril. This indicates that species of *Gastrolobium* such as *G. calycinum* have developed the strategy of protecting the developing seedlings with fluorinated toxicants and is supportive of the reports which describe the seedlings as one of the most toxic growth stages (Gardner and Bennetts, 1956).

The use of the fluoride-specific electrode used by Hall (1972) has been a critical experimental decision. To have made the assumption that all fluorine in *Gastrolobium* seeds is fluoroacetate and utilised a fluoroacetate specific analysis method (such as used by Twigg *et al.*, 1996; Twigg *et al.*, 1999) would have been a major error. At an early stage in the seed analysis it was apparent that the seed has to contain organo-fluorine

compounds other than fluoroacetate alone, to account for the 'total fluorine' being measured in multiple chromatographic fractions. In addition, use of the electrode in attempted purification processes allowed for following of the 'total fluorine' and hence the determination that it was more easily "misplaced" or "lost" than retained. Analysis of organo-fluorine compounds is however very difficult, with the two primary techniques being ion chromatography (eg. O'Hagan *et al.*, 1993) and the fluoride-specific electrode. Although the fluoride-specific electrode is very sensitive, it requires destruction of the sample and this was found to be very inhibiting of the fractionation and purification processes. That is, to determine which fraction has retained the 'total fluorine' requires destruction of some of that sample. As samples were further purified this became very expensive in terms of sample material.

In the analysis of petroleum spirit and chloroform fractions, the presence of 'total fluorine' was suspected to indicate that *Gastrolobium* seeds also contained fluorinated fatty acids as per *Dichapetalum toxicarium* (Peters and Hall, 1960; Hamilton and Harper, 1997). Analysis of these solvent extracts utilising ^{19}F NMR has found evidence for 6 fluorine resonances in the non-polar extract, suggesting possibly 6 new fluorinated compounds, not likely however to be the omega-fluoro fatty acids of *Dichapetalum toxicarium* (Hamilton and Harper, 1997). The saponification experiment to extract these fatty acids, in conjunction with use of the electrode, determined that almost 75% of the fluorine was missing. Some of this was found adsorbed to the sodium sulphate used to dry the extracts and the recovery and NMR analysis of this fluorine provided the closest identification of one of these new fluorinated compounds (see 4.3.4).

Extraction of the seeds with solvents of increasing polarity has separated a number of different organo-fluorine compounds. The majority of the 'total fluorine' is extracted in the final methanol extract. ^{19}F NMR indicates a triplet characteristic of the CH_2F-moiety, and being almost coincident with the triplet of the sodium monofluoroacetate standard, seems most likely to be the same compound. However, the lack of agreement between the HPLC and ^{19}F NMR results for the presence of fluoroacetate is difficult to explain. Different fluoroacetylated standards such as fluoroacetamide, monofluoroacetic acid and sodium monofluoroacetate did have different chemical shifts when analysed by ^{19}F NMR, supporting the primary triplet as being from fluoroacetate salt(s). In contrast and based on the standards injected into both HPLC detectors, no explanation can be given for the absence of a peak at the MFA retention time, apart from absence of the compound itself. Although fluoroacetate was detected in the seeds of *G bilobum* (Quindanning) by GC-MS, the water extraction of the seeds and the derivitisation process may, as has been previously argued, produce the desired fluoroacetyl derivative largely independent of the fluoroacetylated parent compound.

One possible explanation is that the seed methanol extracts contain compounds which have an identical ^{19}F NMR chemical shift to fluoroacetate, but are not themselves fluoroacetate. However, the ^{19}F NMR and GC-MS results support the presence of fluoroacetate, with only the HPLC results indicating otherwise. Although this suggests fluoroacetate is the primary organo-fluorine compound in these seeds, further analysis utilising an LC-Q (a HPLC with a quadrapole mass spectrometer) with a single wavelength detector and more concentrated seed extracts, is recommended.

The presence in the cool methanol extracts of triplets additional to that ascribed to fluoroacetate suggests the other triplets, being both the large and small triplets associated with the primary triplet and also the small downfield triplets at –72.7 and –76.2ppm, are not fluoroacetate. In addition, the presence of a singlet at 78.2ppm indicates that trifluoroacetic acid (TFA) may also be present in these seeds, as an injection of this compound gave a coincident singlet.

4.5 Conclusion

Taken collectively, the results from the analysis of *Gastrolobium* seed for organo-fluorine compounds has detected the presence of numerous additional compounds to the reported fluoroacetate. The presence of fluorinated compounds in addition to fluoroacetate is considered evidence to reject the null hypothesis: that fluoroacetate is the only organo-fluorine compound in *Gastrolobium* seeds. It is unfortunate that the solid state NMR was not successful, as it would have confirmed that the ^{19}F signals additional to the fluoroacetate triplet were not artifacts of the extraction and analysis process without additional chemistry being required.

The purification and identification of fluorinated compounds is very difficult, especially for these new compounds producing the ^{19}F singlets and doublets. Unlike the CH_2F producing triplets, no obvious fragment is readily apparent for mass spectral analysis. Although analysis using GC-MS and GC-ECD were undertaken on separate GC's, access to a GC which possesses both detectors and a sample splitter would enable establishment of which peaks (ie. compounds) give a response on the ECD and thus contain a halogen – possibly fluorine. This would then allow for comparison with the mass spectrum of the identical peak. It is anticipated that this would form the best approach to identification of these new fluorinated compounds. Use of preparative HPLC could then be utilised to collect enough of the compounds for NMR and high resolution mass spectrometry (HRMS) in order to confirm the identifications and establish compound structures. Toxicity and skeletal retention studies could then be undertaken to see if any of these new fluorinated compounds are rapidly toxic and bone retentive, as described in the historical anecdotes.

The investigation of the small triplets at –76.2ppm as being fluoroacetylated sugars is described in the following chapter, Chapter 5.

Chapter 5: Fluoroacetylated Sugars in *Gastrolobium* Seed

5.1 Introduction

The extensive analysis of *Gastrolobium* seed within this study has established the presence of multiple unidentified organo-fluorine compounds. As any of these compounds could explain the rapid death and toxic bone anecdotes, it was necessary to identify as many of these compounds as possible.

The exploratory work of Hall (1972) suggested the possible presence of a fluorinated carbohydrate or amino acid in the seeds of *G. bilobum*. Preliminary column and thin-layer chromatography undertaken in this study (4.2.4) suggested the presence of organo-fluorine compounds additional to the reported fluoroacetate. Using ^{19}F NMR, additional weak triplets downfield of the main fluoroacetate triplet were observed in the methanol extracts of *G. bilobum* (Tambellup) and *G. parviflorum* (Jacup) seeds. As the methanol extracts had a very crystalline, sugar-like appearance and had 'total fluorine' recorded in multiple fractions, it was proposed that these triplets and 'total fluorine' may be from fluoroacetylated sugars, as hypothesised by Hall (1972).

The presence of fluoroacetylated sugars in *Gastrolobium* seeds could help explain the historical anecdotes. Any inhibition of glycolysis may cause rapid death such as described in the anecdotes, with Taylor (1972) stating some fluoro-sugars to competitively inhibit enzyme activity. In addition, the fluoro-sugar fluoroacetyl glucosamine is reported to be incorporated into hyaluronic acid (Kent and Winterbourne, 1977; Winterbourne *et al.*, 1979), a polysaccharide component of cartilage and synovial joint fluid. The presence of fluoroacetyl glucosamine, or other fluoroacetyl sugars if they

exhibit similar chemical properties, may offer an explanation for the toxic bone anecdotes.

The following chapter describes the investigation of the presence of fluoroacetylated sugars in the methanol extracts of *Gastrolobium* seeds and was undertaken in collaboration with Dr Thomas Spande, Laboratory of Bioorganic Chemistry, National Institutes of Health, DHHS, Bethesda Maryland, USA.

5.2 Methods

5.2.1 Source of *Gastrolobium* seed

(see 4.2.1)

5.2.2 Solvent extraction of *Gastrolobium* seed

Seed was firstly hand cleaned of any contaminating material which remained in the purchased seed. It was then milled in a Cyclotec 1093 sample mill (Tecator) and then successively extracted at room temperature with petroleum spirit (AR grade B.R. 60-80^{0}C, BDH), chloroform (AR grade, BDH) and methanol (HPLC grade, BDH). Mixtures were filtered under suction through a Whatman No. 42 ashless filter paper with 2 washes of the seed marc with the appropriate solvent, and the filtrate further filtered through a 0.45µm PTFE hydrophobic syringe filter (Millipore) on a Terumo disposable syringe. Solvents were removed on a Büchi rotary evaporator with the water bath held at 35±1^{0}C. Methanol extracts were transferred to a pre-weighed beaker using distilled water, snap frozen using liquid nitrogen and freeze-dried. Additional samples of milled *G. parviflorum* (Jacup) seed were extracted with ethanol (AR grade, BDH) or distilled water and *G. bilobum* (Tambellup) also with distilled water. Ethanol extract treated as above, with water extracts centrifuged (10mins @ 6000rpm), filtered using 0.45µm cellulose-nitrate filters (Sartorius) with suction, frozen and freeze-dried.

5.2.3 Fluoride selective electrode

Total fluorine content of the intact seed and solvent extracts was determined using the alkali fusion method for total fluorine analysis (see 4.1.2).

5.2.4 High Pressure Liquid Chromatography (HPLC)

A Hewlett-Packard 1100 LC using the solvent system 55% A/ 45%B→25%A/ 75%B over 60 min. at 0.5mL/min and a Phenomenex "Aqua" 4.6 mm x 25 cm C-18 RP column was interfaced with a Finnigan LCQ in the atmospheric pressure chemical ionisation (APCI) mode. APCI fragments result from losses of neutral moieties (always from $[M+H^+]$). One characteristic ion was 331, a protonated tetraacetyl anhydro-hexose, with other ions being 211 (331-2HOAc) and 169 (211-2HOAc-CH_2C=O). These accompanied the major peracetylated RFO sugars. These ions were seen to change to m/z 349, 229 and 187 in the peracetylated MFA-sugars. LC-MS with an APCI interface and the vaporiser heater set at 560 or 580^0C, gave molecular ions that were $[M+NH4^+]$ adducts. This was confirmed by replacing water in the HPLC solvent with D_2O, whereby the molecular ion masses increased by 4 a.m.u., and also by using known standards such as sucrose octaacetate. Solvent A is 0.05% HOAc in water; B is 0.05% HOAc in acetonitrile. A two-step acetylation of ca. 20mg of the seed methanol extracts was performed in 2mL vials using 10 drops each of acetic anhydride and 2,6-lutidine and stirring for 3 days at room temperature. This was followed by the addition of an equivalent volume of pyridine and stirring at room temperature for a further day. Reagents were then removed using a stream of nitrogen, the residue redissolved in acetonitrile and the evaporation process repeated twice more. 20 drops of acetonitrile were then added to prepare the stock solution for ca. 1 to 10 dilution and mass spectral analysis. 10μL of the acetylated seed extract in acetonitrile was injected.

5.2.5 ^{19}F nuclear magnetic resonance (NMR)

NMR analyses were performed using a 600MHz Oxford magnet with a Varian Unity Inova console using Varian VNMR6.1C software. The probe was a 5mm pulsed field gradient probe tuned to ^{19}F (Sfrq = 564.350MHz; Sweep Width = 310, 078Hz; tof = -8846.1; temp. = 25^{0}C). Spectra were referenced to an external reference of either trifluoroacetic acid in D_2O (set to 78.0ppm, in turn referenced to $CFCl_3$ @ 0.0ppm) or sodium monofluoroacetate in D_2O (set to –63.4ppm). Deuterated water (D_2O) was from Sigma-Aldrich and Pestanal grade sodium monofluoroacetate was from Riedel-deHaën.

5.2.6 Synthesis of fluoroacetylated myo-inositol standard

2.48g monofluoroacetic acid (Merck, re-distilled 167^{0}C fraction) and 1g myo-inositol (Sigma) were mixed in a glass vial. The vial was crimp-capped and heated on a hotplate at temperatures up to 70 ± 5^{0}C for a total of 120 hours, at which time the myo-inositol had reacted and dissolved.

5.2.7 Synthesis of fluoroacetylated sugar standards

a. 1g monofluoroacetic acid (Merck, re-distilled 1670C fraction), 2g glacial acetic acid (APS Ajax Finechem, AR grade) and 1g sucrose (Sigma) were mixed in a glass vial. The vial was crimp-capped and heated in a Pierce Reacti-Therm heating module @ 60 ± 1^{0}C for 72 hours. **b.** 10mg of sucrose (99.5%, Sigma) was mixed with 50mg sodium monofluoroacetate (SMFA; Pestanal grade, Riedel-deHaën) and 1ml methanol (HPLC grade, BDH) in a 2ml glass vial. The vials was capped, sonicated for 30 minutes, shaken for 20hrs, and the methanol removed using a stream of nitrogen and freeze-drying.

Material was then acetylated as for the seed extracts. An additional sucrose/SMFA mixture was analysed by ^{19}F NMR without any prior acetylation.

5.2.8 High resolution mass spectrometry (HRMS)

HRMS analysis was achieved using a JEOL SX-102 direct probe mass spectrometer, with a "magic bullet" matrix containing caesium. Samples were collected by preparative HPLC from *G. parviflorum* (Jacup) or *G. parviflorum* (Tambellup) peracetylated methanol extracts.

5.3 Results

The additional weak ^{19}F NMR triplets observed downfield of the main fluoroacetate triplet in the methanol extracts of *G. bilobum* (Tambellup) and *G. parviflorum* (Jacup), although still unassigned (see 4.3.5), are believed to be due to fluoroacetylated sugars. The synthesised fluoroacetylated sucrose standard had a similar shift, with multiple signals between –75.5 to –77.4ppm.

5.3.1 High pressure liquid chromatography (HPLC)

The presence of oligosaccharides in seed methanol extracts was established using LC-MS with an APCI interface. Peracetylated oligosaccharides were separated using a C-18 RP HPLC column with an acetonitrile-water gradient. Sugars of the raffinose family of oligosaccharides (RFO), typical for seeds of a legume (Peterbauer *et al.*, 2001), were subsequently identified. LC-MS with an APCI interface and the vaporiser heater at 580°C, gave molecular ions that were $[M+NH_4^+]$ adducts. This was confirmed by replacing water in the HPLC solvent with D_2O, whereby the molecular ions increased by 4 a.m.u., and also by using known standards such as sucrose octaacetate. Identifications of peracetylated sucrose, raffinose and stachyose were made through coinjections of peracetylated standards. The mass spectral fragments were superimposable. Verbascose ($(gal)_3$-sucrose) and ajugose ($(gal)_4$-sucrose), assumed by analogy and consistent masses, were also found. The first galactosyl moiety is linked 1α to the 6 hydroxyl of the glucose portion of sucrose and the additional galactoses are linked 1α-6 to one another. Ajugose always gave the weakest ion currents and was often barely detectable. Detection was slightly improved by elevating the vaporiser heater temperature but it is likely little is

there initially. The upper mass limit (2K) with this HPLC technique is not sufficient to detect peracetylated higher oligosaccharides. Within each of the five RFOs, one MFA group was found associated as a 5-29% congener (being always most prominent with sucrose; see Table 5.1). Lesser amounts of sugars with two MFA groups attached can be detected by ion profiling, however at this time it has not been shown conclusively that this is not one difluoroacetyl (DFA) group. In addition to the galactosyl-sucrose oligomers, minor amounts of galactosyl methyl-inositols, galactosyl inositols and glycerol conjugates of all these classes were found, all associated with one fluoroacetyl group. The cyclitols often showed two and occasionally three isomers. No data was obtained for the identification of these cyclitols however, of the many known methyl inositol isomers, derivatives of myo-inositol are most probable as it is commonly found in the RFO (Peterbauer and Richter, 2001). In addition, traces of bis MFA (or DFA) isomers were inconsistently found for the RFO sugars and cyclitols (see Table 5.2). Occasionally, however their levels were significantly higher, approaching 10% of that of the MFA-sugar or cyclitol. Of the seeds examined by HPLC after peracetylation, the extract from *G. parviflorum* (Jacup) (E, Table 5.1) had the highest levels of MFA-sugars and cyclitols, often two to three times the levels of the other *Gatrolobium* species examined. Split peaks are seen also for MFA-substituted cyclitols (see Table 5.1) and similarly for the bis MFA- (or DFA) substituted cyclitols (see Table 5.2).

A room temperature extraction with ethanol (commonly used for the extraction of sugars from seeds (Ganter *et al.*, 1991)) or the use of a Soxhlet extractor with refluxing methanol were less effective in recovering the labile MFA sugars, although they could still be easily detected. Water extraction (eg. *G. bilobum* (Tambellup) water extract total fluorine = 944.7 ± 1.0ppm; mean ± SE; $n = 2$) showed no evidence for MFA-sugars and

Table 5.1 Acylated oligosaccharides detected in the seeds of *G. bilobum* (Quindanning) (A), *G. bilobum* (Tambellup) (B), *G. calycinum* (Mundaring) (C), *G. parviflorum* (Tambellup) (D) and *G. parviflorum* (Jacup) (E).

F = methanol extract B exposed for 3d to 1:1 acetonitrile-con. NH_4OH, then acetylated with 1:1 Ac2O-Py, 1d; G = acylated methanol extract of E (see text) exposed to methanol for 10d at r.t. Parts per thousand quantities tabulated are ion intensities for the ions indicated, divided by total ion current for the region of the mass chromatogram containing the acylated polyhexoses and galactosyl cyclitols. **MFA** = monofluoroacetyl; nominal masses are underlined and where one MFA is observed are indicated in boldface. The nominal masses for verbascose and ajugose have been dropped by one mass unit to compensate for a rounding up by the software. Two or three isomers of some cyclitols are indicated by parentheses and are often a major and one or two minor ones, but occasionally two equivalent isomers. nd = not detected.

Peracetylated sugar	$[M+NH_4^+]$	MFA-peracetylated sugar	Peracetylated methylinositol	MFA-peracetylated methylinositol	Peracetylated inositol	MFA-Peracetylated inositol
Sucrose and congeners	696	**714**	710	**728**	738	**756**
A (ppt)	134.5	0.16	2.50	0.04	5.37	nd
B	136.1	28.6	1.31 (2)	0.06 (2)	1.56 (2)	0.05
C	68.2	5.30	10.3	0.06 (2)	3.01	0.19
D	92.8	9.40	3.00 (2)	0.21 (2)	2.49	0.21
E	50.3	19.0	3.29 (2)	0.66 (2)	3.52 (3)	0.88
F	257.6	1.38	1.65 (2)	nd	1.65 (2)	nd
G	53.6	0.58	2.21 (2)	nd	3.07 (2)	nd
Raffinose and congeners	984	**1002**	998	**1016**	1026	**1044**
A (ppt)	21.6	0.04	6.82	nd	9.72	nd
B	22.0	1.52	7.04 (2)	0.35 (2)	4.12 (2)	0.24 (2)
C	25.5	1.78	3.24	0.17 (2)	2.60	0.17
D	12.5	1.52	7.26 (2)	0.78 (2)	3.00	0.38 (2)
E	19.2	5.44	13.4 (2)	3.52 (3)	4.83	1.49 (2)
F	30.56	0.15	9.08 (2)	nd	5.48 (2)	nd
G	9.47	0.16	6.75 (2)	nd	2.56	0.07
Stachyose and congeners	1272	**1290**	1286	**1304**	1314	**1332**
A (ppt)	28.1	0.09	7.84	nd	13.8	nd
B	51.8	3.80	10.0	0.51 (2)	7.11	0.41 (2)
C	29.2	2.82	2.66	0.20 (2)	1.61	0.15
D	26.9	4.92	5.92	0.79 (2)	3.76	0.49 (2)
E	48.8	17.1	8.05	3.46 (2)	5.2	1.97 (2)
F	66.4	0.33	11.9	nd	7.93	nd
G	23.9	0.51	4.14	nd	2.55	0.09
Verbascose and congeners	1560	**1578**	1574	**1592**	1602	**1620**
A (ppt)	67.0	0.07	6.93	nd	20.6	nd
B	95.2	6.16	7.74	0.52 (2)	7.42	0.49 (2)
C	35.4	2.30	0.60	0.06 (2)	4.31	0.04
D	65.5	8.42	1.36	0.30 (2)	2.12	0.36 (2)
E	71.3	28.5	1.20	1.07 (2)	2.50	1.05 (2)
F	111.1	0.25	8.81	nd	9.00	nd
G	34.3	0.06	1.14	nd	1.04	0.06
Ajugose and congeners	1848	**1866**	1862	**1880**	1890	**1908**
A (ppt)	21.4	0.01	0.58	nd	1.45	nd
B	17.6	0.96	0.42	0.03	0.27	0.03
C	0.66	0.05	0.01	nd	0.01	nd
D	3.06	0.38	0.06	0.01	0.04	nd
E	5.27	1.62	0.08	0.04 (2)	0.05	0.03 (2)
F	18.3	0.03	0.45	nd	0.30	nd
G	0.23	0.05	0.04	nd	0.02	nd

it is speculated that an enzyme, associated with germination, is extracted under these conditions, resulting in MFA- hydrolysis during the extraction. This is supported by the observation of glucose and fructose (but not galactose) in this extract, absent from the methanol extract of this seed, and little MFA-glucose or MFA-fructose. The water-extracted seeds also produced a residue, possibly a lectin complex, which with drying proved difficult to solubilise, even with extensive sonication and addition of dimethylformamide.

All the fluoroacetylated moieties are rapidly lost at room temperature with 1:1 conc.-ammonia-water: acetonitrile, reducing peracetylated MFA-sucrose amounts by 70% in 72 hrs. In methanol alone, the peracetylated MFA-sugars slowly lose MFA groups and in about ten days are detected only by ion profiling (96% of MFA-sucrose was methanolised: Table 5.1). These results suggest that strongly alkaline conditions sometimes used in previously reported analyses are likely to have significantly reduced the presence of these compounds. The peracetylated MFA oligosaccharides are stable at room temperature for several months in dry acetonitrile.

5.3.2 High resolution mass spectrometry (HRMS)

Four of the fluoroacetylated sugars, those from sucrose, stachyose, verbascose and raffinose, were acquired using preparative HPLC and analysed by high resolution mass spectrometry (HRMS). HRMS measurements indicated the expected mono-MFA peracetylated formulas for the caesium adducts (Table 5.2).

Mono-fluoroacetylated-peracetylated-Sugar	Formula of Caesium Adduct	Calculated Mass	Observed Mass	Error (ppm)
Sucrose	$C_{28}H_{37}O_{19}F$-Cs	829.0967	829.0906	–7.4
Raffinose	$C_{40}H_{53}O_{27}F$-Cs	1117.1813	1117.1807	-0.5
Stachyose	$C_{52}H_{69}O_{35}F$-Cs	1405.2658	1405.2723	4.7
Verbascose	$C_{64}H_{85}O_{43}F$-Cs	1693.3503	1693.3408	-5.6

Table 5.2 HRMS analysis of fluoroacetylated sugars

5.3.3 Synthesised fluoroacetylated myo-inositol and sugar standards

Synthesis of fluoroacetylated myo-inositol and sucrose standards required heat and time. Multiple fluoroacetylated myo-inositol derivatives (Figure 5.1) and several mono- and polyfluoroacetylated sucrose derivatives were synthesised (Figure 5.2). Replication of the cool methanol extraction process by shaking SMFA with sucrose failed to produce the ^{19}F NMR downfield triplets, with only the SMFA triplet at –63.1ppm evident.

DFA-peracetylated sugar	A (ppt)	B (ppt)	C (ppt)	D (ppt)	E (ppt)
Sucrose-DFA	Not quantified	1.25	1.35	0.36	1.90
Raffinose-DFA	Not quantified	0.06	0.04	0.08	0.67
Stachyose-DFA	Not quantified	0.14	0.10	0.30	2.58
Verbascose-DFA	Not quantified	0.17	0.09	0.56	4.52
Ajugose-DFA	Not quantified	0.03	nd	0.05	0.24

Table 5.3 Acylated difluoroacetylated oligosaccharides detected in the seeds of *G. bilobum* (Quindanning) (A), *G. bilobum* (Tambellup) (B), *G. calycinum* (Mundaring) (C), *G. parviflorum* (Tambellup) (D) and *G. parviflorum* (Jacup) (E)

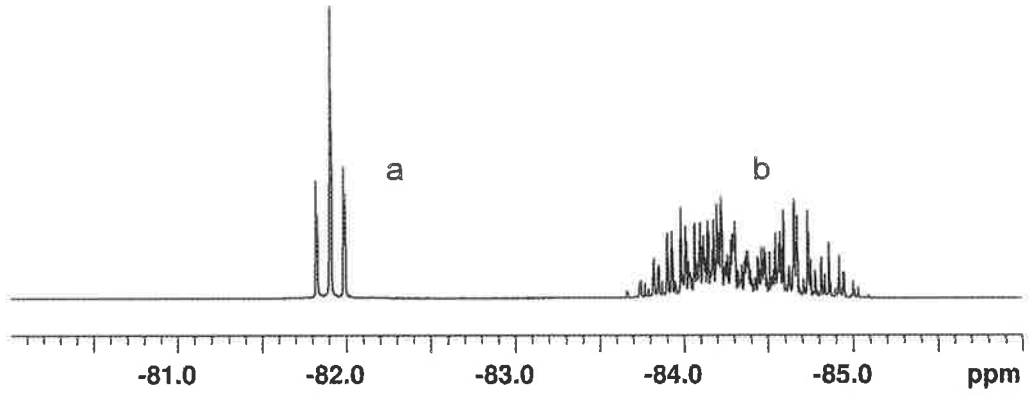


Figure 5.1 ^{19}F NMR of fluoroacetylated myo-inositol

a = monofluoroacetic acid; **b** = fluoroacetylated myo-inositol derivatives; solvent is acetonitrile.

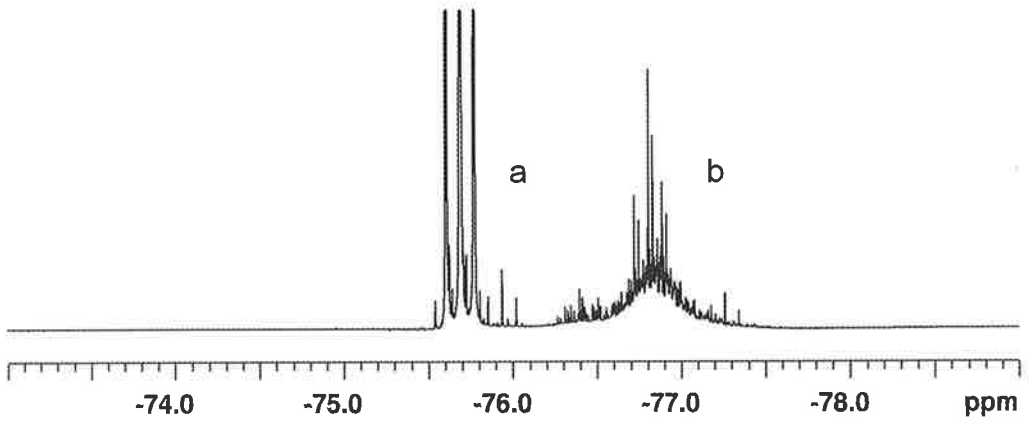


Figure 5.2 ^{19}F NMR of fluoroacetylated sucrose

a = monofluoroacetic acid; **b** = fluoroacetylated sucrose derivatives; solvent is D_2O.

5.4 Discussion

The primary argument against the MFA sugars described in this chapter as being present in *Gastrolobium* seeds, is that they are in fact artifacts of the extraction or analysis process. HRMS demonstrates conclusively (Table 5.2) that the fluoroacetylated sugars are the compounds proposed by HPLC analysis, however it does not establish their presence prior to the acetylation process.

The sucrose/SMFA control described in 5.3.3 indicates that extracting milled *Gastrolobium* seed containing significant quantities of fluoroacetate and sugar does not produce the downfield triplets consistent with MFA-sucrose. Additional findings further support the existence of natural MFA-sugars (rather than artifacts) and these are:

1) the RFOs show primarily a single MFA moiety as identified by HPLC retention times and fragmentations; multiple derivatives would be expected if a trans-esterification with MFA and the sugar were occurring in methanol during the extraction;

2) the seed cool methanol extracts from *G. bilobum* (Tambellup) and *G calycinum*, with consistent amounts of 'total fluorine' (Table 4.9), were extracted identically however have significantly different amounts of MFA sugars (Table 5.1). If the MFA-sugars are artifacts, the concentrations being recorded do not reflect the concentrations of sugars and fluoroacetate (indicated by 'total fluorine') from which they were produced. For example, although *G. bilobum* (Tambellup) has approximately twice as much sucrose as *G calycinum*, it has seven times as much MFA-sucrose. This is a similar result to Hall's observation for *G. bilobum* and *G. parviflorum* seeds using ammoniacal propanol (Hall, 1972).

Sucrose controls using SMFA or MFA, when treated with the seed extract-acetylation protocol, do produce extensive mono-MFA sucrose (and substantial di-MFA

sucrose as well) but differ significantly from the seed results in having two 1:1 monofluoroacetylated sucrose isomers (with different fragmentation patterns) and much more disubstitution than observed with the seed extracts. It is postulated that a mixed anhydride intermediate ($CH_2FC(=O)$-OAc) rapidly forms and competes with acetic anhydride (present in roughly 150 molar excess over MFA or its salt) in reacting at equal rates with the 6-positions of fructose or glucose. The di-substituted MFA-sucrose is considered to be sucrose substituted at both of these two primary hydroxyl groups. The case for naturally occurring MFA-sugars is consequently complicated by these observations but at the present time, the conclusion that MFA-sugars are naturally occurring in some *Gastrolobium* species is still suggested.

At this stage, only tentative structures based upon mass spectrometry can be proposed. Since sucrose hepta-acetyl monofluoroacetate gave the same 349, 219 and 187 ions as detected by the higher galactose oligomers (some detected only by ion profiling of these three ions compared with the usual 331, 211, 169 ions), it is assumed at this stage that the MFA is attached to the 6 hydroxyl of fructose (Figure 5.3). This proposition is supported by research examining the recognition of a fluoro-sucrose by a sucrose transport protein (Card *et al.*, 1986; Hitz *et al.*, 1986). These studies lend support to the proposition that the addition of fluoroacetyl groups to the fructose portion of the RFO family would still enable binding and movement by transport protein(s). It is assumed that in the cyclitols the MFA is attached to a methyl-inositol or inositol moiety. Since their amounts are much less, we have been unsuccessful in reaching any conclusions from the fragments, which do not however appear to parallel those of the RFOs (see Table 5.1).

It is likely that these fluoroacetylated sugars are being synthesised in mesophyll cells by the plant (Grobbelaar and Marion Meyer, 1989) and are being exported by sugar transport proteins, as shown with 1'-fluorosucrose (Hitz *et al.*, 1985; Hitz *et al.*, 1986), to the 'sink' seed tissue (Williams *et al.*, 2000). It is also likely that the synthesis of these fluoroacetylated sugars is under enzymatic control, commencing with a fluorinase (O'Hagan *et al.*, 2002). As *Gastrolobium* plants have symbiotic mycorrhizal fungi associations (Lamont *et al.*, 1985), confirmation of the synthesis of these fluoroacetylated sugars in mesophyll cells would allow for their passage by sugar transporters to the fungi (Harrison, 1996). Their presence in the fungi may help explain the high fluoroacetate tolerance (Oliver *et al.*, 1979) of the predominantly mycophagous brush-tailed bettong (*Bettongia penicillata* Gray, 1837) (Christensen, 1980). Although they are known to both bury (Anon, 1906; Christensen, 1980) and eat seeds (Christensen, 1980), no evidence is available for their consumption of *Gastrolobium* seed, although this is assumed in order to explain their very high fluoroacetate tolerance. However there is a significant disparity if the bettong, an occasional seed eater with a fluoroacetate LD_{50} of ~100mg kg^{-1} (Oliver *et al.*, 1979) is compared with the common bronzewing pigeon, a true granivore but with only approximately half the fluoroacetate tolerance (LD_{50} of ~40mg kg^{-1} - Twigg and King, 1989). Another possibility may be that the mycorrhizal fungi do receive fluoroacetylated sugars from the *Gastrolobium* and that these are then stored in their reproductive bodies as they are in the *Gastrolobium* seed. These fruiting bodies are then consumed by the bettongs as their primary food source, requiring and resulting in the high fluoroacetate tolerance of this species.

It is also likely that *in vivo* fluorocitrate may be ultimately produced when animals ingest, either directly or indirectly, *Gastrolobium* seeds containing these sugars (Bremer and Davis, 1973; Arellano *et al.*, 1998). However a review of the metabolism and enzymology of a range of other fluorinated sugars by Taylor (1972), suggests enzyme inhibition may also be possible. If enzyme inhibition occurs, for example inhibiting phosphofructokinase-1 (PFK-1), considered the rate-limiting enzyme of glycolysis, then these fluoroacetylated sugars may help explain the rapid death anecdotes. As this study proposes the fluoroacetyl group to be attached to one of the hydroxyls of fructose, rather than the glucose molecule, its *in vivo* behaviour may not reflect that of an altered glucose molecule. Also, if these sugars behave in a similar way to *N*-fluoroacetylglucosamine (Kent and Winterbourne, 1977), then their incorporation into hyaluronic acid may also occur. As hyaluronic acid is a component of cartilage and synovial joint fluid, the incorporation of these fluoroacetylated sugars into the bones of bronzewing pigeons may help to explain their toxicity, as stated by the historical anecdotes.

(glucose unit)

(fructose unit)

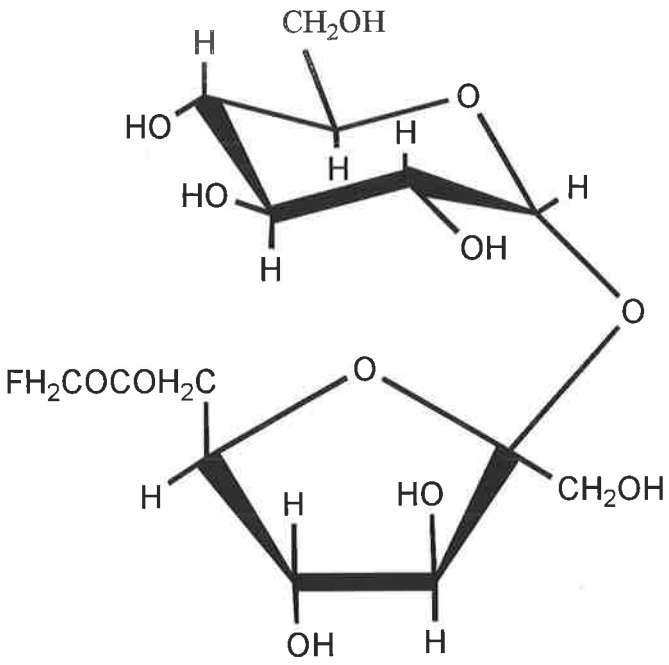


Figure 5.3 Monofluoroacetyl sucrose - modification of sucrose figure from Petrucci and Harwood (1993). The fluoroacetyl is believed to be bonded at the 6 hydroxyl of the fructose unit.

5.5 Conclusion

The toxicity of the leaves and seeds of many species of *Gastrolobium* has been attributed to the presence of monofluoroacetate (Baron *et al.*, 1987; Twigg *et al.*, 1996; Twigg *et al.*, 1999). The results presented in this chapter may indicate that the methanol extracts of *G. bilobum*, *G. calycinum* and *G. parviflorum* seeds also contain fluoroacetylated sugars. Five classes totaling 15 peracetylated sugars are described with the presence of numerous others identified. The fluoroacetyl groups are extremely labile and would be lost with conventional extraction procedures. The identification of these sugars almost doubles the known fluorinated natural products and the known classes of fluorinated natural products (from 6 to 11). It represents the first report of a new fluorinated natural product since the discovery of 4-fluorothreonine in 1986 and suggests that these sugars could exist in other plants where fluoroacetate has been described. In light of these findings, it is recommended that the plant tissues, particularly seeds, of fluoroacetate-containing plants be re-examined. The suggestion by Hall (1972) of the possible presence of a fluorinated carbohydrate in the seeds of *G. bilobum* is supported by this study. His similar suggestion for *Dichapetalum toxicarium* Baill. seed may therefore also prove valid.

The analysis of these MFA-sugars is still being undertaken and is anticipated will more conclusively show that these toxic sugars are a natural product within *Gastrolobium* seeds. Their synthesis may provide another natural toxicant for use against introduced predators, possessing all the benefits of fluoroacetate but also being sweet, for MFA-sucrose, and possibly more rapidly toxic than fluoroacetate.

Chapter 6: Physiological Response of the Common Bronzewing Pigeon, *Phaps chalcoptera* (Latham, 1790) to Sodium Fluoroacetate

6.1 Introduction

In parallel with seed analysis, it was important to investigate the aspect of skeletal retention of toxicants discussed in the historical anecdotes, skeletal retention considered to be of significant potential value as a more physiologically stable site of toxicant retention.

The common bronzewing pigeon (*Phaps chalcoptera*) has been recorded consuming the seeds of *Gastrolobium bilobum* (Webb, 1885; Serventy and Whittell, 1976), *G. calycinum* (Drummond, 1840) and *G. parviflorum* (Herbert, 1921). *Gastrolobium bilobum* is stated to be one of the most toxic of the Australian fluoroacetate producing plants (Aplin, 1971; Twigg *et al.*, 1996), with its seeds reported to contain at least 6500 mg kg^{-1} fluoroacetate (R. Mead pers. com.). There are numerous anecdotes of dogs and cats being poisoned after eating bronzewing pigeons. This poisoning has been attributed to the animals feeding on the entrails (Drummond, 1840; Whittell, 1938) or bones (Webb, 1885; Anon, 1912a; b; 1917; Wansbrough and Anon, 1922) of the pigeons. The records of Whittell (1942) and Serventy and Whittell (1976) of bronzewing pigeon entrails and bones poisoning dogs and cats appear derived from Herbert (1921, p. 11), summarising observations 'collected by Mr. W. C. Grasby, of the "Western Mail"'.
The observation of bronzewing pigeon entrails killing dogs and cats could be attributed to the presence of *Gastrolobium* seed in various stages of digestion. The anecdotes of

bronzewing pigeon bones killing dogs and cats to which they were fed suggest however the possible presence of a bone retentive toxicant in the skeletal material.

The observation of Webb (1885) is most interesting, because of its age and also for what is proposed as the possible source of the skeletal retained toxin. It is reproduced here:

> "I have just had a valuable dog poisoned by eating the breast bone of a bronzewing pigeon. I skinned the bird for stuffing, and as it was a fine plump fellow I roasted it for my breakfast, and threw all the bones in the fire except the breast bone, which the dog managed to get. Ten minutes after eating it he was ... running about and yelping ... I caught him and poured castor oil down his throat, which caused him to vomit the contents of his stomach. This however did not ease him as in a quarter of an hour the poor brute died ... These birds in this locality feed almost exclusively on the seeds of the poison bush, *Gastralobium Bilobum* [sic], and there is every reason to believe that they owe their poisonous properties to this circumstance" (Webb, 1885, p. 3).

Most previous examinations of the physiological fate of fluoroacetate, often dosed as the sodium salt, sodium monofluoroacetate (Compound 1080), have excluded an examination of skeletal tissue (eg. McIntosh *et al.*, 1959; Eason *et al.*, 1993; Rammell, 1993; Eason *et al.*, 1994; Gooneratne *et al.*, 1995; Eason *et al.*, 1996). Studies that have examined skeletal tissue (Wallace-Durbin, 1954; Gal *et al.*, 1961; Sykes *et al.*, 1987) utilised laboratory rats and mice, which have not co-evolved with fluoroacetate-producing vegetation. In addition, these studies used radiolabeled fluoroacetate with

either a ^{18}F or ^{14}C label and as a consequence the reported concentrations and distributions of these tracers do not necessarily identify the chemical structure of the associated compounds. All three studies report a significant and rapid skeletal uptake of the radioactive tracer. For example, Sykes (1987) report 25% of their ^{18}F tracer dose to be in osseous tissue 2 hours after dosing. A diagnostic technique such as mass spectroscopy was not used in any of the studies to identify the compound present in the osseous tissue. Therefore the possibility that this compound was the dosed fluoroacetate, or fluorocitrate (the toxic metabolite of fluoroacetate), or the reported fluoride cannot be excluded. Peters (1969), in a very brief statement reports finding trace amounts of fluorocitrate in fluorosed cattle bones which he was unable to determine as being derived from within the animal or ingested within feed material. Whatever the source, if the observation is valid it supports the skeletal retention of fluorocitrate. In addition, the capacity of fluoroacetate to bind to tissue (Livanos and Milham, 1984; Frost *et al.*, 1989; Parker and Frost, 1991) and fluorocitrate to mitochondrial proteins (Kirsten *et al.*, 1978), gives rise to the possibility that both these compounds may also possess the capacity to bind to osseous tissue and be incorporated in the physiological process of bone mineral layer development. If fluoroacetate is the toxicant being retained in the pigeon bones, its binding to protein may explain the anecdotes which mention toxic bones after the pigeons were stewed (Anon, 1912a; 1917). Fluoroacetate is very water-soluble and would be expected to have been extracted into the stew water, however protein-bound fluoroacetate is likely more insoluble, as proposed by Parker and Frost (1991). This may also be the situation with fluoroacetate retained, and thus encased, within a bone.

The experiment reported in this chapter was undertaken on the assumption that fluoroacetate was the only recognised toxicant within *Gastrolobium* seed. Based on this assumption, this experiment examined the capacity of the skeletal tissue of the South Australian common bronzewing pigeon to retain the toxic compounds fluoroacetate and fluorocitrate. The null hypothesis was therefore that the tissues and bones of fluoroacetate dosed common bronzewing pigeons would contain the same concentrations of fluoroacetate and fluorocitrate as control birds.

6.2 Methods

6.2.1 Source and housing of pigeons

Ten common bronzewing pigeons (6 males and 4 females) were collected using mist-nets erected around Moore's Dam, 'Balah' station, near Morgan, South Australia (Lat. -33^{0}45'45"; Long. 139^{0}55'20"). An additional 5 birds (3 male and 2 female) were obtained from local South Australian breeders and 3 birds (male) were captured at Bradbury, South Australia (Lat. -35^{0}03'40"; Long. 138^{0}43'30") while feeding at a residential chicken coop. The birds were firstly housed in 2 outside aviaries at Adelaide University Waite campus animal house before being moved into individual cages in a temperature-controlled room. Room temperature was maintained at 21 ± 1^{0}C with a 12hr day/night cycle. The birds were given 3 days of acclimatisation before dosing during which their water supplies were changed to demineralised water. The birds were supplied *ad libitum* with a commercial small-seed mix and water.

6.2.2 Experimental design

The birds were uniquely identified using Size 6 (9mm) coloured leg bands. Three birds (1M, 2F) were randomly assigned as controls, with the remaining 15 birds (10M, 5F) receiving the same dose of aqueous 1080. All 15 treatment and 3 control birds were dosed at time 0 (6am). Three randomly selected treatment birds were euthanased at time 2 hours (8am), 4 hours (10am), 8 hours (2pm), 16 hours (10pm) and 32 hours (2pm day 2). One of the 3 control birds was euthanased at time 0, 16 hours (halfway) and 32 hours (experiment end).

6.2.3 Dosing and blood sampling

Dosing and blood sampling was based on the methodology and results of McIlroy (1984) and Twigg and King (1989). An approximate LD_{50} for South Australian common bronzewing pigeons is 25mg 1080 kg^{-1} (Twigg and King, 1989). A stock solution of 10,000 mg ml^{-1} aqueous sodium monofluoroacetate was produced using technical grade sodium monofluoroacetate (BDH Chemicals Ltd, Poole England 85% purity) dissolved in demineralised water.

At time 0, between 0630 and 0900hrs, treatment birds were given 15mg 1080 kg^{-1} (0.37-0.56ml dose) with control birds given an equivalent dose of demineralised water. Dosing was by intramuscular injection to avoid any loss of aqueous 1080 by vomiting (McIlroy, 1984). Although normal ingestion of fluoroacetate would be oral, the route of administration is not considered to significantly affect the toxicity of fluoroacetate or the sensitivity of the dosed animals (Quin and Clark, 1947; Atzert, 1971; McIlroy, 1981a). Blood samples (0.3mls) were taken from the jugular or brachial vein of live birds using 1ml sodium heparin coated syringes at 0, 2, 4, 8, 16 and 32 hours. The birds were euthanased by cervical dislocation.

6.2.4 Tissue and bone analysis

Bird carcasses, tissue and plasma samples were stored at $-18^{0}C$ to prevent any bacterial defluorinating activity (Soiefer and Kostyniak, 1983). Plasma samples were separated using a Clements Orbital 100 centrifuge (Phoenix Scientific Industries Ltd) and frozen immediately. Bone samples with erythropoietic bone marrow (radius, ulna, tibia and tarsus - Schepelmann, 1990) were cleaned of muscle, tendon and cartilage using a

scalpel and forceps. The identification and quantification of fluoroacetate in tissue and bone samples was carried out by the Queensland Department of Natural Resources using a GC-MS method based on Ozawa and Tsukioka (1989).

The identification and quantification of fluorocitrate in bone samples was by the method set out in Appendix 2 and was based on the methodology of Booth *et al.* (1999). It was developed by Bob Parker and Martin Hannan-Jones of the Queensland Department of Natural Resources.

6.2.5 Plasma citrate and total calcium analysis

The *in vivo* physiological response to fluoroacetate intoxication is stated to be its conversion to fluorocitrate and disruption of the tricarboxylic acid cycle with one result being citrate accumulation (Buffa and Peters, 1949; Lindenbaum *et al.*, 1951; Peters, 1972). Monitoring plasma citrate concentrations is therefore considered a method of monitoring fluoroacetate metabolism (eg. Twigg *et al.*, 1986; Twigg and King, 1989).

Plasma citrate concentrations were determined by the Western Australian Department of Agriculture Animal Health Laboratories using the method of Dagley (1974). Plasma samples were deproteinated with 1M perchloric acid and neutralised with potassium carbonate. Analysis was then undertaken using a Cobas Mira Autoanalyser, to which the Boehringer Mannheim citric acid test kit had been adapted.

Bosakowski and Levin (1986) observed a significant decline in serum total calcium in dogs dosed with fluorocitrate and associated this with the known chelation of citrate on calcium. With the stated metabolism of fluoroacetate to fluorocitrate resulting in citrate accumulation, it was hypothesised that any chelation of blood calcium could facilitate the

skeletal retention of fluoroacetate, fluorocitrate or other *Gastrolobium* seed toxicant. To assess whether the chelation of blood calcium occurred in the pigeons, plasma total calcium was also determined by the Western Australian Department of Agriculture Animal Health Laboratories. Deproteinated plasma samples were analysed on a Cobas Mira Autoanalyser using methylthymol blue.

6.3 Results

6.3.1 Plasma citrate and total calcium analysis

Plasma citrate and total calcium results are described in Table 6.1 and graphically represented in Figures 6.1 and 6.2.

Sample Time (hours post-dosing)	Citrate (µg/mL)			Calcium (mmol/l)		
	Mean	SE	n	Mean	SE	n
0	55.8	2.6	18	2.2	0.1	16
2.5	207.0	9.3	15	2.4	0.1	14
4.5	158.1	15.2	12	2.1	0.1	12
8	79.3	3.6	9	2.1	0.1	9
16	59.4	3.9	6	2.1	0.1	6
32	46.3	5.5	3	2.0	0.1	3

Table 6.1 Plasma citrate and total calcium concentrations

Plasma citrate and total calcium concentrations were analysed for statistical significance by a two-sample paired T-test. No significant differences were detected for plasma calcium concentrations:

0,2.5hrs; $t = 0.38$; 14 d. f.; $p = 0.712$

0,4.5hrs; $t = 0.91$; 11 d. f.; $p = 0.382$

Plasma citrate concentrations of treatment birds were significantly different to control birds up to 8 hours post dosing:

0,2.5hrs; $t = 19.45$; 14 d. f.; $p < 0.001$

0,4.5hrs; $t = 7.71$; 11 d. f.; $p = < 0.001$

0,8hrs; t = 6.91; 8 d. f.; p = < 0.001

0,16hrs; t = 1.40; 5 d. f.; p = 0.221

6.3.2 Muscle and bone fluoroacetate concentrations

Muscle and bone fluoroacetate results are presented in Table 6.2 and graphically represented in Figures 6.3 and 6.4. The report with the original data is included as Appendix 3.

Sample Time (hours post-dosing)	Muscle Fluoroacetate (µg/g)			Bone Fluoroacetate (µg/g)		
	Mean	SE	n	Mean	SE	n
0	0.0	0.0	2	1.1	0.3	3
2	1.9	0.6	3	2.9	1.3	3
4	0.6	0.2	3	1.3	0.3	3
8	0.1	0.1	3	1.0	0.6	3
16	0.0	0.0	3	0.0	0.0	3
32	0.0	0.0	3	0.0	0.0	3

Table 6.2 Muscle and bone fluoroacetate concentrations

Muscle fluoroacetate concentrations indicate a significant increase at 2 hours post-dosing with a subsequent decline to undetectable levels at 16 hours. Results could not be analysed for statistical significance by a two-sample paired T-test due to the limited sample size and variation.

The concentrations of fluoroacetate in pigeon bone samples show a similar decline curve to that recorded for muscle fluoroacetate and plasma citrate concentrations.

However, there is substantial variation within each group of 3 treatment birds, with one bird in the 2hr group (#6) having the extreme concentration of 5.4ppm. Exclusion of this bird would suggest minimal inter-dose group variation, with overlapping standard errors.

Bone fluoroacetate results were not statistically analysed due to the testing laboratory reporting fluoroacetate concentrations from the control birds (Time 0) bone samples. This apparent contamination requires this data set to be considered only as a possible indicator of the ability of bronzewing pigeons to retain fluoroacetate in their skeletal tissue.

6.3.3 Muscle and bone fluorocitrate concentrations

The extraction and analysis of pigeon leg bones for fluorocitrate using the method devised for this study failed to detect any fluorocitrate in these bones or in the fluorocitrate controls. It is now known what aspect of this analysis failed.

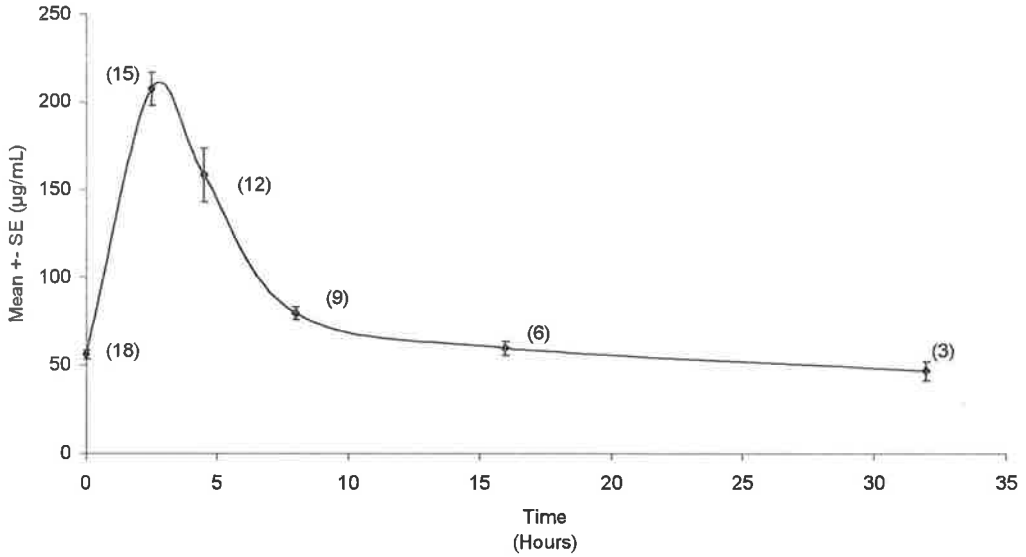


Figure 6.1 Plasma citrate concentration

(Number in brackets = *n*)

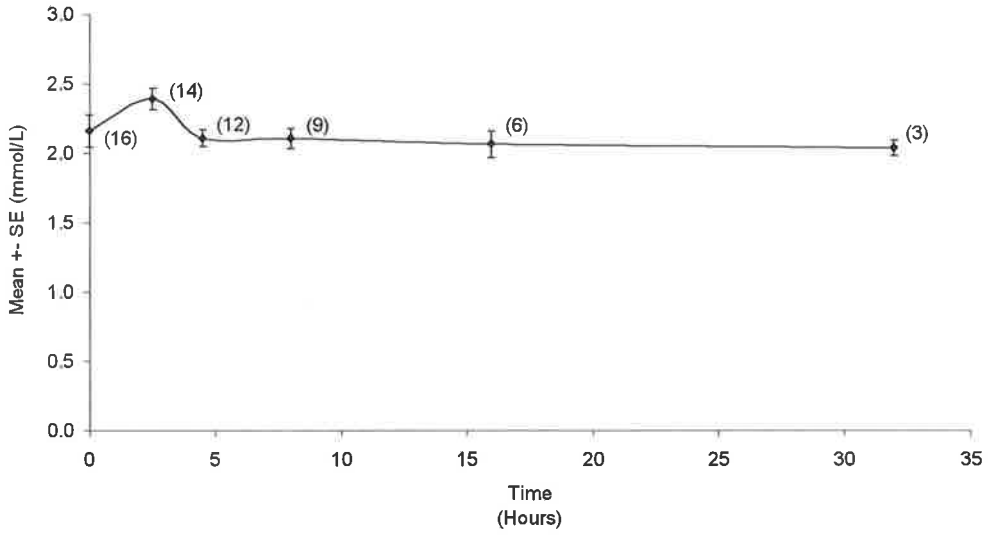


Figure 6.2 Plasma calcium concentration

(Number in brackets = *n*)

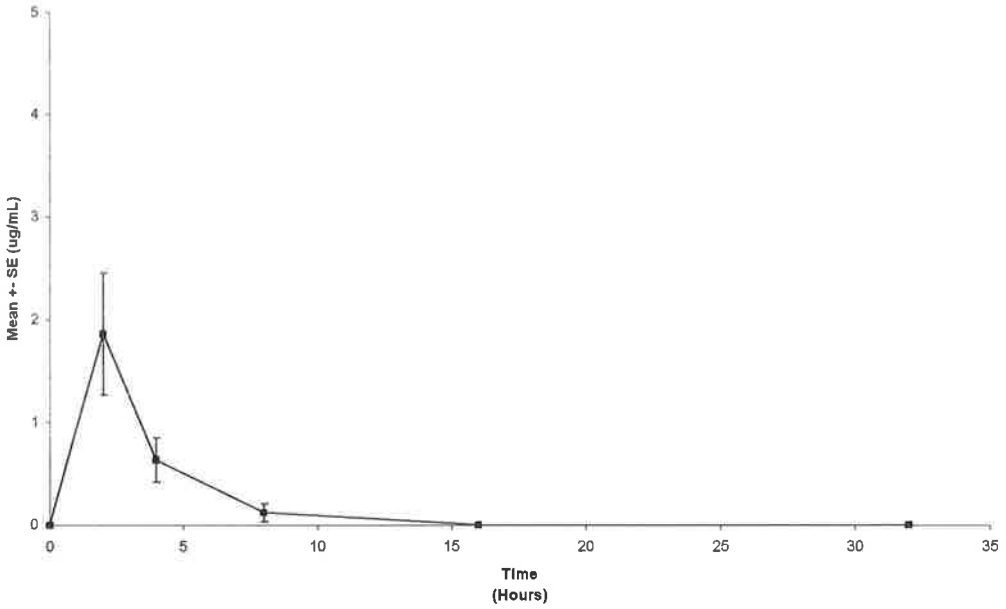


Figure 6.3 Muscle fluoroacetate concentration

(n=2 for Time 0; n=3 for all other data points)

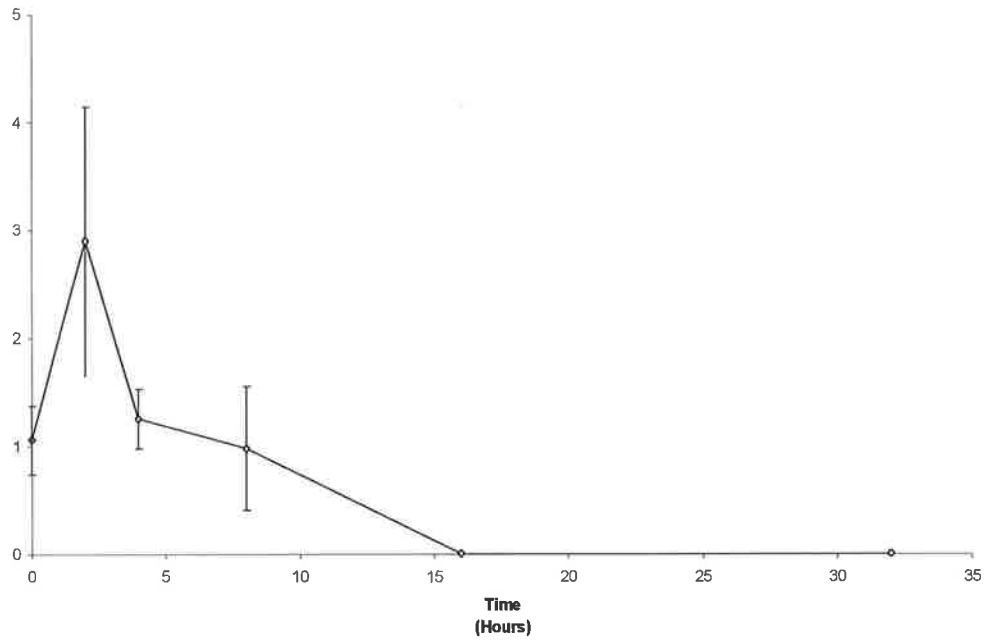


Figure 6.4 Bone fluoroacetate concentration

(n=3 for all data points)

6.4 Discussion

Detection of fluoroacetate at significant concentrations in the 2, 4 and 8 hour bone samples would have indicated this compound to have a bone retentive capacity, although only lasting approximately 10hrs post-dosing. Unfortunately the laboratory conducting the analyses reported a similar result for the control birds, suggesting a contamination issue. The question of the skeletal retention of fluoroacetate must therefore remain unanswered. The similarity in the decline curves for the three samples (plasma, muscle and bone) provides some inter-sample reliability and as such suggests an authenticity to the bone samples, and the possibility that only the samples from the control birds were contaminated. However, the requirement to analyse additional samples is unavoidable. As two separate batches of bones containing erythropoietic bone marrow were analysed at the testing laboratory, and neither produced robust results, significant logistical difficulties inhibit this requirement being undertaken. None of the principal bones which contain erythropoietic bone marrow remain from these pigeons, and with no useable data from 2 analyses, an alternative testing laboratory is necessary but does not exist in Australia.

The basic biochemical and physiological factors involved in the reported skeletal retention anecdotes are believed to be the same in all birds. For example, avian bone is considered to be largely the same between species, with the same general physiology. The use of another bird species such as the domestic pigeon, *Columba livia*, could therefore have replicated much of this experiment but also allowed for the use of a non-native species and also enabled the use of a larger sample size. Both of these experimental modifications would be greatly desirable. The variation recorded in tissue and bone results and the subsequently inconclusive statistical analyses highlight the requirement for future studies to have an

increased sample size, although there are ethical difficulties for non-survival studies requiring wild-caught birds. The selection of the common bronzewing pigeon as the experimental species was however primarily to examine the anecdotes in the most robust way possible, thereby minimising the likelihood of a Type I error. That is, toxic bones had been stated for this species and use of another species may fail to consider an intirinsic capacity possessed by this species. One of the benefits of the use of common bronzewing pigeons is the established high tolerance of these birds to fluoroacetate, and thus the ability to use higher doses, and provide a greater opportunity to test the null hypothesis without killing the test birds. The primary concern was however the use of a native species specifically referred to within historical anecdotes.

The plasma citrate curve for this study (Figure 6.1) is very similar to that determined from three South Australian common bronzewing pigeons dosed at 15 mg kg^{-1} by Twigg and King (1989). The concentration of the maximum recorded citrate peak for this study was 207 µg ml^{-1} for the first sample at 2.5 hours post dosing (n=15). This equates to 1078µM, in comparison with a peak of 1128 µM at 3 hours, the first sample post dosing, for Twigg and King (1989).

The lack of statistical significance between the 16 hour plasma citrate concentrations as compared with 'Time 0' in this study, and an apparent similar result for Twigg and King (1989) at 12hrs, suggests that South Australian common bronzewing pigeons are able to rapidly metabolise fluoroacetate. The rapid metabolism of fluoroacetate by these pigeons would suggest that any possible skeletal retention of the fluoroacetate will occur within approximately 10 hours. This is supported by the bone fluoroacetate analyses.

The digestibility of poultry legs (age and consumption details unknown) was only 52% in mink (Jarosz, 1996 - citing Leoschke, 1987). However if the anecdote of a bronzewing pigeon breastbone killing a dog (Webb, 1885) was caused by digestive release and absorption of fluoroacetate from the bone substrate, adequate digestion of bone would need to have occurred. The sternum from pigeon #1, a 345g (at dosing) female bird, was extracted, cleaned and weighed. Its' 4g weight was used as an indicator for the Webb (1885) anecdote. If the dog was 10kg, at an LD_{50} of 0.06mg kg^{-1} (McIlroy, 1981b) the dog would require at least 0.6mg fluoroacetate in a 4g sternum, suggesting fluoroacetate as the toxicant would need to be at a substantial 150mg kg^{-1}. An area of significance within this anecdote is the speed (~25 minutes) at which the dog is reported to have died. Symptoms of fluoroacetate poisoning in the dog are stated by Rammell and Fleming (1978) to arise 4-5 hours after ingestion. Similarly, in the closely related but less fluoroacetate sensitive dingo (LD_{50} 0.11mg kg^{-1}), McIlroy (1981b) found that the minimum time until death was 5.3 hours. If massive doses of fluoroacetate did kill dogs in the times stated in the rapid death anecdotes, the required concentration in the pigeon bones would be excessively massive.

It is still unknown whether the toxic fluoroacetate metabolic product fluorocitrate is bone retentive, due to failure of the analysis. However if it was fluorocitrate present in the pigeon's sternum, the speed of death still appears excessively rapid. Bosakowski and Levin (1986) found dogs (adults 7.3-10.6kg) did not exhibit symptoms until 1-2 hours after dosing with 8-32mg kg^{-1} fluorocitrate, with death occurring at approximately 4 hours. Even if a massive dose of fluoroacetate was ingested by the dog, in the various species tested by Quin and Clark (1947) this was not found to produce an immediate effect but to reduce the latent period. Dr Charles Eason (Landcare NZ), a toxicologist with extensive experience

researching fluoroacetate toxicology, suggested that a possible explanation for the toxic bone anecdotes could be the blood-transported fluoroacetate remaining *in situ* after death. That is, the blood in the bones transporting the fluoroacetate from the ingested *Gastrolobium* seeds stays where it is when the birds die, thereby resulting in toxic bones. This explanation requires bones to have erythropoietic bone marrow and in the 6 month old pigeon this is largely restricted to the radius, ulna, tibia and tarsus (Schepelmann, 1990 - Fig. 8E, p. 31). The "breast bone" stated by Webb (1885) is anticipated to have been from an adult bird as it was a "fine plump fellow" and was collected for a taxidermic skin, suggesting the need for adult plumage. Based on Schepelmann (1990) this breast bone lacks the necessary blood supply, being almost completely ossified and pneumatised and thus the suggestion of Eason is not considered a plausible explanation for this principal toxic bone anecdote.

The lack of any statistically significant differences in plasma total calcium concentrations indicates that the chelation of blood calcium is not occurring in these birds and is therefore not a factor in any skeletal retention of toxicant. The hypothesis that chelation of blood calcium, through the process of the birds ingesting *Gastrolobium* seed containing large concentrations of fluoroacetate, stimulating a calcium concentration gradient and/or chemical imbalance, which then facilitates the skeletal retention of a seed toxicant, is not supported by the results of this experiment.

West *et al.* (1996) found bone remodeling to be minimal in 9 month old pigeons (species not stated but probably *Columba livia*), however also states egg laying is established as affecting bone remodeling. This raises the breeding process as another possible factor in skeletal toxicant retention. It may be that bone modeling in growing nestlings is the time for toxicant retention, rather than any remodeling in mature birds. Most breeding of common

bronzewing pigeons in southern Western Australia occurs from August to October (Carter, 1923; Masters and Milhinch, 1974), which coincides with the flowering and seed-set in most of the toxic *Gastrolobium* species (Chandler *et al.*, 2002). The birds are known to feed on the highly toxic *Gastrolobium* seed (see Table 1.1), with *G. bilobum* specifically mentioned by Webb (1885) and Serventy and Whittell (1976), and they almost certainly coincide their breeding with the abundant supply of seed, including *Gastrolobium*, at this time of the year. Higgins and Davies (1996) cite Lea and Gray (no year or reference) as recording 27 grains of wheat found in one crop. Although *G. bilobum* seeds would be at least five times smaller than a grain of wheat, this suggests that at least 27 *G. bilobum* seeds could be regurgitated at one time from the crop to a nestling. At a median potential seed fluoroacetate concentration of approximately 2000µg g^{-1} (see Figure 4.4) and 0.007g $seed^{-1}$ (this study), 27 *G. bilobum* seeds would contain 378µg fluoroacetate. The LD_{50} of the Western Australian common bronzewing pigeon is approximately 40mg kg^{-1} (Twigg and King, 1989). Therefore a hatchling weighing an average 8.8g ("Frith" cited in Higgins and Davies, 1996) could be killed by 0.352mg fluoroacetate (ie. the 1 crop of 27 *G. bilobum* seeds at 2000µg g^{-1}). Notwithstanding the question of how these nestlings survive this fluoroacetate if they are being fed these conservative quantities of *Gastrolobium* seed, a significant ingestion of fluoroacetate, and any other seed toxicants could be occurring at this time of rapid growth and bone modeling. "Frith" cited in Higgins and Davies (1996) states that the mean daily increase in tarsus length for 10 common bronzewing nestlings was 0.9mm day^{-1}. This rapid period of bone modeling may be the period when a *Gastrolobium* seed toxicant is being incorporated into the bone matrix, thereby making the pigeon skeleton toxic to cats and dogs.

6.5 Conclusion

The results obtained from this experiment suggest the common bronzewing pigeon may retain fluoroacetate in its bones, but only for possibly 10-15 hours post-dosing. Lack of robust laboratory analyses does however require replication of this study with additional birds.

The lack of reliable laboratory results from 2 attempts to have pigeon bone samples analysed for fluoroacetate and one attempt for fluorocitrate, are indicative of the inherent difficulties when analysing for these compounds. Although the GC-MS methods are very sensitive, the multiple steps in extraction of a biological sample, derivitisation and analysis offer several possibilities for errors to arise. In terms of future research, the preferred method of analysis would be similar to the relatively simple Minnaar *et al.* (2000a; 2000b), used in conjunction with a single wavelength detector and a mass spectrometer. Use of Western Australian common bronzewing pigeons with a higher fluoroacetate tolerance, and *ad libitum* supply of *Gastrolobium* seed, should provide the possibility for the retention of fluoroacetate such that it can be detected by this HPLC-MS method. In addition, based on the results of this experiment, the birds should be sampled more intensively in the first 5 hours. The anecdotes of toxic pigeon bones may be due to the presence of fluoroacetate, however if this is the case, the bones must have been eaten soon after the birds were feeding on the *Gastrolobium* seeds, as this toxicant seems to have a short half-life in bones just as in the other tissues.

Any further attempts to examine for the bone retention of fluorocitrate will require development of an appropriate analytical method, possibly based on that used in this experiment and described in Appendix 2.

Chapter 7: Toxicity and Skeletal Retention of Alkaloids and Organo-Fluorine Compounds in the Laboratory Rat (*Rattus norvegicus*) Fed *Gastrolobium bilobum* Seed

7.1 Introduction

The search for a chemical defense strategy for reintroduced fauna has identified numerous historical anecdotes which report mammals such as the boodie [sic], kangaroo rat [sic] and opossum [sic] to have caused the deaths of cats and dogs. As mammals are of principal interest in reintroduction programs, their capacity to retain a chemical toxicant from *Gastrolobium* seeds is of primary significance. However, the conservation status of reintroduction mammals generally precludes them from being used in experiments examining the retention of any seed toxicants. The laboratory rat was nominated as a model for the following reasons:

1. No significant differences in mammal skeletal physiology could be identified, therefore it was considered to satisfactorily model native mammals;
2. It was considered to be of sufficient size to enable the extraction of its' skeleton;
3. It was considered to have a sufficiently high enough fluoroacetate LD_{50} to enable significant ingestion of *Gastrolobium* seeds, and therefore allow any toxicant retention to occur without being poisoned by the fluoroacetate;
4. It was readily available from the university breeding facility.

Preliminary seed analysis had identified the presence of numerous fluorinated compounds and putative alkaloids, being the Dragendorff responsive compounds, in *Gastrolobium* seeds. This study therefore examined the capacity of the laboratory rat to retain fluorinated and Dragendorff responsive compounds within its' skeletal tissue.

Two null hypotheses were proposed for this experiment. Firstly:

- That the skeletal extracts of treatment rats would lack any Dragendorff responsive compounds; and
- That skeletal extracts from treatment and control rats would not show a significant difference in fluoride concentrations.

7.2 Methods

7.2.1 Source and housing of rats

Thirty-three 7 week old Sprague-Dawley strain *Rattus norvegicus* were purchased from The University of Adelaide animal house and housed in individual cages in a temperature (20 ± 3^{0}C) and light (6am – 6pm day) controlled room with water and feed pellets (Joint Stock II, Ridley Agriproducts Pty Ltd) available *ad libitum*. The ingredients in the feed pellets are listed as: wheat, barley, oats, triticale, corn, peas, lupins, meat meal, blood meal, fish meal, tallow, vegetable oil, soyabean meal, canola meal, cottonseed meal, sunflower meal, rice pollard, lucerne meal, millrun, pea pollard, calcite, salt, rock phosphate, dicalcium phosphate, kynofos, synthetic amino acids, vitamins, minerals and antioxidants.

7.2.2 Source of *Gastrolobium* seed

G. bilobum seed was purchased from Nindethana Seed Service Pty. Ltd. having been collected from Quindanning, Western Australia (Nindethana provenance NS-19238) in January 2000 (see 3.2.1). Seed was milled in a Tecator 'Cyclotec 1093 Sample Mill'. The fluorine analyses described in 4.3 showed this seed to have 10.4 ± 3.7ppm ($\bar{x}$ ± SE, n=6) fluoride. The analysis in 4.3 describes the extraction with hot sodium hydroxide showing only 3.3 ± 0.3ppm ($\bar{x}$ ± SE, n=3) to be from inorganic fluoride. Seed was assayed for fluoroacetate by the Queensland Department of Natural Resources, using a method based on Ozawa and Tsukioka (1989), and showed a level of 6.5ppm fluoroacetate.

7.2.3 Experimental design

Three rats were randomly assigned as 'Time 0' control; 5 as both 'Day 5' control and treatment; 5 as both 'Day 10' control and treatment; and 5 as both 'Day 20' control and treatment.

All rats were given 1g 'Kraft Smooth Peanut Butter' the day before dosing to familiarise them with the bait substrate and then their feed pellets were removed in the evening.

Rats were fasted overnight and their feed pellets returned the following morning following consumption of the dose sample.

From Day 1 to Day 5 all control rats were given 1g peanut butter in a glass petri dish and all treatment rats 100mg milled seed (~14 seeds) thoroughly stirred into 1g peanut butter and formed into a single pellet in a glass petri dish.

From Day 6 to Day 10 control rats were given 2.5g peanut butter in a glass petri dish and all treatment rats 500mg milled seed (~70 seeds) thoroughly stirred into 2g peanut butter and formed into a single pellet in a glass petri dish.

From Day 11 to Day 15 control rats were given 4g peanut butter in a glass petri dish and all treatment rats 1g milled seed (~138 seeds) thoroughly stirred into 3g peanut butter and formed into a single pellet in a glass petri dish.

From Day 16 to Day 20 control rats were given 5.5g peanut butter in a glass petri dish and all treatment rats 2g milled seed (~276 seeds) thoroughly stirred into 4.5g peanut butter and formed into a single pellet in a glass petri dish.

All rats were euthanased by cervical dislocation.

The 3 'Time 0' control rats were euthanased in the afternoon of Day 1; 'Day 5' rats were euthanased the morning of Day 6; 'Day 10' rats the morning of Day 11 and 'Day 20' rats the morning of Day 21.

Before euthanasia rats were weighed and after death a blood sample was removed using a 2ml lithium heparin coated vacutainer. The liver and kidneys were removed into separate tissue vials and stored, with the carcass, at -19^0C. Blood samples were centrifuged and the plasma sample removed into a separate vial. Plasma and red blood cells were stored at -19^0C.

7.2.4 Extraction of skeletal and soft tissue

Beginning with the 'Day 20' dose group, rats in a treatment and control pair were thawed and their gastro-intestinal tract, remaining organs and as much muscle as possible removed using forceps and a scalpel. The skeletal carcasses were then placed within individual fly-wire cages and these cages placed within a large predator and rain-limiting cage on meat ant nests. The individual fly-wire cages enabled the ants to remove the remaining soft tissue without also removing any bones. This process took 17 to 58 days.

The normal practice of rotting the flesh from bones in a water bath was considered unsuitable due to the possible leaching out of any retained seed toxicants.

The head and feet of each rat was excised to remove non-skeletal material. The remaining skeleton was then milled in a 'freezer mill', being placed within a mortar, covered with liquid nitrogen and then reduced to a fine particle size with a pestle.

In replication of the extraction of the *Gastrolobium* seed, the milled skeleton was transferred to a pre-weighed 30mm x 80mm cellulose extraction thimble (Whatman),

capped with glass wool and extracted with 250ml of chloroform (BDH, AR grade), plus boiling chips, in a soxhlet apparatus for 7 hours. After allowing the thimble and contents to air dry, extraction was repeated with 250ml of methanol (BDH, HPLC grade).

Solvents were then reduced to a few millilitres using a rotary evaporator over a $35\pm1^{0}C$ water bath, centrifuged for 10 minutes at 6000rpm, with the supernatant transferred to a pre-weighed round-bottom flask and the solvent removed. The extract was then frozen, freeze-dried and a sample analysed for fluorine and alkaloids.

Samples of muscle tissue were freeze-dried and analysed for their total fluorine in order to determine the contribution possible from any residual muscle in the skeletal solvent extracts.

If results for the 'Day 20' dose group were significant it was proposed to similarly analyse the 'Day 10' and 'Day 5' dose groups.

7.2.5 Tissue and bone analysis for the presence of organo-fluorine compounds

Duplicate solvent extract samples and triplicate muscle samples were analysed for the presence of 'total fluorine' using the alkali fusion method described in 4.1.2.

7.2.6 Tissue and bone analysis for the presence of alkaloids

The presence of alkaloids was determined through thin layer chromatography (TLC) and application of Dragendorff's Reagent (Sigma), giving an orange spot on a yellow background. Solvent extracts of the rat skeletons and muscle samples were applied in replicate to a Kieselgel 0.25mm silica on aluminium (Merck) TLC plate and

compared to a chloroform extract of *G. bilobum* seed applied to the same plate. Plates were developed in a 60:40 chloroform:methanol solvent and 5:2:4 1-butanol:acetic acid:water.

7.3 Results

At 2g seed per day, rats were ingesting 0.013mg fluoroacetate per day. Average weight of treatment rats was 326g when dosed with 2g seed per day. White laboratory rats (*Rattus norvegicus* - Sprague-Dawley strain) have a fluoroacetate LD_{50} of 3.7 mg kg^{-1} (Kostyniak, 1979). At 3.7mg kg^{-1}, a 326g rat has an LD_{50} of 1.21mg (~ 186g seed). Thus the consumption of 2g milled seed per day was well below the rat's LD_{50} with rats showing no aversion to the milled seed in peanut butter, generally eating the dose within minutes.

7.3.1 Presence and quantification of organo-fluorine compounds in muscle and bone

The chloroform extracts of treatment and control rats from the '20 Day' dose group are presented in Table 7.1. The analysis of these results using an ANOVA was unsuccessful due to the significant variability between rats. A comparison of means ± SE indicates no significant difference between treatment and control groups (treatment = 5.6 ± 1.2ppm; control = 5.7 ± 1.8ppm), however this should only be taken as an indication as it masks the variation which prevented analysis by an ANOVA.

The methanol extracts of treatment and control rats from the '20 Day' dose group are presented in Table 7.2. The analysis of these results using an ANOVA was also unsuccessful due to the variation involved. A comparison of means ± SE indicates some difference (treatment = 6.8 ± 2.1ppm; control = 4.1 ± 1.0ppm). As with the chloroform extracts, this should only be seen as an indicator, since it masks the variation which

prevented analysis by an ANOVA and therefore no test of significance has been undertaken.

The 'total fluorine' analysis of muscle samples from the treatment and control rats from the '20 Day' dose group is presented in Table 7.3. The analysis of these results using an ANOVA was also unsuccessful due to the significant intra- and inter-rat variation. A comparison of means ± SE shows a significant difference (treatment = 6.5 ± 0.5ppm; control = 11.9 ± 3.5ppm), however the bias is towards the control rats. This is difficult to explain with their diet lacking the organo-fluorine containing milled seed. As with the chloroform and methanol extracts, this difference in means should only be taken as an indication as it masks the variation which prevented analysis by an ANOVA, and as such no test of significance has been undertaken.

7.3.2 Presence and quantification of alkaloids in muscle and bone

TLC of both the chloroform and methanol extracts of the rat skeletons gave Dragendorff positive results. Identical spots, being a streak from the origin to 0.9 Rf, were observed in both the treatment and control rats. This was similar to the chloroform seed extract, having one spot at the origin and one at 0.11 Rf. Addition of 4 drops of 36% hydrochloric acid to the TLC solvent reduced the pH to ~1.0 and moved all spots to higher Rf's. Although this caused some streaking, it showed a difference in Rf between the Dragendorff positive compounds from the rats and those from the seed extract. This indicates the Dragendorff positive compounds in the *Gastrolobium* seed (Chapter 3) are not being retained in the rat skeletons.

Table 7.1 'Total fluorine' analysis of the chloroform extract of rat skeletons

	Rat	Sample Weight (mg)	Total Fluorine (ppm)
Treatment	#21	0.2089	3.1
		0.2061	2.4
	#22	0.2125	4.6
		0.2251	2.8
	#23	0.3071	5.3
		0.3335	4.2
	#24	0.2056	4.8
		0.2095	3.7
	#25	0.2035	12.3
		0.2012	12.4
Control	#26	0.2066	19.2
		0.2037	13.3
	#27	0.2033	3.2
		0.2197	2.3
	#28	0.2065	3.5
		0.2007	2.8
	#29	0.205	2.5
		0.2033	2.5
	#30	0.4169	4.9
		0.4965	3.2

Table 7.2 'Total fluorine' analysis of the methanol extract of rat skeletons

	Rat	Sample Weight (mg)	Total Fluorine (ppm)
Treatment	#21	0.2692	2.2
		0.2447	2.2
	#22	0.1508	5.5
		0.1513	5.3
	#23	0.0723	20.8
		0.0858	17.6
	#24	0.155	3.6
		0.1507	2.9
	#25	0.1513	4.3
		0.1569	3.3
Control	#26	0.151	3.2
		0.1506	2.8
	#27	0.1533	3.2
		0.1509	3.2
	#28	0.152	2.9
		0.1517	1.6
	#29	0.1529	2.5
		0.1553	2.5
	#30	0.1315	10.7
		0.1476	8.8

Table 7.3 'Total fluorine' analysis of muscle

	Rat	Sample Weight (mg)	Total Fluorine (ppm)
Treatment	#21	0.2373	4.8
		0.2507	4.2
		0.2515	4.6
	#22	0.201	7.0
		0.2086	4.1
		0.2039	4.0
	#23	0.2014	9.9
		0.2092	8.2
		0.2066	7.9
	#24	0.2033	7.7
		0.2008	7.6
		0.201	6.3
	#25	0.2041	7.4
		0.2051	8.4
		0.209	4.6
Control	#26	0.2065	5.1
		0.2045	4.9
		0.2052	3.6
	#27	0.2041	6.2
		0.2035	3.3
		0.2032	3.5
	#28	0.2034	9.7
		0.202	6.5
		0.2017	23.5
	#29	0.203	48.6
		0.2081	35.8
		0.204	7.0
	#30	0.2222	6.9
		0.2012	6.6
		0.2024	7.8

7.4 Discussion

This experiment was primarily an examination of the skeletal retention of the putative alkaloids in *Gastrolobium* seed and thus highly fluorinated seed was avoided. As discussed in Chapter 3, alkaloids have been identified previously from the leaves of species of *Gastrolobium*. In fact the findings of Mann and Ince (1905; 1906) were describing alkaloids so highly toxic that this experiment was commenced using a very conservative dose of seed. At the time of undertaking this experiment the presence of Dragendorff responsive compounds in *Gastrolobium* seeds was believed to indicate the presence of alkaloids, Dragendorff's reagent being the principal alkaloid-indicating reagent. However it is also used as an indicator of quaternary nitrogen compounds. The identification of the putative alkaloids in *Gastrolobium* seeds as being phosphatidyl cholines (lecithins) occurred after this experiment was concluded. No studies were found that reported the use of this reagent for staining lecithins. As phosphatidyl cholines are ubiquitous compounds in an organism and were found to be completely soluble in chloroform, stain with Dragendorffs, and would be extracted from skeletal material, it is considered most likely that they are the Dragendorff responsive compounds extracted from the rat skeletons.

Another possibility is that the Dragendorff responsive compounds could in fact be alkaloids, but instead had been sourced from the lupins in the feed pellets. It was subsequently discovered that the feed pellets contain lupins as a primary ingredient, a legume known to contain quinolizidine alkaloids. As these pellets were given to both treatment and control animals, the Dragendorff responsive compounds extracted from the skeletons of both groups of rats could be these lupin alkaloids. The '20-Day' rats had

ingested the pellets for approximately 70 days, however alkaloid retention may have begun with suckling, as Panariti (1996) detected the alkaloid colchicine unchanged in the milk of a lactating sheep. Establishing the presence of lupin alkaloids in the rat skeletons would be beneficial to the hypothesis of alkaloids as a bone retentive toxicant. However, having been unable to confirm the presence of alkaloids in *Gastrolobium* seeds, thereby effectively ruling them out as the bone-retentive toxicants targeted within this study, it was considered beyond the scope of this research to explore this area further.

Chapter 4 describes the presence of numerous fluorinated compounds in *Gastrolobium* seeds, with chapter 5 outlining the possible presence of fluoroacetylated sugars. These are all present in *G. bilobum* (Quindanning) seed but in much lower concentrations compared to other analysed seed. In hindsight, with the subsequent identification of the putative alkaloids as phosphatidyl cholines, thereby making irrelevant the concerns of alkaloid toxicity referred to by Mann and Ince (1905; 1906), the treatment rats could have been dosed with a more fluorinated seed. This would inherently enable much higher doses and have allowed for more thorough testing of the hypothesis of skeletal retention of fluorinated compounds from ingested *Gastrolobium* seeds.

The limitation of the fluoride selective electrode approach used in this experiment is that it lacks the sensitivity of analytical techniques such as GC-MS, which target a specific compound. The results from the pigeon bone analysis (6.3.2) suggest it is possible for fluoroacetate to be retained and then extracted and analysed, with the reported concentrations likely to be missed in the inherent variation measured by the electrode. However, this GC-MS method is restricted to fluoroacetate, with other

fluoroacetylated compounds such as fluoroacetylated sugars, also possibly derivitised and described as fluoroacetate. The benefit then of the electrode approach is that if the treatment rats had retained any of the organo-fluorine compounds represented by the ^{19}F NMR resonances, or fluoride itself, the potential existed for this retention to be detected. Until all the fluorinated compounds are identified, such a general approach to their detection and measurement is necessary.

With confirmation of the identity of fluoroacetylated sugars, a future experiment would be to replicate the study but this time using MFA-sucrose, the most abundant fluoroacetylated sugar. This would require a cool methanol extraction of the skeletons (see 5.2.2) and utilisation of the HPLC-MS method outlined in 5.2.4, in order to determine the presence of MFA-sucrose. Utilising ^{18}F or ^{14}C radio-labeled MFA-sucrose as per the studies of Gal *et al.* (1961) and Sykes *et al.* (1986; 1987) is not considered a suitable method for verifying the skeletal retention of this fluoroacetylated sugar. It is a criticism of these studies that establishing the presence of the radio-label does not automatically establish the identity of the associated compound, if there is one. It may simply indicate the presence of the radio-label. Although these studies demonstrate a rapid and significant skeletal uptake of the radio-labeled fluorine, there is no data provided to confirm the identity of any associated compound. Gal *et al.* (1961) suggest that their data supports the skeletal retention of fluoride from the metabolised fluoroacetate, however no definitive data is provided to support this conclusion.

7.5 Conclusion

Although Dragendorff's Reagent is the principal stain for alkaloids, its use with bone extracts is limited by the presence of lecithins. A positive staining reaction was always going to be evident, due to the ubiquitous presence of lecithins, thereby making it difficult to detect the presence of alkaloids. The use of Dragendorff's Reagent as an initial indicator of alkaloids when staining bone (and seed) extracts is therefore considered inappropriate. Future experiments examining the bone retention of alkaloids should be restricted to an approach like Panariti (1996), where a specific alkaloid is targeted. This could also be said for future analysis of bone retained organo-fluorine compounds. The situation faced in this study was the presence of multiple unidentified organo-fluorine compounds in the seeds, any or all of which may have been bone retentive. The examination of the bone-retentive capacity of these compounds may have to follow the identification of these compounds, enabling their specific extraction and analysis.

Seed analysis undertaken in parallel to this experiment described the potential existence of fluoroacetylated sugars. These compounds may be bone retentive if they behave in the manner reported for fluoroacetyl glucosamine in becoming incoportated with hyaluronic acid (Kent and Winterbourne, 1977; Winterbourne *et al.*, 1979). The seed analysis findings post-dated this rat experiment and in avoiding high 'total fluorine' and hence high fluoroacetate seed, the rats were dosed with seed that would be low in these sugars. Future studies examining the skeletal retention of these compounds should utilise extraction and detection techniques specific for these compounds, described in Chapter 5.

Similarly, the possible retention of fluoroacetate in pigeon bones described in Chapter 6, suggests that this may also occur in mammal bones. Use of a native mammal species with a high tolerance to fluoroacetate, and of a sufficient size to allow for extraction of its bones, in conjunction with an established GC-MS method, would enable this hypothesis to be tested. However, the significant variations in 'total fluorine' concentrations obtained in this experiment suggest any future studies should utilise a larger number of animals. The intra- and inter-rat variation observed in this experiment indicates that animals can have a significant difference in their propensity to retain organ-fluorine compounds or fluoride. Use of larger groups of animals may minimse this variation and therefore enables more robust statistical analysis of any results, however ethical issues may prevent this if these animals are required to be native.

Chapter 8: Toxicity and Physiological Effect of *Gastrolobium* Seed on the Domestic Cat (*Felis silvestris catus*)

8.1 Introduction

Cats are the introduced predators for which this project received funding to investigate a control technique. They are stated to be quite unique in their physiology – for example, they lack the ability to synthesise a significant amount of arachidonate from linoleate in the liver and also have low levels of glucuronyl transferase in the liver (MacDonald *et al.*, 1984) which makes them less tolerant to drugs/toxins. Another example of their unique physiology is their inability to desaturate fatty acids (Rivers *et al.*, 1975). Domestic cats also have unique dietary factors. For example, they require taurine, arginine and arachidonate in their diet (MacDonald *et al.*, 1984) however they can obtain water requirements from their prey (Prentiss *et al.*, 1959). Diet and physiology comprise some of the most fundamental issues involved in attempting cat control and therefore use of cats in feeding experiments is both necessary and ethical. Use of other species requires potentially incorrect supposition regarding the relationship between their response and the response of cats, or eventual testing on cats being required to confirm their response.

The primary aim of this study was to identify a toxicant which would help mitigate the catastrophic predation, primarily by just 1 or 2 feral cats, on reintroduced fauna. This research objective is outlined in Chapter 1.

Chapter 2 includes 32 historical anecdotes which discuss the toxicity of animals believed to be due to their feeding on plants of the *Gastrolobium* genus. Of these, 15 discuss the toxicity of bronzewing pigeons (*Phaps chalcoptera* and *P. elegans*) to cats and dogs, with 6 particularly mentioning the birds' bones as being toxic.

The seed analysis detailed in Chapters 3, 4 and 5 outlines the search for a toxicant contained in the seed which could explain the historical anecdotes and enable replication of this toxic wildlife as a feral predator management strategy.

The study detailed in this chapter describes an examination of the toxicity and physiological response of the domestic cat to milled *Gastrolobium* seed and a chloroform extract. The primary aim of this experiment was to determine if *Gastrolobium* seed, and selected fractions, caused any physiological effect on cats. The study objectives were to replicate the historical accounts of rapid deaths, establish a seed solvent extract which also produced rapid death and examine a broad spectrum of physiological parameters to attempt to explain the cause of death and the possible toxicant(s) involved.

A proposal for a replicated study using significant numbers of feral cats dosed under veterinary-assisted anesthesia, enabling use of additional species of seed, seed fractions and replicated doses was applied for however did not obtain ethics approval. Approval was given for a very restricted study. The study outlined here has therefore been limited to a pilot study, detailing a preliminary examination of the toxicity of 3 species (provenances) of *Gastrolobium* seed and the chloroform fraction of *G. parviflorum* (Jacup) seed to the domestic cat.

8.2 Methods

8.2.1 Source and housing of cats

Four 10-12 month old male homozygous normal laboratory bred cats were purchased from the Institute of Medical and Veterinary Science (IMVS) Gilles Plains Animal Resource Centre (GPARC), South Australia. They had been bred at GPARC, housed in pens which incorporated a room and outside run, and fed daily with IAMS dry biscuits (adult). When required for dosing experimentation they were transported in an individual cat carry-box directly to The University of Adelaide medical school.

8.2.2 Source of *Gastrolobium* seed

(See 3.2.1)

8.2.3 Dosing and physiological monitoring of cats

Dosing experimentation was done in a medical room with a temperature of 21 ± 2^{0}C and with lighting provided by standard fluorescent lights. Cats were fasted for 12 hours prior to dosing experimentation at which time they were weighed and then placed under light anesthesia with an intravenous injection of 0.7ml kg^{-1} Saffan (Pitman-Moore). Animals were then intubated and maintained under a light plane of anesthesia with 1.5% isofluorane gas. A 3mm gastric catheter was then inserted down the oesophagus and through the pyloric sphincter into the cat's stomach for dosing. Electrocardiogram (ECG) and electrocephalogram (EEG) probes were connected and a pulse oximeter was attached to an ear. Two femoral catheters were inserted for blood pressure monitoring and blood sampling. A baseline blood sample and physiological data (eg. pulse, blood pressure, heart rate) were collected prior to dosing.

The size of the milled seed dose was based on the belief that 15g of seed (5g in the crop and 10g in the gastro-intestinal tract) would be the maximum amount in a bronzewing pigeon (Ron Johnstone, Curator of Birds, Western Australian Museum, personal communication). Use of the bronzewing pigeon as a guide was based on the anecdote of the insides of a shot bronzewing pigeon causing the death of a retriever dog in 20 minutes (Le Souëf, 1907).

Cat #1, a 4.4kg tabby, was dosed with 3.5g kg^{-1} (15.4g) milled *G. bilobum* (Tambellup) seed flour flushed into the stomach with 70mls water.

Cat #2, a 4.56kg tabby, was dosed with 3.5g kg^{-1} (15.96g) milled *G. parviflorum* (Jacup) seed flour flushed into the stomach with 80mls water.

Cat #3, a 3.8kg tabby, was dosed with 3.5g kg^{-1} (13.3g) milled *G. calycinum* (Mundaring) seed flour flushed into the stomach with 70mls water.

Cat #4, a 4.57kg black and white, was dosed with 0.8633g chloroform extract from milled *G. parviflorum* (Jacup) seed, adsorbed to 5g plain wholemeal wheat flour (Bi-Lo) and flushed into the stomach with 30mls water. From a previous milled seed extraction this equated to the extract from 15.96g milled seed, guided by the results from Cat #2. The dose was prepared by re-dissolving the chloroform extract in a few millilitres of chloroform, transferring the solution to the flour, adding additional washes of chloroform flask to the flour and then ensuring the flour was uniformly mixed. The chloroform was then removed on a Buchi rotary evaporator with the water bath held at 35 ± 1^{0}C.

The doses were prepared as a suspension and transferred to a 60ml catheter tip disposable syringe (Terumo). The syringe was inserted into the top of the catheter tube and the suspension dose slowly delivered directly into the stomach. The syringe and

catheter tube were then flushed with an additional 5-10ml water wash. Dosed cats were observed and monitored constantly during anaesthesia to ensure there was no vomiting & potential for choking and to monitor any physiological response.

While under anaesthesia the cats were monitored for their pulse and heart rate, blood pressure, respiration rate and depth, heart and brain activity and blood gases.

If dosing caused a lethal response, this occurred while the animal was under anaesthesia. If dosing caused no lethal response, the cat was monitored under anaesthesia for at least 2 hours post-dosing before being euthanased with lethabarb (Virbac).

Euthanased animals were autopsied with tissue samples extracted for possible histopathological analysis.

8.2.4 Tissue analysis

Tissue and plasma samples were stored at -18^0C to prevent any bacterial defluorinating activity (Soiefer and Kostyniak, 1983). Plasma samples were separated using a Clements Orbital 100 centrifuge (Phoenix Scientific Industries Ltd) and immediately frozen.

8.2.5 Plasma citrate and biochemistry

Plasma citrate concentrations were determined by the Western Australian Department of Agriculture Animal Health Laboratories (see 6.2.4). Plasma biochemistry (feline body function) was undertaken by IDEXX Veterinary Pathology Services, Adelaide, with IDEXX veterinary pathologist Martin Copland providing an interpretation of the results provided.

8.2.6 Liver and kidney fluoroacetate analysis

Liver and kidney fluoroacetate concentrations were determined by the Queensland Department of Natural Resources using a method based on Ozawa and Tsukioka (1989) – see Appendix 3.

8.2.7 Blood gas analysis

1ml blood samples were taken from a femoral artery catheter and immediately analysed for blood gases by an ABL System 625 (Radiometer). Measured variables are:

pCO_2 (mmHg) – Carbon dioxide partial pressure

pO_2 (mmHg) – Oxygen partial pressure

HCO_3 (mmol/L) - Bicarbonate

ABE (mmol/L) - Acid base excess

SBE (mmol/L) - Standard base excess

SBC (mmol/L) - Standard bicarbonate concentration

tHb (g/dL) - Total haemoglobin

sO_2 (%) - Oxygen saturation

Hct (%) - Haematocrit

8.3 Results

8.3.1 Seed toxicity

Cats 01 and 04 showed no significant physiological abnormalities at 120 minutes post-dosing and were euthansed. Cat 02 was observed to have ceased respiration at 82 minutes post-dosing with brain and heart activity continuing but deteriorating until euthanased at 120 minutes. Cat 03 was observed to be very close to death on 2 occasions before being euthanased at 180 minutes post dosing.

It was planned to randomly select one of the 4 cats to be a control and receive plain wholemeal flour as a test to ensure the dosing and monitoring procedure was not causing an adverse response. However the survival of Cat 01, and subsequently Cats 03 and 04, suggests the dosing and monitoring methodology were not the cause of the cessation of respiration in Cat 02. Similarly Cat 04 acts as a control for the ingestion of fluoroacetylated compounds (fluoroacetate and any MFA-sugars), as these are absent from the chloroform extract ingested by Cat 04, but present in the milled seed given to Cats 01, 02 and 03.

8.3.2 Plasma citrate and biochemistry

Plasma citrate and biochemistry results outside the normal range are presented in Figures 8.1 to 8.8. Due to the large number of biochemical analyses undertaken, results considered within normal limits or a consequence of cat age, are located in Appendix 4, although it is recognised normality can be both diagnostic and significant. Horizontal dashed lines indicate IDEXX upper, and when provided, lower normal concentration limits.

8.3.3 Analysis of blood gases

Results of blood gas analysis are presented in Figures 8.9 to 8.12 with blood oxygen (O_2) partial pressure presented in Figure 8.13. Cats 01, 03 and 04 show normal blood gas levels, with the exception of Cat 03 showing an elevated blood carbon dioxide (CO_2) partial pressure in the final 30 minutes before euthanasia. Similarly, blood O_2 levels for Cat 02 began very low compared to the other cats, resulting in an apparent peak to a level within the range of the other test animals, before a significant decline occurred. This low starting level may have been real, but is considered more likely to have resulted from a technical fault in the collection or analysis of blood O_2 in these 2 samples, as no corresponding elevation in CO_2 partial pressure is observed (Figure 8.10). The O_2 saturation (Figure 8.10) shows no change from pre- to post-dosing, in contrast to the blood O_2 levels, understandable as O_2 partial pressure always exceeded 150mm Hg.

8.3.4 Electroencephalogram (EEG) monitoring

EEG monitoring recorded a large number of brain activity indexes, however the bispectral (BIS) index was recommended as a general index for brain activity and this data for the 4 cats is presented in Figures 8.14 to 8.17. Excluding some apparent technical abnormalities indicated by outlier data points, Cats 01 and 04 show relatively 'normal' brain activity. The excessive variability in the first 60 minutes of data for Cat 02 is possibly due to poor electrode conductivity, but could be a true record of serious brain electrical dysfunction. The overall trend in the data does however demonstrate a significant decline in brain activity. Cat 03 shows a decline in brain activity at 110 minutes post dosing and then a major decline at 150 minutes.

8.3.5 Electrocardiogram (ECG) monitoring

The intricacies of interpretation of an ECG requires specialist analysis and nobody experienced in reading a feline ECG could be located. They are therefore not reproduced here. However, the ECG of Cat 01 appears largely unchanged except for a slowing of the heart rate, which could simply be a result of the extent of time under anesthesia. The ECG for Cat 02 changes significantly at the 45 minute printout with additional small peaks, then a reduction in peak height at 60 minutes, a substantial negative peak arising at 65 and 70 minutes, then a significant slowing and reduction in intensity of peaks from 77 to 100 minutes. The ECG of Cat 03 shows a strong and regular heart rate from 0 to 180 minutes maintaining a consistent pattern. The heart rate did slow significantly, which would be expected to some degree after 3 hours of anaesthesia, with a deepening in the intensity of the peaks. The ECG of Cat 04 was unchanged with only a minor slowing in heart rate from 0 to 120 minutes.

8.3.6 Blood pressure monitoring

The use of a femoral catheter for the monitoring of blood pressure required regular flushing of the catheter with heparin, to eliminate blood clotting. This is indicated by large fluctuations in recorded blood pressure as the monitoring is impeded and then restored. As blood pressure was recorded every second, the average was made of each 10 recordings to simplify the enormous data sets. The results for each of the 4 cats is presented in Figures 8.18 to 8.21. Cat 01 shows no significant deterioration in blood pressure, although the decline in the final 20 minutes before euthanasia may be real rather than another gradual blockage of the catheter. Cat 02 shows a severe decline from

approximately 60 minutes post dosing, with Cat 03 in decline from approximately 68 minutes and Cat 04 showing a slight decline from 70 minutes post dosing. As Cat 04 showed little physiological or biochemical abnormality in other analyses, the slight decline in Cat 04 may simply have been a response to the anesthesia.

8.3.7 Tissue fluoroacetate concentrations

The analysis of liver and kidney samples from each of the four cats detected fluoroacetate in Cats 01, 02 and 03, with concentrations reflecting the seed 'total fluorine' and therefore potential seed fluoroacetate dosed to each of these cats. The liver and kidney samples from Cat 04, having been dosed with the chloroform fraction of *G. parviflorum* (Jacup) seed which lacked any ^{19}F NMR triplets, understandably resulted in the detection of no fluoroacetate. Results are presented in Table 8.1.

Cat #	Liver		Kidney	
	Sample Weight (g)	**Fluoroacetate (µg/g)**	**Sample Weight (g)**	**Fluoroacetate (µg/g)**
01	9.712	0.416	21.395	0.218
02	14.196	4.08	21.351	0.926
03	10.428	1.10	10.183	0.375
04	12.120	ND	48.197	ND

Table 8.1 Liver and kidney fluoroacetate concentrations

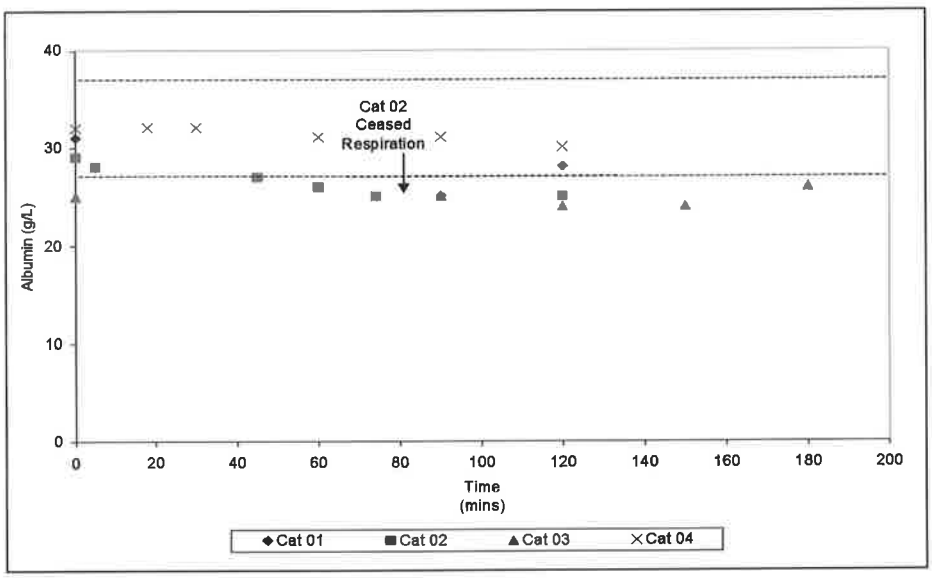


Figure 8.1 Plasma albumin concentration. Reduced levels in Cat 02 and 03 may indicate impaired liver function or albumin loss through the gastrointestinal tract or kidneys.

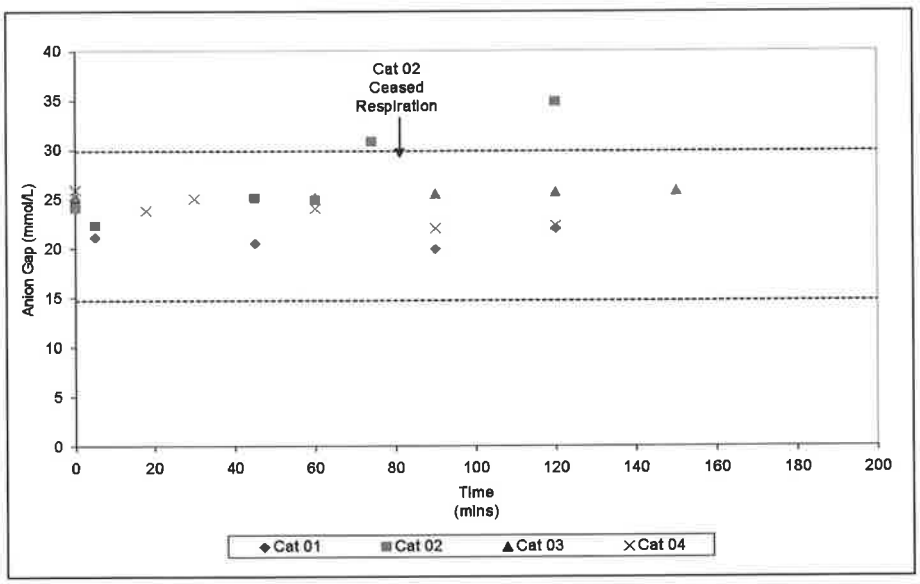


Figure 8.2 Plasma anion gap concentration. Elevated levels in Cat 02 taken with reduced plasma bicarbonate (Figure 8.6) suggest metabolic acidosis.

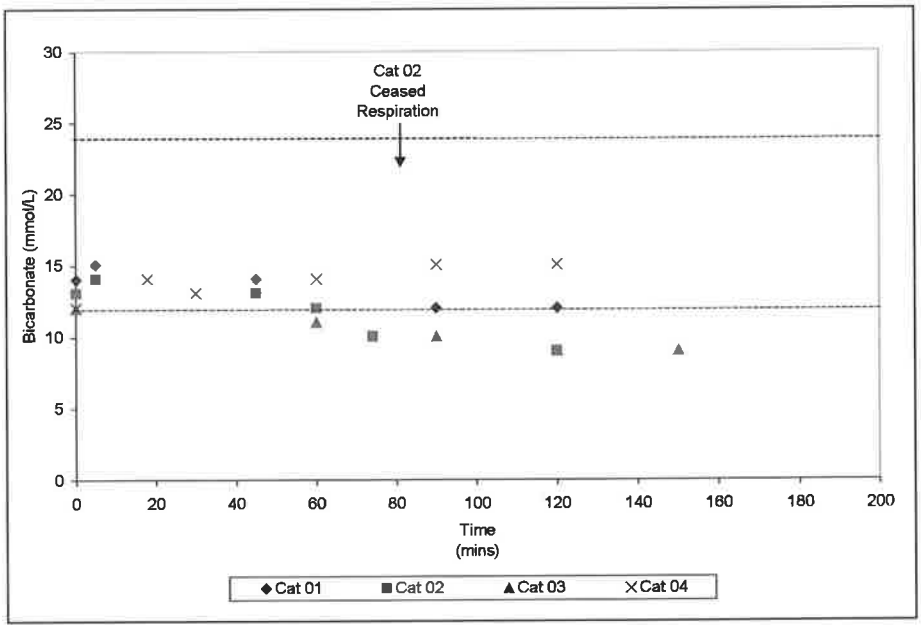


Figure 8.3 Plasma bicarbonate concentration. Reduced levels in Cat 02 taken with elevated plasma anion gap (Figure 8.4) suggest metabolic acidosis.

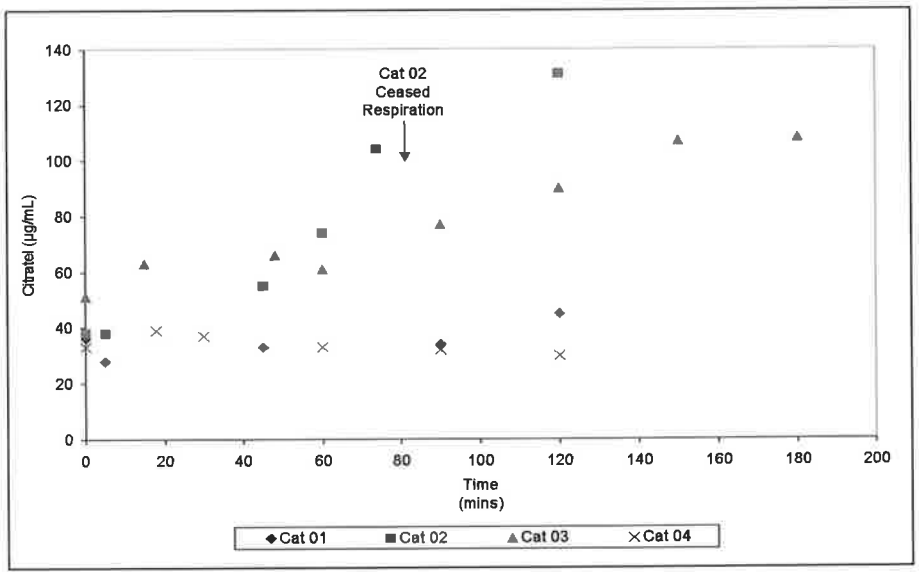


Figure 8.4 Plasma citrate concentration. Elevated plasma citrate is evident in Cats 01 to 03 indicative of the metabolism of fluoroacetate. Citrate accumulation reflects seed total fluorine and hence fluoroacetate (more = quicker).

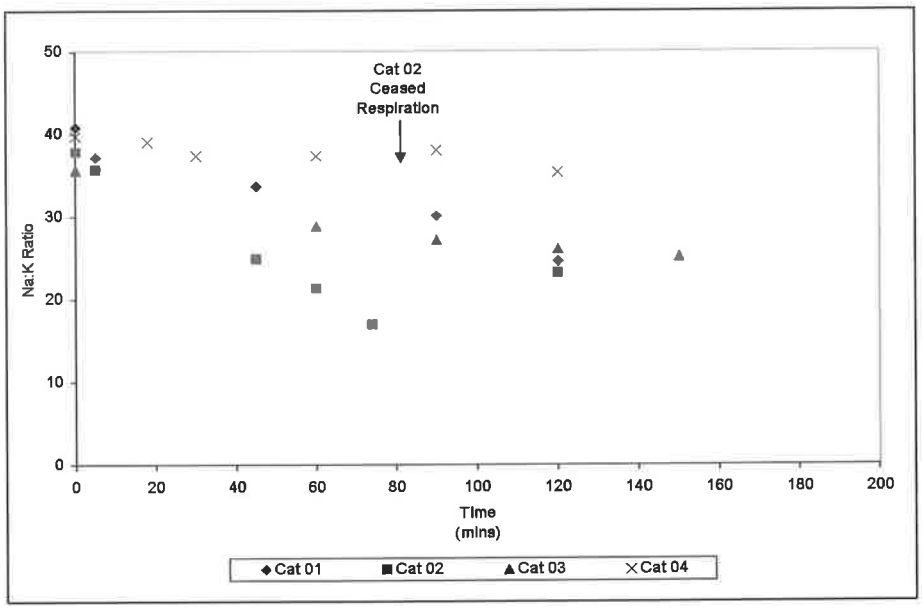


Figure 8.5 Plasma sodium : potassium ratio. Decline in Cats 01 to 03 reflects marginal increase in plasma potassium (see Figure 8.18).

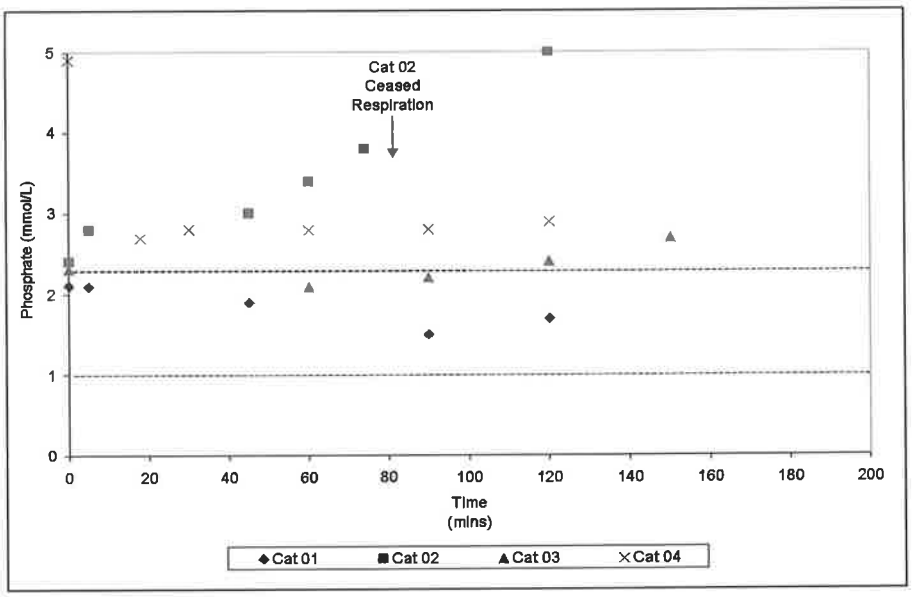


Figure 8.6 Plasma phosphate concentration. Time 0 value for Cat 04 due to haemolysed sample (see also Figure 8.20). Cat 02 showing reduced kidney function.

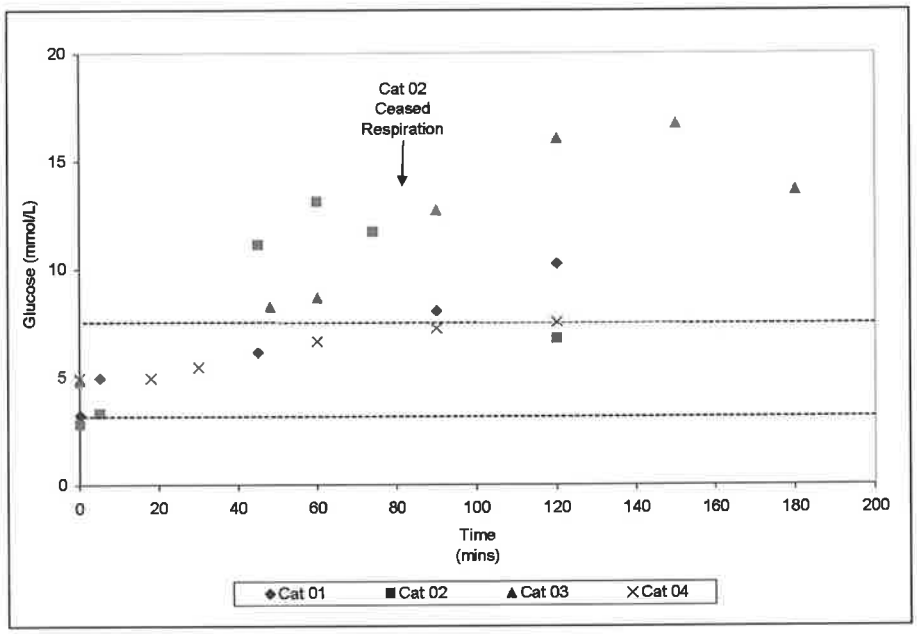


Figure 8.7 Plasma glucose concentration. Elevated levels are a stress response to the experimental procedure, highest in Cat 03, the longest under anaesthesia.

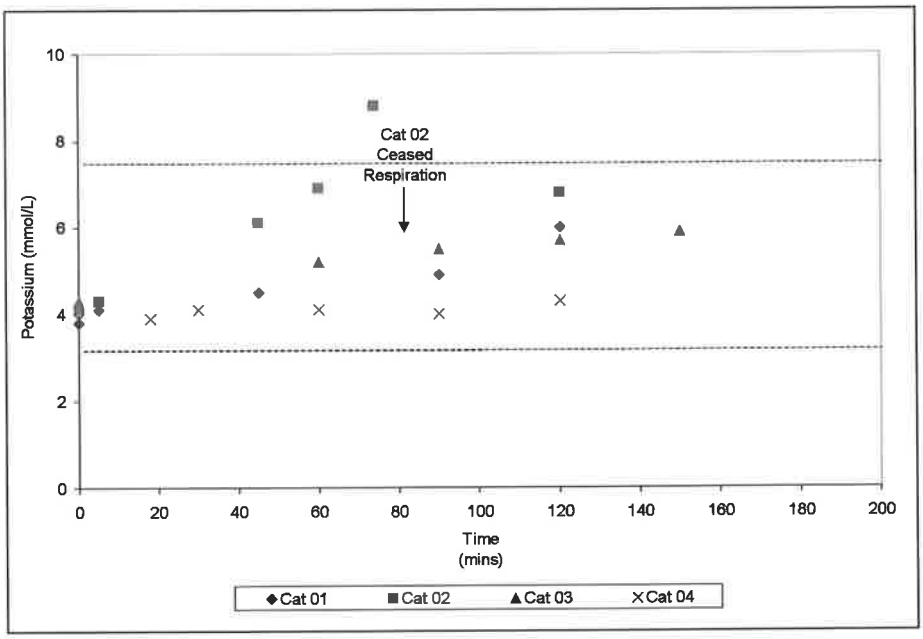


Figure 8.8 Plasma potassium concentration. Levels are largely within normal limits but show an upward trend indicating the destruction of cell membranes.

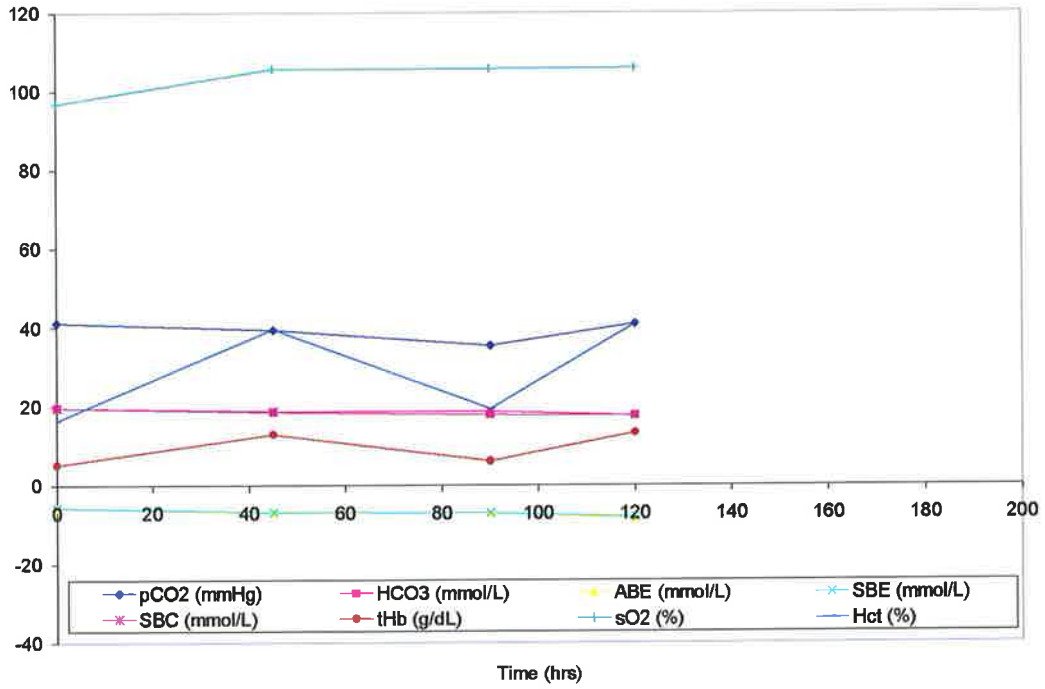


Figure 8.9 Cat 01 Blood gases

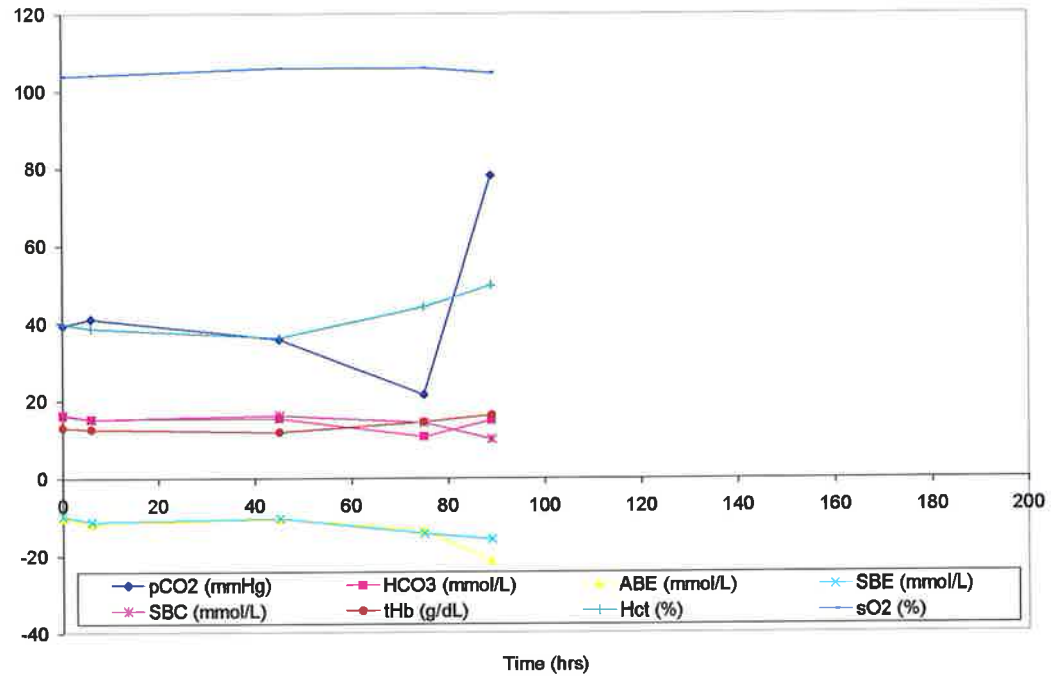


Figure 8.10 Cat 02 Blood gases

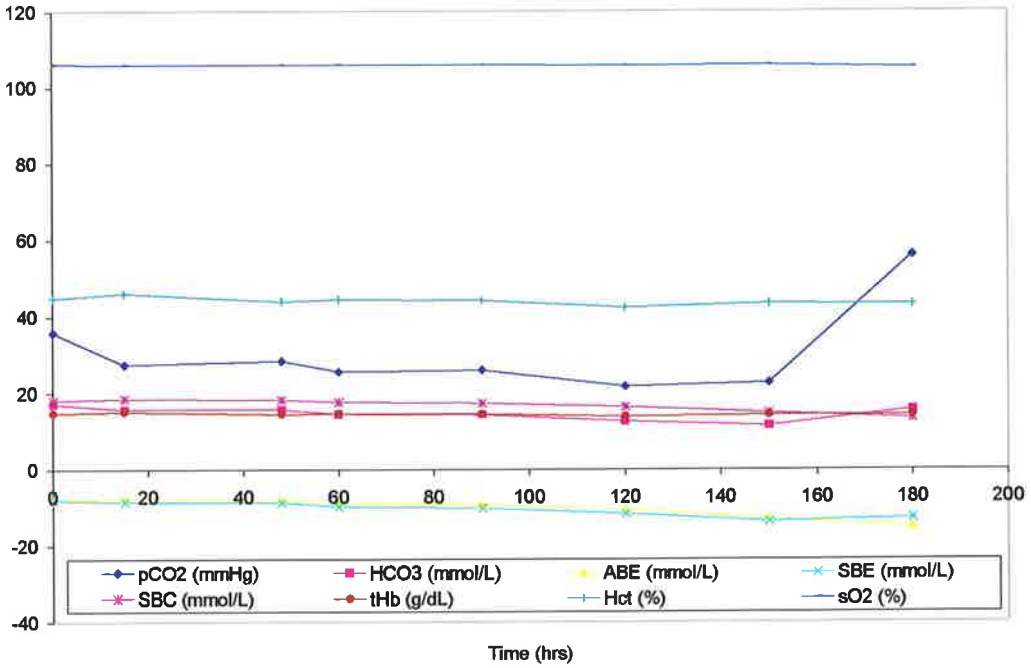


Figure 8.11 Cat 03 Blood gases

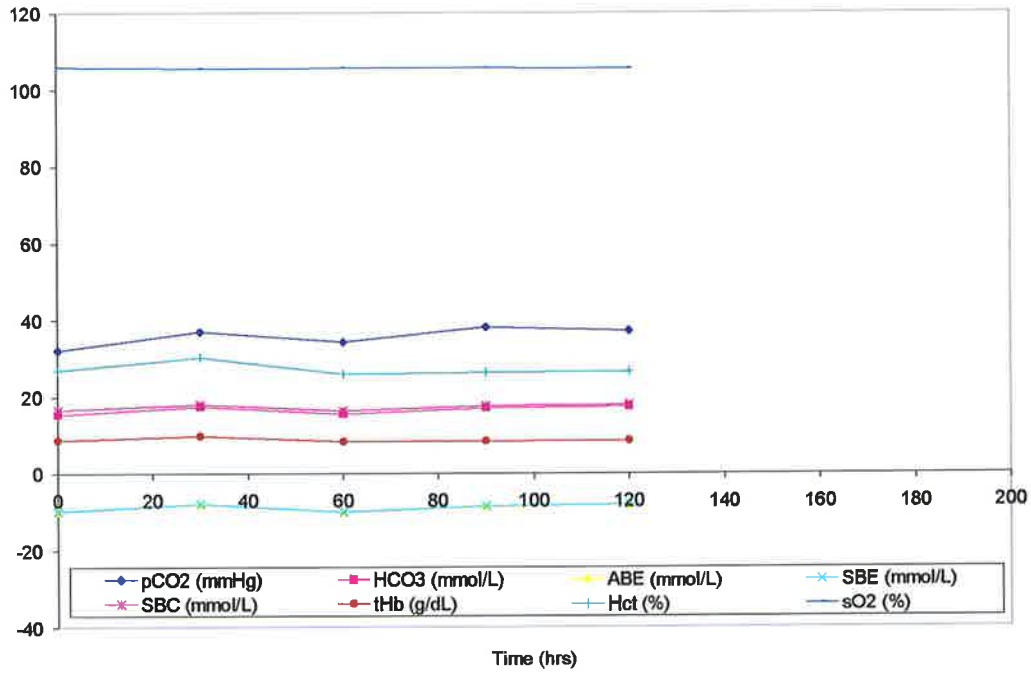


Figure 8.12 Cat 04 Blood gases

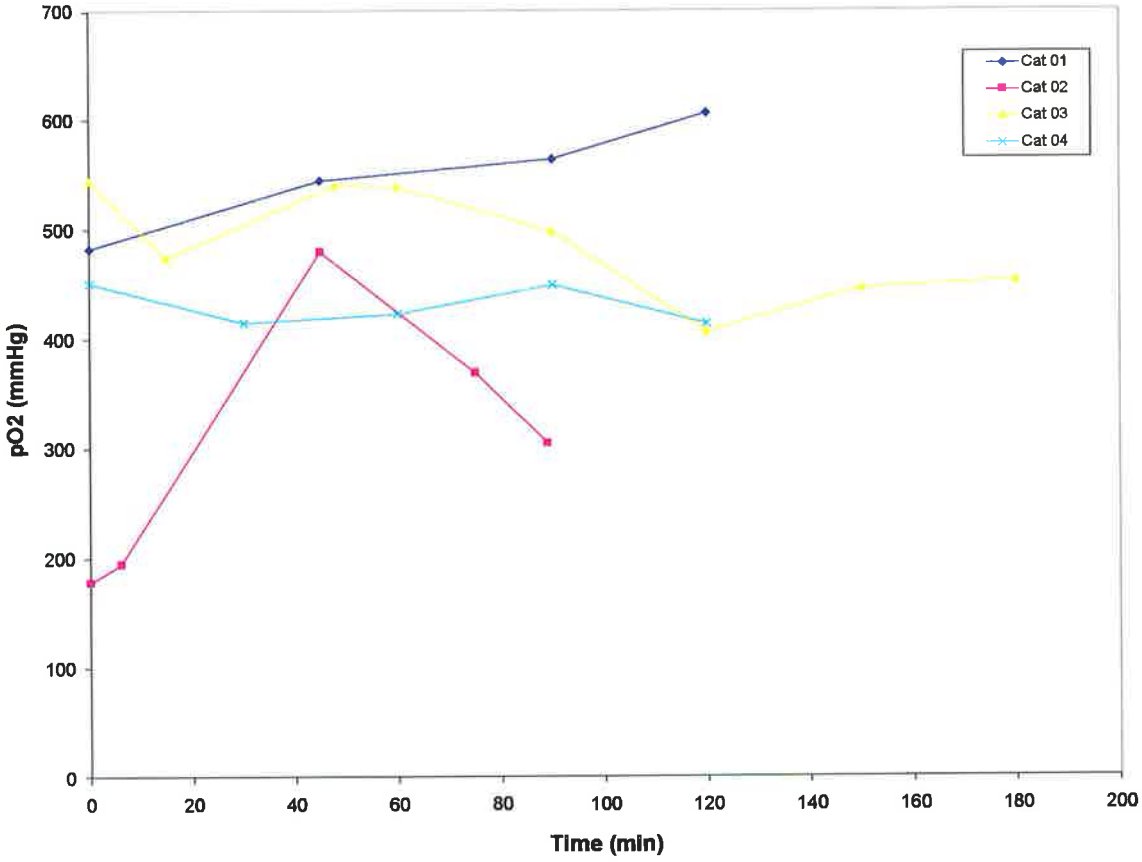


Figure 8.13 Cat blood oxygen partial pressure

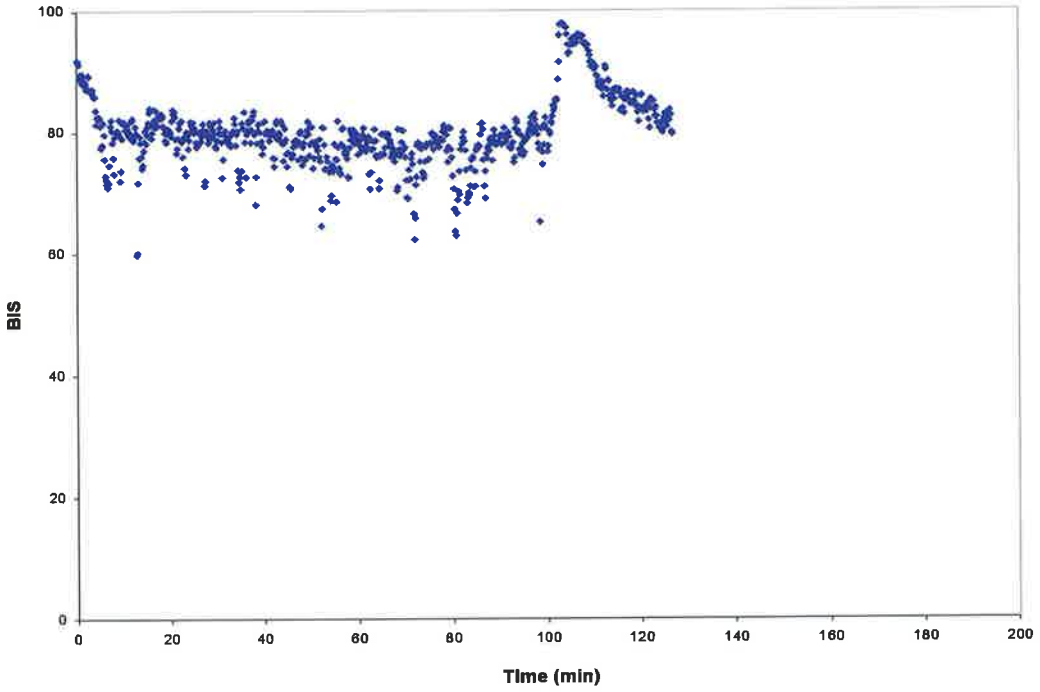


Figure 8.14 Cat 01 electroencephalogram (EEG) bispectral (BIS) index

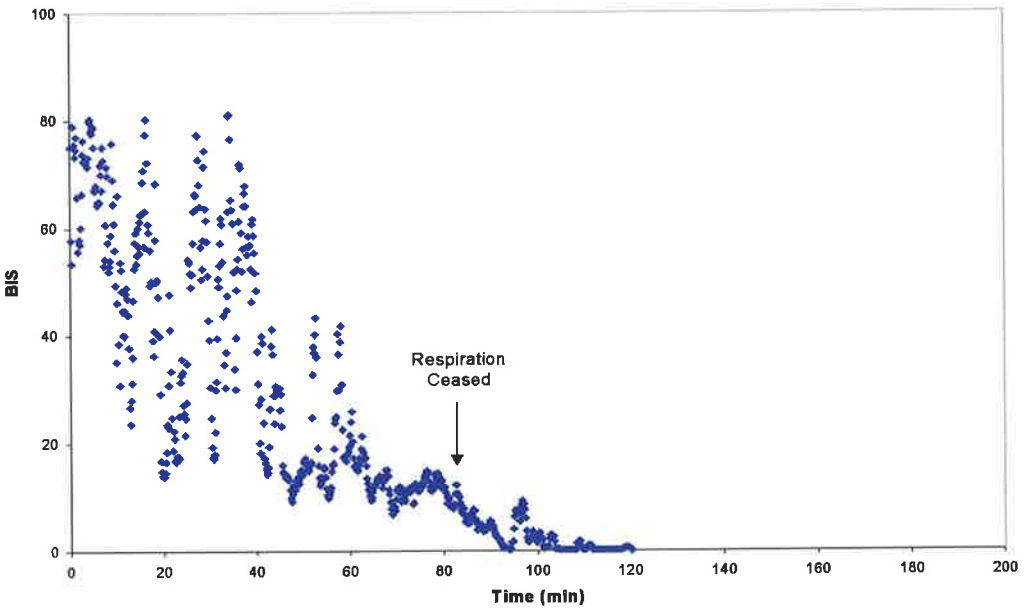


Figure 8.15 Cat 02 electroencephalogram (EEG) bispectral (BIS) index

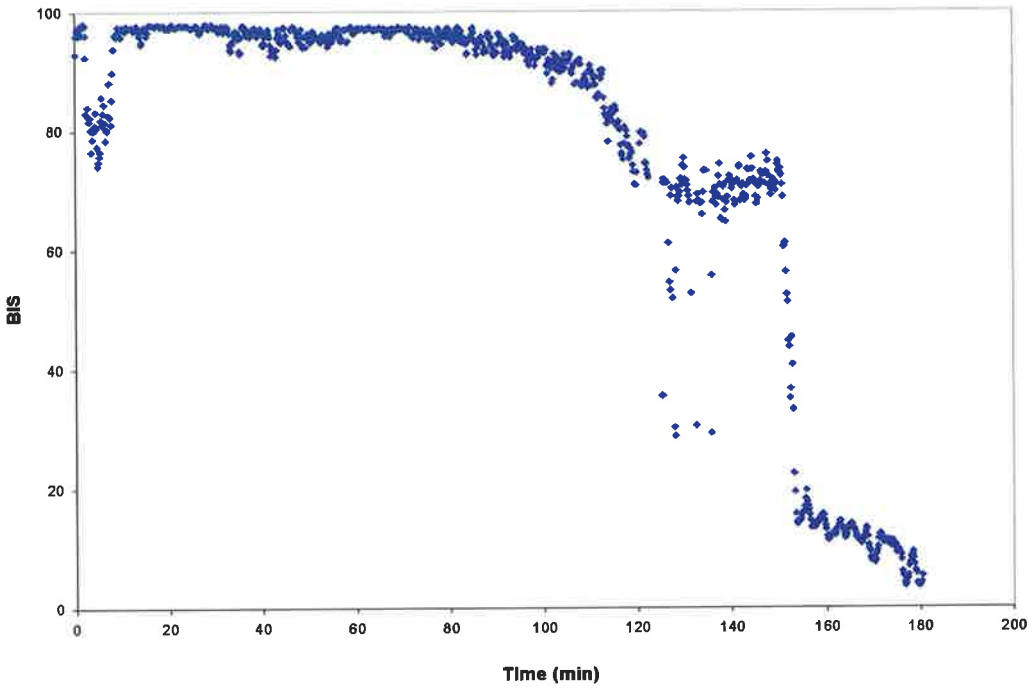


Figure 8.16 Cat 03 electroencephalogram (EEG) bispectral (BIS) index

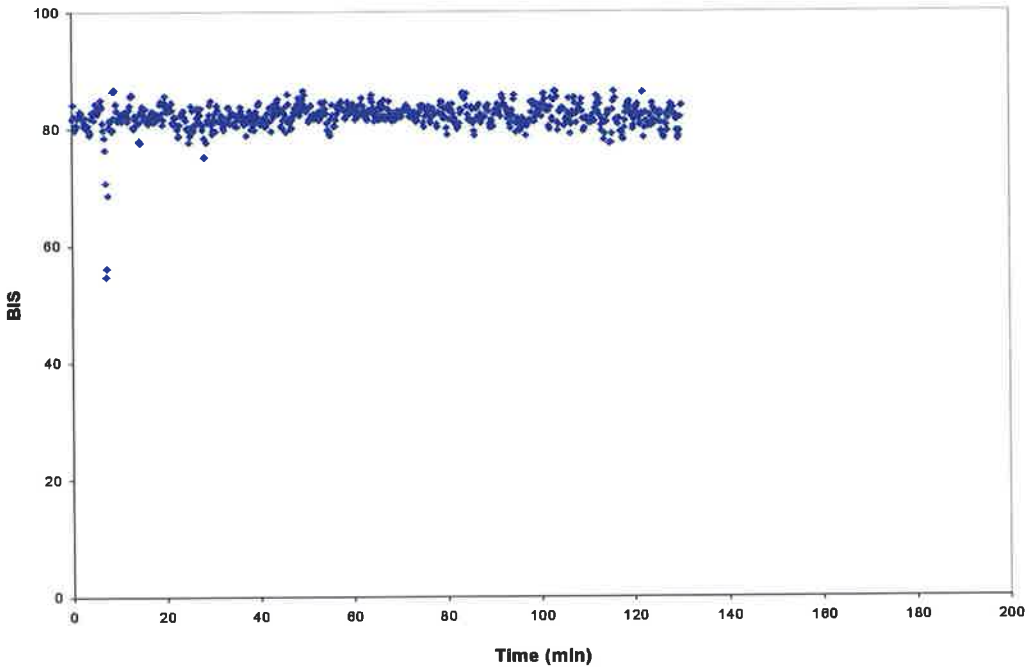


Figure 8.17 Cat 04 electroencephalogram (EEG) bispectral (BIS) index

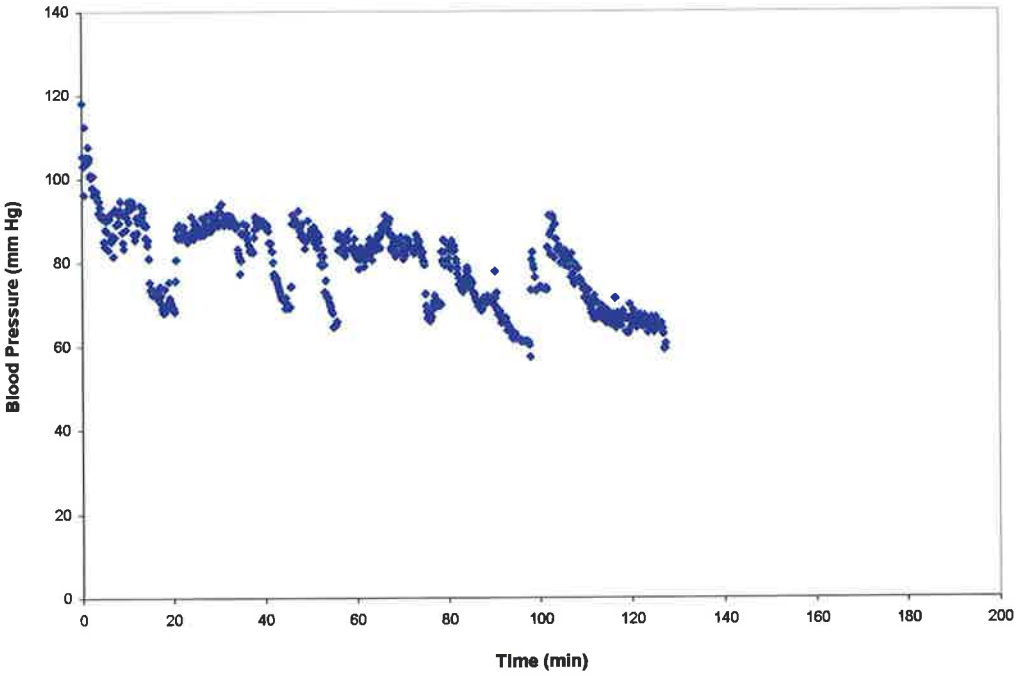


Figure 8.18 Cat 01 blood pressure monitoring

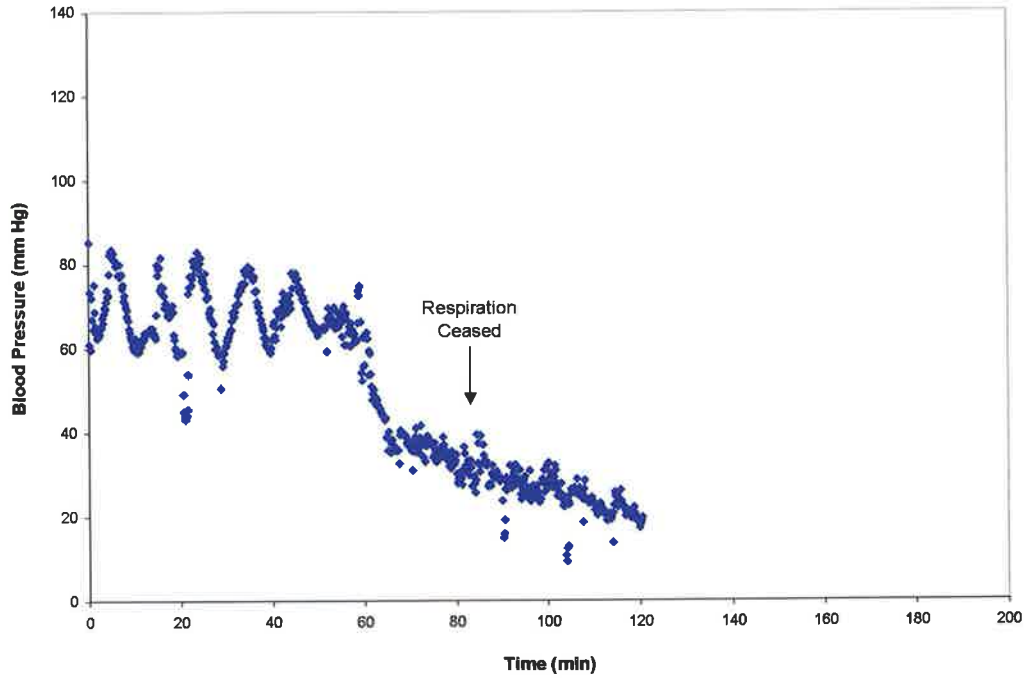


Figure 8.19 Cat 02 blood pressure monitoring

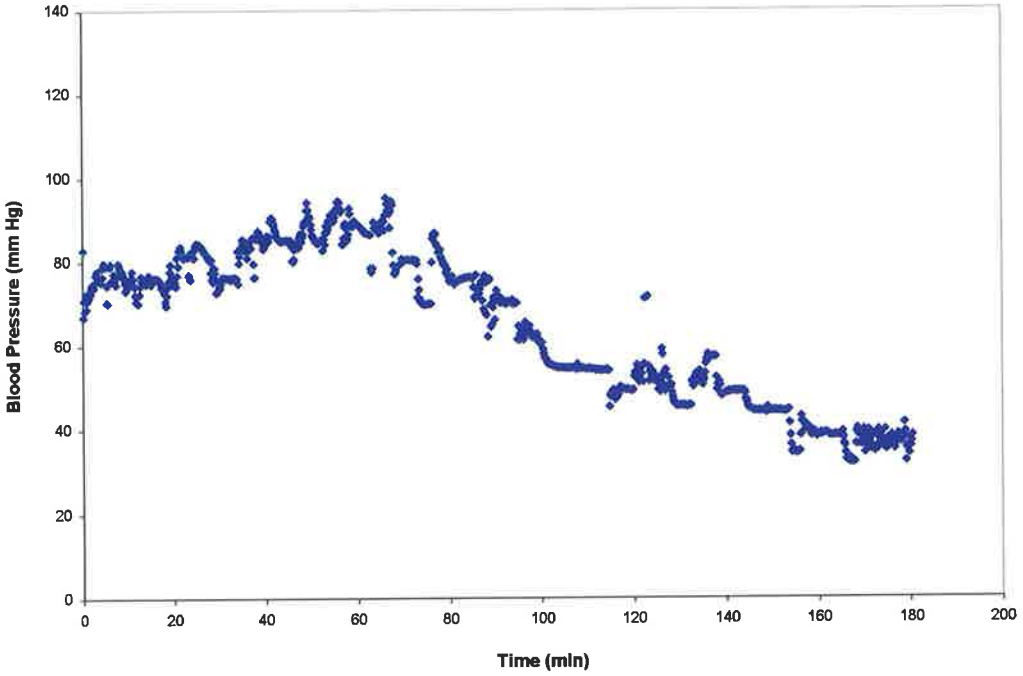


Figure 8.20 Cat 03 blood pressure monitoring

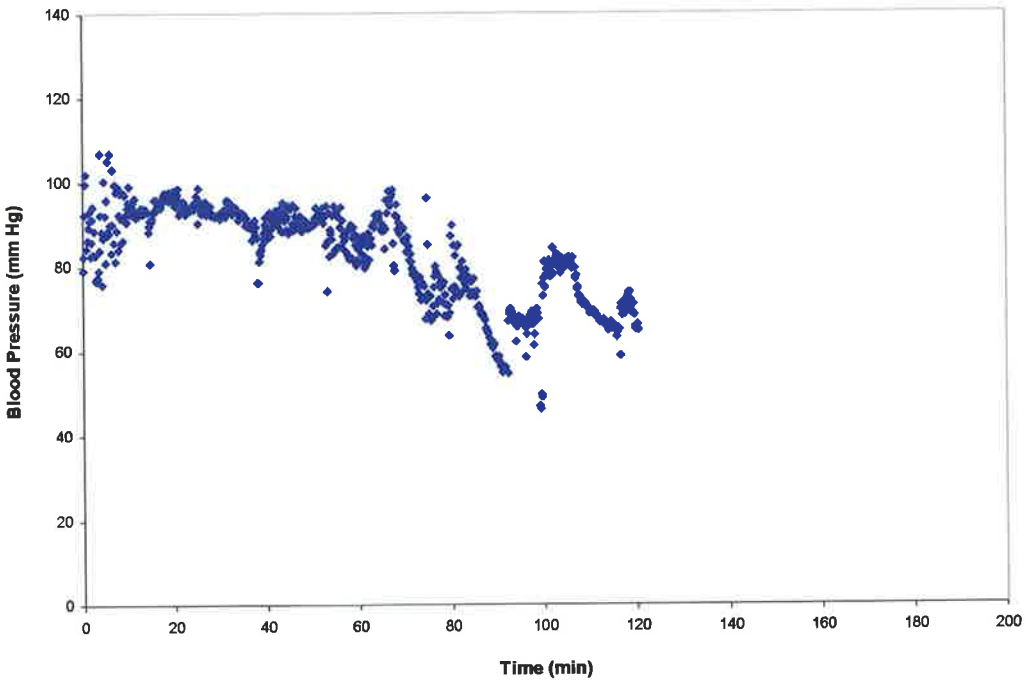


Figure 8.21 Cat 04 blood pressure monitoring

8.4 Discussion

The biochemical and physiological response of Cat 02 to the 15.96g of milled *G. parviflorum* seed was broadscale and led to fatal deterioration of function. Biochemistry suggests function at the cell level (Na/K pump) failed, with the kidneys deprived of blood, central nervous system failure and organ/cell shutdown (Martin Copland, IDEXX veterinary pathologist, personal communication). This resulted in the cessation of respiration at 82 minutes post-dosing, presumably due to failure of the brain's respiratory centre. Responses were very similar to those observed by Chenoweth and Gilman (1946) for unanaesthetised cats intravenously dosed with methyl fluoroacetate. They found either a cardiac or central nervous system response may arise and 'predominate' in an individual cat, with unanaesthetised cats dying primarily due to depression of the respiratory centre, with a heart rate still evident after cessation of breathing. This observation similarly describes the physiological response and subsequent death of Cat 02, anaesthetised and dosed with a gastric lavage of milled *G. parviflorum* seed. (Anaesthetised cats in the Chenoweth study were found to have minimal depression of their respiration, however all died when injected with at least 0.5mg kg^{-1} methyl fluoroacetate).

The biochemical and physiological responses of Cats 01, 02 and 03 may have been a response to large doses of fluoroacetate, and any other fluoroacetylated compounds present. The detection of high concentrations of fluoroacetate in the liver samples and moderate concentrations in the kidney samples of these cats supports these compounds as being the primary toxicant(s) in these seeds. Physiological distress, biochemical imbalance, tissue fluoroacetate and the accumulation of plasma citrate appear directly

related to dose amounts. Elevated citrate is considered to indicate the conversion of fluoroacetate to fluorocitrate, citrate accumulation, cell death, organ failure and generally the subsequent death of the animal. The results suggest Cat 02's rapid death was due to a rapid conversion of fluoroacetate, or other fluoroacetylated compounds, to fluorocitrate, with Cats 01 and 03 affected in a similar way, but not so quickly. The rate of conversion (ie. citrate level) therefore appears directly related to dose level. However, the whole 'lethal synthesis' theory (Peters, 1954; Peters, 1963) requires time for the fluoroacetate to fluorocitrate process and then the cell death and organ failure. The time to dose relationship cannot therefore be linear. Eason and Frampton (1991) gave 8 cats (mean weight 3.6kg) one bait each dosed with 1.6mg sodium fluoroacetate and had a mean time to death of 15hrs, with one cat dying at 7hrs post-dosing. There did appear therefore to be a time to death dose response. McIlroy (1981) orally dosed 20 cats (mean weight of 1kg; dose not specified) with sodium monofluoroacetate, and all cats died at approximately 21hrs post-dosing. A study utilising "spinal animals" in a stimulus-response experiment with a maximum intravenous dose of 4mg kg^{-1}, states a mean time to death of 3hrs (Foss, 1948). Chenoweth (1946) states time to death in rabbits to relate to dose, with intravenous 0.20-0.25mg kg^{-1} resulting in a delay of up to 24hrs, while 2.0mg kg^{-1} may cause death in 30-60 minutes. This was not however the case in dogs, with the 1-2hr latent period "somewhat, but not markedly, shortened" when using higher doses (Chenoweth and Gilman, 1946, p. 98). A massive dose of fluoroacetate, such as given to Cat 02, may cause a marked reduction in the time to death, however not likely to the degree described in some of the historical anecdotes, with dogs stated for example as dying in 25 and 20 minutes (Webb, 1885; Le Souëf, 1907).

The cat LD_{50} is stated to be 0.28mg kg^{-1} (Eason and Frampton, 1991). Therefore 1.5mg would be enough to kill all the cats in this study. Cat 02, which ceased respiration in 82 minutes, was dosed with *G. parviflorum* seed which had a mean 'total fluorine' of 975.5ppm. Thus in the 15.96g dose there would have been approximately 16mg 'total fluorine'. If all of this was incorporated as potassium fluoroacetate (KMFA; potassium being the most abundant cation), there would be a massive total of 93mg. Half of the dose appeared however to have remained in the stomach at death, but even a 47mg dose is huge and unparalleled. Cat 03 survived 13.3g of *G. calycinum* seed with a mean 'total fluorine' of 250ppm, which equates to approximately 20mg KMFA, (10mgs if it was only half digested and absorbed). In response to this large dose, the cat was in serious physiological distress, was monitored for an additional hour past the proposed 2hr limit but did not die. Cat 01 survived 15.4g of *G. bilobum* seed with a mean 'total fluorine' of 145ppm, which equates to about 13mg KMFA. If only half was digested and absorbed, 6.5mgs is also still a significant dose - and this cat did not die within the 2 hour testing time period, or even exhibit a physiological parameter to suggest it was in trouble.

8.5 Conclusion

The results from this study indicate that the toxicant(s) within *Gastrolobium* seed is not within the chloroform fraction and most probably within the methanol fraction. This suggests the rapidly toxic alkaloids reportedly extracted from the leaves of *G. parviflorum* and *G. calycinum* (Mann, 1905; 1906) are not present in these seeds. Assuming that the only fluoroacetylated compound within the *Gastrolobium* seed dosed to Cats 01, 02 and 03 is fluoroacetate, the preliminary data from this experiment does suggest that the larger the dose, the quicker the death of cats. This supports the trend seen in the data of Eason *et al.* (1991) but is difficult to explain with respect to the 'lethal synthesis' hypothesis, with its required time factor. It is unknown whether this trend is also evident in dogs when the doses are of the magnitude used here. This would be contrary to published studies which report a lengthy latent period before symptoms arise and any susequent death, but this has never been tested.

It is possible that the account of 3 cats dead in under an hour from the "insides" of bronzewing pigeons (Knight, 1912) could be explained by a larger or more fluorinated dose of *Gastrolobium* seed than used in this experiment. Although anesthesia can slow digestion, thereby slowing any cat deaths, the seed used in this study was milled and in an aqueous suspension, which would greatly quicken extraction of the fluoroacetate. Also, the *G. parviflorum* seed dosed to Cat 02 was one of the most fluorinated recorded. As cats are stated to eat smaller meals more often (Bradshaw *et al.*, 1996), it seems unlikely that the cats in the Knight (1912) anecdote would each have consumed more "insides" than the equivalent from one pigeon. However, to achieve the reported deaths in under an hour, presumably from largely intact (except in the gizzard) and hard-coated seed, the

results from this study would suggest ingestion of seed more fluorinated than is recorded in this present study (see Chapter 4), and/or ingestion of more seed than believed likely within a bronzewing pigeon (R. Johnstone personal communication, 2001). Both of these proposed conditions are considered improbable.

It is therefore considered unlikely that the Knight (1912) anecdote, and very unlikely that the anecdotes reporting dog deaths in 20-25 minutes - even with a 100% error in the times to death - are explained by the seed toxicant being solely fluoroacetate. As one of these dog anecdotes also involves the toxicant having been bone retained (Webb, 1885), a previously unrecognised capacity of fluoroacetate, it is suggested that the presence of an additional toxicant would better explain these anecdotes. If the approximately 47mgs of fluoroacetate proposed to be digested by Cat 02 was sufficient to cause the 25 minute dog death in the Webb (1885) anecdote, it would equate to the unlikely concentration of almost 12,000ppm contained in the pigeon's breast-bone. A replicated study using a canid model would better test whether the seed doses used in this experiment can cause their deaths in times equivalent to the anecdotes.

The presence of fluoroacetylated sugars in the seed is yet to be verified. Their presence may cause an inhibition in glycolysis and possibly the more rapid citrate accumulation seen in Cat 02 compared with Cats 03 and 01. This may form an alternative explanation for Cat 02's fast death within this pilot study, especially when considered in conjunction with the rapid deaths and bone retention reported in historical anecdotes. With confirmation of the presence of fluoroacetylated sugars another experiment would be beneficial, dosing with MFA-sucrose for example, again seeking to replicate the rapid death anecdotes.

Chapter 9: Discussion, Future Research and Conclusion

9.1 Discussion

The object of this study was to determine if the seeds of species of *Gastrolobium* contain a toxicant that is bone retentive and rapidly toxic to predating cats and foxes, with such a phenomenon reported to have occurred in south-west Western Australia during agricultural and pastoral expansion. The sourcing of the many historical anecdotes of toxic native fauna in south-west Western Australia, detailed in Chapter 2, is considered one of the significant discoveries of this research. These observations taken as a collective suggest that it was established knowledge for people cohabiting with *Gastrolobium* plants, that many of the native animal species with which they had regular contact could be toxic to their cats and dogs. This knowledge included specifically the toxicity of the bones of the bronzewing pigeons. Although these 'stories' have survived to modern times as 'verbal folklore', the knowledge has never progressed to any form of scientific study. As such this investigation for a bone retentive toxicant was often met with comments regarding the disparity between the 'verbal folklore' and the established presence of fluoroacetate. That is, some of the verbal anecdotes were seen as being in error because they could not be explained by the established toxic action of fluoroacetate. Fluoroacetate is not known to be bone retentive and is recognised as having a 'lengthy' time to death. The possibility that the *Gastrolobium* could have additional toxicants that would explain its' toxicity and also explain the anecdotes, has rarely been considered. It therefore seems very likely that some of these historical anecdotes are indicating the presence of an additional unknown toxicant(s) within *Gastrolobium* seed, which is both rapidly toxic and bone retentive. The search for this

toxicant was a very extensive and difficult task and unfortunately largely inconclusive at this point in time.

The established knowledge at the commencement of this project was that fluoroacetate is the sole toxicant within the *Gastrolobium* genus. Aside from the alkaloid work of Mann and Ince (1905; 1906), Webb (1949) and Cannon and Williams (1982), which has been published, the presence of alkaloids as an additional toxicant within *Gastrolobium* receives no mention in modern research (eg. Twigg *et al.*, 1996; Twigg and Socha, 1996). The sourcing of these alkaloid research papers and the research describing the bone (Panariti, 1996; McIntyre *et al.*, 2000) and feather (Dumbacher *et al.*, 1992; Escalante and Daly, 1994; Dumbacher *et al.*, 2000) retentive capacity of alkaloids, indicated initially that these compounds may best explain the historical anecdotes. The analyses detailed in Chapter 3 suggested however that the *Gastrolobium* seeds do not contain any significant alkaloids. It is disappointing that the numerous ethics applications requesting an appropriate bioassay were not approved. The eventual restricted approval for laboratory bred cats indicates, through the survival of Cat 04 with no significant physiological abnormalities when euthanased, that there is no toxicity to explain the anecdotes within the chloroform seed fraction (Chapter 8), the fraction containing the putative alkaloids. The use of aquatic invertebrates (data not presented) and laboratory rats as bioassays is not considered valid as these species may lack sensitivity to the seed toxicants, thereby providing false negatives. The results of the limited pilot study detailed in Chapter 8 suggests through the death of Cat 02 and survival of Cat 04 that the seed toxicity lies in the seed methanol extract. As no additional cats were available to dose with this methanol extract, this remains an

assumption.

The physiological and biochemical monitoring supports the view that Cat 02 died quite rapidly from a fluoroacetate induced toxicosis. It is however considered very likely that all fluoroacetylated compounds present in the seeds will metabolise to fluoroacetate (eg. Arellano *et al.*, 1998) or fluorocitrate (eg. Bremer and Davis, 1973) and cause biochemical responses such as elevated plasma citrate. It may be that the Le Souëf (1907) rapid toxicity anecdote is due to a synergism between the various organo-fluorine toxicants within the seed (Vining, 1990), as suggested for the fluoroacetate containing *Palicourea marcgravii* (Kemmerling, 1996). However, it would be unlikely for all the various organo-fluorine toxicants within the seed to have been retained in a pigeon breast-bone, also reported to have caused rapid toxicity (Webb, 1885). The presence of significant concentrations of fluoroacetate in the pigeon leg bones does support this compound being bone retentive, if only for up to 15hrs without replenishment. However fluoroacetate being reported in the leg bones of control birds, which could not be adequately explained by the testing laboratory, necessitates caution in interpreting this data. The highest concentration detected would still require an approximately 4g pigeon breastbone to be 100 times more concentrated to kill most dogs (~2mg). As the adult pigeon breastbone lacks significant bone marrow and a 2mg fluoroacetate dose should cause a dog death measured in hours (not minutes), this result still fails to explain some of the anecdotes.

The hypothesis regarding the chelation of blood calcium (6.2.5) occurring with fluorocitrate intoxication (Bosakowski and Levin, 1986), and that this was facilitating the skeletal retention of a toxicant, was not supported. The lack of any statistical

significance in the pigeon and cat plasma total calcium concentrations provides no support for this hypothesis. It is therefore concluded that fluoroacetate ingestion, and its metabolic conversion to fluorocitrate, causes no known calcium imbalance which could facilitate the skeletal retention of a *Gastrolobium* seed toxicant.

The discovery of a ^{19}F triplet in the seed methanol extract after only a few scans was seen to support the established presence of fluoroacetate. Further analysis of this extract suggested additional organo-fluorine compounds, therefore more extensive scanning was undertaken which indicated the additional small downfield triplets. Pursuing the hypothesis of possible compounds such as fluoroacetyl glucosamine suggested a room temperature extraction as appropriate. This coupled with long scan times displayed triplets indicative of multiple fluoroacetylated compounds in the seed methanol extract. HPLC-MS analysis of this methanol extract then detected the presence of additional fragmentation ions associated with RFO sugars, and also indicated the presence of a fluoroacetyl group. This was consistent with the presence of minor downfield triplets in the ^{19}F NMR analyses, which were at the same shift as a synthesised fluoroacetylated sucrose standard. Further research has however determined that the process of acetylation to enable liquid chromatographic analysis can produce the MFA sugars. That is, MFA sugars of the raffinose family can be produced during the acetylation by a mixed anhydride formed between the sodium fluoroacetate present in the seeds and acetic anhydride. So far all of the controls indicate that this is occurring, although questions remain regarding certain aspects of the results, such as why is there so much disubstitution in the controls compared to the seed methanol extracts and why do seeds with similar 'total fluorine' show MFA sugars which do not reflect the seed sugar

concentrations? It is possible that both naturally occurring and artifactural MFA sugars are present in the acetylated extract.

In summary, the results of this research indicate that significantly more work is required in this area. The NMR and preliminary research data presented supports the presence of organo-fluorine compounds additional to the reported fluoroacetate, however it has also been found that the process of acetylation to enable liquid chromatography analysis can produce MFA sugars.

The undescribed fluorinated compounds, being the unassigned ^{19}F NMR resonances in the petroleum spirit/chloroform and methanol extracts, may be unique chemical toxicants. The focus of further work should be to purify, identify and establish the structure of at least some of these compounds and then determine their toxicity to target species. If they are likely to accumulate in bone, or cause rapid disruption to biochemical pathways, (especially those of the nervous system), then they may provide the basis for "chemical defense" of reintroduced fauna vulnerable to catastrophic cat and fox predation.

9.2 Future Research

This study has raised many questions yet to be answered and identified numerous areas of possible future research. Some of these include:

Identification of the new organo-fluorine compounds present in *Gastrolobium* seeds.

The unassigned ^{19}F NMR resonances in the petroleum spirit, chloroform and methanol extracts await identification. There is evidence to suggest the existence of fluorinated fatty acids and fluoroacetylated sugars, however this needs to be confirmed through the identification of these compounds and establishment of their existence as natural products. This is also the case for the additional ^{19}F triplets seen in the methanol extracts.

Examination of the toxicity of the putative fluoroacetylated sugars in *Gastrolobium* seeds.

The preference of many species for sugar is well recognised, including pest species as diverse as the coyote (Mason and McConnell, 1997), fox (Saunders and Harris, 2000), rodents (Marsh, 1988) and ant. With confirmation of the MFA-sugars as natural products within the seeds, investigation of their toxicity and mode of action would determine their ability to explain the rapid death anecdotes. The mode of action of these toxic sugars, yet to be determined for any species, may possibly be through inhibiting glycolysis (Taylor, 1972), as well as causing Peters' (1963) 'lethal synthesis' . As it is anticipated that these sugars would be lethal to most species, they may have an application for controlling a wide variety of pest species such as canids, felids, mustelids, rodents, birds and insects.

The role of the granite outcrops and granite derived soils as a fluorine source for the *Gastrolobium*.

The high organo-fluorine concentrations in the *Gastrolobium* appear related to the granite outcrops common in south-west Western Australia. Chandler (personal communication, 2002) defines species in the toxic "*Gastrolobium bilobum* group", such as the aptly named *G. graniticum* (Chandler *et al.*, 2002, p. 709), as the granite outcrop endemics. Harnisch *et al.* (2000) confirms the presence of various organo-fluorine compounds (CF_4, CF_2Cl_2, $CFCl_3$ and SF_6) in granite and fluorite samples with Wainwright and Supharungsun (1984) identifying soil fungi capable of releasing fluoride ions (F^-) from insoluble fluorides. It therefore seems highly likely that a fluorine association exists between the *Gastrolobium* and these rocks and minerals, and research in this area will further our understanding of the *Gastrolobium*/fluorine story and possibly help explain the intra- and inter-species variation in these fluorinated compounds.

The role of mycorrhizal fungi in the synthesis and hyper-accumulation of organo-fluorine compounds by species of *Gastrolobium*.

Since *Gastrolobium* plants have symbiotic mycorrhizal fungi associations (Warcup, 1980; Lamont *et al.*, 1985), confirmation of the presence and synthesis of fluoroacetylated sugars in the seeds and plant tissues would allow for the possibility of their passage by sugar transporters to the fungi (Harrison, 1996). This pathway and any metabolism of these sugars by the fungi form additional areas for future research.

During the course of this research, a hypothesis has developed that the concept of *Gastrolobium* plants hyper-accumulating fluoride ions from the soil and synthesising

often massive concentrations of primarily fluoroacetate, may be an over-simplification of a more complex process. It seems likely that there is a genetic factor in the ability of *Gastrolobium* plants to produce toxic concentrations of organo-fluorine compounds, as the species recorded to contain the higher concentrations are primarily grouped together in two taxonomic clades (Chandler *et al.*, 2002, Figure 2, p. 709). However, there is obviously an overriding environmental influence, seen in the inter- and intra-species variation and the doubling of 'total fluorine' in *G. cuneatum* at Torbay between seasons just one year apart.

It is assumed that the *Gastrolobium* plants themselves synthesise the fluorinated organic molecules, however this has never been supported by experimental data. It seems highly likely that mycorrhizal fungi may play a very significant role in the *Gastrolobium*/fluorine story, as indicated by the following factors:

- The well established role of mycorrhizal fungi in the uptake of trace nutrients and metals from the soil and their association with species of *Gastrolobium* (Warcup, 1980; Lamont *et al.*, 1985);
- The ability of some fungi to release fluoride ions from mineral fluorine sources (Wainwright and Supharungsun, 1984);
- Species such as *G. calycinum* reported as being particularly dangerous when suckering from the root system (Gardner and Bennetts, 1956). A fungi and/or root source of the toxicants may explain such a rapid provision of the toxicants to the plant sucker.
- The bio-transformation of compounds with an aromatic carbon-fluorine bond by ectomycorrhizal fungi is supported by the study of Green (1999).

It therefore seems very probable that mycorrhizal fungi play a significant role in the release and uptake of fluoride ions and the synthesis of fluorinated organic molecules by plants of the *Gastrolobium* genus. Future research examining the role of mycorrhizal fungi should significantly improve our understanding of the *Gastrolobium*/fluorine story.

Whether vegetative parasites of species of *Gastrolobium* also accumulate organo-fluorine compounds.

Dell and Burbidge (1981) recorded the parasitic plant *Pilostyles* parasitising *Gastrolobium velutinum* and *Oxylobium atropurpurea and O. linearifolium* (reclassified as *Gastrolobium leakeanum* and *G. ebracteolosum* respectively (Chandler *et al.*, 2002)). Robert Davis, Western Australian Herbarium, has observed *P. hamiltonii* parasitising *G. spinosum* (Davis pers. com., 2003). The invasion of *Pilostyles hamiltonii* tissues into the phloem and xylem tissues of *Daviesia* stems (Dell *et al.*, 1982) suggests this would also be the process of the parasitism of *Gastrolobium*. Assuming the organo-fluorine compounds found in *Gastrolobium* are transported around within the *Gastrolobium* plant in phloem and xylem tissues, the suggestion is that the *Pilostyles* could be accessing tissues transporting organo-fluorine compounds. The *Pilostyles* therefore may also possess organo-fluorine compounds within its' tissues. Dell *et al.* (1982) suggest *Pilostyles* is dependent on the host plant for its' carbohydrate requirements, and with the fluoroacetylated sugars tentatively identified in this study as present in *Gastrolobium* seeds, it is possible that the *Pilostyles* is also obtaining these sugars.

Use of radio-labeled fluorine (^{18}F) with ^{19}F in a pot trial would enable monitoring of the passage of this ion through the *Gastrolobium* plant, from its application in solution

to the soil, to its passage through the plant. Any eventual synthesis into organo-fluorine compounds and storage in the *Gastrolobium* plant tissue and seeds, and possibly also into any associated parasitic plants, could be confirmed using ^{19}F NMR, as per this study.

9.3 Conclusion

In conclusion, this study has found historical literature to support the concept of reintroduced native animals becoming toxic to introduced predators. All the evidence from the seed analysis indicates *Gastrolobium* seeds to contain multiple fluorinated compounds, but no significant concentration of alkaloids. Although the specific toxicant that could explain the rapid death and toxic bone anecdotes was not identified, the evidence from this study does suggest fluoroacetate and/or other fluoroacetylated compounds to be responsible, as indicated by analysis of the death of Cat 02. The analysis of pigeon leg bones did report the presence of fluoroacetate, often at a much higher concentration than muscle tissue, however the laboratory reporting of positive control birds necessitates caution and the need for additional studies.

The results, questions and new hypotheses arising from this research have reopened the *Gastrolobium* as an area of research. Numerous new organo-fluorine natural products await identification and investigation as potential toxicants, with the plant physiology and mycorrhizal involvement offering additional areas of unique scientific research. Future research into these seed toxicants and the bones of bronzewing pigeons feeding regularly on *Gastrolobium* will hopefully confirm the bronzewing pigeon as another rare toxic bird.

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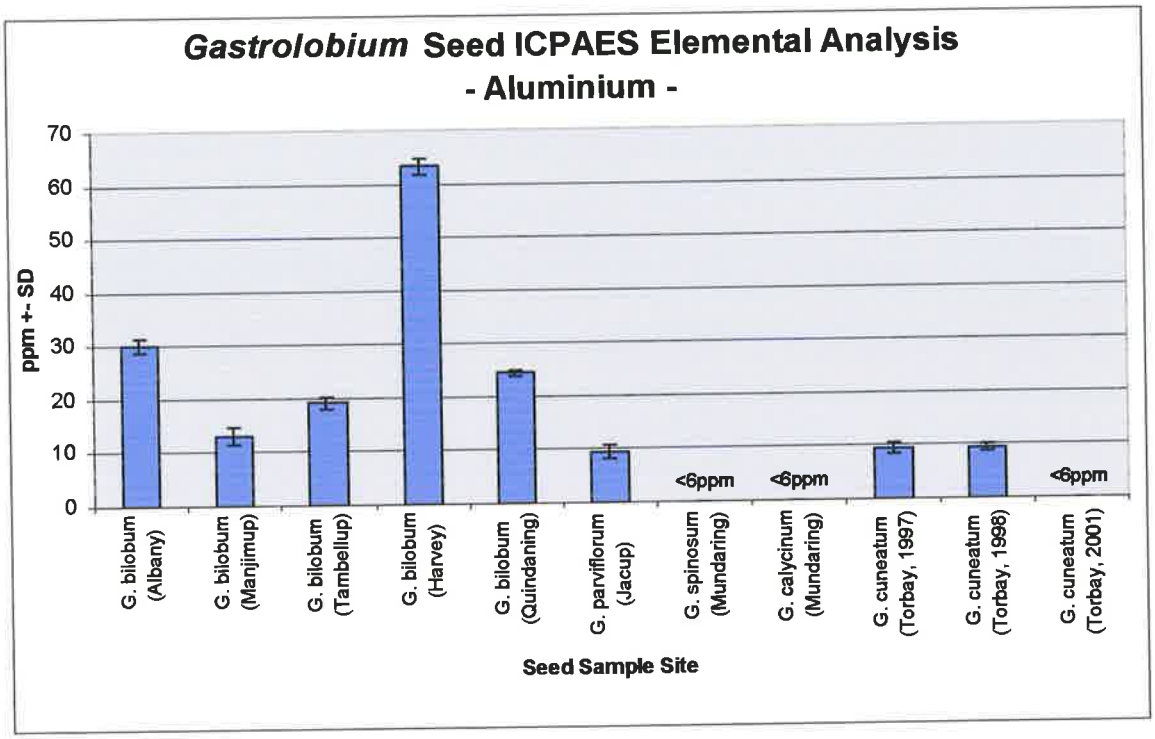
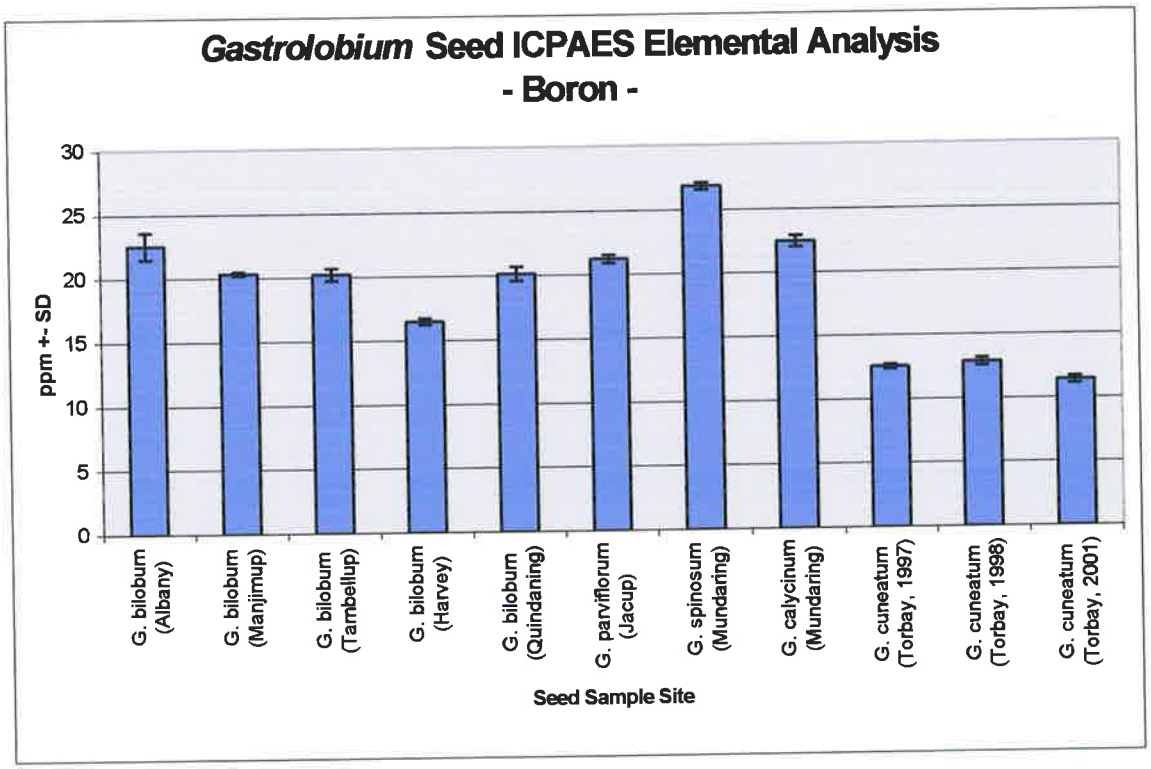
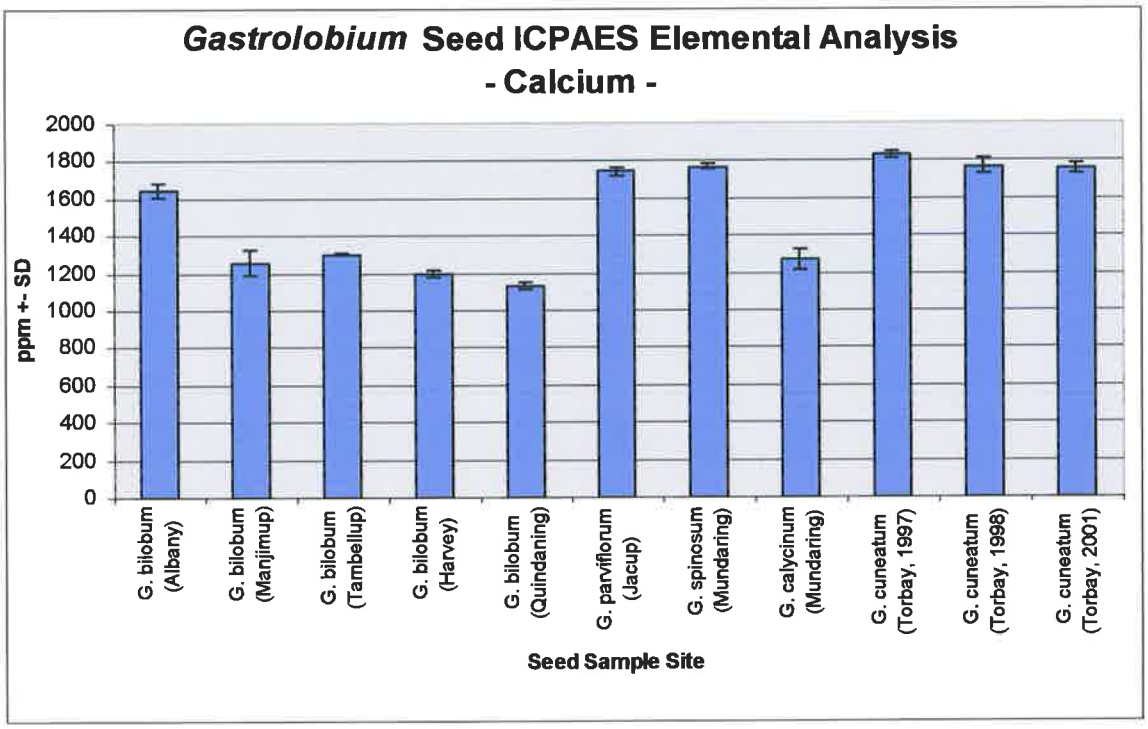
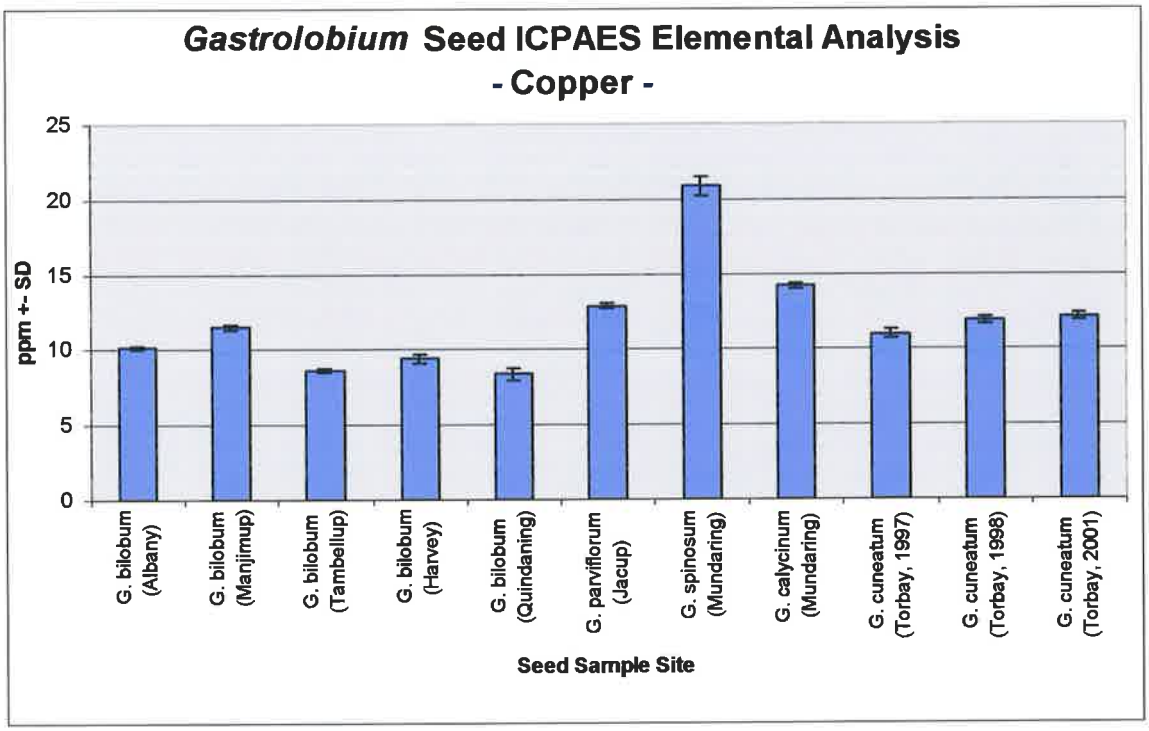
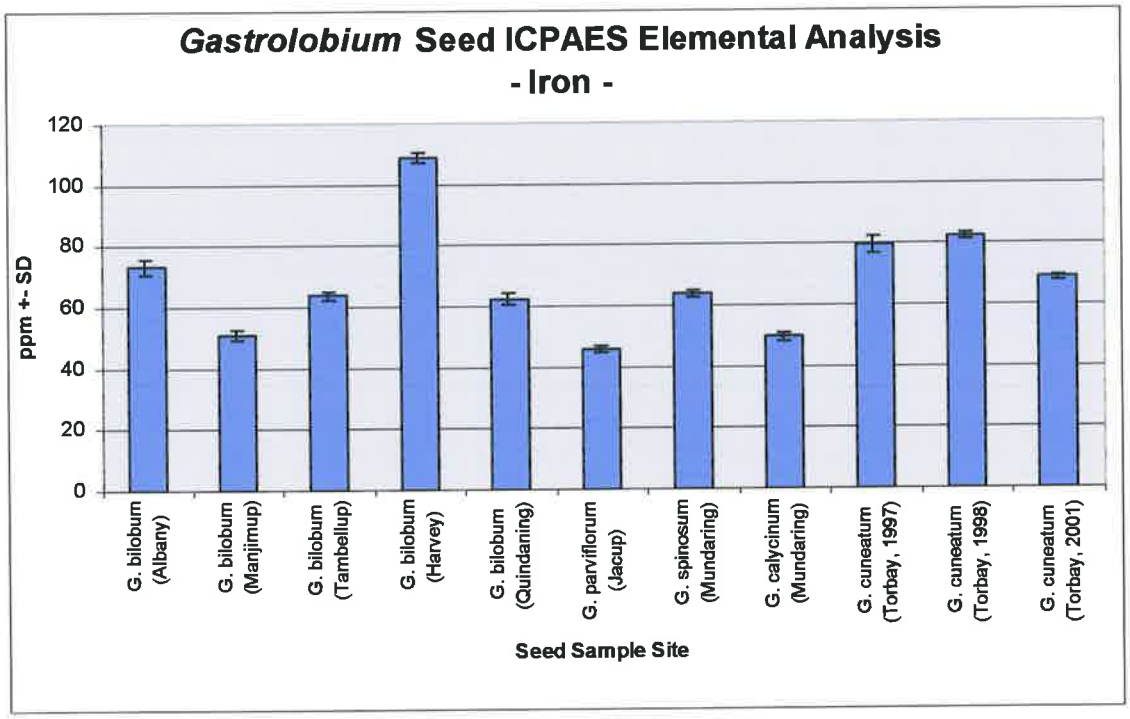
Appendix 1: Inductively Coupled Plasma – Atomic Emission Spectrometer (ICP-AES) Seed Elemental Analysis.
Graphs of elemental analysis results.

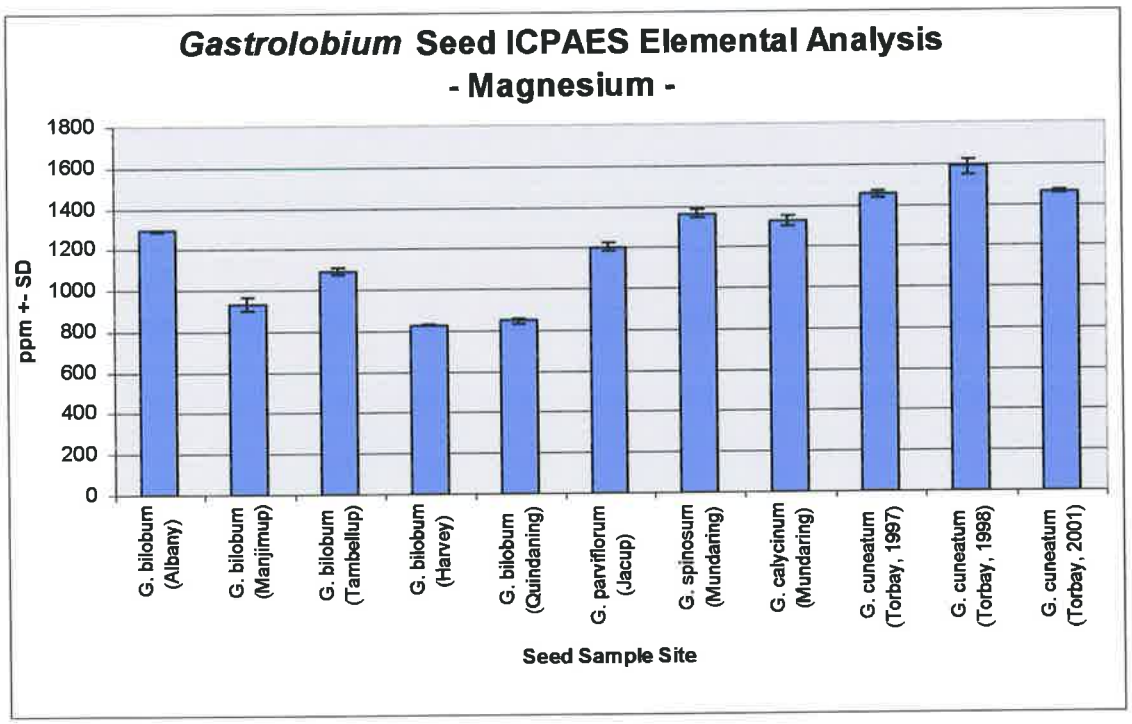

Gastrolobium Seed ICPAES Elemental Analysis
- Aluminium -
ppm +- SD
70
60
50
40
30
20
10
0
<6ppm
<6ppm
<6ppm
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

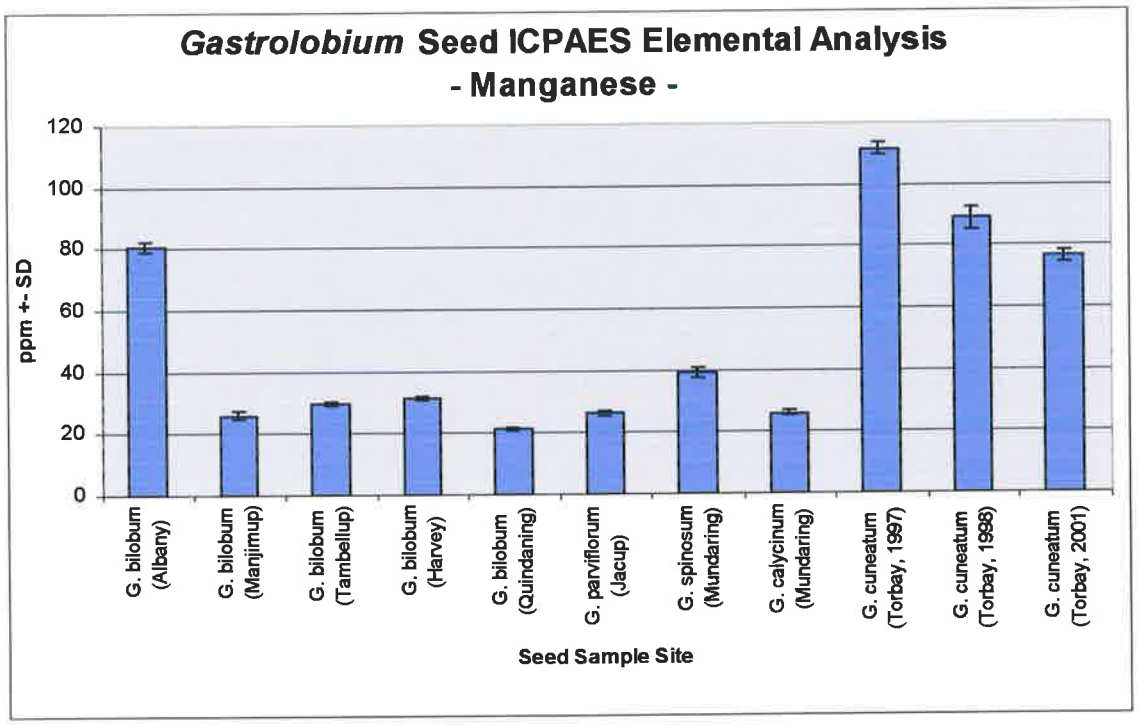

Gastrolobium Seed ICPAES Elemental Analysis
- Boron -
ppm +- SD
30
25
20
15
10
5
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

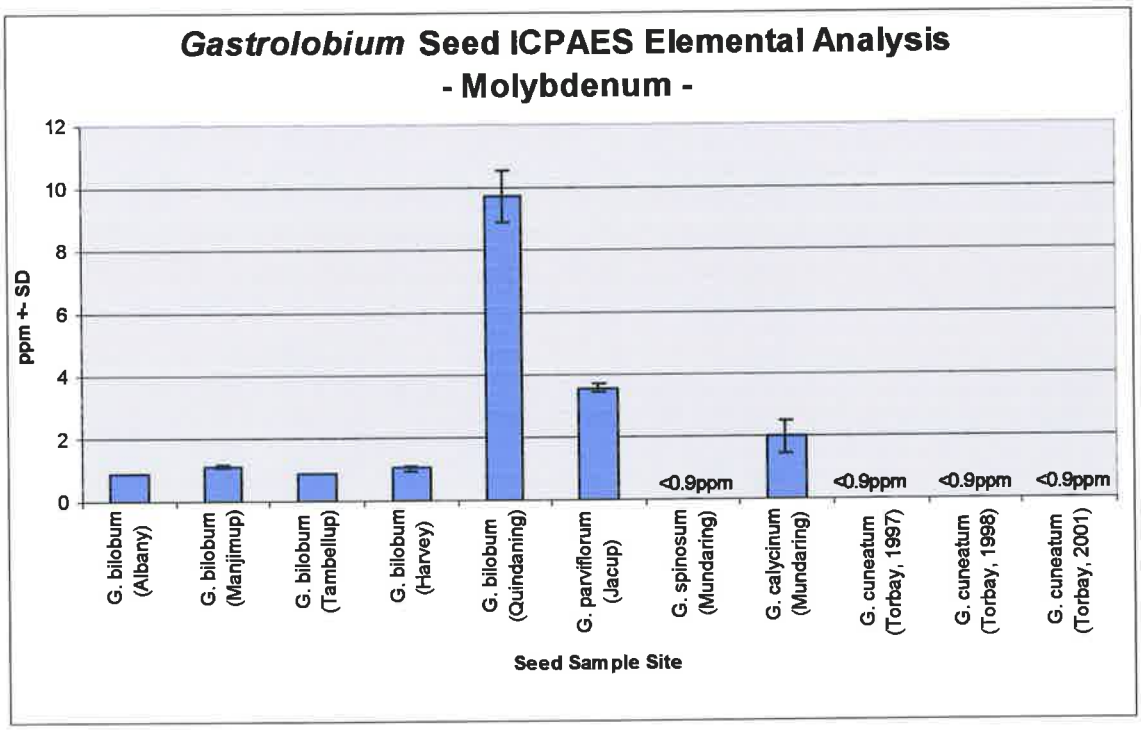

Gastrolobium Seed ICPAES Elemental Analysis
- Calcium -
ppm +- SD
2000
1800
1600
1400
1200
1000
800
600
400
200
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

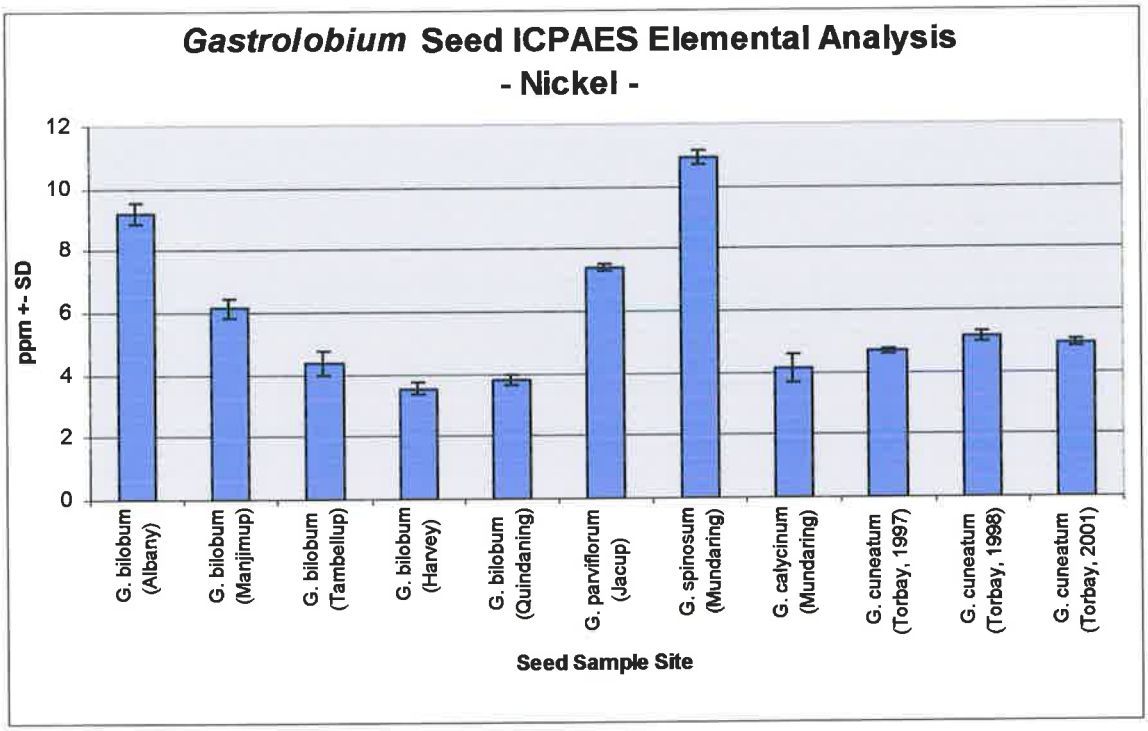

Gastrolobium Seed ICPAES Elemental Analysis
- Copper -
ppm +- SD
25
20
15
10
5
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

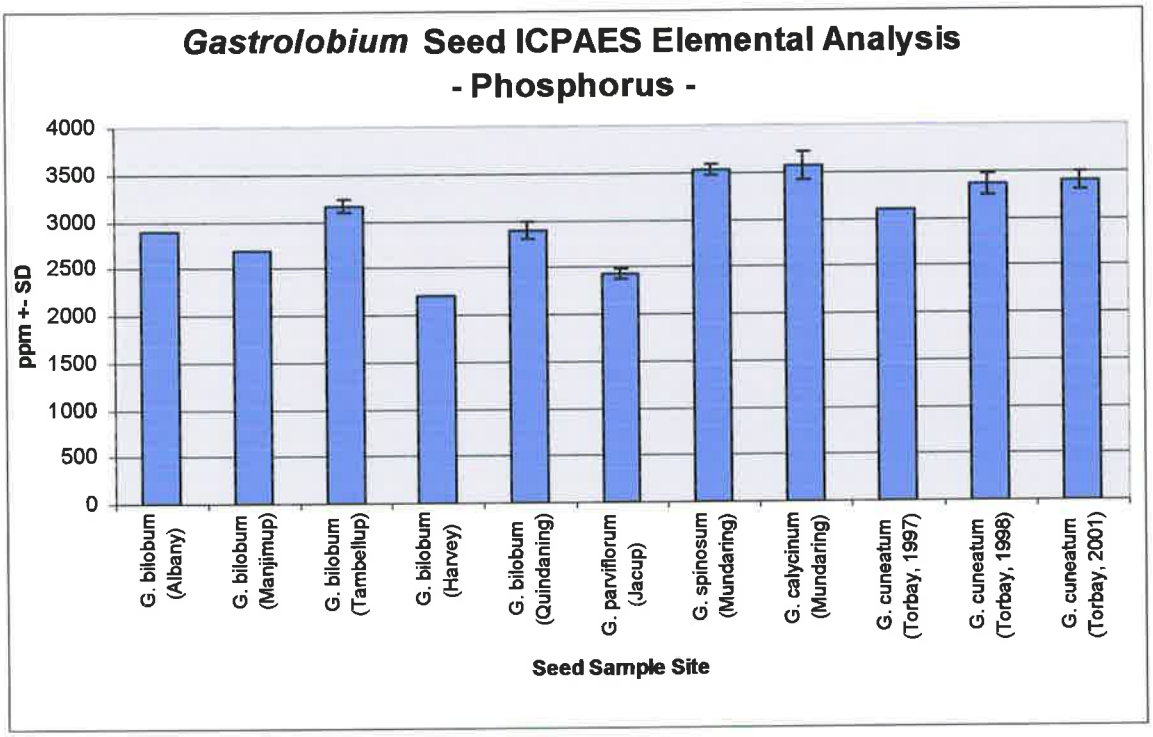

Gastrolobium Seed ICPAES Elemental Analysis
- Iron -
ppm +- SD
120
100
80
60
40
20
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

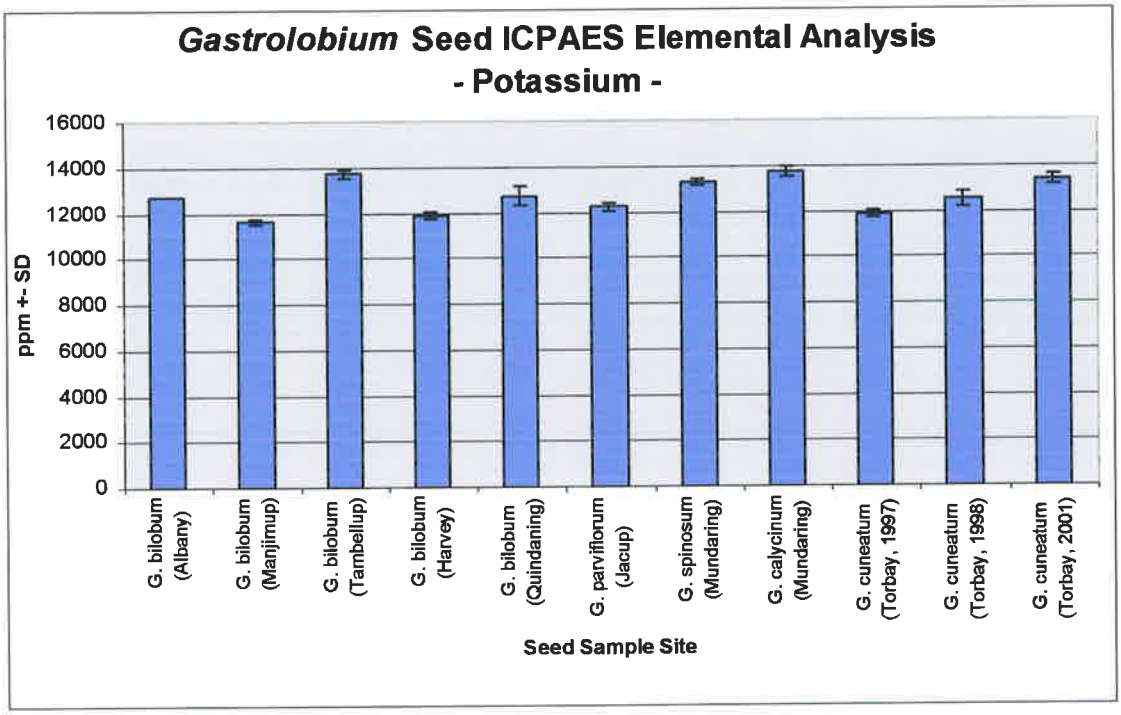

Gastrolobium Seed ICPAES Elemental Analysis
- Magnesium -
ppm +- SD
1800
1600
1400
1200
1000
800
600
400
200
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

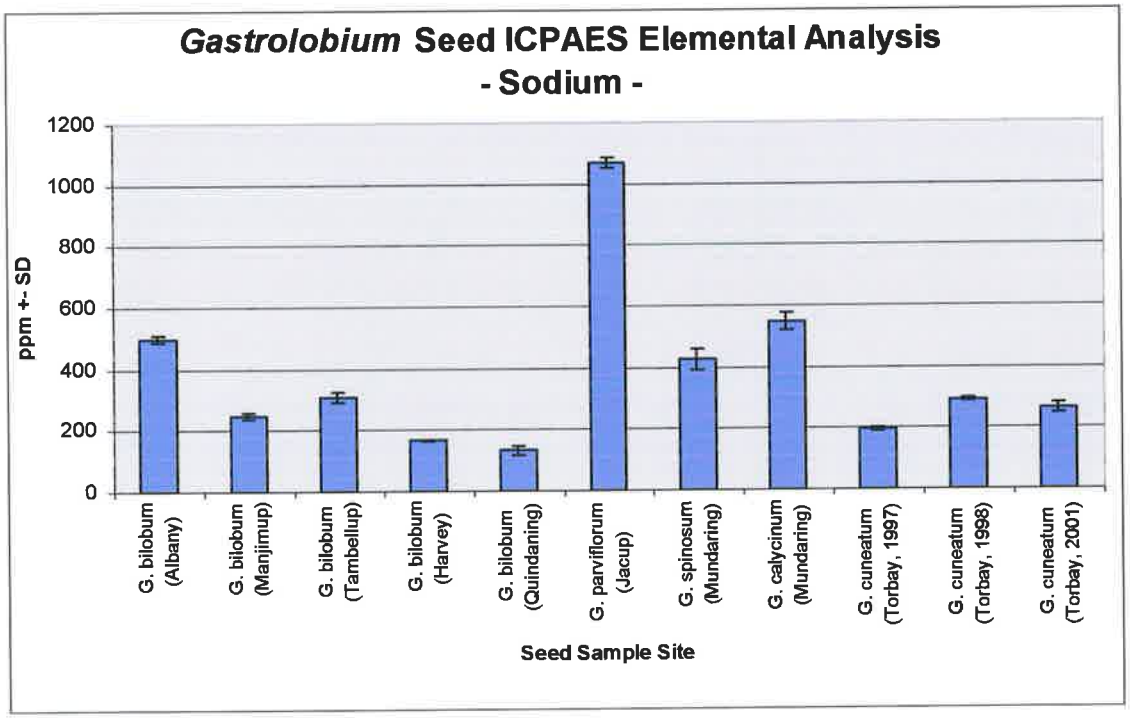

Gastrolobium Seed ICPAES Elemental Analysis
- Manganese -
ppm +- SD
120
100
80
60
40
20
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

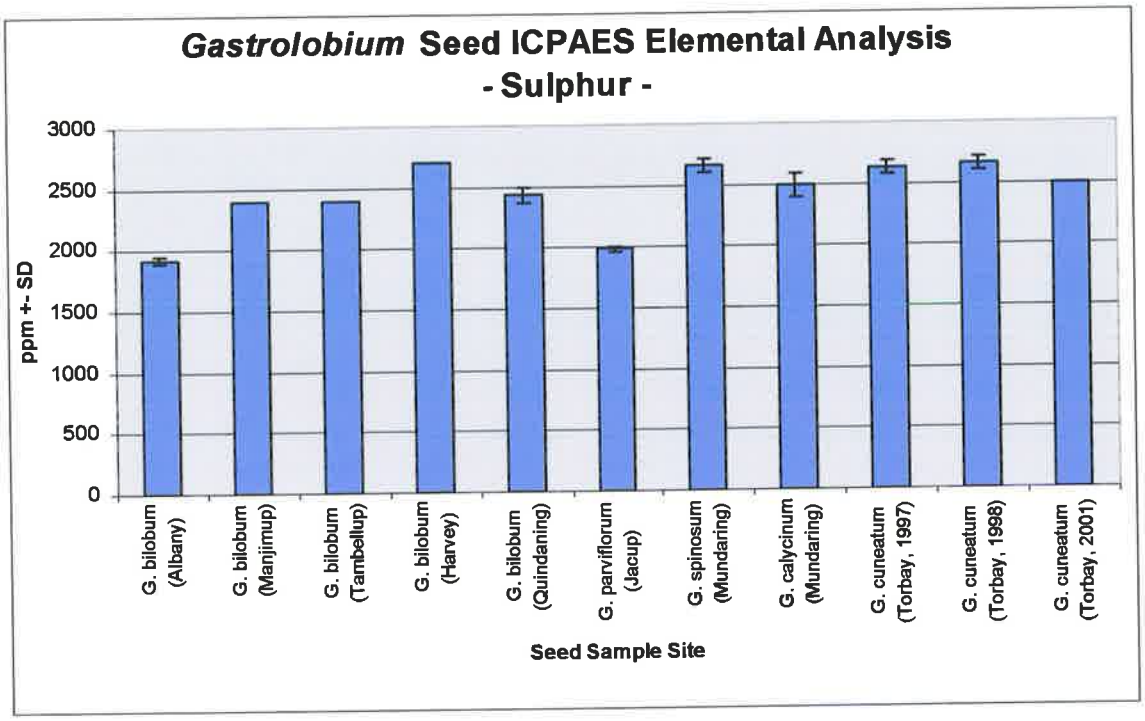

Gastrolobium Seed ICPAES Elemental Analysis
- Molybdenum -
ppm +- SD
12
10
8
6
4
2
0
<0.9ppm
<0.9ppm
<0.9ppm
<0.9ppm
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

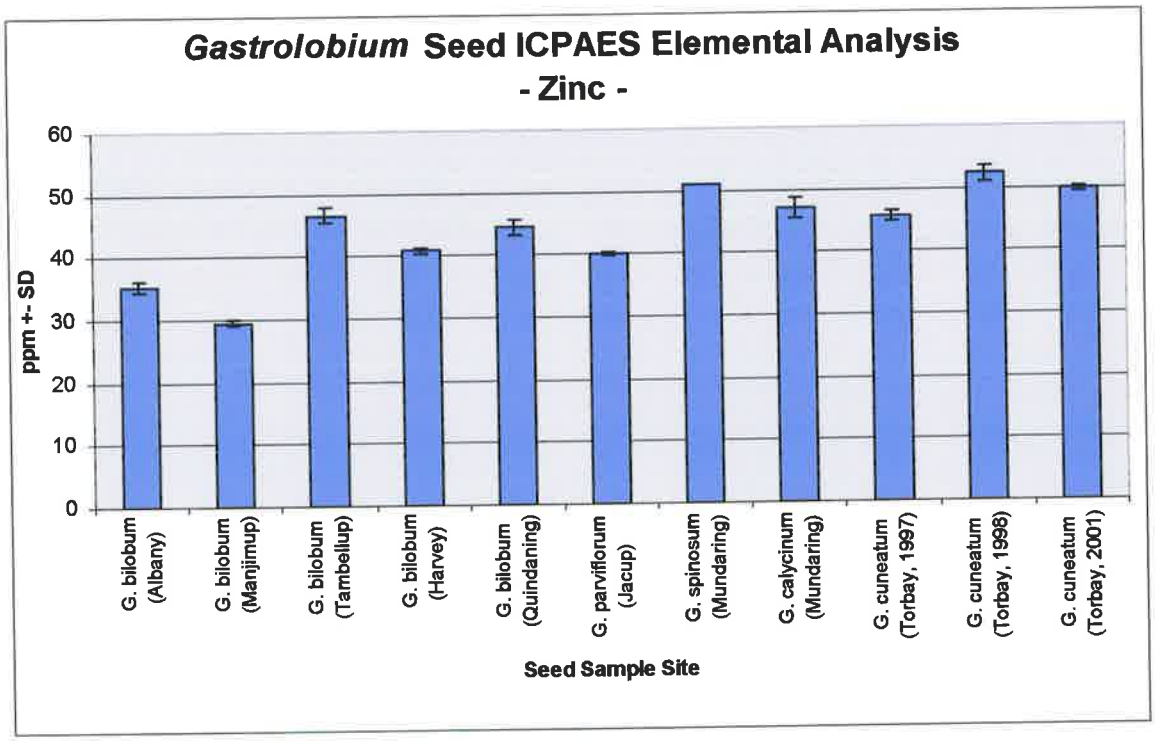

Gastrolobium Seed ICPAES Elemental Analysis
- Nickel -
ppm +- SD
12
10
8
6
4
2
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site


Gastrolobium Seed ICPAES Elemental Analysis
- Phosphorus -
ppm +- SD
4000
3500
3000
2500
2000
1500
1000
500
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site


Gastrolobium Seed ICPAES Elemental Analysis
- Potassium -
ppm +- SD
16000
14000
12000
10000
8000
6000
4000
2000
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site


Gastrolobium Seed ICPAES Elemental Analysis
- Sodium -
ppm +- SD
1200
1000
800
600
400
200
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site


Gastrolobium Seed ICPAES Elemental Analysis
- Sulphur -
ppm +- SD
3000
2500
2000
1500
1000
500
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site


Gastrolobium Seed ICPAES Elemental Analysis
- Zinc -
ppm +- SD
60
50
40
30
20
10
0
G. bilobum (Albany)
G. bilobum (Manjimup)
G. bilobum (Tambellup)
G. bilobum (Harvey)
G. bilobum (Quindaning)
G. parviflorum (Jacup)
G. spinosum (Mundaring)
G. calycinum (Mundaring)
G. cuneatum (Torbay, 1997)
G. cuneatum (Torbay, 1998)
G. cuneatum (Torbay, 2001)
Seed Sample Site

Appendix 2: Fluorocitrate Extraction and Analysis Method. Developed by Bob Parker and Martin Hannan-Jones of the Queensland Department of Natural Resources and based on the methodology of Booth *et al.* (1999).

Extraction

1. Cut bone samples up into pieces ~10mm long and record weight.
2. In a pre-weighed 125ml conical flask add 20ml distilled water and 40ml 2M HCl (record weight of water + acid)
3. Place bone sample and acid/water solution into an Omni-Mixer blender chamber and macerate for 2 minutes on moderate speed (~3).
4. Filter sample solution through glass wool in a polypropylene funnel back into the 125ml conical flask. Collect ~50ml filtrate.
5. Check that pH is 1-2.
6. Add solution to a 50ml plastic centrifuge tube and adjust weights so that all tubes are ± 0.1g.
7. Record weight of flasks + remaining solution.
8. Centrifuge tubes for 30 minutes at 15,000rpm.
9. Pipette off into a separate 50ml glass screw-top tube ~15ml of the middle solution layer (ie. not the top ~12mm or the bottom ~10mm).
10. Using nitrogen gas, blow down the tube contents to dryness (>11.5hrs), but do not heat past this point.
11. Add 5ml of boron tri-fluoro methanol and incubate for 16-20 hours.
12. Heat tube contents in an 80^0C water bath for 2 minutes.
13. Dilute tube contents with 5ml distilled water.
14. Slowly add 10ml of saturated sodium carbonate.
15. Check that pH is 5-8.
16. Add 5ml ethyl acetate; shake. If necessary, centrifuge for ~5mins at ~1000rpm then, using a pasteur pipette, remove the top layer into a separate 50ml glass screw-top tube.
17. Repeat Step 15 twice, adding 5mls of ethyl acetate, shaking the tube and if necessary, centrifuging for ~5mins at ~1000rpm.
18. To the contents of the 50ml tube, add ~1g anhydrous sodium carbonate; shake for 1 minute; centrifuge for ~5mins at ~1650rpm.
19. Pour off the filtrate into a separate 50ml glass screw-top tube.
20. Add 5ml ethyl acetate to the anhydrous sodium carbonate precipitate; shake; centrifuge for ~5mins at ~1000rpm then pour off the ethyl acetate and add to the 50ml tube with the other ethyl acetate samples.
21. Using nitrogen gas, blow down the tube contents to 5-7ml.
22. Make up to 10ml in a volumetric glass centrifuge tube.
23. Sonicate for ~10 minutes.

Analysis

2ml of the tube contents were added to a disposable 3ml syringe with filter and the filtrate collected in 2ml glass GC vial and capped with a lid and silicon septa. The derivitised sample was analysed by an undisclosed GC-MS method.

Appendix 3: Fluoroacetate Extraction and Analysis Report for Pigeon and Cat Samples. Report Provided by the Independent Analytical Laboratory.

Alan Fletcher Research Station pest management chemistry – report 2003, *PMC 03/059: study of fluoroacetate (1080) residues in pigeons and cats,* Queensland Government.

Appendix 4: Cat Biochemistry Results Additional to Those Presented in 8.3.2.

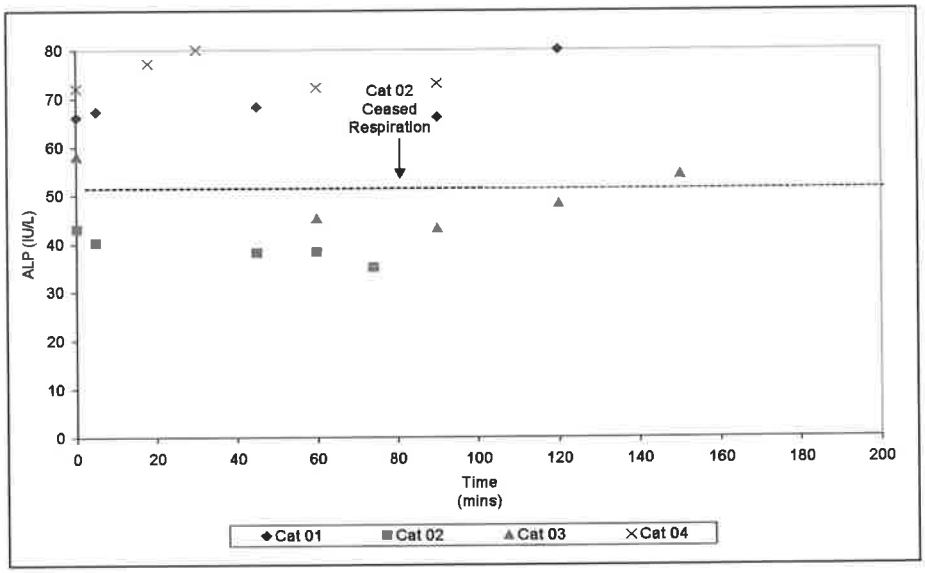


Figure A3.1 Plasma alkaline phosphatase (ALP) concentration. Elevated levels in Cats 01 and 04 likely a function of bone physiology in cats of this age.

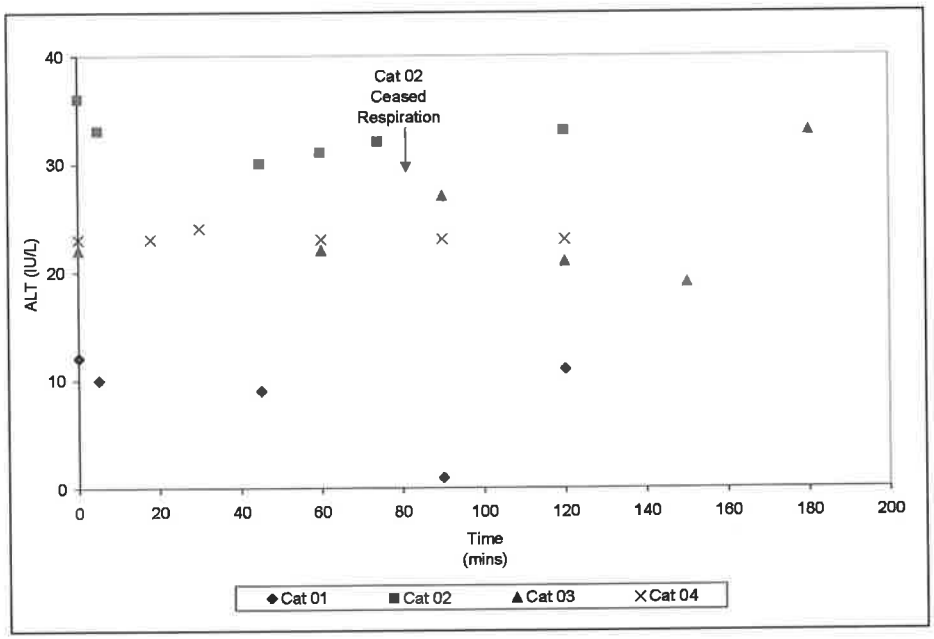


Figure A3.2 Plasma alanine transferase (ALT) concentration (IDEXX normal concentration <91 IU/L). Levels within normal limits.

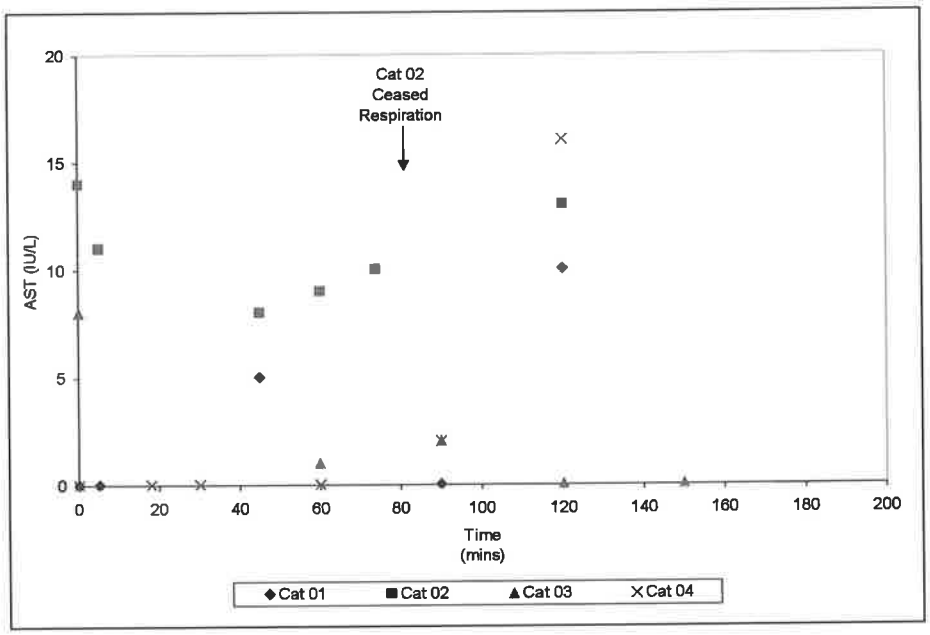


Figure A3.3 Plasma aspartate amino transferase (AST) concentration (IDEXX normal concentration <66 IU/L). Levels within normal limits.

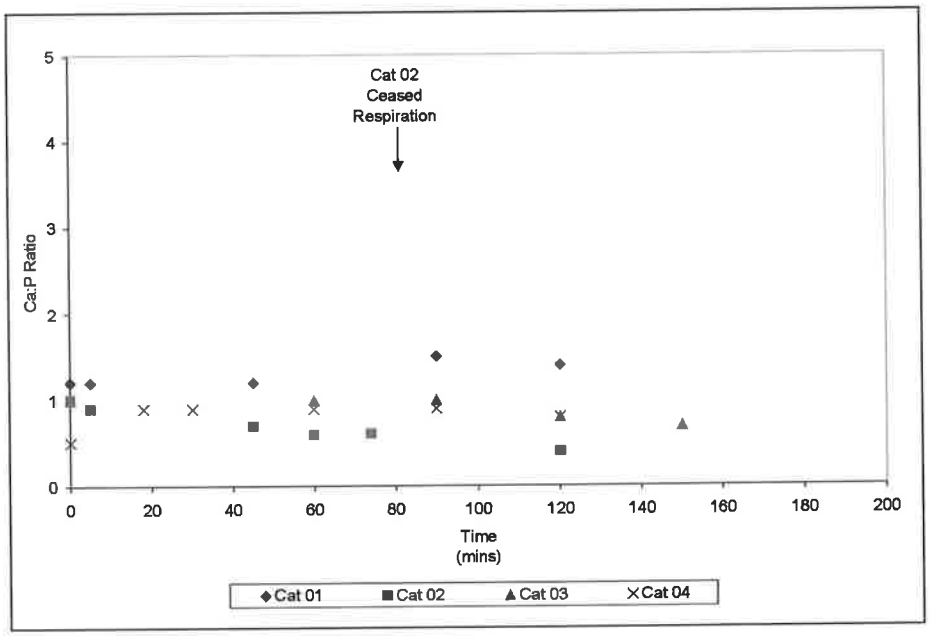


Figure A3.4 Plasma calcium : phosphate ratio. Levels within normal limits.

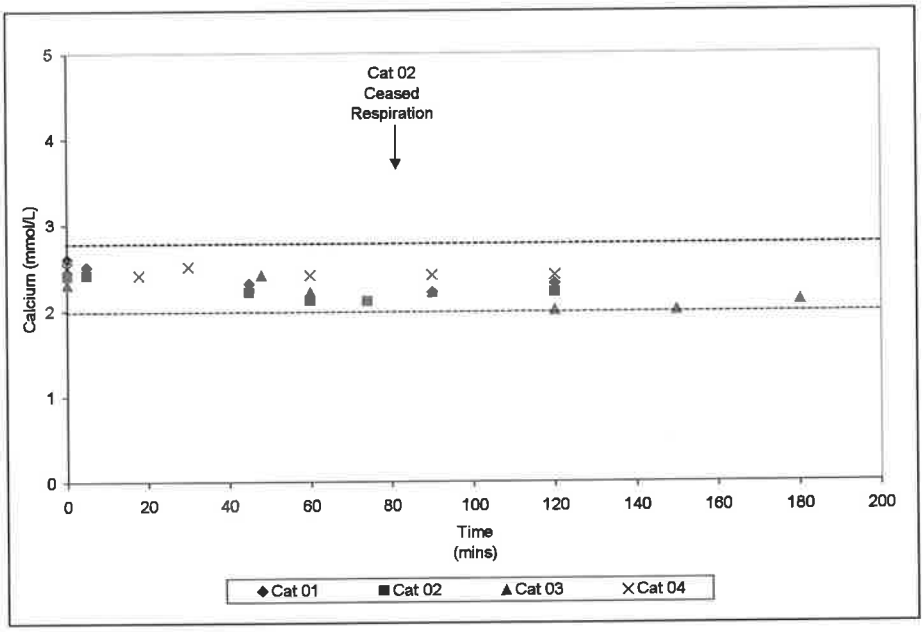


Figure A3.5 Plasma calcium concentration. Levels within normal limits.

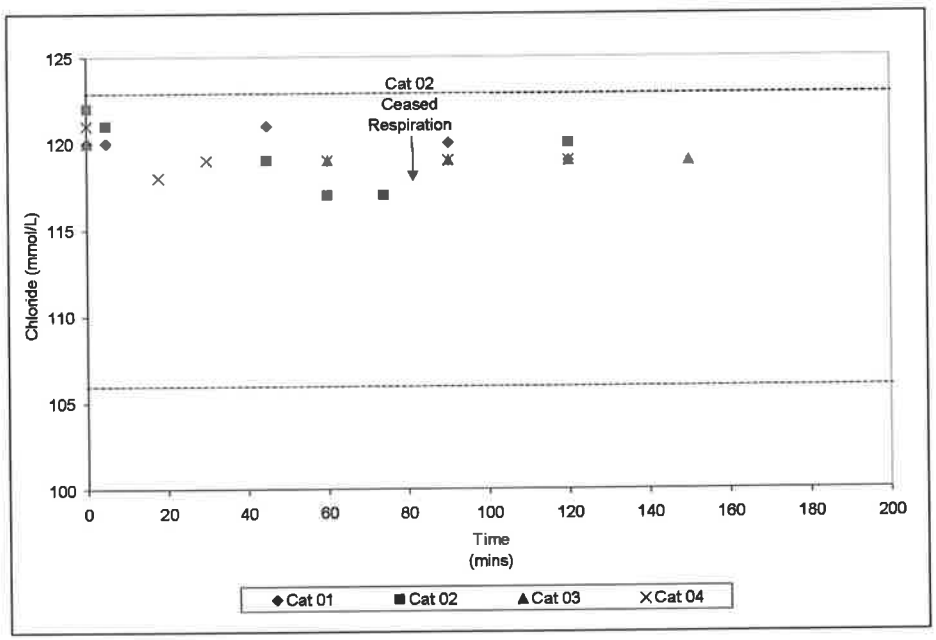


Figure A3.6 Plasma chloride concentration. Levels within normal limits.

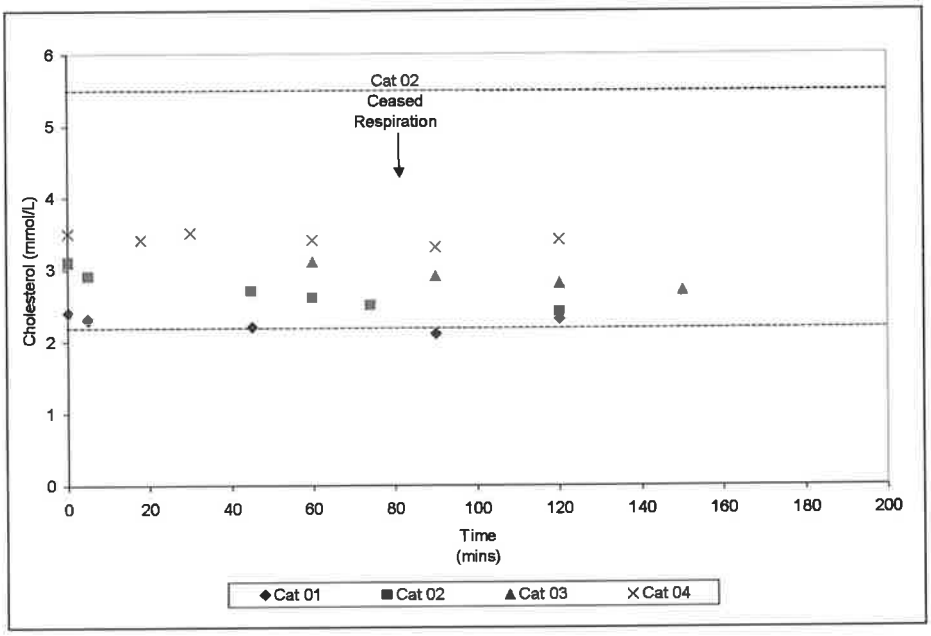


Figure A3.7 Plasma cholesterol concentration. Levels within normal limits.

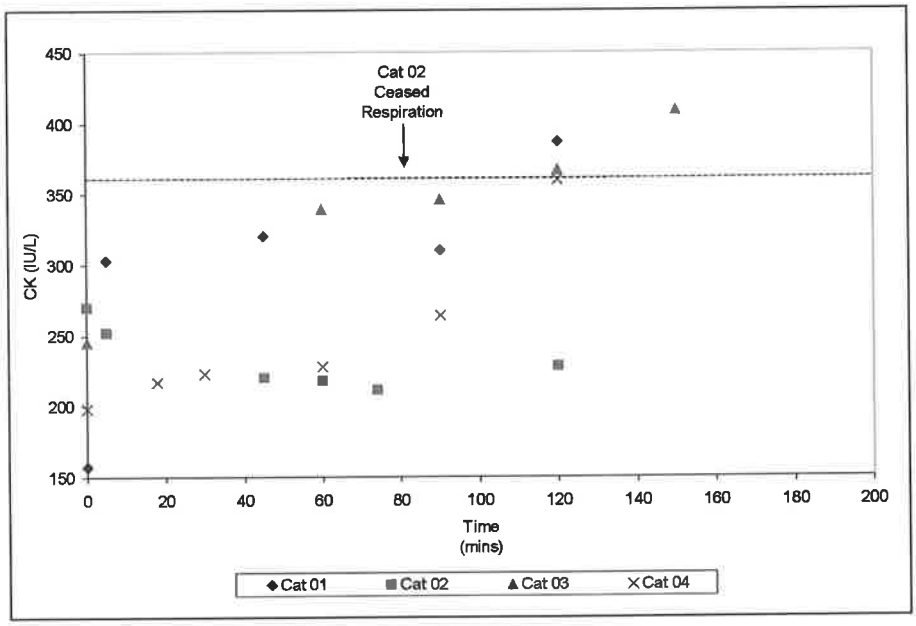


Figure A3.8 Plasma creatinine kinase (CK) concentration. Levels not significant with increase in Cat 03 likely due to minor muscle ascaemia from immobility.

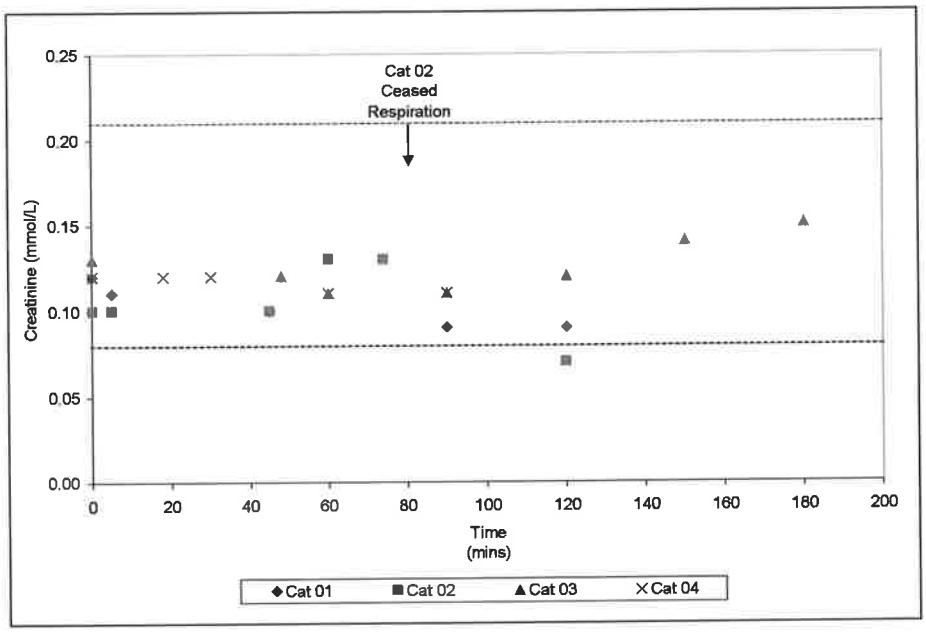


Figure A3.9 Plasma creatinine concentration. Levels within normal limits.

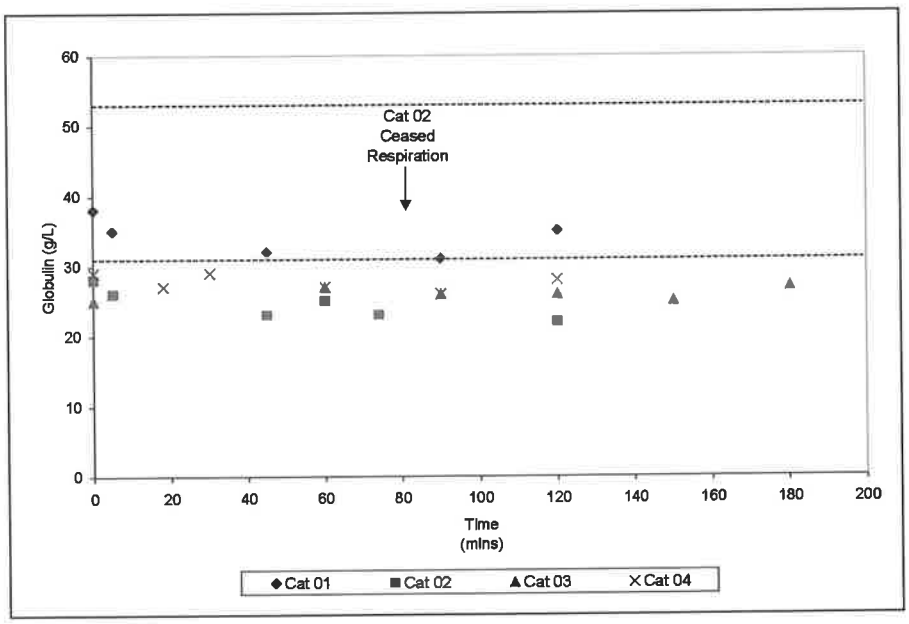


Figure A3.10 Plasma globulin concentration. Reduced levels in Cats 02 to 04 likely a function of younger cats with little disease exposure.

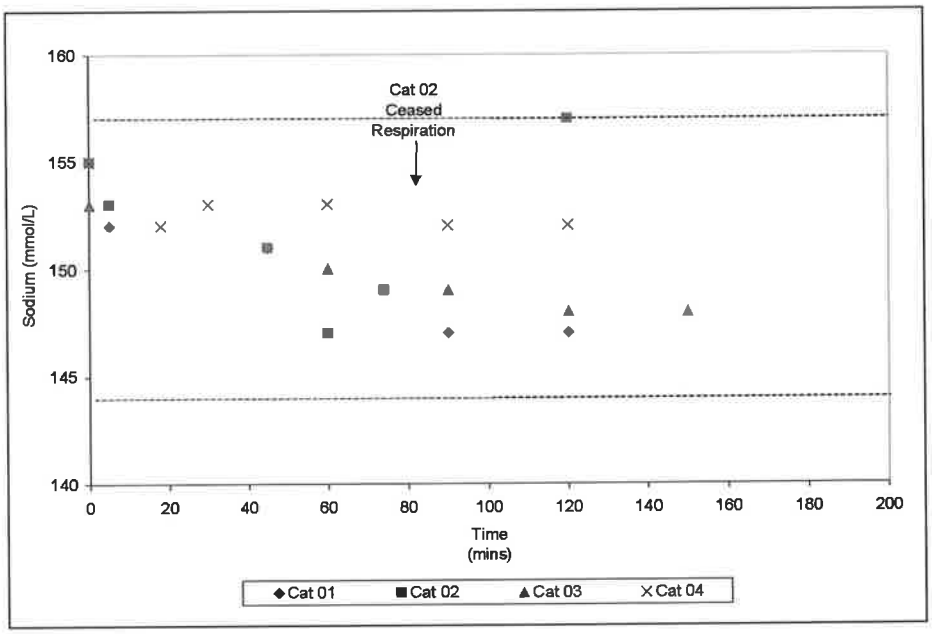


Figure A3.11 Plasma sodium concentration. Levels within normal limits.

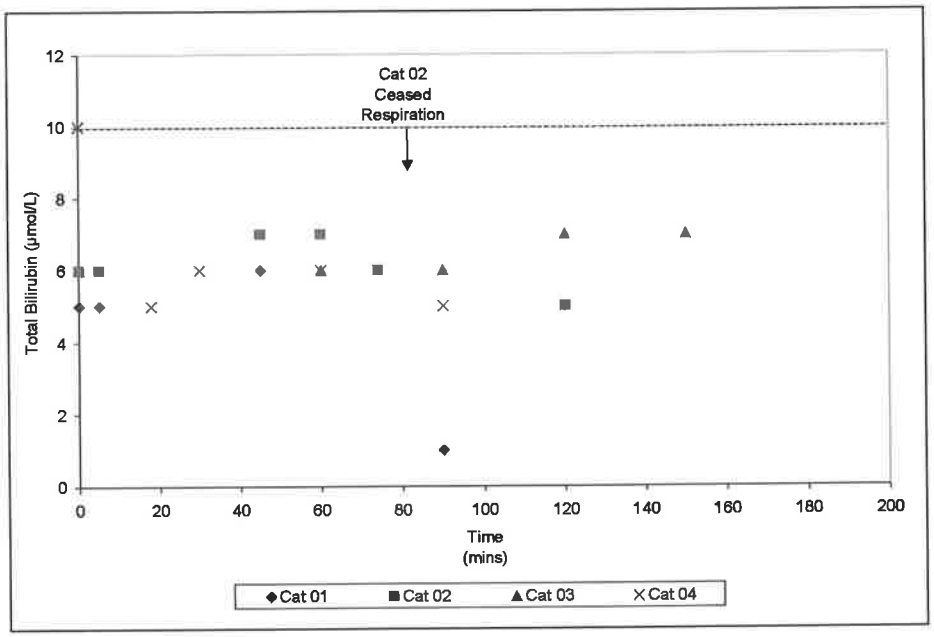


Figure A3.12 Plasma total bilirubin concentration. Levels within normal limits. Time 0 value for Cat 04 due to haemolysed sample (see also Figure 8.16).

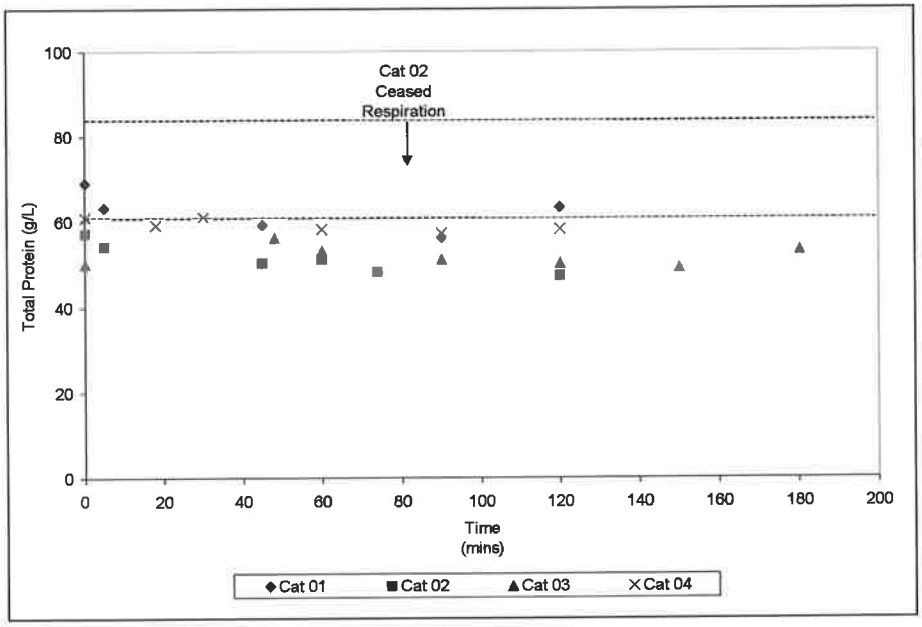


Figure A3.13 Plasma total protein concentration. Levels are low due to younger age of cats.

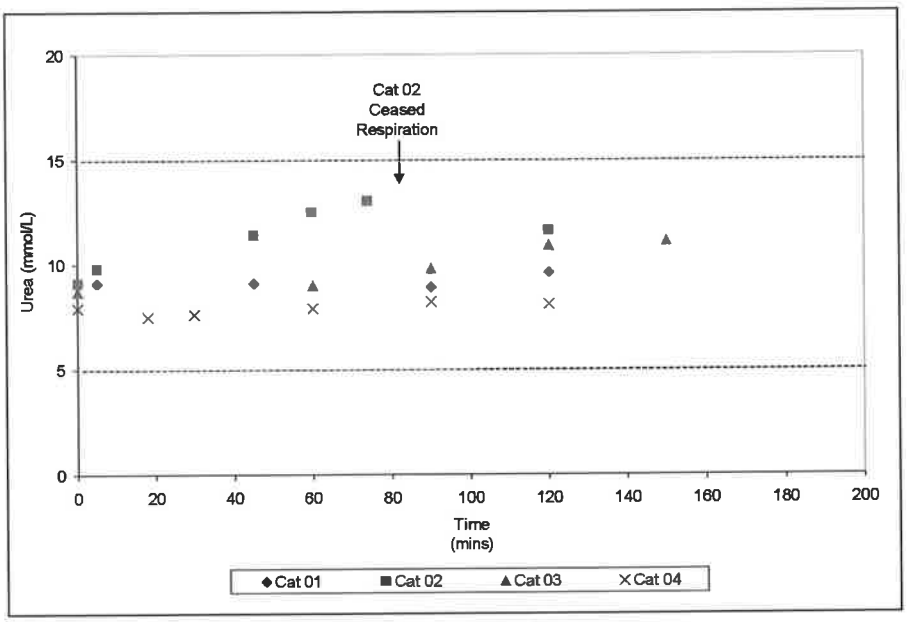


Figure A3.14 Plasma urea concentration. Levels within normal limits.